

# **ICOS LIGAND VARIANT IMMUNOMODULATORY PROTEINS AND USES THEREOF**

## **Cross-Reference to Related Applications**

[0001] This application claims priority from U.S. provisional application No. 62/323,608 filed April 15, 2016, entitled “ICOS Ligand Variant Immunomodulatory Proteins and Uses Thereof,” U.S. provisional application No. 62/394,745 filed September 14, 2016, entitled “ICOS Ligand Variant Immunomodulatory Proteins and Uses Thereof,” U.S. provisional application No. 62/410,842 filed October 20, 2016, entitled “ICOS Ligand Variant Immunomodulatory Proteins and Uses Thereof,” U.S. provisional application No. 62/472,568 filed March 16 2017, entitled “ICOS Ligand Variant Immunomodulatory Proteins and Uses Thereof,” and U.S. provisional application No. 62/475,162 filed March 22, 2017, entitled “ICOS Ligand Variant Immunomodulatory Proteins and Uses Thereof,” the contents of each of which are incorporated by reference in their entirety.

## **Incorporation by Reference of Sequence Listing**

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled 761612000340SeqList.txt, created April 13, 2017, which is 979,474 bytes in size. The information in the electronic format of the Sequence Listing is incorporated by reference in its entirety.

## **Field**

[0003] The present disclosure relates to therapeutic compositions for modulating immune response in the treatment of cancer and immunological diseases. In some aspects, the present disclosure relates to particular variants of ICOS Ligand (ICOSL) that exhibit improved binding affinity for one or both of the cognate binding partner proteins ICOS or CD28.

## **Background**

[0004] Modulation of the immune response by intervening in the processes that occur in the immunological synapse (IS) formed by and between antigen-presenting cells (APCs) or target cells and lymphocytes is of increasing medical interest. Mechanistically, cell surface proteins in the IS can involve the coordinated and often simultaneous interaction of multiple protein targets with a single protein to which they bind. IS interactions occur in close association with the junction of two cells, and a single protein in this structure can interact with both a protein on the same cell (cis) as well as a protein on the associated cell (trans), likely at the same time.

Although therapeutics are known that can modulate the IS, improved therapeutics are needed. Provided are immunomodulatory proteins, including soluble proteins or transmembrane immunomodulatory proteins capable of being expressed on cells, that meet such needs.

## **Summary**

[0005] Disclosed herein is a variant ICOS Ligand (ICOSL) polypeptide, comprising an IgV domain or specific binding fragment thereof, an IgC domain or a specific binding fragment thereof, or both, that comprises one or more amino acid modifications at one or more positions in an unmodified ICOSL or a specific binding fragment thereof, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of human ICOS and the ectodomain of human CD28 each with increased affinity compared to the binding of the unmodified ICOSL polypeptide to the ectodomain of human ICOS and human CD28. Also disclosed herein is a variant ICOS Ligand (ICOSL) polypeptide, comprising an IgV domain or specific binding fragment thereof, an IgC domain or specific binding fragment thereof, or both, wherein the variant ICOSL polypeptide contains one or more amino acid substitutions in an unmodified ICOSL or a specific binding fragment thereof corresponding to position(s) selected from 52, 57, 100, 10, 11, 13, 16, 18, 20, 25, 27, 30, 33, 37, 38, 42, 43, 47, 54, 61, 62, 67, 71, 72, 74, 75, 77, 78, 80, 84, 89, 90, 92, 93, 94, 96, 97, 98, 99, 102, 103, 107, 109, 110, 111, 113, 115, 116, 117, 119, 120, 121, 122, 126, 129, 130, 132, 133, 135, 138, 139, 140, 142, 143, 144, 146, 148, 151, 152, 153, 154, 155, 156, 158, 161, 164, 166, 168, 172, 173, 175, 190, 192, 193, 194, 198, 201, 203, 207, 208, 210, 212, 217, 218, 220, 221, 224, 225, or 227 with reference to SEQ ID NO:32. In some embodiments, the unmodified ICOSL is a mammalian ICOSL or a specific binding fragment thereof. In some embodiments, the unmodified ICOSL is a human ICOSL or a specific

binding fragment thereof. In some embodiments, the unmodified ICOSL includes (i) the sequence of amino acids set forth in SEQ ID NO:32, (ii) a sequence of amino acids that has at least 95% sequence identity to SEQ ID NO:32; or (iii) a portion thereof comprising an IgV domain or IgC domain or specific binding fragments thereof or both.

**[0005a]** Disclosed herein is a variant ICOS Ligand (ICOSL) polypeptide, comprising one or more amino acid substitutions N52H, N57Y or Q100R, in an unmodified ICOSL set forth in SEQ ID NO:32 or a portion of SEQ ID NO:32 comprising an IgV domain, wherein the IgV domain is amino acids 19-129 of SEQ ID NO:5; wherein: the variant ICOSL exhibits at least 95% sequence identity to SEQ ID NO:32 or the portion thereof comprising the IgV domain; and the variant ICOSL polypeptide specifically binds to the ectodomain of human CD28 with increased affinity compared to the binding of the unmodified ICOSL to the ectodomain of human CD28.

**[0006]** In some embodiments of any one of the variant ICOSL polypeptides described above, the specific binding fragment of the IgV domain or IgC domain has a length of at least 50, 60, 70, 80, 90, 100, 110 or more amino acids; or the specific binding fragment of the IgV domain includes a length that is at least 80% of the length of the IgV domain set forth as amino acids 19-129 of SEQ ID NO:5 and/or the specific binding fragment of the IgC domain includes a length that is at least 80% of the length of the IgC domain set forth as amino acids 141-227 of SEQ ID NO:5. In some embodiments, the variant ICOSL polypeptide includes up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid modifications, optionally amino acid substitutions, insertions and/or deletions. In some embodiments, the ICOSL variant includes a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:32 or a specific binding fragment thereof. In some embodiments, any of the variant ICOSL polypeptides provided herein exhibit altered binding to the ectodomain of ICOS, CD28, or CTLA-4 compared to the unmodified ICOSL. In some embodiments, any of the variant ICOSL polypeptides exhibit altered binding to the ectodomain of ICOS or CD28 compared to the unmodified ICOSL. In some embodiments, the altered binding is altered binding affinity and/or altered binding selectivity.

**[0007]** In some embodiments of any one of the variant ICOSL polypeptides described above, the one or more amino acid substitutions are selected from M10V, M10I, V11E, S13G, E16V, S18R, A20V, S25G, F27S, F27C, N30D, Y33del, Q37R, K42E, T43A, Y47H, N52H, N52D, N52S, N52Q, N52Y, N52K, S54A, S54P, N57Y, N57D, R61S, R61C, Y62F, L67P, A71T,

G72R, L74Q, R75Q, D77G, F78L, L80P, N84Q, D89G, E90A, K92R, F93L, H94E, H94D, L96F, L96I, V97A, L98F, S99G, Q100R, Q100K, Q100P, L102R, G103E, V107A, V107I, S109G, S109N, V110D, V110N, V110A, E111del, T113E, H115R, H115Q, V116A, A117T, N119Q, F120I, F120S, S121G, V122A, V122M, S126T, S126R, H129P, S130G, S132F, Q133H, E135K, F138L, T139S, C140D, C140del, S142F, I143V, I143T, N144D, Y146C, V151A, Y152C, Y152H, W153R, I154F, N155H, N155Q, K156M, D158G, L161P, L161M, L166Q, N168Q, F172S, L173S, M175T, T190A, T190S, S192G, V193M, N194D, C198R, N201S, L203P, L203F, N207Q, L208P, V210A, S212G, D217V, I218T, I218N, E220G, R221G, R221I, I224V, T225A, N227K or a conservative amino acid substitution thereof.

**[0008]** In some embodiments, the one or more amino acid modifications are selected from among N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52S/Y146C/Y152C, N52H/C198R, N52H/C140D/T225A, N52H/C198R/T225A, N52H/K92R, N52H/S99G, N57Y/Q100P, N52S/G103E, N52S/S130G/Y152C, N52S/Y152C, N52S/C198R, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52S/T113E, N52D/S54P, N52K/L208P, N52S/Y152H, N52D/V151A, N52H/I143T, N52S/L80P, F120S/Y152H/N201S, N52S/R75Q/L203P, N52S/D158G, N52D/Q133H, N52S/N57Y/H94D/L96F/L98F/Q100R, N52S/N57Y/H94D/L96F/L98F/Q100R/G103E/F120S, N52H/F78L/Q100R, N52H/N57Y/Q100R/V110D, N52H/N57Y/R75Q/Q100R/V110D, N52H/N57Y/Q100R, N52H/N57Y/L74Q/Q100R/V110D, N52H/Q100R, N52H/S121G, A20V/N52H/N57Y/Q100R/S109G, N52H/N57Y/R61S/Q100R/V110D/L173S, N52H/N57Y/Q100R/V122A, N52H/N57Y/Q100R/F172S, N52H/N57Y, N52S/F120S, N52S/V97A, N52S/G72R, N52S/A71T/A117T, N52S/E220G, Y47H/N52S/V107A/F120S, N52H/N57Y/Q100R/V110D/S132F/M175T, E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R, Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G, N52H/N57Y/Q100R/V110D/C198R, N52H/N57Y/Q100R/V110D/V116A/L161M/F172S/S192G/C198R, F27S/N52H/N57Y/V110N, N52S/H94E/L96I/S109N/L166Q, S18R/N52S/F93L/I143V/R221G, A20T/N52D/Y146C/Q164L, V11E/N30D/N52H/N57Y/H94E/L96I/L98F/N194D/V210A/I218T, N52S/H94E/L96I/V122M, N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N, M10V/S18R/N30D/N52S/S126R/T139S/L203F, S25G/N30D/N52S/F120S/N227K,

N30D/N52S/L67P/Q100K/D217G/R221K/T225S,  
 N52H/N57Y/Q100R/V110D/A117T/T190S/C198R, N52H/N57Y/Q100R/V110D/F172S/C198R,  
 S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R,  
 N52H/N57Y/V110A/C198R/R221I,  
 M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M,C198R,  
 N52H/N57Y/R61C/Y62F/Q100R/V110N/F120S/C198R,  
 N52H/N57Y/Q100R/V110D/H115R/C198R,  
 N52H/N57Y/Q100R/V110D/N144D/F172S/C198R, N52S/H94E/L98F/Q100R, N52S/E90A,  
 N30D/K42E/N52S, N52S/F120S/I143V/I224V, N52H/ N57Y/Q100R/V110D/C198R/S212G,  
 N52H/N57Y/Q100R/C198R, N52S/N194D, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R,  
 N52S/S54P, T38P/N52S/N57D, E111del, Y33del, N52H/C140del/T225A,  
 N52H/F78L/Q100R/C198R, N52H/N57Y/R75Q/Q100P/V110D,  
 N52H/N57Y/L74Q/V110D/S192G, N52H/S121G/C198R, N52S/F120S/N227K,  
 N52S/A71T/A117T/T190A/C198R, T43A/N52H/N57Y/L74Q/D89G/V110D/F172S,  
 N52H/N57Y/Q100R/V110D/S132F/M175T,  
 N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G, N84Q, N119Q, N168Q, N207Q,  
 N52Q, N52Q/N207Q, N168Q/N207Q, N52Q/N168Q, N84Q/N207Q, N155Q/N207Q,  
 N119Q/N168Q, N119Q/N207Q, N119Q/N155Q, N52Q/N84Q, N52Q/N119Q, N84Q/N119Q,  
 N52Q/N84Q/N168Q, N52Q/N84Q/N207Q, N84Q/N155Q/N168Q, N84Q/N168Q/N207Q,  
 N84Q/N155H/N207Q, N155Q/N168Q/N207Q, N119Q N155Q/N168Q, N119Q/N168Q/N207Q,  
 N84Q/N119Q/N207Q, N119Q/N155H/N207Q, N84Q/N119Q/N155Q, N52Q/N119Q/N155Q,  
 N52H/N84Q/N119Q, N52H/N84Q, N52H/N84Q/N168Q, N52H/N84Q/N207Q,  
 N52H/N84Q/N168Q/N207Q, N52Q/N84Q/N155Q, N52Q/N84Q/N168Q,  
 N52Q/N84Q/N155Q/N168Q, N52Q/N84Q/N119Q/N168Q, N84Q/N119Q/N155Q/N168Q,  
 N84Q/N155Q/N168Q/N207Q, N84Q/N119Q/N155Q/N207Q, N52Q/N84Q/N119Q/N207Q,  
 N52Q/N84Q/N119Q/N155Q, N52Q/N84Q/N119Q/N155Q/N207Q,  
 N84Q/N119Q/N155Q/N168Q/N207Q, Q100R, F138L/L203P, N52Y/F138L/L203P,  
 N57Y/Q100R/C198R, N57Y/F138L/L203P, Q100R/F138L, L203P,  
 N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/Q100R/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/F172S/C198R, N52H/N57Y/Q100R/H115R/I143V/F172S/C198R,  
 N52H/N57Y/Q100R/L102R/H115R/F172S/C198R, N52H/V122A/F172S/C198R,

N52H/N57Y/Q100R/H115R/F172S/N194D, N52H/N57Y/H115R/F172S/C198R,  
N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/H115R, N52H/N57Y/Q100R/H115R,  
N52H/N57Y/Q100R/H115R/F172S/I224V, N52H/N57Y/Q100R/H115R/F172S,  
N52H/N57Y/Q100R/F172S, N52H/Q100R/H115R/I143T/F172S,  
N52H/N57Y/Q100P/H115R/F172S, N52Y/N57Y/Q100P/F172S,  
E16V/N52H/N57Y/Q100R/V110D/H115R/C198R,  
E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R, N52S/E90A/H115R,  
N30D/K42E N52S/H115R, N30D/K42E/N52S/H115R/C198R/R221I,  
N30D/K42E/N52S/H115R/C198R, N30D/K42E/N52S/H115R/F172S/N194D,  
N52S/H115R/F120S/I143V/C198R, N52S/H115R/F172S/C198R, N52H/N57Y/Q100P/C198R,  
N52H/N57Y/Q100P H115R/F172S/C198R, N52H/N57Y/Q100P/F172S/C198R,  
N52H/N57Y/Q100P/H115R, N52H/N57Y/Q100P/H115R/C198R, N52H/Q100R/C198R,  
N52H/Q100R/H115R/F172S, N52H/Q100R/ F172S/C198R,  
N52H/Q100R/H115R/F172S/C198R, or N52H/N57Y/Q100R/F172S/C198R.

**[0009]** In some of any such embodiments, the one or more amino acid modifications are at a position(s) corresponding to a position selected from 52, 57, 100, 110, or 198. In some of any such embodiments, the one or more amino acid modifications are selected from N52H, N52D, N52S, N52K, N52Q, S54A, S54P, N57Y, Q100P, Q100R, V110A, V110D, C198R, or a conservative amino acid substitution thereof. In some embodiments, the variant ICOSL further includes one or more additional modification, such as any as described herein. In some of any such embodiments, the variant ICOSL polypeptide further includes one or more amino acid modifications selected from V11E, E16V, N30D, K42E, N52H, N52S, N52Y, N57Y, E90A, H94E, L96I, L98F, Q100R, Q100P, L102R, V110A, V110D, H115R, F120S, V122A, F138L, I143V, V152C, K156M, K156R, F172S, N194D, C198R, L203P, V210A, S212G, I218T, R221I, I224V, or a conservative amino acid substitution thereof.

**[0010]** In some of any such embodiments, the one or more amino acid modifications are N52H/N57Y/Q100R/C198R, N52H/N57Y/Q100R/V122A, N52H/N57Y/Q100R/F172S, N52Y/N57Y/F138L/L203P, V11E/N30D/N52H/N57Y/H94E/L96I/L98F/N194D/V210A/I218T, N52H/N57Y/Q100R, N52H/Q100R, N52H/N57Y/Q100R/V110D/C198R/S212G, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R, E16V/N52H/N57Y/Q100R/V110D/H115R/V152C/K156M/C198R, N30D/K42E/N52S,

N52S/F120S/I143V/I224V, N52S/E90A, N52H/N57Y/V110A/C198R/R221I, N52H/N57Y/Q100P, or N52S/N194D.

**[0011]** In some embodiments of any one of the variant ICOSL polypeptides described above, the variant ICOSL polypeptide includes the IgV domain or a specific fragment thereof and the IgC domain or a specific fragment thereof. In some embodiments of any one of the variant ICOSL polypeptides described above, the variant ICOSL polypeptide includes the sequence of amino acids set forth in any of SEQ ID NOS: 109-142, 239, 280-325, 364-381, 387-424, 427-433, 435-470 or a specific binding fragment thereof, or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS: 109-142, 239, 280-325, 364-381, 387-424, 427-433, 435-470 or a specific binding fragment thereof and that contains the one or more of the amino acid substitutions.

**[0012]** In some embodiments of any one of the variant ICOSL polypeptides described above, the variant ICOSL polypeptide includes the IgV domain or a specific binding fragment thereof. In some embodiments, the IgV domain or specific binding fragment thereof is the only ICOSL portion of the variant ICOSL polypeptide. In some embodiments, the IgC domain or specific binding fragment thereof is the only ICOSL portion of the variant ICOSL polypeptide.

**[0013]** In some embodiments of any one of the variant ICOSL polypeptides described above, the variant ICOSL polypeptide includes the sequence of amino acids set forth in any of SEQ ID NOS: 197-199, 201-208, 210, 212, 240, 326-340, 382-386, 425-426, and 434 or a specific binding fragment thereof, a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS: 197-199, 201-208, 210, 212, 240, 326-340, 382-386, 425-426, and 434 or a specific binding fragment thereof and that contains the one or more of the amino acid substitutions.

**[0014]** In some embodiments of any one of the variant ICOSL polypeptides described above, the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS, CD28, or CTLA-4 with increased affinity compared to the unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS or CD28 with increased affinity compared to the unmodified ICOSL. In some embodiments, the variant ICOSL

polypeptide specifically binds to the ectodomain of ICOS and the ectodomain of CD28 each with increased affinity compared to the unmodified ICOSL.

**[0015]** In some of any such embodiments, the variant ICOSL polypeptide specifically binds to the ectodomain of CD28 with increased affinity compared to the unmodified ICOSL. In some of any such embodiments, the increased affinity to the ectodomain of CD28 is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold or 60-fold compared to the unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS with increased affinity compared to the unmodified ICOSL. In some of any such embodiments, the increased affinity to the ectodomain of ICOS is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 40-fold, 50-fold, 60-fold or 70-fold compared to the unmodified ICOSL.

**[0016]** In some embodiments of any one of the variant ICOSL polypeptides, the one or more amino acid substitutions correspond to position(s) selected from 52, 54 or 57. In some embodiments, the one or more amino acid modifications are selected from N52H, N52D, N52Q, N52S, N52Y, N52K, S54A, S54P, N57D, N57Y or a conservative amino acid substitution thereof. In some of any such embodiments, the one or more amino acid substitutions/modifications correspond to position(s) selected from 52 or 57. In some embodiments, the one or more amino acid substitutions are selected from N52H, N52D, N52S, N52K or N57Y. In some embodiments, any one of the variant ICOSL polypeptides further includes one more additional amino acid modification, such as any as described. In some embodiments of any one of the variant ICOSL polypeptides described above, the variant ICOSL polypeptide further includes one or more amino acid modifications selected from N52H, N52D, N52S, N52Y, N52K, S54A, S54P, N57Y, R75Q, L80P, K92R, S99G, H94D, L96F, L98F, L96I, S99G, Q100R, Q100P, G103E, T113E, F120S, H129P, S130G, Q133H, F138L, C140D, C140del, I143T, Y146C, V151A, Y152C, Y152H, D158G, L161P, C198R, N201S, L203P, L208P or T225A, or a conservative amino acid substitution thereof.

**[0017]** In some of any such embodiments, the one or more amino acid modifications are selected from N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52S/Y146C/Y152C, N52H/C198R, N52H/C140del/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52S/C198R, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R,

N52S/T113E, N52S/S54P, N52K/L208P, N52S/Y152H, N52H/I143T, N52S/R75Q/L203P,  
N52S/D158G, N52D/Q133H,  
N52H/N57Y/Q100R/V110D/C198R/S212G, N52H/N57Y/Q100R/C198R, N52S/N194D,  
N52H/N57Y/Q100R/L102R/V110D/H115R/C198R, N52S/S54P, T38P/N52S/N57D,  
N52H/C140del/T225A, N52H/F78L/Q100R/C198R, N52H/N57Y/R75Q/Q100P/V110D,  
N52H/N57Y/L74Q/V110D/S192G, N52H/S121G/C198R, N52S/F120S/N227K,  
N52S/A71T/A117T/T190A/C198R, T43A/N52H/N57Y/L74Q/D89G/V110D/F172S,  
N52H/N57Y/Q100R/V110D/S132F/M175T,  
N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G, N52Q/N207Q, N52Q/N168Q,  
N52Q/N84Q, N52Q/N119Q, N52Q/N84Q/N168Q, N52Q/N84Q/N207Q,  
N52Q/N119Q/N155Q, N52H/N84Q/N119Q, N52H/N84Q, N52H/N84Q/N168Q,  
N52H/N84Q/N207Q, N52H/N84Q/N168Q/N207Q, N52Q/N84Q/N155Q, N52Q/N84Q/N168Q,  
N52Q/N84Q/N155Q/N168Q, N52Q/N84Q/N119Q/N168Q, N52Q/N84Q/N119Q/N207Q,  
N52Q/N84Q/N119Q/N155Q, N52Q/N84Q/N119Q/N155Q/N207Q, N52Y/F138L/L203P,  
N57Y/Q100R/C198R, N57Y/F138L/L203P, N52H/N57Y/Q100R/H115R/C198R,  
N52H/N57Y/Q100R/F172S/C198R, N52H/N57Y/Q100R/H115R/F172S/C198R,  
N52H/N57Y/Q100R/H115R/I143V/F172S/C198R,  
N52H/N57Y/Q100R/L102R/H115R/F172S/C198R, N52H/V122A/F172S/C198R,  
N52H/N57Y/Q100R/H115R/F172S/N194D, N52H/N57Y/H115R/F172S/C198R,  
N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/H115R, N52H/N57Y/Q100R/H115R,  
N52H/N57Y/Q100R/H115R/F172S/I224V, N52H/N57Y/Q100R/H115R/F172S,  
N52H/N57Y/Q100R/F172S, N52H/Q100R/H115R/I143T/F172S,  
N52H/N57Y/Q100P/H115R/F172S, N52Y/N57Y/Q100P/F172S,  
E16V/N52H/N57Y/Q100R/V110D/H115R/C198R,  
E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R, N52S/E90A/H115R,  
N30D/K42E/N52S/H115R, N30D/K42E/N52S/H115R/C198R/R221I,  
N30D/K42E/N52S/H115R/C198R, N30D/K42E/N52S/H115R/F172S/N194D,  
N52S/H115R/F120S/I143V/C198R, N52S/H115R/F172S/C198R, N52H/N57Y/Q100P/C198R,  
N52H/N57Y/Q100P H115R/F172S/C198R, N52H/N57Y/Q100P/F172S/C198R,  
N52H/N57Y/Q100P/H115R, N52H/N57Y/Q100P/H115R/C198R, N52H/Q100R/C198R,

N52H/Q100R/H115R/F172S, N52H/Q100R/ F172S/C198R,  
N52H/Q100R/H115R/F172S/C198R, or N52H/N57Y/Q100R/F172S/C198R.

**[0018]** In some of any such embodiments, the one or more amino acid modifications are selected from among N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52S/Y146C/Y152C, N52H/C198R, N52H/C140del/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52S/C198R, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52S/T113E, N52S/S54P, N52K/L208P, N52S/Y152H, N52H/I143T, N52S/R75Q/L203P, N52S/D158G, or N52D/Q133H. In some of any such embodiments, the one or more amino acid modifications are selected from N52H/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52H/K92R, N52H/C140del/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52K/L208P or N52H/I143T.

**[0019]** In some of any such embodiments, the variant ICOSL polypeptide specifically binds to the ectodomain of CTLA-4 with increased affinity compared to the unmodified ICOSL. In some aspects, the increased affinity to the ectodomain of CTLA-4 is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 40-fold, 50-fold, 60-fold or 70-fold compared to the unmodified ICOSL.

**[0020]** In some of any such embodiments, the variant polypeptide specifically binds to the ectodomain of ICOS, CD28 or CTLA4 with increased selectivity compared to the unmodified ICOSL. In some embodiments, the increased selectivity includes a greater ratio of binding of the variant polypeptide for one cognate binding partner selected from among ICOS, CD28 and CTLA4 versus another of the cognate binding partner compared to the ratio of binding of the unmodified ICOSL polypeptide for the one cognate binding partner versus the another of the cognate binding partner. In some cases, the ratio is greater by at least or at least about 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5-fold, 10-fold, 15-fold, 20-fold, 30-fold, 40-fold, 50-fold or more.

**[0021]** In some embodiments of any one of the variant ICOSL polypeptides, the variant ICOSL polypeptide further includes one or more amino acid selected from M10V, M10I, V11E, S13G, E16V, S18R, A20V, S25G, F27S, F27C, N30D, Q37R, K42E, T43A, Y47H, N52H, N52D, N52S, N52Q, N52Y, N52K, S54A, S54P, N57D, N57Y, R61S, R61C, Y62F, L67P, A71T, G72R, L74Q, R75Q, D77G, F78L, L80P, N84Q, D89G, E90A, K92R, F93L, H94E, H94D, L96F, L96I, V97A, L98F, S99G, Q100R, Q100K, Q100P, L102R, G103E, V107A, V107I, S109G, S109N, V110D, V110N, V110A, T113E, H115R, H115Q, V116A, A117T,

F120S, F120I, S121G, V122A, V122M, S126T, S126R, H129P, S130G, S132F, Q133H, E135K, F138L, T139S, C140D, C140del, S142F, I143V, I143T, N144D, Y146C, V151A, Y152C, Y152H, W153R, I154F, N155H, N155Q, K156M, D158G, L161P, L161M, L166Q, N168Q, F172S, L173S, M175T, T190A, T190S, S192G, V193M, N194D, C198R, N201S, L203P, L203F, N207Q, L208P, V210A, S212G, D217V, I218T, I218N, E220G, R221G, R221I, I224V, T225A, N227K, or a conservative amino acid substitution thereof. In some embodiments of any one of the variant ICOSL polypeptides, the variant ICOSL polypeptide further includes one or more amino acid deletions corresponding to position 140 of SEQ ID NO: 32.

**[0022]** In some embodiments of any one of the variant ICOSL polypeptides, the one or more amino acid substitutions are selected from among N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52S/Y146C/Y152C, N52H/C198R, N52H/C140D/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52S/C198R, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52S/T113E, N52D/S54P, N52K/L208P, N52S/Y152H, N52H/I143T, N52S/R75Q/L203P, N52S/D158G, N52D/Q133H, N52H/N57Y/Q100R/V110D/C198R/S212G, N52H/N57Y/Q100R/C198R, N52S/N194D, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R, N52S/S54P, T38P/N52S/N57D, N52H/C140del/T225A, N52H/F78L/Q100R/C198R, N52H/N57Y/R75Q/Q100P/V110D, N52H/N57Y/L74Q/V110D/S192G, N52H/S121G/C198R, N52S/F120S/N227K, N52S/A71T/A117T/T190A/C198R, T43A/N52H/N57Y/L74Q/D89G/V110D/F172S, N52H/N57Y/Q100R/V110D/S132F/M175T, or N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G.

**[0023]** In some embodiments of any one of the variant ICOSL polypeptides, the one or more amino acid substitutions are selected from N52H/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52H/K92R, N52H/C140del/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52K/L208P, N52H/I143T, A20V/N52H/N57Y/Q100R/S109G, N52H/N57Y/R61S/Q100R/V110D/L173S, N52H/N57Y/Q100R/V107I/V110D/S132F/I154F/C198R/R221G, Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G, N52H/N57Y/Q100R/V110D/C198R, F27S/N52H/N57Y/V110N, S18R/N52S/F93L/I143V/R221G, A20T/N52D/Y146C/Q164L, N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N,

N52H/N57Y/Q100R/V110D/F172S/C198R,  
S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R, or  
M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M/C198R.

**[0024]** In some embodiments of any one of the variant ICOSL polypeptides, the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS or CD28 with increased affinity and specifically binds to the ectodomain of the other of ICOS or CD28 with decreased affinity compared to the unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS with increased affinity and specifically binds to the ectodomain of CD28 with decreased affinity compared to the unmodified ICOSL. In some embodiments, the one or more amino acid substitutions are selected from N57Y/Q100P, N52S/S130G/Y152C, N52S/Y152C, N52Y/N57Y/Y152C, N52H/L161P/C198R, N52H/L161P/C198R, N52S/L80P, A20V/N52H/N57Y/Q100R/S109G, N52H/N57Y/R61S/Q100R/V110D/L173S, N52H/N57Y/Q100R/V107I/V110D/S132F/I154F/C198R/R221G, Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G, N52H/N57Y/Q100R/V110D/C198R, F27S/N52H/N57Y/V110N, S18R/N52S/F93L/I143V/R221G, A20T/N52D/Y146C/Q164L, N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N, N52H/N57Y/Q100R/V110D/F172S/C198R, S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R, or M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M/C198R.

**[0025]** In some embodiments, the variant ICOSL polypeptide specifically binds to the ectodomain of CD28 with increased affinity and specifically binds to the ectodomain of ICOS with decreased affinity compared to the unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide includes the amino acid substitutions N52S/R75Q/L203P or N30D/K42E/N52S.

**[0026]** In some embodiments of any one of the variant ICOSL polypeptides, the ICOS is a human ICOS. In some embodiments, the CD28 is a human CD28.

**[0027]** In some embodiments of any one of the variant ICOSL polypeptides, the binding is altered (increased or decreased) more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold,

7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold compared to the unmodified ICOSL.

**[0028]** In some embodiments of any one of the variant ICOSL polypeptides, the variant ICOSL polypeptide is a soluble protein.

**[0029]** In some embodiments of any one of the variant ICOSL polypeptides, the variant ICOSL polypeptide is linked to a multimerization domain. In some embodiments, the variant ICOSL polypeptide is a multimeric polypeptide, optionally a dimeric polypeptide, comprising a first variant ICOSL polypeptide linked to a multimerization domain and a second variant ICOSL polypeptide linked to a multimerization domain. In some embodiments, the first variant ICOSL polypeptide and the second variant ICOSL polypeptide are the same or different. In some embodiments, the multimerization domain is an Fc domain or a variant thereof with reduced effector function. In some embodiments of any one of the variant ICOSL polypeptides, the variant ICOSL polypeptide is linked to a moiety that increases biological half-life of the polypeptide.

**[0030]** In some embodiments, the variant ICOSL polypeptide is linked to an Fc domain or a variant thereof with reduced effector function. In some embodiments, the Fc domain is mammalian, optionally human; or the variant Fc domain includes one or more amino acid modifications compared to an unmodified Fc domain that is mammalian, optionally human. In some embodiments, the Fc domain or variant thereof includes the sequence of amino acids set forth in SEQ ID NO:226 or SEQ ID NO:227 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:226 or SEQ ID NO:227.

**[0030a]** Disclosed herein is a variant ICOSL-Fc fusion protein comprising a variant ICOSL polypeptide as defined herein and an Fc domain.

**[0030b]** Disclosed herein is a variant ICOSL-Fc fusion protein, comprising a variant ICOSL polypeptide linked via a linker to an Fc domain, wherein: (i) the variant ICOSL polypeptide comprises the amino acid substitutions N52H/N57Y/Q100R in the amino acid sequence set forth in SEQ ID NO:32 or a portion thereof comprising an IgV domain, wherein the IgV domain is amino acids 19-129 of SEQ ID NO:5; and (ii) the Fc domain is a variant human IgG1 Fc with reduced effector function.

**[0031]** In some embodiments, the Fc domain includes one or more amino acid modifications selected from among E233P, L234A, L234V, L235A, L235E, G236del, G237A, S267K, R292C, N297G and V302C, each by EU numbering. In some aspects, the Fc domain includes the amino acid modification C220S by EU numbering. In some embodiments, the Fc domain includes the sequence of amino acids set forth in any of SEQ ID NOS:474, 476, 477, 478 or a sequence of amino acids that exhibits at least 85% sequence identity to any of SEQ ID NOS: 474, 476, 477, 478 and that contains the one or more amino acid modifications and/or exhibits reduced effector function.

**[0032]** In some embodiments, the variant ICOSL polypeptide is linked indirectly to the multimerization domain or Fc via a linker, optionally a G4S linker.

**[0033]** In some embodiments of any one of the variant ICOSL polypeptides, the variant ICOSL polypeptide is a transmembrane immunomodulatory protein that further contains a transmembrane domain linked to the extracellular domain (ECD) or specific binding fragment thereof of the variant ICOSL polypeptide. In some embodiments, the transmembrane domain includes the sequence of amino acids set forth as residues 257-277 of SEQ ID NO:5 or a functional variant thereof that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to residues 257-277 of SEQ ID NO:5. In some embodiments, the variant ICOSL polypeptide further includes a cytoplasmic signaling domain linked to the transmembrane domain. In some embodiments, the cytoplasmic signaling domain includes the sequence of amino acids set forth as residues 278-302 of SEQ ID NO:5 or a functional variant thereof that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to residues 278-302 of SEQ ID NO:5.

**[0034]** In some of any such embodiments, the variant ICOSL polypeptide comprising the sequence of amino acids set forth in any of SEQ ID NOS:494-503 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS:494-503 and that comprises one or more amino acid modifications as described.

**[0035]** In some embodiments of any one of the variant ICOSL polypeptides, the ICOSL variant increases IFN-gamma (interferon-gamma) expression relative to the unmodified ICOSL in an in vitro primary T-cell assay. In some embodiments of any one of the variant ICOSL

polypeptides described above, the variant ICOSL decreases IFN-gamma (interferon-gamma) expression relative to the unmodified ICOSL in an in vitro primary T-cell assay. In some embodiments of any one of the variant ICOSL polypeptides described above, the variant ICOSL polypeptide is deglycosylated.

**[0036]** In some embodiments, provided herein is an immunomodulatory protein comprising the variant ICOSL polypeptide according to any one of the provided embodiments linked to a second polypeptide comprising an immunoglobulin superfamily (IgSF) domain. In some embodiments, the IgSF domain is affinity modified and exhibits altered binding to one or more of its cognate binding partner(s) compared to the unmodified or wild-type IgSF domain. In some embodiments, the IgSF domain exhibits increased binding to one or more of its cognate binding partner(s) compared to the unmodified or wild-type IgSF domain. In some embodiments, the variant ICOSL polypeptide is a first variant ICOSL polypeptide and the IgSF domain of the second polypeptide is an IgSF domain from a second variant ICOSL polypeptide according to any one of the provided embodiments, wherein the first and second variant ICOSL are the same or different. In some embodiments, the variant ICOSL polypeptide is capable of specifically binding to CD28 or ICOS and the IgSF domain of the second polypeptide is capable of binding to a cognate binding partner other than one specifically bound by the variant ICOSL polypeptide. In some embodiments, the IgSF domain is from a member of the B7 family. In some embodiments, the IgSF domain is a tumor-localizing moiety that binds to a ligand expressed on a tumor or is an inflammatory-localizing moiety that binds to a ligand expressed on a cell or tissue of an inflammatory environment. In some embodiments, the IgSF domain is a tumor-localizing moiety that binds to a ligand expressed on a tumor. In some embodiments, the ligand is B7H6. In some embodiments, the IgSF domain is from NKp30. In some embodiments, the IgSF domain is or includes an IgV domain. In some embodiments, the variant ICOSL polypeptide is or includes an IgV domain.

**[0037]** In some embodiments according to any one of the immunomodulatory proteins, the immunomodulatory protein includes a multimerization domain linked to one or both of the variant ICOSL polypeptide or the second polypeptide comprising the IgSF domain. In some embodiments, the multimerization domain is an Fc domain or a variant thereof with reduced effector function. In some embodiments, the immunomodulatory protein is dimeric. In some

embodiments, the immunomodulatory protein is homodimeric. In some cases, the immunomodulatory protein is heterodimeric.

**[0038]** In some embodiments, provided herein is a conjugate comprising a variant ICOSL polypeptide according to any one of the embodiments or an immunomodulatory polypeptide according to any one of the embodiments linked to a moiety. Disclosed herein is a conjugate comprising a variant ICOSL polypeptide according to any one of the embodiments or a variant ICOSL-Fc fusion protein according to any one of the embodiments linked to a moiety. In some embodiments, the moiety is a targeting moiety that specifically binds to a molecule on the surface of a cell. In some embodiments, the targeting moiety specifically binds to a molecule on the surface of an immune cell. In some embodiments, the immune cell is an antigen presenting cell or a lymphocyte. In some embodiments, the targeting moiety is a tumor-localizing moiety that binds to a molecule on the surface of a tumor. In some embodiments, the moiety is a protein, a peptide, nucleic acid, small molecule or nanoparticle. In some embodiments, the moiety is an antibody or antigen-binding fragment. In some embodiments, the conjugate is divalent, tetravalent, hexavalent or octavalent.

**[0039]** In some embodiments, provided herein is a nucleic acid molecule encoding a variant ICOSL according to any one of the provided embodiments or an immunomodulatory polypeptide according to any one of the provided embodiments. Disclosed herein is a nucleic acid molecule encoding a variant ICOSL according to any one of the provided embodiments or a variant ICOSL-Fc fusion protein according to any one of the provided embodiments. In some embodiments, the nucleic acid molecule is a synthetic nucleic acid. In some embodiments, the nucleic acid is cDNA.

**[0040]** Disclosed herein is a vector comprising the nucleic acid of any one of the provided embodiments. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a mammalian expression vector or a viral vector.

**[0041]** Disclosed herein is a cell comprising the nucleic acid or vector according to any one of the provided embodiments. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell.

**[0042]** In some embodiments, provided herein is a method of producing a variant ICOSL polypeptide or an immunomodulatory protein, comprising introducing the nucleic acid molecule according to any one of the provided embodiments or vector according to any one of the

provided embodiments into a host cell under conditions to express the protein in the cell. Disclosed herein is a method of producing a variant ICOSL polypeptide or a variant ICOSL-Fc fusion protein, comprising introducing the nucleic acid molecule according to any one of the provided embodiments or vector according to any one of the provided embodiments into a host cell under conditions to express the protein in the cell. In some embodiments, the method further includes isolating or purifying the variant ICOSL polypeptide or immunomodulatory protein from the cell.

**[0043]** In some embodiments, provided herein is a method of engineering a cell expressing a variant ICOSL polypeptide, comprising introducing a nucleic acid molecule encoding the variant ICOSL polypeptide or immunomodulatory polypeptide according to any one of the provided embodiments into a host cell under conditions in which the polypeptide is expressed in the cell. Disclosed herein is an *in vitro* method of engineering a cell expressing a variant ICOSL polypeptide, comprising introducing a nucleic acid molecule encoding the variant ICOSL polypeptide or a variant ICOSL-Fc fusion protein according to any one of the provided embodiments into a host cell under conditions in which the polypeptide is expressed in the cell. In some embodiments, provided herein is an engineered cell, expressing the variant ICOSL polypeptide, immunomodulatory polypeptide, the nucleic acid molecule, or the vector according to any one of the provided embodiments. Disclosed herein is an engineered cell, expressing the variant ICOSL polypeptide, variant ICOSL-Fc fusion protein, the nucleic acid molecule, or the vector according to any one of the provided embodiments.

**[0044]** In some embodiments, the variant ICOSL polypeptide or immunomodulatory polypeptide includes a signal peptide. In some aspects, the variant ICOSL polypeptide or immunomodulatory polypeptide does not contain a transmembrane domain and/or is not expressed on the surface of the cell. In some cases, the variant ICOSL polypeptide or immunomodulatory polypeptide is secreted from the engineered cell. In some aspects, the engineered cell contains a variant ICOSL polypeptide that contains a transmembrane domain and/or is the transmembrane immunomodulatory protein according to any of the provided embodiments. In some embodiments, the variant ICOSL polypeptide is expressed on the surface of the cell.

**[0045]** In some embodiments, the engineered cell is an immune cell. In some embodiments, the immune cell is an antigen presenting cell (APC) or a lymphocyte. In some embodiments, the

engineered cell is a primary cell. In some embodiments, the engineered cell is a mammalian cell. In some embodiments, the engineered cell is a human cell. In some embodiments, the lymphocyte is a T cell. In some embodiments, the APC is an artificial APC. In some embodiments, the engineered cell further contains a chimeric antigen receptor (CAR) or an engineered T-cell receptor (TCR).

**[0046]** Also provided is an infectious agent, comprising a nucleic acid molecule encoding a variant ICOSL polypeptide or an immunomodulatory polypeptide according to any of the provided embodiments. Also disclosed herein is an infectious agent, comprising a nucleic acid molecule encoding a variant ICOSL polypeptide or a variant ICOSL-Fc fusion protein according to any of the provided embodiments. In some embodiments, the encoded variant ICOSL polypeptide or immunomodulatory polypeptide does not contain a transmembrane domain and/or is not expressed on the surface of a cell in which it is expressed. In some cases, the encoded variant ICOSL polypeptide or immunomodulatory polypeptide is secreted from a cell in which it is expressed. In some cases, the encoded variant ICOSL polypeptide contains a transmembrane domain. In some embodiments, the encoded variant ICOSL polypeptide is expressed on the surface of a cell in which it is expressed.

**[0047]** In some of any such embodiments, the infectious agent is a bacteria or a virus. In some examples, the virus is an oncolytic virus. In some aspects, the oncolytic virus is an adenoviruses, adeno-associated viruses, herpes viruses, Herpes Simplex Virus, Vesticular Stomatitis virus, Reovirus, Newcastle Disease virus, parvovirus, measles virus, vesticular stomatitis virus (VSV), Coxsackie virus or a Vaccinia virus. In some embodiments, the virus specifically targets dendritic cells (DCs) and/or is dendritic cell-tropic. In some embodiments, the virus is a lentiviral vector that is pseudotyped with a modified Sindbis virus envelope product.

**[0048]** In some of any such embodiments, the infectious agent further comprises a nucleic acid molecule encoding a further gene product that results in death of a target cell or that can augment or boost an immune response. In some embodiments, the further gene product is selected from an anticancer agent, anti-metastatic agent, an antiangiogenic agent, an immunomodulatory molecule, an immune checkpoint inhibitor, an antibody, a cytokine, a growth factor, an antigen, a cytotoxic gene product, a pro-apoptotic gene product, an anti-apoptotic gene

product, a cell matrix degradative gene, genes for tissue regeneration or a reprogramming human somatic cells to pluripotency.

**[0049]** In some embodiments, provided herein is a pharmaceutical composition, comprising the variant ICOSL polypeptide according to any one of the provided embodiments, an immunomodulatory protein according to any one of the provided embodiments, a conjugate according to any one of the provided embodiments, an engineered cell according to any one of the provided embodiments, or an infectious agent according to any one of the provided embodiments, and a pharmaceutically acceptable excipient. Disclosed herein is a pharmaceutical composition, comprising the variant ICOSL polypeptide according to any one of the provided embodiments, a variant ICOSL-Fc fusion protein according to any one of the provided embodiments, a conjugate according to any one of the provided embodiments, an engineered cell according to any one of the provided embodiments, or an infectious agent according to any one of the provided embodiments, and a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition is sterile.

**[0050]** Disclosed herein is an article of manufacture includes any of the provided pharmaceutical compositions in a vial. In some embodiments, the vial is sealed. In some embodiments, also provided is a kit including any of the pharmaceutical compositions and instructions for use. In some aspects, provided is a kit including the article of manufacture and instructions for use. Disclosed herein is a kit comprising the pharmaceutical composition according to any one of the provided embodiments, the article of manufacture of according to any one or the provided embodiments, and instructions for use according to any one of the provided embodiments.

**[0051]** In some embodiments, provided herein is a method of modulating an immune response in a subject, comprising administering the pharmaceutical composition according to any one of the provided embodiments to the subject. In some embodiments, the method includes administering the engineered cells as provided according to any one of the provided embodiments. In some embodiments, the engineered cells are autologous to the subject. In some embodiments, the engineered cells are allogenic to the subject. In some embodiments, modulating the immune response treats a disease or condition in the subject.

**[0051a]** Disclosed herein is the use of the pharmaceutical composition according to any one of the provided embodiments for the preparation of a medicament for the treatment of an

autoimmune response or inflammatory disease in a mammalian subject. Disclosed herein is a pharmaceutical composition according to any one of the provided embodiments for use in modulating an immune response in a mammalian subject, wherein an autoimmune or inflammatory disease is to be treated by modulating the immune response.

**[0052]** In some embodiments, the immune response is increased. In some embodiments, an immunomodulatory protein or conjugate comprising a variant ICOSL polypeptide linked to a tumor-localizing moiety is administered to the subject. In some cases, the tumor-localizing moiety is or includes a binding molecule that recognizes a tumor antigen. In some aspects, the binding molecule includes an antibody or an antigen-binding fragment thereof or includes a wild-type IgSF domain or variant thereof.

**[0053]** In some of any such embodiments, a pharmaceutical composition comprising the immunomodulatory protein according to any one of the provided embodiments or the conjugate according to any one of the provided embodiments is administered to the subject. In some embodiments, an engineered cell comprising a variant ICOSL polypeptide that is a transmembrane immunomodulatory protein is administered to the subject and/or the engineered cell according to any one of the embodiments described above.

**[0054]** In some embodiments, an infectious agent encoding a variant ICOSL polypeptide that is a transmembrane immunomodulatory protein is administered to the subject, optionally under conditions in which the infectious agent infects a tumor cell or immune cell and the transmembrane immunomodulatory protein is expressed on the surface of the infected cell. In some aspects, the transmembrane immunomodulatory protein is any provided according to any one of the provided embodiments.

**[0055]** In some embodiments, the disease or condition is a tumor or cancer. In some embodiments, the disease or condition is selected from melanoma, lung cancer, bladder cancer, a hematological malignancy, liver cancer, brain cancer, renal cancer, breast cancer, pancreatic cancer, colorectal cancer, spleen cancer, prostate cancer, testicular cancer, ovarian cancer, uterine cancer, gastric carcinoma, a musculoskeletal cancer, a head and neck cancer, a gastrointestinal cancer, a germ cell cancer, or an endocrine and neuroendocrine cancer.

**[0056]** In some embodiments, the immune response is decreased by the provided methods of modulating the immune response. In some embodiments, a variant ICOSL polypeptide or immunomodulatory protein that is soluble is administered to the subject. In some cases, the

soluble polypeptide or immunomodulatory protein is an Fc fusion protein. In some embodiments, a pharmaceutical composition comprising a variant ICOSL polypeptide according to any one of the provided embodiments, or the immunomodulatory protein according to any one of the provided embodiments is administered to the subject.

**[0057]** In some of any such embodiments, an engineered cell comprising a secretable variant ICOSL polypeptide is administered to the subject. In some embodiments, an engineered cell according to any one of the provided embodiments is administered to the subject. In some embodiments, an infectious agent encoding a variant ICOSL polypeptide that is a secretable immunomodulatory protein is administered to the subject, optionally under conditions in which the infectious agent infects a tumor cell or immune cell and the secretable immunomodulatory protein is secreted from the infected cell.

**[0058]** In some embodiments, the disease or condition is an inflammatory or autoimmune disease or condition. In some embodiments, the disease or condition is an Antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, a vasculitis, an autoimmune skin disease, transplantation, a Rheumatic disease, an inflammatory gastrointestinal disease, an inflammatory eye disease, an inflammatory neurological disease, an inflammatory pulmonary disease, an inflammatory endocrine disease, or an autoimmune hematological disease. In some embodiments, the disease or condition is selected from inflammatory bowel disease, transplant, Crohn's disease, ulcerative colitis, multiple sclerosis, asthma, rheumatoid arthritis, or psoriasis.

**[0059]** In some of any such embodiments of the variant ICOSL polypeptide, the amino acid modification is an amino acid substitution, insertion or a deletion.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0060] FIG. 1** depicts impedance results reflecting cytotoxic killing activity of cells engineered with an anti-CD19 chimeric antigen receptor (CAR) alone or with an exemplary transmembrane immunomodulatory TIP (CD80-TIP or ICOSL-TIP) or the corresponding CD80 or ICOSL wild-type transmembrane protein following co-culture with target antigen-expressing cells. Impedance was assessed using the Acea Real-Time Cell Analyzer (RTCA), which measures the impedance variations in the culture media of a 96-well microelectronic plate (E-plate).

**[0061] FIG. 2A** depicts that primary T cells are effectively transduced with viruses encoding both the CAR and TIP proteins. Primary human T cells activated 48 hours with anti-CD3 plus anti-CD28 beads and were then transduced with a Lenti-virus encoding an anti-CD19 CAR with a BFP reporter, plus a second Lenti-virus encoding and ICOSL TIP with a GFP reporter. The FACs plot shows BFP expression on the y-axis and GFP expression on the x-axis and the percentage of T cells that fall into each quadrant are indicated. Results show that the cultures include CAR only transduced cells (upper left quadrant), TIP only transduced cells (lower right quadrant), cells transduced with both viruses (upper right quadrant) and cells that were not transduced with either (lower left). In **FIG. 2B**, TIPs expressed on CAR-T cells provide co-stimulation to the CAR-T cells. CAR-T cells with or without TIP co-transduction were labeled with Cell-Trace Far Red and incubated with the CD19+ NALM6 cell line to engage the CAR. Proliferation was assessed by the percentage of CAR-expressing cells that had diluted out the fluorescent dye. Cells transduced with mutated TIPs showed an increase proliferation of CAR+ T cells compared to those without TIPs or those transduced with wild-type ICOSL. Mock transduced cells that lacked CAR expression failed to proliferate in this assay.

**[0062] FIG. 3A-3B** demonstrate, via cytokine release, the costimulatory capacity of wild-type (WT) or variant ICOSL when coimmobilized with anti-CD3. 10 nM anti-CD3 was wet coated to the wells of 96-well flat bottomed polystyrene tissue culture plates with 40 nM (arrows) or 10 nM WT or variant ICOSL. 100,000 purified CD4<sup>+</sup> and CD8<sup>+</sup> (pan) T-cells were added and supernatant was harvested 72 hours later for ELISA analysis for cytokine release. **FIG. 3A** shows IFN-gamma and **FIG. 3B** shows IL-17 protein levels secreted from pan T-cells. Graphs are representative of typical IFN-gamma and IL-17 responses from pan T-cell costimulation.

**[0063] FIG. 4A-4B** demonstrate, via proliferation, the costimulatory capacity of wild-type (WT) or variant ICOSL when coimmobilized with anti-CD3. CFSE-labeled pan T-cells were incubated in anti-CD3 and ICOSL coated plates as previously described for 72 hours. Cells were harvested, washed, stained with fluorescently conjugated anti-CD4 or anti-CD8 antibodies, and analyzed by flow cytometry. Gates and cytometer voltages were set using non-stimulated control CFSE-labeled T-cells. Proliferation was determined by CFSE dilution from control. **FIG. 4A** shows percent of total proliferating (arrows), CD4<sup>+</sup> (solid bar), and CD8<sup>+</sup> cells (hatched bar) T-cells following 40 nM ICOSL costimulation. **FIG. 4B** shows percent of total pan T-cell

proliferation following 10 nM ICOSL costimulation. Graphs are representative of typical proliferative response from pan T-cell costimulation.

**[0064] FIG. 5** depicts ICOSL vIgD candidate function in a human Mixed-Lymphocyte-Reaction (MLR). ICOSL variants and their mutations are listed on the x-axis, along with wild-type ICOSL, negative controls PDL2-Fc and human IgG, as well as the positive control benchmark molecule CTLA-Ig Belatacept. The line across the graph represents the baseline amount of IFN-gamma detected in the supernatants of negative control cultures. For each ICOSL variant candidate or control, three different concentrations were tested with arrows indicating the highest concentration of protein in cultures at 40nM. The majority of ICOSL variant candidates show superior antagonistic activity at all three concentrations tested compared to belatacept as reflected by the lower concentration of IFN-gamma in those cultures.

**[0065] FIG. 6** depicts the inhibition of soluble ICOSL Fc-fusion proteins on B and T cell responses in a B-T co-culture assay. **FIG. 6A** depicts soluble ICOSL Fc-fusion proteins inhibition of T cell-driven B cell proliferation. Purified CD4+ T cells and B cells from a single donor were CFSE-labeled and co-incubated at a 1:1 ratio in the presence or absence of the indicated mitogens with or without the indicated ICOSL Fc-fusion proteins. Cells were stimulated with Staph enterotoxin B (SEB) at 100 ng/ml, Pokeweed mitogen (PWM) at 1 mg/ml, or both. ICOSL Fc-fusion proteins were included at a final concentration of 40 nM and cultures were incubated for 7 days and subjected to FACS analysis. The number of divided B cells was determined from the number of cells in the cultures that had diluted their CFSE. All of the ICOSL Fc-fusion proteins tested except for wild-type reduced B cell proliferation. **FIG. 6B-D** show ICOSL Fc-fusion proteins inhibited cytokine T cell cytokine production in B-T co-cultures. Supernatants from the cultures described above were harvested on day 7 and analyzed for cytokine content using a LEGENDplex Human Th Cytokine Panel (Biolegend). T cell production of IL-5 (**FIG. 6B**), IL-13 (**FIG. 6C**) and IL-21 (**FIG. 6D**) is attenuated by inclusion of ICOSL Fc-fusion proteins.

**[0066] FIG 7A-7D** depicts different endpoints in a mouse model of Graft Versus Host Disease (GVHD) where human PBMC cells were adoptively transferred into immunodeficient NSG murine hosts. **FIG. 7A** shows survival curves of the treated animals. Aggressive disease course and subsequent mortality was observed in the saline control animals, with similar survival observed in the animals treated with wild-type ICOSL-Fc, as well as the N52H/I143T ICOSL

variant. Variant N52H/N57Y/Q100P had improved survival rates comparable to the clinical benchmark belatacept. **FIG. 7B** shows similar trends in body weight loss, with ICOSL variant N52H/N57Y/Q100P demonstrating similar weight maintenance as animals treated with belatacept, even though all other groups experienced rapid weight loss. **FIG. 7C** shows clinical scores from standardized GVHD Disease Activity Index (DAI) observations, again showing lower scores in animals treated with the ICOSL variant N52H/N57Y/Q100P that are comparable to the clinical benchmark belatacept while the other groups of animals experienced higher DAI scores. **FIG. 7D** depicts a flow cytometric measurement of CD4 and CD8 percentages in blood from experimental animals measured on day 14. The percentage of CD8 cells between experimental groups was largely the same, however, animals treated with ICOSL variant N52H/N57Y/Q100P and belatacept have lower percentages of CD4 cells compared to the other experimental groups.

**[0067] FIG. 8** shows localized costimulatory activity conveyed by the indicated variant stack molecule vIgD C-L, where C represents an ICOSL costimulatory domain and L represents a NKp30 localizing domain. In this assay, target K562 cells expressing the localizing surface protein, B7-H6, were cultured in the presence of anti-CD3 with human T cells and T cell activation was assessed by IFN-gamma levels in culture supernatants. Including anti-CD3 alone or no stack variant Fc molecules did not induce T cell activation. Similarly, cells cultured with only the wild-type localizing NKp30 domain alone or the wild-type costimulatory ICOSL domain alone as Fc fusion proteins did not result in T cell activation. A stacked domain containing the wild-type version of both the costimulatory domain and localizing domain induced measurable IFN-gamma at the highest concentration tested, however, the variant localizing costimulatory stack induced greater than two fold higher IFN-gamma levels at the highest concentration, and IFN-gamma levels that were still observed as the concentrations were titrated down.

**[0068] FIG. 9** summarizes changes in ear thickness in mice from a standard model of Delayed-Type Hypersensitivity (DTH). PBS treated animals sensitized with ovalbumin and subsequently challenged in the ear with the same protein, show the highest level of measured ear swelling. Mice treated with clinical benchmark Abatacept have slightly reduced ear swelling following ear challenge. All five ICOSL variant treatment groups demonstrated equal or improved reductions in ear swelling compared to Abatacept.

[0069] **FIG. 10A-C** depicts various exemplary configurations of a variant IgSF domain (vIgD) conjugated to an antibody (V-Mab). **FIG. 10A** shows various configurations in which a vIgD is linked, directly or indirectly, to the N- and/or C-terminus of the light chain of an antibody. **FIG. 10B** shows various configurations in which a vIgD is linked, directly or indirectly, to the N- and/or C-terminus of the heavy chain of an antibody. **FIG. 10C** depicts the resulting V-Mab configurations when a light chain of **FIG. 10A** and a heavy chain of **FIG. 10B** are co-expressed in a cell.

[0070] **FIG. 11A-11B** demonstrate V-Mab specificity for cognate binding partners. Binding assays were performed on Expi293 cells transiently transfected with DNA for mammalian surface expression of human HER2, CD28, CTLA-4, or ICOS. 200,000 transfected cells were incubated with 100,000 pM to 100 pM parental antibody (C1) or various V-Mabs (C2-9). Unbound antibody was removed, bound antibody detected with fluorescently conjugated anti-human IgG, and the cells were analyzed by flow cytometry for MFI and percentage positive based on Fc controls. **FIG. 11A** shows binding of the V-Mabs to HER2 transfectants at levels similar to the parental antibody. Binding to mock transfected cells is observed with all V-Mabs, though not WT ICOSL, due to low levels of endogenous HER2 expression on Expi293 parental cells. **FIG. 11B** shows binding of the parental IgSF-domain (N52H/N57Y/Q100P) to its cognate partners is maintained or increased (C2, C3, C4, C5, C6, C8, C9) by V-Mabs.

[0071] **FIG. 12** demonstrates V-Mab costimulatory and proliferative capacity when coimmobilized with anti-CD3. 10 nM anti-CD3 was wet coated to the wells of 96-well flat bottomed polystyrene tissue culture plates with 30 nM to 3 nM parental antibody, V-Mabs, or Fc controls. CFSE-labeled pan T-cells were added for 72 hours. IFN-gamma secretion was measured by ELISA and total T-cell proliferation was measured by flow cytometric analysis of CFSE-dilution. IFN-gamma secretion and proliferation of IgSF-domain (N52H/N57Y/Q100P) is greater than WT ICOSL. V-Mabs demonstrate increased cytokine and proliferative costimulatory capacity over the parental IgSF.

[0072] **FIG. 13A-13C** depicts various formats of the provided variant IgSF domain molecules. **FIG. 13A** depicts soluble molecules, including: (1) a variant IgSF domain (vIgD) fused to an Fc chain; (2) a stack molecule containing a first variant IgSF domain (first vIgD) and a second IgSF domain, such as a second variant IgSF domain (second vIgD); (3) a tumor

targeting IgSF molecule containing a first variant IgSF domain (vIgD) and an IgSF domain that targets to a tumor antigen, such as an Nkp30 IgSF domain; and (4) a variant IgSF domain (vIgD) linked to an antibody (V-Mab). **FIG. 13B** depicts a transmembrane immunomodulatory protein (TIP) containing a variant IgSF domain (vIgD), e.g. variant ICOSL, expressed on the surface of a cell. In an exemplary embodiment, the cognate binding partner of the transmembrane bound vIgD is a costimulatory receptor, e.g. CD28, and the TIP containing the vIgD (e.g. ICOSL vIgD) agonizes the costimulatory receptor such that the TIP induces a positive signal in the cell expressing the costimulatory receptor. **FIG. 13C** depicts a secreted immunomodulatory protein (SIP) in which a variant IgSF domain (vIgD), e.g. variant ICOSL, is secreted from a cell, such as a first T cell (e.g. CAR T cell). In an exemplary embodiment, the cognate binding partner of the secreted vIgD is an activating receptor, e.g. CD28, which can be expressed on the first cell (e.g. T cell) and/or on a second cell (e.g. T cell; either endogenous or engineered, such as a CAR T cell). Upon binding of the SIP with its cognate binding partner, signaling via the activating receptor is blocked. In all cases, the vIgD can be a V-domain (IgV) only, the combination of the V-domain (IgV) and C-domain (IgC), including the entire extracellular domain (ECD), or any combination of Ig domains of the IgSF superfamily member.

**[0073] FIG. 14** depicts an exemplary schematic of the activity of a variant IgSF domain (vIgD) fused to an Fc (vIgD-Fc) in which the vIgD is a variant of an IgSF domain of ICOSL. As shown, a soluble vIgD of ICOSL interacts with its cognate binding partners to block interactions of CD80 (B7-1)/CD86 (B7-2) or ICOSL with CD28 or ICOS, respectively, thereby blocking costimulation by the CD28 and/or ICOS costimulatory receptors.

**[0074] FIG. 15** depicts an exemplary schematic of a stack molecule for localizing the variant IgSF domain (vIgD) to a tumor cell. In this format, the stack molecule contains a first variant IgSF domain (first vIgD) and a second IgSF domain (e.g. a second vIgD) in which the second IgSF domain (e.g. a second vIgD) is a tumor-targeted IgSF domain that binds to a tumor antigen. An exemplary tumor-targeted IgSF domain is an IgSF domain of Nkp30, which binds to the tumor antigen B7-H6. In this depiction, the vIgD is a variant of an IgSF domain of ICOSL. As shown, binding of tumor-targeted IgSF domain to the surface of the tumor cell localizes the first vIgD on the tumor cell surface where it can interact with one or more of its cognate binding partners (e.g. CD28 or ICOS) expressed on the surface of an adjacent immune cell (e.g. T cell) to stimulate the costimulatory receptor.

**[0075] FIG. 16** depicts various exemplary configurations of a stack molecule containing a first variant IgSF domain (first vIgD), e.g. variant ICOSL, and a second IgSF domain, such as a second variant IgSF domain (second vIgD). As shown, the first vIgD and second IgSF domain are independently linked, directly or indirectly, to the N- or C-terminus of an Fc subunit. For generating a homodimeric Fc molecule, the Fc subunit is one that is capable of forming a homodimer with a matched Fc subunit by co-expression of the individual Fc subunits in a cell. For generating a heterodimeric Fc molecule, the individual Fc subunits contain mutations (e.g. “knob-into-hole” mutations in the CH3 domain), such that formation of the heterodimer is favored compared to homodimers when the individual Fc subunits are co-expressed in a cell.

**[0076] FIG. 17** depicts an exemplary schematic of the activity of a variant IgSF domain (vIgD) conjugated to an antibody (V-Mab) in which the antibody (e.g. anti-HER2 antibody) binds to an antigen on the surface of the tumor cell. In this depiction, the vIgD is a variant of an IgSF domain of ICOSL. As shown, binding of the antibody to the surface of the tumor cell localizes the vIgD on the tumor cell surface where it can interact with one or more of its cognate binding partners expressed on the surface of an adjacent immune cell (e.g. T cell) to agonize receptor signaling. In an exemplary embodiment as shown, the variant IgSF domain (vIgD) is a variant of an IgSF domain of ICOSL. Binding of the ICOSL vIgD to CD28 or ICOS costimulatory receptors provides an agonist or costimulatory signal.

**[0077] FIG. 18** depicts the Nanostring transcriptional signature of primary human T cells when incubated 10 nM anti-CD3 with 40 nM of an Fc-control protein, wild-type ICOSL-Fc, wild-type CD80-Fc, both of these proteins, or a variant ICOSL-Fc-fusion proteins with mutations as indicated. Total RNA from samples was prepared from harvested cells and the RNA was transferred to Nanostring and a Cancer Immune chip was used to quantitate transcripts of 750 gene in each sample. Altered transcripts include those whose level is above or below the diagonal, including the noted transcripts.

**[0078] FIG. 19** depicts transcript levels of exemplary transcripts upon incubation as described in FIG. 18 for the indicated times in the presence of the various immunomodulatory proteins.

**[0079] FIG. 20A-B** demonstrates VmAb mediated T-cell proliferation when co-cultured with HER2 expressing targets. CFSE-labeled pan T-cells were activated with K562-derived artificial target cells displaying cell surface anti-CD3 single chain Fv (OKT3) and HER2 in the presence

of VmAbs or control proteins. Proliferation was measured by flow cytometric analysis of CFSE-dilution on CD4<sup>+</sup> (left panel) or CD8<sup>+</sup> (right panel) stained T-cells. In **FIG. 20A**, K562 cells were titrated and plated with T-cells for an effector:target (E:T) ratio of 40 to 1280:1. VmAbs, parental IgSF domain, or WT ICOSL were added at 1000pM. In **FIG. 20B**, K562 cells were added to T-cells for an E:T ratio of 160:1. VmAbs or control proteins were titrated and added at 3000 to 37pM.

### **Detailed Description**

**[0080]** Provided herein are immunomodulatory proteins that are or comprise variants or mutants of ICOS ligand (ICOSL) or specific binding fragments thereof that exhibit activity to bind to at least one target ligand cognate binding partner (also called counter-structure protein). In some embodiments, the variant ICOSL polypeptides contain one or more amino acid modifications (e.g. amino acid substitutions, deletions or additions) compared to an unmodified or wild-type ICOSL polypeptide. In some embodiments, the one or more amino acid modifications (e.g. amino acid substitutions, deletions or additions) are in an IgSF domain (e.g. IgV) of an unmodified or wild-type ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide exhibits altered, such as increased or decreased, binding activity or affinity for at least one cognate binding partner, such as at least one of ICOS, CD28, or CTLA-4. In some embodiments, the immunomodulatory proteins are soluble. In some embodiments, the immunomodulatory proteins are transmembrane immunomodulatory proteins capable of being expressed on the surface of cells. In some embodiments, also provided herein are one or more other immunomodulatory proteins that are conjugates or fusions containing a variant ICOSL polypeptide provided herein and one or more other moiety or polypeptide.

**[0081]** In some embodiments, the variant ICOSL polypeptides and immunomodulatory proteins modulate an immunological immune response, such as an increased or decreased immune response. In some embodiments, the variant ICOSL polypeptides and immunomodulatory proteins provided herein can be used for the treatment of diseases or conditions that are associated with a dysregulated immune response.

**[0082]** In some embodiments, the provided variant ICOSL polypeptides modulate T cell activation via interactions with costimulatory signaling molecules. In general, antigen specific T-cell activation requires two distinct signals. The first signal is provided by the interaction of

the T-cell receptor (TCR) with major histocompatibility complex (MHC) associated antigens present on antigen presenting cells (APCs). The second signal is costimulatory to TCR engagement and necessary to avoid T-cell apoptosis or anergy.

**[0083]** In some embodiments, under normal physiological conditions, the T cell-mediated immune response is initiated by antigen recognition by the T cell receptor (TCR) and is regulated by a balance of co-stimulatory and inhibitory signals (e.g., immune checkpoint receptors). The immune system relies on immune checkpoint receptors to prevent autoimmunity (i.e., self-tolerance) and to protect tissues from excessive damage during an immune response, for example during an attack against a pathogenic infection. In some cases, however, these immunomodulatory proteins can be dysregulated in diseases and conditions, including tumors, as a mechanism for evading the immune system.

**[0084]** In some embodiments, among known T-cell costimulatory receptors is CD28, which is the T-cell costimulatory receptor for the ligands B7-1 (CD80) and B7-2 (CD86) both of which are present on APCs. These same ligands can also bind to the inhibitory T-cell receptor CTLA4 (cytotoxic T-lymphocyte-associated protein 4) with greater affinity than for CD28; the binding to CTLA-4 acts to down-modulate the immune response. ICOS (inducible costimulator) is another T-cell costimulatory receptor which binds to ICOS ligand (ICOSL) on APCs. In some cases, CD28 and CTLA-4 also are known to interact with ICOSL at a binding site that overlaps with the binding of ICOSL to the T-cell costimulatory receptor ICOS (Yao et al. (2011) *Immunity*, 34:729-740). Although CD28 and ICOS are related CD28 family activating receptors and share some intracellular signaling motifs, costimulatory effects between CD28 and ICOS differ. For example, CD28 is expressed on both unactivated and activated T cells and its signaling is important for IL-2 production and subsequent T cell effector function. ICOS is generally not expressed on the surface of T cells until after T cell activation, and signaling through ICOS on activated T cells supports specialized T cell subset differentiation. Thus, in some cases, costimulation by CD28 and ICOS yields overlapping and complementary effects.

**[0085]** In some embodiments, the T-cell costimulatory receptors CD28 and ICOS have distinct but complementary roles in modulating an immune response. Enhancement or suppression of the activity of these receptors has clinical significance for treatment of inflammatory and autoimmune disorders, cancer, and viral infections. In some cases, however, therapies to intervene and alter the costimulatory effects of both receptors are constrained by the

spatial orientation requirements as well as size limitations imposed by the confines of the immunological synapse. In some aspects, existing therapeutic drugs, including antibody drugs, may not be able to interact simultaneously with the multiple target proteins involved in modulating these interactions. In addition, in some cases, existing therapeutic drugs may only have the ability to antagonize but not agonize an immune response. Additionally, pharmacokinetic differences between drugs that independently target one or the other of these two receptors can create difficulties in properly maintaining a desired blood concentration of such drug combinations throughout the course of treatment.

**[0086]** In some embodiments, the provided variant ICOSL polypeptides or immunomodulatory proteins modulate (e.g. increase or decrease) immunological activity induced by costimulatory receptors CD28 or ICOS. Thus, in some embodiments, the provided polypeptides overcome these constraints by providing variant ICOSL (inducible costimulator ligand) with altered (e.g. increased or decreased) binding affinities to both CD28 and ICOS, and, in some cases, CTLA-4, thereby agonizing or antagonizing the complementary effects of costimulation by receptors. Methods of making and using these variant ICOSL are also provided.

**[0087]** All publications, including patents, patent applications scientific articles and databases, mentioned in this specification are herein incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, including patent, patent application, scientific article or database, were specifically and individually indicated to be incorporated by reference. If a definition set forth herein is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth herein prevails over the definition that is incorporated herein by reference.

**[0088]** The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

## **I. DEFINITIONS**

**[0089]** Unless defined otherwise, all terms of art, notations and other technical and scientific terms or terminology used herein are intended to have the same meaning as is commonly understood by one of ordinary skill in the art to which the claimed subject matter pertains. In

some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art.

**[0090]** The terms used throughout this specification are defined as follows unless otherwise limited in specific instances. As used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms, acronyms, and abbreviations used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Unless indicated otherwise, abbreviations and symbols for chemical and biochemical names is per IUPAC-IUB nomenclature. Unless indicated otherwise, all numerical ranges are inclusive of the values defining the range as well as all integer values in-between.

**[0091]** The term “affinity modified” as used in the context of an immunoglobulin superfamily domain, means a mammalian immunoglobulin superfamily (IgSF) domain having an altered amino acid sequence (relative to the corresponding wild-type parental or unmodified IgSF domain) such that it has an increased or decreased binding affinity or avidity to at least one of its cognate binding partners (alternatively “counter-structures”) compared to the parental wild-type or unmodified (i.e., non-affinity modified) IgSF control domain. Included in this context is an affinity modified ICOSL IgSF domain. In some embodiments, the affinity-modified IgSF domain can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acid differences, such as amino acid substitutions, in a wildtype or unmodified IgSF domain. An increase or decrease in binding affinity or avidity can be determined using well known binding assays such as flow cytometry. Larsen et al., American Journal of Transplantation, Vol 5: 443-453 (2005). See also, Linsley et al., Immunity, 1: 7930801 (1994). An increase in a protein’s binding affinity or avidity to its cognate binding partner(s) is to a value at least 10% greater than that of the wild-type IgSF domain control and in some embodiments, at least 20%, 30%, 40%, 50%, 100%, 200%, 300%, 500%, 1000%, 5000%, or 10000% greater than that of the wild-type IgSF domain control value. A decrease in a protein’s binding affinity or avidity to at least one of its cognate binding partner is to a value no greater than 90% of the control but no less than 10% of the wild-type IgSF domain control value, and in some embodiments no greater than 80%, 70% 60%, 50%, 40%, 30%, or 20% but no less

than 10% of the wild-type IgSF domain control value. An affinity-modified protein is altered in primary amino acid sequence by substitution, addition, or deletion of amino acid residues. The term “affinity modified IgSF domain” is not be construed as imposing any condition for any particular starting composition or method by which the affinity-modified IgSF domain was created. Thus, the affinity modified IgSF domains of the present invention are not limited to wild type IgF domains that are then transformed to an affinity modified IgSF domain by any particular process of affinity modification. An affinity modified IgSF domain polypeptide can, for example, be generated starting from wild type mammalian IgSF domain sequence information, then modeled in silico for binding to its cognate binding partner, and finally recombinantly or chemically synthesized to yield the affinity modified IgSF domain composition of matter. In but one alternative example, an affinity modified IgSF domain can be created by site-directed mutagenesis of a wild-type IgSF domain. Thus, affinity modified IgSF domain denotes a product and not necessarily a product produced by any given process. A variety of techniques including recombinant methods, chemical synthesis, or combinations thereof, may be employed.

**[0092]** The term “allogeneic” as used herein means a cell or tissue that is removed from one organism and then infused or adoptively transferred into a genetically dissimilar organism of the same species. In some embodiments of the invention, the species is murine or human.

**[0093]** The term “autologous” as used herein means a cell or tissue that is removed from the same organism to which it is later infused or adoptively transferred. An autologous cell or tissue can be altered by, for example, recombinant DNA methodologies, such that it is no longer genetically identical to the native cell or native tissue which is removed from the organism. For example, a native autologous T-cell can be genetically engineered by recombinant DNA techniques to become an autologous engineered cell expressing a transmembrane immunomodulatory protein and/or chimeric antigen receptor (CAR), which in some cases involves engineering a T-cell or TIL (tumor infiltrating lymphocyte). The engineered cells are then infused into a patient from which the native T-cell was isolated. In some embodiments, the organism is human or murine.

**[0094]** The terms “binding affinity,” and “binding avidity” as used herein means the specific binding affinity and specific binding avidity, respectively, of a protein for its counter-structure under specific binding conditions. In biochemical kinetics avidity refers to the accumulated strength of multiple affinities of individual non-covalent binding interactions, such as between

ICOSL and its counter-structures ICOS and/or CD28. As such, avidity is distinct from affinity, which describes the strength of a single interaction. An increase or attenuation in binding affinity of a variant ICOSL containing an affinity modified ICOSL IgSF domain to its counter-structure is determined relative to the binding affinity of the unmodified ICOSL, such as an unmodified ICOSL containing the native or wild-type IgSF domain, such as IgV domain. Methods for determining binding affinity or avidity are known in art. See, for example, Larsen et al., American Journal of Transplantation, Vol 5: 443-453 (2005). In some embodiments, a variant ICOSL of the invention (i.e. a ICOSL protein containing an affinity modified IgSF domain) specifically binds to CD28 and/or ICOS measured by flow cytometry with a binding affinity that yields a Mean Fluorescence Intensity (MFI) value at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than a wild-type ICOSL control in a binding assay such as described in Example 6.

**[0095]** The term “biological half-life” refers to the amount of time it takes for a substance, such as an immunomodulatory polypeptide comprising a variant ICOSL of the present invention, to lose half of its pharmacologic or physiologic activity or concentration. Biological half-life can be affected by elimination, excretion, degradation (e.g., enzymatic) of the substance, or absorption and concentration in certain organs or tissues of the body. In some embodiments, biological half-life can be assessed by determining the time it takes for the blood plasma concentration of the substance to reach half its steady state level (“plasma half-life”). Conjugates that can be used to derivatize and increase the biological half-life of polypeptides of the invention are known in the art and include, but are not limited to, polyethylene glycol (PEG), hydroxyethyl starch (HES), XTEN (extended recombinant peptides; see, WO2013130683), human serum albumin (HSA), bovine serum albumin (BSA), lipids (acylation), and poly-Pro-Ala-Ser (PAS), polyglutamic acid (glutamylolation).

**[0096]** The term “chimeric antigen receptor” or “CAR” as used herein refers to an artificial (i.e., man-made) transmembrane protein expressed on a mammalian cell comprising at least an ectodomain, a transmembrane, and an endodomain. Optionally, the CAR protein includes a “spacer” which covalently links the ectodomain to the transmembrane domain. A spacer is often a polypeptide linking the ectodomain to the transmembrane domain via peptide bonds. The CAR is typically expressed on a mammalian lymphocyte. In some embodiments, the CAR is expressed on a mammalian cell such as a T-cell or a tumor infiltrating lymphocyte (TIL). A

CAR expressed on a T-cell is referred to herein as a “CAR T-cell” or “CAR-T.” In some embodiments the CAR-T is a T helper cell, a cytotoxic T-cell, a natural killer T-cell, a memory T-cell, a regulatory T-cell, or a gamma delta T-cell. When used clinically in, e.g. adoptive cell transfer, a CAR-T with antigen binding specificity to the patient's tumor is typically engineered to express on a native T-cell obtained from the patient. The engineered T-cell expressing the CAR is then infused back into the patient. The CAR-T is thus often an autologous CAR-T although allogeneic CAR-T are included within the scope of the invention. The ectodomain of a CAR comprises an antigen binding region, such as an antibody or antigen binding fragment thereof (e.g. scFv), that specifically binds under physiological conditions with a target antigen, such as a tumor specific antigen. Upon specific binding a biochemical chain of events (i.e., signal transduction) results in modulation of the immunological activity of the CAR-T. Thus, for example, upon specific binding by the antigen binding region of the CAR-T to its target antigen can lead to changes in the immunological activity of the T-cell activity as reflected by changes in cytotoxicity, proliferation or cytokine production. Signal transduction upon CAR-T activation is achieved in some embodiments by the CD3-zeta chain (“CD3-z”) which is involved in signal transduction in native mammalian T-cells. CAR-Ts can further comprises multiple signaling domains such as CD28, 41BB or OX40, to further modulate immunomodulatory response of the T-cell. CD3-z comprises a conserved motif known as an immunoreceptor tyrosine-based activation motif (ITAM) which is involved in T-cell receptor signal transduction.

**[0097]** The term “collectively” or “collective” when used in reference to cytokine production induced by the presence of two or more variant ICOSL of the invention in an in vitro assay, means the overall cytokine expression level irrespective of the cytokine production induced by individual variant ICOSL. In some embodiments, the cytokine being assayed is IFN-gamma in an in vitro primary T-cell assay such as described in Example 7.

**[0098]** The term “cognate binding partner” (used interchangeably with “counter-structure”) in reference to a polypeptide, such as in reference to an IgSF domain of a variant ICOSL, refers to at least one molecule (typically a native mammalian protein) to which the referenced polypeptide specifically binds under specific binding conditions. In some aspects, a variant ICOSL containing an affinity modified IgSF domain specifically binds to the counter-structure of the corresponding native or wildtype ICOSL but with increased or attenuated affinity. A species of ligand recognized and specifically binding to its cognate receptor under specific binding

conditions is an example of a counter-structure or cognate binding partner of that receptor. A “cognate cell surface binding partner” is a cognate binding partner expressed on a mammalian cell surface. A “cell surface molecular species” is a cognate binding partner of ligands of the immunological synapse (IS), expressed on and by cells, such as mammalian cells, forming the immunological synapse.

**[0099]** As used herein, “conjugate,” “conjugation” or grammatical variations thereof refers the joining or linking together of two or more compounds resulting in the formation of another compound, by any joining or linking methods known in the art. It can also refer to a compound which is generated by the joining or linking together two or more compounds. For example, a variant ICOSL polypeptide linked directly or indirectly to one or more chemical moieties or polypeptide is an exemplary conjugate. Such conjugates include fusion proteins, those produced by chemical conjugates and those produced by any other methods.

**[0100]** The term “competitive binding” as used herein means that a protein is capable of specifically binding to at least two cognate binding partners but that specific binding of one cognate binding partner inhibits, such as prevents or precludes, simultaneous binding of the second cognate binding partner. Thus, in some cases, it is not possible for a protein to bind the two cognate binding partners at the same time. Generally, competitive binders contain the same or overlapping binding site for specific binding but this is not a requirement. In some embodiments, competitive binding causes a measurable inhibition (partial or complete) of specific binding of a protein to one of its cognate binding partner due to specific binding of a second cognate binding partner. A variety of methods are known to quantify competitive binding such as ELISA (enzyme linked immunosorbent assay) assays.

**[0101]** The term “conservative amino acid substitution” as used herein means an amino acid substitution in which an amino acid residue is substituted by another amino acid residue having a side chain R group with similar chemical properties (e.g., charge or hydrophobicity). Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine, and isoleucine; 2) aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains: asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; 6) acidic side chains: aspartic acid and glutamic acid; and 7) sulfur-containing side chains: cysteine and methionine. Conservative amino acids substitution groups

are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine.

**[0102]** The term, “corresponding to” with reference to positions of a protein, such as recitation that nucleotides or amino acid positions “correspond to” nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence based on structural sequence alignment or using a standard alignment algorithm, such as the GAP algorithm. For example, corresponding residues can be determined by alignment of a reference sequence with the sequence set forth in SEQ ID NO:32 (ECD domain) or set forth in SEQ ID NO: 196 (IgV domain) by structural alignment methods as described herein. By aligning the sequences, one skilled in the art can identify corresponding residues, for example, using conserved and identical amino acid residues as guides.

**[0103]** The terms “decrease” or “attenuate” “or suppress” as used herein means to decrease by a statistically significant amount. A decrease can be at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%.

**[0104]** The terms “derivatives” or “derivatized” refer to modification of a protein by covalently linking it, directly or indirectly, to a composition so as to alter such characteristics as biological half-life, bioavailability, immunogenicity, solubility, toxicity, potency, or efficacy while retaining or enhancing its therapeutic benefit. Derivatives of immunomodulatory polypeptides of the invention are within the scope of the invention and can be made by, for example, glycosylation, pegylation, lipidation, or Fc-fusion.

**[0105]** As used herein, domain (typically a sequence of three or more, generally 5 or 7 or more amino acids, such as 10 to 200 amino acid residues) refers to a portion of a molecule, such as a protein or encoding nucleic acid, that is structurally and/or functionally distinct from other portions of the molecule and is identifiable. For example, domains include those portions of a polypeptide chain that can form an independently folded structure within a protein made up of one or more structural motifs and/or that is recognized by virtue of a functional activity, such as binding activity. A protein can have one, or more than one, distinct domains. For example, a domain can be identified, defined or distinguished by homology of the primary sequence or structure to related family members, such as homology to motifs. In another example, a domain

can be distinguished by its function, such as an ability to interact with a biomolecule, such as a cognate binding partner. A domain independently can exhibit a biological function or activity such that the domain independently or fused to another molecule can perform an activity, such as, for example binding. A domain can be a linear sequence of amino acids or a non-linear sequence of amino acids. Many polypeptides contain a plurality of domains. Such domains are known, and can be identified by those of skill in the art. For exemplification herein, definitions are provided, but it is understood that it is well within the skill in the art to recognize particular domains by name. If needed appropriate software can be employed to identify domains.

**[0106]** The term “ectodomain” as used herein refers to the region of a membrane protein, such as a transmembrane protein, that lies outside the vesicular membrane. Ectodomains often comprise binding domains that specifically bind to ligands or cell surface receptors, such as via a binding domain that specifically binds to the ligand or cell surface receptor. The ectodomain of a cellular transmembrane protein is alternately referred to as an extracellular domain.

**[0107]** The terms “effective amount” or “therapeutically effective amount” refer to a quantity and/or concentration of a therapeutic composition of the invention, including a protein composition or cell composition, that when administered ex vivo (by contact with a cell from a patient) or in vivo (by administration into a patient) either alone (i.e., as a monotherapy) or in combination with additional therapeutic agents, yields a statistically significant decrease in disease progression as, for example, by ameliorating or eliminating symptoms and/or the cause of the disease. An effective amount may be an amount that relieves, lessens, or alleviates at least one symptom or biological response or effect associated with a disease or disorder, prevents progression of the disease or disorder, or improves physical functioning of the patient. In the case of cell therapy, the effective amount is an effective dose or number of cells administered to a patient by adoptive cell therapy. In some embodiments the patient is a mammal such as a non-human primate or human patient.

**[0108]** The term “endodomain” as used herein refers to the region found in some membrane proteins, such as transmembrane proteins, that extends into the interior space defined by the cell surface membrane. In mammalian cells, the endodomain is the cytoplasmic region of the membrane protein. In cells, the endodomain interacts with intracellular constituents and can be play a role in signal transduction and thus, in some cases, can be an intracellular signaling

domain. The endodomain of a cellular transmembrane protein is alternately referred to as a cytoplasmic domain, which, in some cases, can be a cytoplasmic signaling domain.

**[0109]** The terms “enhanced” or “increased” as used herein in the context of increasing immunological activity of a mammalian lymphocyte means to increase one or more activities the lymphocyte. An increased activity can be one or more of increase cell survival, cell proliferation, cytokine production, or T-cell cytotoxicity, such as by a statistically significant amount. In some embodiments, reference to increased immunological activity means to increase interferon gamma (IFN-gamma) production, such as by a statistically significant amount. In some embodiments, the immunological activity can be assessed in a mixed lymphocyte reaction (MLR) assay. Methods of conducting MLR assays are known in the art. Wang et al., *Cancer Immunol Res.* 2014 Sep; 2(9):846-56. Other methods of assessing activities of lymphocytes are known in the art, including any assay as described herein. In some embodiments an enhancement can be an increase of at least 10%, 20%, 30%, 40%, 50%, 75%, 100%, 200%, 300%, 400%, or 500% greater than a non-zero control value.

**[0110]** The term “engineered cell” as used herein refers to a mammalian cell that has been genetically modified by human intervention such as by recombinant DNA methods or viral transduction. In some embodiments, the cell is an immune cell, such as a lymphocyte (e.g. T cell, B cell, NK cell) or an antigen presenting cell (e.g. dendritic cell). The cell can be a primary cell from a patient or can be a cell line. In some embodiments, an engineered cell of the invention comprises a variant ICOSL of the invention engineered to modulate immunological activity of a T-cell expressing CD28, ICOS, or CTLA-4 to which the variant ICOSL specifically binds. In some embodiments, the variant ICOSL is a transmembrane immunomodulatory protein (hereinafter referred to as “TIP”) containing the extracellular domain or a portion thereof containing the IgV domain linked to a transmembrane domain (e.g. a ICOSL transmembrane domain) and, optionally, an intracellular signaling domain. In some cases, the TIP is formatted as a chimeric receptor containing a heterologous cytoplasmic signaling domain or endodomain. In some embodiments, an engineered cell is capable of expressing and secreting a immunomodulatory protein as described herein. Among provided engineered cells also are cells further containing an engineered T-cell receptor (TCR) or chimeric antigen receptor (CAR).

**[0111]** The term “engineered T-cell” as used herein refers to a T-cell such as a T helper cell, cytotoxic T-cell (alternatively, cytotoxic T lymphocyte or CTL), natural killer T-cell, regulatory

T-cell, memory T-cell, or gamma delta T-cell, that has been genetically modified by human intervention such as by recombinant DNA methods or viral transduction methods. An engineered T-cell comprises a variant ICOSL transmembrane immunomodulatory protein (TIP) of the present invention that is expressed on the T-cell and is engineered to modulate immunological activity of the engineered T-cell itself, or a mammalian cell to which the variant ICOSL expressed on the T-cell specifically binds. The term “engineered T-cell receptor” or “engineered TCR” refers to a T-cell receptor (TCR) engineered to specifically bind with a desired affinity to a major histocompatibility complex (MHC)/peptide target antigen that is selected, cloned, and/or subsequently introduced into a population of T-cells, often used for adoptive immunotherapy. In contrast to engineered TCRs, CARs are engineered to bind target antigens in a MHC independent manner.

**[0112]** The term “expressed on” as used herein is used in reference to a protein expressed on the surface of a cell, such as a mammalian cell. Thus, the protein is expressed as a membrane protein. In some embodiments, the expressed protein is a transmembrane protein. In some embodiments, the protein is conjugated to a small molecule moiety such as a drug or detectable label. Proteins expressed on the surface of a cell can include cell-surface proteins such as cell surface receptors that are expressed on mammalian cells.

**[0113]** The term “half-life extending moiety” refers to a moiety of a polypeptide fusion or chemical conjugate that extends the half-life of a protein circulating in mammalian blood serum compared to the half-life of the protein that is not so conjugated to the moiety. In some embodiments, half-life is extended by greater than or greater than about 1.2-fold, 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5.0-fold, or 6.0-fold. In some embodiments, half-life is extended by more than 6 hours, more than 12 hours, more than 24 hours, more than 48 hours, more than 72 hours, more than 96 hours or more than 1 week after *in vivo* administration compared to the protein without the half-life extending moiety. The half-life refers to the amount of time it takes for the protein to lose half of its concentration, amount, or activity. Half-life can be determined for example, by using an ELISA assay or an activity assay. Exemplary half-life extending moieties include an Fc domain, a multimerization domain, polyethylene glycol (PEG), hydroxyethyl starch (HES), XTEN (extended recombinant peptides; see, WO2013130683), human serum albumin (HSA), bovine serum albumin (BSA), lipids (acylation), and poly-Pro-Ala-Ser (PAS), and polyglutamic acid (glutamylation).

**[0114]** The term “immunological synapse” or “immune synapse” as used herein means the interface between a mammalian cell that expresses MHC I (major histocompatibility complex) or MHC II, such as an antigen-presenting cell or tumor cell, and a mammalian lymphocyte such as an effector T cell or Natural Killer (NK) cell.

**[0115]** An Fc (fragment crystallizable) region or domain of an immunoglobulin molecule (also termed an Fc polypeptide) corresponds largely to the constant region of the immunoglobulin heavy chain, and is responsible for various functions, including the antibody’s effector function(s). The Fc domain contains part or all of a hinge domain of an immunoglobulin molecule plus a CH2 and a CH3 domain. The Fc domain can form a dimer of two polypeptide chains joined by one or more disulfide bonds. In some embodiments, the Fc is a variant Fc that exhibits reduced (e.g. reduced greater than 30%, 40%, 50%, 60%, 70%, 80%, 90% or more) activity to facilitate an effector function. In some embodiments, reference to amino acid substitutions in an Fc region is by EU numbering system unless described with reference to a specific SEQ ID NO. EU numbering is known and is according to the most recently updated IMGT Scientific Chart (IMGT®, the international ImMunoGeneTics information system® [http://www.imgt.org/IMGTScientificChart/Numbering/Hu\\_IGHGnber.html](http://www.imgt.org/IMGTScientificChart/Numbering/Hu_IGHGnber.html) (created: 17 May 2001 , last updated: 10 Jan 2013) and the EU index as reported in Kabat, E.A. et al. Sequences of Proteins of Immunological interest. 5th ed. US Department of Health and Human Services, NIH publication No. 91-3242 (1991 ).

**[0116]** An immunoglobulin Fc fusion (“Fc-fusion”), such as an immunomodulatory Fc fusion protein, is a molecule comprising one or more polypeptides (or one or more small molecules) operably linked to an Fc region of an immunoglobulin. An Fc-fusion may comprise, for example, the Fc region of an antibody (which facilitates effector functions and pharmacokinetics) and a variant ICOSL. An immunoglobulin Fc region may be linked indirectly or directly to one or more variant ICOSL or small molecules (fusion partners). Various linkers are known in the art and can optionally be used to link an Fc to a fusion partner to generate an Fc-fusion. Fc-fusions of identical species can be dimerized to form Fc-fusion homodimers, or using non-identical species to form Fc-fusion heterodimers. In some embodiments, the Fc is a mammalian Fc such as a murine or human Fc.

**[0117]** The term “host cell” refers to a cell that can be used to express a protein encoded by a recombinant expression vector. A host cell can be a prokaryote, for example, E. coli, or it can be

a eukaryote, for example, a single-celled eukaryote (e.g., a yeast or other fungus), a plant cell (e.g., a tobacco or tomato plant cell), an animal cell (e.g., a human cell, a monkey cell, a hamster cell, a rat cell, a mouse cell, or an insect cell) or a hybridoma. Examples of host cells include Chinese hamster ovary (CHO) cells or their derivatives such as Veggie CHO and related cell lines which grow in serum-free media or CHO strain DX-B11, which is deficient in DHFR. In some embodiments, a host cell is a mammalian cell (e.g., a human cell, a monkey cell, a hamster cell, a rat cell, a mouse cell, or an insect cell).

**[0118]** The term “immunoglobulin” (abbreviated “Ig”) as used herein refers to a mammalian immunoglobulin protein including any of the five human classes of antibody: IgA (which includes subclasses IgA1 and IgA2), IgD, IgE, IgG (which includes subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. The term is also inclusive of immunoglobulins that are less than full-length, whether wholly or partially synthetic (e.g., recombinant or chemical synthesis) or naturally produced, such as antigen binding fragment (Fab), variable fragment (Fv) containing  $V_H$  and  $V_L$ , the single chain variable fragment (scFv) containing  $V_H$  and  $V_L$  linked together in one chain, as well as other antibody V region fragments, such as Fab', F(ab)<sub>2</sub>, F(ab')<sub>2</sub>, dsFv diabody, Fc, and Fd polypeptide fragments. Bispecific antibodies, homobispecific and heterobispecific, are included within the meaning of the term.

**[0119]** The term “immunoglobulin superfamily” or “IgSF” as used herein means the group of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells. Molecules are categorized as members of this superfamily based on shared structural features with immunoglobulins (i.e., antibodies); they all possess a domain known as an immunoglobulin domain or fold. Members of the IgSF include cell surface antigen receptors, co-receptors and co-stimulatory molecules of the immune system, molecules involved in antigen presentation to lymphocytes, cell adhesion molecules, certain cytokine receptors and intracellular muscle proteins. They are commonly associated with roles in the immune system. Proteins in the immunological synapse are often members of the IgSF. IgSF can also be classified into “subfamilies” based on shared properties such as function. Such subfamilies typically consist of from 4 to 30 IgSF members.

**[0120]** The terms “IgSF domain” or “immunoglobulin domain” or “Ig domain” as used herein refers to a structural domain of IgSF proteins. Ig domains are named after the immunoglobulin molecules. They contain about 70-110 amino acids and are categorized

according to their size and function. Ig-domains possess a characteristic Ig-fold, which has a sandwich-like structure formed by two sheets of antiparallel beta strands. Interactions between hydrophobic amino acids on the inner side of the sandwich and highly conserved disulfide bonds formed between cysteine residues in the B and F strands, stabilize the Ig-fold. One end of the Ig domain has a section called the complementarity determining region that is important for the specificity of antibodies for their ligands. The Ig like domains can be classified (into classes) as: IgV, IgC1, IgC2, or IgI. Most Ig domains are either variable (IgV) or constant (IgC). IgV domains with 9 beta strands are generally longer than IgC domains with 7 beta strands. Ig domains of some members of the IgSF resemble IgV domains in the amino acid sequence, yet are similar in size to IgC domains. These are called IgC2 domains, while standard IgC domains are called IgC1 domains. T-cell receptor (TCR) chains contain two Ig domains in the extracellular portion; one IgV domain at the N-terminus and one IgC1 domain adjacent to the cell membrane. ICOSL contains two Ig domains: IgV and IgC.

**[0121]** The term “IgSF species” as used herein means an ensemble of IgSF member proteins with identical or substantially identical primary amino acid sequence. Each mammalian immunoglobulin superfamily (IgSF) member defines a unique identity of all IgSF species that belong to that IgSF member. Thus, each IgSF family member is unique from other IgSF family members and, accordingly, each species of a particular IgSF family member is unique from the species of another IgSF family member. Nevertheless, variation between molecules that are of the same IgSF species may occur owing to differences in post-translational modification such as glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation. Additionally, minor sequence differences within a single IgSF species owing to gene polymorphisms constitute another form of variation within a single IgSF species as do wild type truncated forms of IgSF species owing to, for example, proteolytic cleavage. A “cell surface IgSF species” is an IgSF species expressed on the surface of a cell, generally a mammalian cell.

**[0122]** The term “immunological activity” as used herein in the context of mammalian lymphocytes such as T-cells refers to one or more cell survival, cell proliferation, cytokine production (e.g. interferon-gamma), or T-cell cytotoxicity activities. In some cases, an immunological activity can mean the cell expression of cytokines, such as chemokines or interleukins. Assays for determining enhancement or suppression of immunological activity include the MLR (mixed lymphocyte reaction) assays measuring interferon-gamma cytokine

levels in culture supernatants (Wang et al., Cancer Immunol Res. 2014 Sep; 2(9):846-56), SEB (staphylococcal enterotoxin B) T cell stimulation assay (Wang et al., Cancer Immunol Res. 2014 Sep; 2(9):846-56), and anti-CD3 T cell stimulation assays (Li and Kurlander, J Transl Med. 2010; 8: 104). Since T cell activation is associated with secretion of IFN-gamma cytokine, detecting IFN-gamma levels in culture supernatants from these in vitro human T cell assays can be assayed using commercial ELISA kits (Wu et al, Immunol Lett 2008 Apr 15; 117(1): 57-62). Induction of an immune response results in an increase in immunological activity relative to quiescent lymphocytes. An immunomodulatory protein, such as a variant ICOSL polypeptide containing an affinity modified IgSF domain, as provided herein can in some embodiments increase or, in alternative embodiments, decrease IFN-gamma (interferon-gamma) expression in a primary T-cell assay relative to a wild-type IgSF member or IgSF domain control. Those of skill will recognize that the format of the primary T-cell assay used to determine an increase in IFN-gamma expression will differ from that employed to assay for a decrease in IFN-gamma expression. In assaying for the ability of an immunomodulatory protein or affinity modified IgSF domain of the invention to decrease IFN-gamma expression in a primary T-cell assay, a Mixed Lymphocyte Reaction (MLR) assay can be used as described in Example 6. Conveniently, a soluble form of an affinity modified IgSF domain of the invention can be employed to determine its ability to antagonize and thereby decrease the IFN-gamma expression in a MLR as likewise described in Example 6. Alternatively, in assaying for the ability of an immunomodulatory protein or affinity modified IgSF domain of the invention to increase IFN-gamma expression in a primary T-cell assay, a co-immobilization assay can be used. In a co-immobilization assay, a T-cell receptor signal, provided in some embodiments by anti-CD3 antibody, is used in conjunction with a co-immobilized affinity modified IgSF domain, such as variant ICOSL, to determine the ability to increase IFN-gamma expression relative to a wild-type IgSF domain control. Methods to assay the immunological activity of engineered cells, including to evaluate the activity of a variant ICOSL transmembrane immunomodulatory protein, are known in the art and include, but are not limited to, the ability to expand T cells following antigen stimulation, sustain T cell expansion in the absence of re-stimulation, and anti-cancer activities in appropriate animal models. Assays also include assays to assess cytotoxicity, including a standard <sup>51</sup>Cr-release assay (see e.g. Milone et al., (2009) Molecular Therapy 17:

1453-1464) or flow based cytotoxicity assays, or an impedance based cytotoxicity assay (Peper et al. (2014) Journal of Immunological Methods, 405:192-198).

**[0123]** An “immunomodulatory polypeptide” is a polypeptide that modulates immunological activity. By “modulation” or “modulating” an immune response is meant that immunological activity is either increased or decreased. An immunomodulatory polypeptide can be a single polypeptide chain or a multimer (dimers or higher order multimers) of at least two polypeptide chains covalently bonded to each other by, for example, interchain disulfide bonds. Thus, monomeric, dimeric, and higher order multimeric polypeptides are within the scope of the defined term. Multimeric polypeptides can be homomultimeric (of identical polypeptide chains) or heteromultimeric (of non-identical polypeptide chains). An immunomodulatory polypeptide of the invention comprises a variant ICOSL.

**[0124]** The term “increase” as used herein means to increase by a statistically significant amount. An increase can be at least 5%, 10%, 20%, 30%, 40%, 50%, 75%, 100%, or greater than a non-zero control value.

**[0125]** An “isoform” of ICOSL (inducible costimulator ligand; CD275) is one of a plurality naturally occurring ICOSL polypeptides that differ in amino acid sequence. Isoforms can be the product of splice variants of an RNA transcript expressed by a single gene, or the expression product of highly similar but different genes yielding a functionally similar protein such as may occur from gene duplication. As used herein, the term “isoform” of ICOSL also refers to the product of different alleles of an ICOSL gene (e.g., ICOSLG).

**[0126]** The term “lymphocyte” as used herein means any of three subtypes of white blood cell in a mammalian immune system. They include natural killer cells (NK cells) (which function in cell-mediated, cytotoxic innate immunity), T cells (for cell-mediated, cytotoxic adaptive immunity), and B cells (for humoral, antibody-driven adaptive immunity). T cells include: T helper cells, cytotoxic T-cells, natural killer T-cells, memory T-cells, regulatory T-cells, or gamma delta T-cells. Innate lymphoid cells (ILC) are also included within the definition of lymphocyte.

**[0127]** The terms “mammal,” or “patient” specifically includes reference to at least one of a: human, chimpanzee, rhesus monkey, cynomolgus monkey, dog, cat, mouse, or rat.

**[0128]** The term “membrane protein” as used herein means a protein that, under physiological conditions, is attached directly or indirectly to a lipid bilayer. A lipid bilayer that

forms a membrane can be a biological membrane such as a eukaryotic (e.g., mammalian) cell membrane or an artificial (i.e., man-made) membrane such as that found on a liposome. Attachment of a membrane protein to the lipid bilayer can be by way of covalent attachment, or by way of non-covalent interactions such as hydrophobic or electrostatic interactions. A membrane protein can be an integral membrane protein or a peripheral membrane protein. Membrane proteins that are peripheral membrane proteins are non-covalently attached to the lipid bilayer or non-covalently attached to an integral membrane protein. A peripheral membrane protein forms a temporary attachment to the lipid bilayer such that under the range of conditions that are physiological in a mammal, peripheral membrane protein can associate and/or disassociate from the lipid bilayer. In contrast to peripheral membrane proteins, integral membrane proteins form a substantially permanent attachment to the membrane's lipid bilayer such that under the range of conditions that are physiological in a mammal, integral membrane proteins do not disassociate from their attachment to the lipid bilayer. A membrane protein can form an attachment to the membrane by way of one layer of the lipid bilayer (monotopic), or attached by way of both layers of the membrane (polytopic). An integral membrane protein that interacts with only one lipid bilayer is an "integral monotopic protein". An integral membrane protein that interacts with both lipid bilayers is an "integral polytopic protein" alternatively referred to herein as a "transmembrane protein".

**[0129]** The terms "modulating" or "modulate" as used herein in the context of an immune response, such as a mammalian immune response, refer to any alteration, such as an increase or a decrease, of existing or potential immune responses that occurs as a result of administration of an immunomodulatory polypeptide comprising a variant ICOSL of the present invention or as a result of administration of engineered cells expresses an immunomodulatory protein, such as a variant ICOSL transmembrane immunomodulatory protein of the present invention. Thus, it refers to an alteration, such as an increase or decrease, of an immune response as compared to the immune response that occurs or is present in the absence of the administration of the immunomodulatory protein comprising the variant ICOSL or cells expressing such an immunomodulatory polypeptide. Such modulation includes any induction, activation, suppression or alteration in degree or extent of immunological activity of an immune cell. Immune cells include B cells, T cells, NK (natural killer) cells, NK T cells, professional antigen-presenting cells (APCs), and non-professional antigen-presenting cells, and inflammatory cells

(neutrophils, macrophages, monocytes, eosinophils, and basophils). Modulation includes any change imparted on an existing immune response, a developing immune response, a potential immune response, or the capacity to induce, regulate, influence, or respond to an immune response. Modulation includes any alteration in the expression and/or function of genes, proteins and/or other molecules in immune cells as part of an immune response. Modulation of an immune response or modulation of immunological activity includes, for example, the following: elimination, deletion, or sequestration of immune cells; induction or generation of immune cells that can modulate the functional capacity of other cells such as autoreactive lymphocytes, antigen presenting cells, or inflammatory cells; induction of an unresponsive state in immune cells (i.e., anergy); enhancing or suppressing the activity or function of immune cells, including but not limited to altering the pattern of proteins expressed by these cells. Examples include altered production and/or secretion of certain classes of molecules such as cytokines, chemokines, growth factors, transcription factors, kinases, costimulatory molecules, or other cell surface receptors or any combination of these modulatory events. Modulation can be assessed, for example, by an alteration in IFN-gamma (interferon gamma) expression relative to the wild-type ICOSL control in a primary T cell assay (see, Zhao and Ji, Exp Cell Res. 2016 Jan1; 340(1) 132-138). Modulation can be assessed, for example, by an alteration of an immunological activity of engineered cells, such as an alteration in cytotoxic activity of engineered cells or an alteration in cytokine secretion of engineered cells relative to cells engineered with a wild-type ICOSL transmembrane protein.

**[0130]** The term “molecular species” as used herein means an ensemble of proteins with identical or substantially identical primary amino acid sequence. Each mammalian immunoglobulin superfamily (IgSF) member defines a collection of identical or substantially identical molecular species. Thus, for example, human ICOSL is an IgSF member and each human ICOSL molecule is a molecule species of ICOS. Variation between molecules that are of the same molecular species may occur owing to differences in post-translational modification such as glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation. Additionally, minor sequence differences within a single molecular species owing to gene polymorphisms constitute another form of variation within a single molecular species as do wild type truncated forms of a single molecular species owing to, for example, proteolytic cleavage. A “cell surface molecular species” is a molecular species expressed on the surface of a

mammalian cell. Two or more different species of protein, each of which is present exclusively on one or exclusively the other (but not both) of the two mammalian cells forming the IS, are said to be in “cis” or “cis configuration” with each other. Two different species of protein, the first of which is exclusively present on one of the two mammalian cells forming the IS and the second of which is present exclusively on the second of the two mammalian cells forming the IS, are said to be in “trans” or “trans configuration.” Two different species of protein each of which is present on both of the two mammalian cells forming the IS are in both cis and trans configurations on these cells.

**[0131]** The term, a “multimerization domain” refers to a sequence of amino acids that promotes stable interaction of a polypeptide molecule with one or more additional polypeptide molecules, each containing a complementary multimerization domain, which can be the same or a different multimerization domain to form a stable multimer with the first domain. Generally, a polypeptide is joined directly or indirectly to the multimerization domain. Exemplary multimerization domains include the immunoglobulin sequences or portions thereof, leucine zippers, hydrophobic regions, hydrophilic regions, and compatible protein-protein interaction domains. The multimerization domain, for example, can be an immunoglobulin constant region or domain, such as, for example, the Fc domain or portions thereof from IgG, including IgG1, IgG2, IgG3 or IgG4 subtypes, IgA, IgE, IgD and IgM and modified forms thereof.

**[0132]** The terms “nucleic acid” and “polynucleotide” are used interchangeably to refer to a polymer of nucleic acid residues (e.g., deoxyribonucleotides or ribonucleotides) in either single- or double-stranded form. Unless specifically limited, the terms encompass nucleic acids containing known analogues of natural nucleotides and that have similar binding properties to it and are metabolized in a manner similar to naturally-occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary nucleotide sequences as well as the sequence explicitly indicated (a “reference sequence”). Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues. The term nucleic acid or polynucleotide encompasses cDNA or mRNA encoded by a gene.

**[0133]** The term “non-competitive binding” as used herein means the ability of a protein to specifically bind simultaneously to at least two cognate binding partners. Thus, the protein is

able to bind to at least two different cognate binding partners at the same time, although the binding interaction need not be for the same duration such that, in some cases, the protein is specifically bound to only one of the cognate binding partners. In some embodiments, the binding occurs under specific binding conditions. In some embodiments, the simultaneous binding is such that binding of one cognate binding partner does not substantially inhibit simultaneous binding to a second cognate binding partner. In some embodiments, non-competitive binding means that binding a second cognate binding partner to its binding site on the protein does not displace the binding of a first cognate binding partner to its binding site on the protein. Methods of assessing non-competitive binding are well known in the art such as the method described in Perez de La Lastra et al., *Immunology*, 1999 Apr; 96(4): 663–670. In some cases, in non-competitive interactions, the first cognate binding partner specifically binds at an interaction site that does not overlap with the interaction site of the second cognate binding partner such that binding of the second cognate binding partner does not directly interfere with the binding of the first cognate binding partner. Thus, any effect on binding of the cognate binding partner by the binding of the second cognate binding partner is through a mechanism other than direct interference with the binding of the first cognate binding partner. For example, in the context of enzyme-substrate interactions, a non-competitive inhibitor binds to a site other than the active site of the enzyme. Non-competitive binding encompasses uncompetitive binding interactions in which a second cognate binding partner specifically binds at an interaction site that does not overlap with the binding of the first cognate binding partner but binds to the second interaction site only when the first interaction site is occupied by the first cognate binding partner.

**[0134]** The term “pharmaceutical composition” refers to a composition suitable for pharmaceutical use in a mammalian subject, often a human. A pharmaceutical composition typically comprises an effective amount of an active agent (e.g., an immunomodulatory polypeptide comprising a variant ICOSL or engineered cells expressing a variant ICOSL transmembrane immunomodulatory protein) and a carrier, excipient, or diluent. The carrier, excipient, or diluent is typically a pharmaceutically acceptable carrier, excipient or diluent, respectively.

**[0135]** The terms “polypeptide” and “protein” are used interchangeably herein and refer to a molecular chain of two or more amino acids linked through peptide bonds. The terms do not refer

to a specific length of the product. Thus, “peptides,” and “oligopeptides,” are included within the definition of polypeptide. The terms include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. The terms also include molecules in which one or more amino acid analogs or non-canonical or unnatural amino acids are included as can be synthesized, or expressed recombinantly using known protein engineering techniques. In addition, proteins can be derivatized.

**[0136]** The term “primary T-cell assay” as used herein refers to an in vitro assay to measure interferon-gamma (“IFN-gamma”) expression. A variety of such primary T-cell assays are known in the art such as that described in Example 7. In a preferred embodiment, the assay used is an anti-CD3 coimmobilization assay. In this assay, primary T cells are stimulated by anti-CD3 immobilized with or without additional recombinant proteins. Culture supernatants are harvested at timepoints, usually 24-72 hours. In another embodiment, the assay used is the MLR. In this assay, primary T cells are stimulated with allogeneic APC. Culture supernatants are harvested at timepoints, usually 24-72 hours. Human IFN-gamma levels are measured in culture supernatants by standard ELISA techniques. Commercial kits are available from vendors and the assay is performed according to manufacturer’s recommendation.

**[0137]** The term “purified” as applied to nucleic acids, such as encoding immunomodulatory proteins of the invention, generally denotes a nucleic acid or polypeptide that is substantially free from other components as determined by analytical techniques well known in the art (e.g., a purified polypeptide or polynucleotide forms a discrete band in an electrophoretic gel, chromatographic eluate, and/or a media subjected to density gradient centrifugation). For example, a nucleic acid or polypeptide that gives rise to essentially one band in an electrophoretic gel is “purified.” A purified nucleic acid or protein of the invention is at least about 50% pure, usually at least about 75%, 80%, 85%, 90%, 95%, 96%, 99% or more pure (e.g., percent by weight or on a molar basis).

**[0138]** The term “recombinant” indicates that the material (e.g., a nucleic acid or a polypeptide) has been artificially (i.e., non-naturally) altered by human intervention. The alteration can be performed on the material within, or removed from, its natural environment or state. For example, a “recombinant nucleic acid” is one that is made by recombining nucleic acids, e.g., during cloning, affinity modification, DNA shuffling or other well-known molecular

biological procedures. A “recombinant DNA molecule,” is comprised of segments of DNA joined together by means of such molecular biological techniques. The term “recombinant protein” or “recombinant polypeptide” as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule. A “recombinant host cell” is a cell that contains and/or expresses a recombinant nucleic acid or that is otherwise altered by genetic engineering, such as by introducing into the cell a nucleic acid molecule encoding a recombinant protein, such as a transmembrane immunomodulatory protein provided herein. Transcriptional control signals in eukaryotes comprise “promoter” and “enhancer” elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, i.e., promoters, are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. The terms “in operable combination,” “in operable order” and “operably linked” as used herein refer to the linkage of nucleic acid sequences in such a manner or orientation that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced.

**[0139]** The term “recombinant expression vector” as used herein refers to a DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host cell. Nucleic acid sequences necessary for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals. A secretory signal peptide sequence can also, optionally, be encoded by the recombinant expression vector, operably linked to the coding sequence for the recombinant protein, such as a recombinant fusion protein, so that the expressed fusion protein can be secreted by the recombinant host cell, for easier isolation of the fusion protein from the cell, if desired. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Among the vectors are viral vectors, such as lentiviral vectors.

**[0140]** The term “selectivity” refers to the preference of a subject protein, or polypeptide, for specific binding of one substrate, such as one cognate binding partner, compared to specific binding for another substrate, such as a different cognate binding partner of the subject protein. Selectivity can be reflected as a ratio of the binding activity (e.g. binding affinity) of a subject protein and a first substrate, such as a first cognate binding partner, (e.g.,  $K_{d1}$ ) and the binding activity (e.g. binding affinity) of the same subject protein with a second cognate binding partner (e.g.,  $K_{d2}$ ).

**[0141]** The term “sequence identity” as used herein refers to the sequence identity between genes or proteins at the nucleotide or amino acid level, respectively. “Sequence identity” is a measure of identity between proteins at the amino acid level and a measure of identity between nucleic acids at nucleotide level. The protein sequence identity may be determined by comparing the amino acid sequence in a given position in each sequence when the sequences are aligned. Similarly, the nucleic acid sequence identity may be determined by comparing the nucleotide sequence in a given position in each sequence when the sequences are aligned. Methods for the alignment of sequences for comparison are well known in the art, such methods include GAP, BESTFIT, BLAST, FASTA and TFASTA. The BLAST algorithm calculates percent sequence identity and performs a statistical analysis of the similarity between the two sequences. The software for performing BLAST analysis is publicly available through the National Center for Biotechnology Information (NCBI) website.

**[0142]** The term “soluble” as used herein in reference to proteins, means that the protein is not a membrane protein. In general, a soluble protein contains only the extracellular domain of an IgSF family member receptor, or a portion thereof containing an IgSF domain or domains or specific-binding fragments thereof, but does not contain the transmembrane domain. In some cases, solubility of a protein can be improved by linkage or attachment, directly or indirectly via a linker, to an Fc domain, which, in some cases, also can improve the stability and/or half-life of the protein. In some aspects, a soluble protein is an Fc fusion protein.

**[0143]** The term “species” as used herein with respect to polypeptides or nucleic acids means an ensemble of molecules with identical or substantially identical sequences. Variation between polypeptides that are of the same species may occur owing to differences in post-translational modification such as glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation. Slightly truncated sequences of polypeptides that differ (or encode a

difference) from the full length species at the amino-terminus or carboxy-terminus by no more than 1, 2, or 3 amino acid residues are considered to be of a single species. Such microheterogeneities are a common feature of manufactured proteins.

**[0144]** The term “specific binding fragment” as used herein in reference to a full-length wild-type mammalian ICOSL polypeptide or an IgV or an IgC domain thereof, means a polypeptide having a subsequence of an IgV and/or IgC domain and that specifically binds in vitro and/or in vivo to a mammalian ICOS and/or mammalian CD28 such as a human or murine ICOS or CD28. In some embodiments, the specific binding fragment of ICOSL IgV or ICOSL IgC is at least 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% the sequence length of the full-length wild-type sequence. The specific binding fragment can be altered in sequence to form a variant ICOSL of the invention.

**[0145]** The term “specifically binds” as used herein means the ability of a protein, under specific binding conditions, to bind to a target protein such that its affinity or avidity is at least 5 times as great, but optionally at least 10, 20, 30, 40, 50, 100, 250 or 500 times as great, or even at least 1000 times as great as the average affinity or avidity of the same protein to a collection of random peptides or polypeptides of sufficient statistical size. A specifically binding protein need not bind exclusively to a single target molecule but may specifically bind to a non-target molecule due to similarity in structural conformation between the target and non-target (e.g., paralogs or orthologs). Those of skill will recognize that specific binding to a molecule having the same function in a different species of animal (i.e., ortholog) or to a non-target molecule having a substantially similar epitope as the target molecule (e.g., paralog) is possible and does not detract from the specificity of binding which is determined relative to a statistically valid collection of unique non-targets (e.g., random polypeptides). Thus, a polypeptide of the invention may specifically bind to more than one distinct species of target molecule due to cross-reactivity. Solid-phase ELISA immunoassays or Biacore measurements can be used to determine specific binding between two proteins. Generally, interactions between two binding proteins have dissociation constants ( $K_d$ ) less than  $1 \times 10^{-5}$  M, and often as low as  $1 \times 10^{-12}$  M. In certain embodiments of the present disclosure, interactions between two binding proteins have dissociation constants of  $1 \times 10^{-6}$  M,  $1 \times 10^{-7}$  M,  $1 \times 10^{-8}$  M,  $1 \times 10^{-9}$  M,  $1 \times 10^{-10}$  M or  $1 \times 10^{-11}$  M.

**[0146]** The terms “surface expresses” or “surface expression” in reference to a mammalian cell expressing a polypeptide means that the polypeptide is expressed as a membrane protein. In some embodiments, the membrane protein is a transmembrane protein.

**[0147]** As used herein, “synthetic,” with reference to, for example, a synthetic nucleic acid molecule or a synthetic gene or a synthetic peptide refers to a nucleic acid molecule or polypeptide molecule that is produced by recombinant methods and/or by chemical synthesis methods.

**[0148]** The term “targeting moiety” as used herein refers to a composition that is covalently or non-covalently attached to, or physically encapsulates, a polypeptide comprising a variant ICOSL of the present invention. The targeting moiety has specific binding affinity for a desired counter-structure such as a cell surface receptor (e.g., the B7 family member PD-L1), or a tumor antigen such as tumor specific antigen (TSA) or a tumor associated antigen (TAA) such as B7-H6. Typically, the desired counter-structure is localized on a specific tissue or cell-type. Targeting moieties include: antibodies, antigen binding fragment (Fab), variable fragment (Fv) containing V<sub>H</sub> and V<sub>L</sub>, the single chain variable fragment (scFv) containing V<sub>H</sub> and V<sub>L</sub> linked together in one chain, as well as other antibody V region fragments, such as Fab', F(ab)<sub>2</sub>, F(ab')<sub>2</sub>, dsFv diabody, nanobodies, soluble receptors, receptor ligands, affinity matured receptors or ligands, as well as small molecule (<500 dalton) compositions (e.g., specific binding receptor compositions). Targeting moieties can also be attached covalently or non-covalently to the lipid membrane of liposomes that encapsulate a polypeptide of the present invention.

**[0149]** The term “transmembrane protein” as used herein means a membrane protein that substantially or completely spans a lipid bilayer such as those lipid bilayers found in a biological membrane such as a mammalian cell, or in an artificial construct such as a liposome. The transmembrane protein comprises a transmembrane domain (“transmembrane domain”) by which it is integrated into the lipid bilayer and by which the integration is thermodynamically stable under physiological conditions. Transmembrane domains are generally predictable from their amino acid sequence via any number of commercially available bioinformatics software applications on the basis of their elevated hydrophobicity relative to regions of the protein that interact with aqueous environments (e.g., cytosol, extracellular fluid). A transmembrane domain is often a hydrophobic alpha helix that spans the membrane. A transmembrane protein can pass through the both layers of the lipid bilayer once or multiple times. A transmembrane protein

includes the provided transmembrane immunomodulatory proteins described herein. In addition to the transmembrane domain, a transmembrane immunomodulatory protein of the invention further comprises an ectodomain and, in some embodiments, an endodomain.

**[0150]** The terms “treating,” “treatment,” or “therapy” of a disease or disorder as used herein mean slowing, stopping or reversing the disease or disorders progression, as evidenced by decreasing, cessation or elimination of either clinical or diagnostic symptoms, by administration of a therapeutic composition (e.g. containing an immunomodulatory protein or engineered cells) of the invention either alone or in combination with another compound as described herein. “Treating,” “treatment,” or “therapy” also means a decrease in the severity of symptoms in an acute or chronic disease or disorder or a decrease in the relapse rate as for example in the case of a relapsing or remitting autoimmune disease course or a decrease in inflammation in the case of an inflammatory aspect of an autoimmune disease. As used herein in the context of cancer, the terms “treatment” or, “inhibit,” “inhibiting” or “inhibition” of cancer refers to at least one of: a statistically significant decrease in the rate of tumor growth, a cessation of tumor growth, or a reduction in the size, mass, metabolic activity, or volume of the tumor, as measured by standard criteria such as, but not limited to, the Response Evaluation Criteria for Solid Tumors (RECIST), or a statistically significant increase in progression free survival (PFS) or overall survival (OS). “Preventing,” “prophylaxis,” or “prevention” of a disease or disorder as used in the context of this invention refers to the administration of an immunomodulatory polypeptide or engineered cells of the invention, either alone or in combination with another compound, to prevent the occurrence or onset of a disease or disorder or some or all of the symptoms of a disease or disorder or to lessen the likelihood of the onset of a disease or disorder.

**[0151]** The term “tumor specific antigen” or “TSA” as used herein refers to a counter-structure that is present primarily on tumor cells of a mammalian subject but generally not found on normal cells of the mammalian subject. A tumor specific antigen need not be exclusive to tumor cells but the percentage of cells of a particular mammal that have the tumor specific antigen is sufficiently high or the levels of the tumor specific antigen on the surface of the tumor are sufficiently high such that it can be targeted by anti-tumor therapeutics, such as immunomodulatory polypeptides of the invention, and provide prevention or treatment of the mammal from the effects of the tumor. In some embodiments, in a random statistical sample of cells from a mammal with a tumor, at least 50% of the cells displaying a TSA are cancerous. In

other embodiments, at least 60%, 70%, 80%, 85%, 90%, 95%, or 99% of the cells displaying a TSA are cancerous.

**[0152]** The term “variant” (also “modified” or mutant”) as used in reference to a variant ICOSL means an ICOSL, such as a mammalian (e.g., human or murine) ICOSL created by human intervention. The variant ICOSL is a polypeptide having an altered amino acid sequence, relative to an unmodified or wild-type ICOSL. The variant ICOSL is a polypeptide which differs from a wild-type ICOSL isoform sequence by one or more amino acid substitutions, deletions, additions, or combinations thereof. For purposes herein, the variant ICOSL contains at least one affinity modified domain, whereby one or more of the amino acid differences occurs in an IgSF domain (e.g. IgV domain). A variant ICOSL can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acid differences, such as amino acid substitutions. A variant ICOSL polypeptide generally exhibits at least 50%, 60%, 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a corresponding wild-type or unmodified ICOSL, such as to the sequence of SEQ ID NO:5, a mature sequence thereof or a portion thereof containing the extracellular domain or an IgSF domain thereof. In some embodiments, a variant ICOSL polypeptide exhibits at least 50%, 60%, 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a corresponding wild-type or unmodified ICOSL comprising the sequence set forth in SEQ ID NO:32 or SEQ ID NO: 196. Non-naturally occurring amino acids as well as naturally occurring amino acids are included within the scope of permissible substitutions or additions. A variant ICOSL is not limited to any particular method of making and includes, for example, de novo chemical synthesis, de novo recombinant DNA techniques, or combinations thereof. A variant ICOSL of the invention specifically binds to at least one or more of: CD28, ICOS, or CTLA-4 of a mammalian species. In some embodiments, the altered amino acid sequence results in an altered (i.e., increased or decreased) binding affinity or avidity to ICOS and/or CD28 compared to the wild-type ICOSL protein. An increase or decrease in binding affinity or avidity can be determined using well known binding assays such as flow cytometry. Larsen et al., American Journal of Transplantation, Vol 5: 443-453 (2005). See also, Linsley et al., Immunity, 1: 7930801 (1994). An increase in variant ICOSL binding affinity or avidity to ICOS and/or CD28 is to a value at least 5% greater than that of the wild-type ICOSL and in some embodiments, at least

10%, 15%, 20%, 30%, 40%, 50%, 100% greater than that of the wild-type ICOSL control value. A decrease in ICOSL binding affinity or avidity to ICOS and/or CD28 is to a value no greater than 95% of the of the wild-type control values, and in some embodiments no greater than 80%, 70% 60%, 50%, 40%, 30%, 20%, 10%, 5%, or no detectable binding affinity or avidity of the wild-type ICOS and/or CD28 control values. A variant ICOSL is altered in primary amino acid sequence by substitution, addition, or deletion of amino acid residues. The term “variant” in the context of variant ICOSL is not be construed as imposing any condition for any particular starting composition or method by which the variant ICOSL is created. A variant ICOSL can, for example, be generated starting from wild type mammalian ICOSL sequence information, then modeled in silico for binding to ICOS and/or CD28, and finally recombinantly or chemically synthesized to yield a variant ICOSL of the present invention. In but one alternative example, a variant ICOSL can be created by site-directed mutagenesis of a wild-type ICOSL. Thus, variant ICOSL denotes a composition and not necessarily a product produced by any given process. A variety of techniques including recombinant methods, chemical synthesis, or combinations thereof, may be employed.

**[0153]** The term “wild-type” or “natural” or “native” as used herein is used in connection with biological materials such as nucleic acid molecules, proteins (e.g., ICOSL), IgSF members, host cells, and the like, refers to those which are found in nature and not modified by human intervention.

## **II. VARIANT ICOSL POLYPEPTIDES**

**[0154]** Provided herein are variant ICOSL polypeptides that exhibit altered (increased or decreased) binding activity or affinity for one or more of an ICOSL cognate binding partner. In some embodiments, the ICOSL cognate binding partner is CD28, ICOS, or CTLA-4. In some embodiments, the variant ICOSL polypeptide contains one or more amino acids modifications, such as one or more substitutions (alternatively, “mutations” or “replacements”), deletions or addition, in an immunoglobulin superfamily (IgSF) domain (IgD) relative to a wild-type or unmodified ICOSL polypeptide or a portion of a wild-type or unmodified ICOSL containing an immunoglobulin superfamily (IgSF) domain or a specific binding fragment thereof. Thus, a

provided variant ICOSL polypeptide is or comprises a variant IgD (hereinafter called “vIgD”) in which the one or more amino acid modifications (e.g. substitutions) is in an IgD.

**[0155]** In some embodiments, the IgD comprises an IgV domain or an IgC (e.g. IgC2) domain or specific binding fragment of the IgV domain or the IgC (e.g. IgC2) domain, or combinations thereof. In some embodiments, the IgD can be an IgV only, the combination of the IgV and IgC, including the entire extracellular domain (ECD), or any combination of Ig domains of ICOSL. Table 2 provides exemplary residues that correspond to IgV or IgC regions of ICOSL. In some embodiments, the variant ICOSL polypeptide contains an IgV domain or an IgC domain or specific binding fragments thereof in which the at least one of the amino acid modifications (e.g. substitutions) is in the IgV domain or IgC domain or a specific binding fragment thereof. In some embodiments, by virtue of the altered binding activity or affinity, the IgV domain or IgC domain is an affinity-modified IgSF domain.

**[0156]** In some embodiments, the variant is modified in one more IgSF domains relative to the sequence of an unmodified ICOSL sequence. In some embodiments, the unmodified ICOSL sequence is a wild-type ICOSL. In some embodiments, the unmodified or wild-type ICOSL has the sequence of a native ICOSL or an ortholog thereof. In some embodiments, the unmodified ICOSL is or comprises the extracellular domain (ECD) of ICOSL or a portion thereof containing one or more IgSF domain (see Table 2). In some embodiments, the extracellular domain of an unmodified or wild-type ICOSL polypeptide comprises an IgV domain and an IgC domain or domains. However, the variant ICOSL polypeptide need not comprise both the IgV domain and the IgC domain or domains. In some embodiments, the variant ICOSL polypeptide comprises or consists essentially of the IgV domain or a specific binding fragment thereof. In some embodiments, the variant ICOSL polypeptide comprises or consists essentially of the IgC domain or specific binding fragments thereof. In some embodiments, the variant ICOSL is soluble and lacks a transmembrane domain. In some embodiments, the variant ICOSL further comprises a transmembrane domain and, in some cases, also a cytoplasmic domain.

**[0157]** In some embodiments, the wild-type or unmodified ICOSL sequence is a mammalian ICOSL sequence. In some embodiments, the wild-type or unmodified ICOSL sequence can be a mammalian ICOSL that includes, but is not limited to, human, mouse, cynomolgus monkey, or rat. In some embodiments, the wild-type or unmodified ICOSL sequence is human.

**[0158]** In some embodiments, the wild-type or unmodified ICOSL sequence has (i) the sequence of amino acids set forth in SEQ ID NO:5 or a mature form thereof lacking the signal sequence, (ii) a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:5 or the mature form thereof, or (iii) is a portion of (i) or (ii) containing an IgV domain or IgC domain or specific binding fragments thereof.

**[0159]** In some embodiments, the wild-type or unmodified ICOSL sequence is or comprises an extracellular domain of the ICOSL or a portion thereof. In some embodiments, the unmodified or wild-type ICOSL polypeptide comprises the amino acid sequence set forth in SEQ ID NO:32, or an ortholog thereof. In some cases, the unmodified or wild-type ICOSL polypeptide can comprise (i) the sequence of amino acids set forth in SEQ ID NO:32, (ii) a sequence of amino acids that has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO: 32, or (iii) is a specific binding fragment of the sequence of (i) or (ii) comprising an IgV domain or an IgC domain.

**[0160]** In some embodiments, the wild-type or unmodified ICOSL polypeptide comprises an IgV domain or an IgC domain, or a specific binding fragment thereof. In some embodiments, the IgV domain of the wild-type or unmodified ICOSL polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 196 (corresponding to amino acid residues 19-129 of SEQ ID NO:5), or an ortholog thereof. For example, the IgV domain of the unmodified or wild-type ICOSL polypeptide can contain (i) the sequence of amino acids set forth in SEQ ID NO: 196, (ii) a sequence of amino acids that has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO: 196, or (iii) a specific binding fragment of the sequence of amino acids set forth in SEQ ID NO: 196 or a specific binding fragment of a sequence of (i) or (ii). In some embodiments, the wild-type or unmodified IgV domain is capable of binding one or more ICOSL cognate binding proteins, such as one or more of CD28, ICOS, or CTLA-4.

**[0161]** In some embodiments, the IgC domain of the wild-type or unmodified ICOSL polypeptide comprises the amino acid sequence set forth as residues 141-227 of SEQ ID NO: 5, or an ortholog thereof. For example, the IgC domain of the unmodified or wild-type ICOSL polypeptide can contain (i) the sequence of amino acids set forth residues 141-227 of SEQ ID NO: 5, (ii) a sequence of amino acids that has at least about 85%, 86%, 87%, 88%, 89%, 90%,

91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to residues 141-227 of SEQ ID NO: 5, or (iii) (i) or (ii). In some embodiments, the wild-type or unmodified IgV domain is capable of binding one or more ICOSL cognate binding proteins.

**[0162]** In some embodiments, the wild-type or unmodified ICOSL polypeptide contains a specific binding fragment of ICOSL, such as a specific binding fragment of the IgV domain or the IgC domain. In some embodiments the specific binding fragment can bind CD28, ICOS, and/or CTLA-4. The specific binding fragment can have an amino acid length of at least 50 amino acids, such as at least 60, 70, 80, 90, 100, or 110 amino acids. In some embodiments, the specific binding fragment of the IgV domain contains an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the length of the IgV domain set forth as amino acids 19-129 of SEQ ID NO: 5. In some embodiments, the specific binding fragment of the IgC domain comprises an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the length of the IgC domain set forth as amino acids 141-227 of SEQ ID NO: 5.

**[0163]** In some embodiments, the variant ICOSL polypeptide comprises the ECD domain or a portion thereof comprising one or more affinity modified IgSF domains. In some embodiments, the variant ICOSL polypeptides can comprise an IgV domain or an IgC domain, in which one or more of the IgSF domains (IgV or IgC) or a specific binding fragment of the IgV domain or a specific binding fragment of the IgC domain contains the one or more amino acid modifications (e.g. substitutions). In some embodiments, the variant ICOSL polypeptides can comprise an IgV domain and an IgC domain, or a specific binding fragment of the IgV domain and a specific binding fragment of the IgC domain. In some embodiments, the variant ICOSL polypeptide comprises a full-length IgV domain. In some embodiments, the variant ICOSL polypeptide comprises a full-length IgC domain. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of the IgV domain. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of the IgC domain. In some embodiments, the variant ICOSL polypeptide comprises a full-length IgV domain and a full-length IgC domain. In some embodiments, the variant ICOSL polypeptide comprises a full-length IgV domain and a specific binding fragment of an IgC domain. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of an IgV domain and a full-

length IgC domain. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of an IgV domain and a specific binding fragment of an IgC domain.

**[0164]** In any of such embodiments, the one or more amino acid modifications (e.g. substitutions) of the variant ICOSL polypeptides can be located in any one or more of the ICOSL polypeptide domains. For example, in some embodiments, one or more amino acid substitutions are located in the extracellular domain of the variant ICOSL polypeptide. In some embodiments, one or more amino acid substitutions are located in the IgV domain or specific binding fragment of the IgV domain. In some embodiments, one or more amino acid modifications (e.g. substitutions) are located in the IgC domain or specific binding fragment of the IgC domain.

**[0165]** Generally, each of the various attributes of polypeptides are separately disclosed below (e.g., soluble, secretable and membrane bound polypeptides, affinity of ICOSL for CD28, ICOS, and CTLA-4, number of variations per polypeptide chain, number of linked polypeptide chains, the number and nature of amino acid alterations per variant ICOSL, etc.). However, as will be clear to the skilled artisan, any particular polypeptide can comprise a combination of these independent attributes. It is understood that reference to amino acids, including to a specific sequence set forth as a SEQ ID NO used to describe domain organization of an IgSF domain are for illustrative purposes and are not meant to limit the scope of the embodiments provided. It is understood that polypeptides and the description of domains thereof are theoretically derived based on homology analysis and alignments with similar molecules. Thus, the exact locus can vary, and is not necessarily the same for each protein. Hence, the specific IgSF domain, such as specific IgV domain or IgC domain, can be several amino acids (such as one, two, three or four) longer or shorter.

**[0166]** Further, various embodiments of the invention as discussed below are frequently provided within the meaning of a defined term as disclosed above. The embodiments described in a particular definition are therefore to be interpreted as being incorporated by reference when the defined term is utilized in discussing the various aspects and attributes described herein. Thus, the headings, the order of presentation of the various aspects and embodiments, and the separate disclosure of each independent attribute is not meant to be a limitation to the scope of the present disclosure.

### **Exemplary Modifications**

**[0167]** Provided herein are variant ICOSL polypeptides containing at least one affinity-modified IgSF domain (e.g. IgV or IgC) or a specific binding fragment thereof in an IgSF domain contained in a wild-type or unmodified ICOSL polypeptide such that the variant ICOSL polypeptide exhibits altered (increased or decreased) binding activity or affinity for one or more ligands ICOS, CD28, or CTLA-4 compared to a wild-type or unmodified ICOSL polypeptide. In some embodiments, a variant ICOSL polypeptide has a binding affinity for CD28, ICOS, and/or CTLA-4 that differs from that of a wild-type or unmodified ICOSL polypeptide control sequence as determined by, for example, solid-phase ELISA immunoassays, flow cytometry or Biacore assays. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28, ICOS, and/or CTLA-4. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28, ICOS, and/or CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. The CD28, ICOS and/or the CTLA-4 can be a mammalian protein, such as a human protein or a murine protein.

**[0168]** Binding affinities for each of the cognate binding partners are independent; that is, in some embodiments, a variant ICOSL polypeptide has an increased binding affinity for one, two or three of CD28, ICOS, and/or CTLA-4, and a decreased binding affinity for one, two or three of CD28, ICOS, and CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide.

**[0169]** In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for ICOS, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for ICOS, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide.

**[0170]** In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28 and ICOS, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28 and a

decreased binding affinity for ICOS, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28 and ICOS, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28 and an increased binding affinity for ICOS, relative to a wild-type or unmodified ICOSL polypeptide.

**[0171]** In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28 and CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28 and a decreased binding affinity for CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28 and CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28 and an increased binding affinity for CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide.

**[0172]** In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for ICOS and CTLA-4, relative to a wild-type or unmodified ICOS polypeptide. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for ICOS and a decreased binding affinity for CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for ICOS and CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for ICOS and an increased binding affinity for CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide.

**[0173]** In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28, ICOS, and CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28 and ICOS, and a decreased binding affinity for CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28 and CTLA-4, and a decreased binding affinity for ICOS, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28 and ICOS, and an increased binding

affinity for CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28 and an increased binding affinity for ICOS and CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has an increased binding affinity for CD28, and a decreased binding affinity for ICOS and CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28, CTLA-4, and ICOS, relative to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant ICOSL polypeptide has a decreased binding affinity for CD28, and an increased binding affinity for ICOS and CTLA-4, relative to a wild-type or unmodified ICOSL polypeptide.

**[0174]** In some embodiments, a variant ICOSL polypeptide with increased or greater binding affinity to CD28, ICOS, and/or CTLA-4 will have an increase in binding affinity relative to the wild-type or unmodified ICOSL polypeptide control of at least about 5%, such as at least about 10%, 15%, 20%, 25%, 35%, or 50% for the CD28, ICOS, and/or CTLA-4. In some embodiments, the increase in binding affinity relative to the wild-type or unmodified ICOSL polypeptide is more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold. In such examples, the wild-type or unmodified ICOSL polypeptide has the same sequence as the variant ICOSL polypeptide except that it does not contain the one or more amino acid modifications (e.g. substitutions).

**[0175]** In some embodiments, a variant ICOSL polypeptide with reduced or decreased binding affinity to CD28, ICOS, and/or CTLA-4 will have decrease in binding affinity relative to the wild-type or unmodified ICOSL polypeptide control of at least 5%, such as at least about 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more for the CD28, ICOSL, and/or CTLA-4. In some embodiments, the decrease in binding affinity relative to the wild-type or unmodified ICOSL polypeptide is more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold. In such examples, the wild-type or unmodified ICOSL polypeptide has the same sequence as the variant ICOSL polypeptide except that it does not contain the one or more amino acid modifications, e.g. substitutions.

**[0176]** In some embodiments, the equilibrium dissociation constant ( $K_d$ ) of any of the foregoing embodiments to CD28, ICOS, and/or CTLA-4 can be less than  $1 \times 10^{-5}$  M,  $1 \times 10^{-6}$  M,  $1 \times 10^{-7}$  M,  $1 \times 10^{-8}$  M,  $1 \times 10^{-9}$  M,  $1 \times 10^{-10}$  M or  $1 \times 10^{-11}$  M, or  $1 \times 10^{-12}$  M.

**[0177]** In some embodiments, a variant ICOSL polypeptide has an increased or greater binding affinity to CD28. In some embodiments, a variant ICOSL polypeptide with increased or greater binding affinity to CD28 will have an increase in binding affinity relative to the wild-type or unmodified ICOSL polypeptide control of at least about 25%, such as at least about 30%, 40%, 50%, or 60% for CD28. In some embodiments, a variant ICOSL polypeptide with increased or greater binding affinity to CD28 has an equilibrium dissociation constant ( $K_d$ ) of less than 200 pM, 300 pM, 400 pM, 500 pM, or 600 pM for CD28. In some embodiments, the variant polypeptide specifically binds to the ectodomain of one of ICOS, CD28 or CTLA4 with increased selectivity compared to the unmodified ICOSL. In some embodiments, the increased selectivity is for CD28. In some embodiments, the increased selectivity comprises a greater ratio of binding of the variant ICOSL polypeptide for one cognate binding partner selected from among ICOS, CD28 and CTLA4 versus another of the cognate binding partner compared to the ratio of binding of the unmodified ICOSL polypeptide for the one cognate binding partner versus the another of the cognate binding partner. In some embodiments, the ratio is greater by at least or at least about 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5-fold, 10-fold, 15-fold, 20-fold, 30-fold, 40-fold, 50-fold or more.

**[0178]** The wild-type or unmodified ICOSL sequence does not necessarily have to be used as a starting composition to generate variant ICOSL polypeptides described herein. Therefore, use of the term “modification”, such as “substitution” does not imply that the present embodiments are limited to a particular method of making variant ICOSL polypeptides. Variant ICOSL polypeptides can be made, for example, by *de novo* peptide synthesis and thus does not necessarily require a modification, such as a “substitution”, in the sense of altering a codon to encode for the modification, e.g. substitution. This principle also extends to the terms “addition” and “deletion” of an amino acid residue which likewise do not imply a particular method of making. The means by which the variant ICOSL polypeptides are designed or created is not limited to any particular method. In some embodiments, however, a wild-type or unmodified ICOSL encoding nucleic acid is mutagenized from wild-type or unmodified ICOSL genetic material and screened for desired specific binding affinity and/or induction of IFN-gamma

expression or other functional activity. In some embodiments, a variant ICOSL polypeptide is synthesized *de novo* utilizing protein or nucleic acid sequences available at any number of publicly available databases and then subsequently screened. The National Center for Biotechnology Information provides such information and its website is publicly accessible via the internet as is the UniProtKB database as discussed previously.

**[0179]** Unless stated otherwise, as indicated throughout the present disclosure, the amino acid substitution(s) are designated by amino acid position number corresponding to the numbering of positions of the unmodified ECD sequence set forth in SEQ ID NO:32 or, where applicable, the unmodified IgV sequence set forth in SEQ ID NO:196 (containing residues 19-129 of SEQ ID NO:32) as follows:

DTQEKEVRAMVGSDVELSCACPEGSRFDLNDVYVYWQTSESKTVVTYHIPQNSSLEN  
VDSRYRNRALMSPAGMLRGDFSLRLFNVTQPDEQKFHCLVLSQSLGFQEVLSEVTL  
HVAANFSVPVVSAPHSPSQDELFTTCTTSINGYPRPNVYWINKTDNSLLDQALQNDTVF  
LNMRLGYDVVSVLRIARTPSVNIGCCIEENVLLQQNLTVGSQTGNDIGERDKITENPVS  
TGEKNAAT (SEQ ID NO:32)

DTQEKEVRAMVGSDVELSCACPEGSRFDLNDVYVYWQTSESKTVVTYHIPQNSSLEN  
VDSRYRNRALMSPAGMLRGDFSLRLFNVTQPDEQKFHCLVLSQSLGFQEVLSE  
(SEQ ID NO:196)

**[0180]** It is within the level of a skilled artisan to identify the corresponding position of a modification, e.g. amino acid substitution, in an ICOSL polypeptide, including portion thereof containing an IgSF domain (e.g. IgV) thereof, such as by alignment of a reference sequence with SEQ ID NO:32 or SEQ ID NO:196. In the listing of modifications throughout this disclosure, the amino acid position is indicated in the middle, with the corresponding unmodified (e.g. wild-type) amino acid listed before the number and the identified variant amino acid substitution listed after the number. If the modification is a deletion of the position a “del” is indicated and if the modification is an insertion at the position an “ins” is indicated.

**[0181]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution in a wild-type or unmodified ICOSL sequence. The one or more

amino acid modification, e.g. substitution can be in the ectodomain (extracellular domain) of the wild-type or unmodified ICOSL sequence. In some embodiments, the one or more amino acid modification, e.g. substitution is in the IgV domain or specific binding fragment thereof. In some embodiments, the one or more amino acid modification, e.g. substitution is in the IgC domain or specific binding fragment thereof. In some embodiments of the variant ICOSL polypeptide, some of the one or more amino acid modification, e.g. substitution is in the IgV domain or a specific binding fragment thereof, and some of the one or more amino acid modification, e.g. substitution are in the IgC domain or a specific binding fragment thereof.

**[0182]** In some embodiments, the variant ICOSL polypeptide has up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid modification(s), e.g. substitution. The modification, e.g. substitution can be in the IgV domain or the IgC domain. In some embodiments, the variant ICOSL polypeptide has up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid substitutions in the IgV domain or specific binding fragment thereof. In some embodiments, the variant ICOSL polypeptide has up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid substitutions in the IgC domain or specific binding fragment thereof. In some embodiments, the variant ICOSL polypeptide has at least about 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with the wild-type or unmodified ICOSL polypeptide or specific binding fragment thereof, such as with the amino acid sequence of SEQ ID NO: 32 or 196.

**[0183]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution in an unmodified ICOSL or specific binding fragment thereof corresponding to position(s) 10, 11, 13, 16, 18, 20, 25, 27, 30, 33, 37, 42, 43, 47, 52, 54, 57, 61, 62, 67, 71, 72, 74, 77, 78, 75, 80, 84, 89, 90, 92, 93, 94, 96, 97, 98, 99, 100, 102, 103, 107, 109, 110, 111, 113, 115, 116, 117, 119, 120, 121, 122, 126, 129, 130, 132, 133, 135, 138, 139, 140, 142, 143, 144, 146, 151, 152, 153, 154, 155, 156, 158, 161, 166, 168, 172, 173, 175, 190, 192, 193, 194, 198, 201, 203, 207, 208, 210, 212, 217, 218, 220, 221, 224, 225, or 227 with reference to numbering of SEQ ID NO:32. In some embodiments, such variant ICOSL polypeptides exhibit altered binding affinity to one or more of CD28, ICOS, and/or CTLA-4 compared to the wild-type or unmodified ICOSL polypeptide. For example, in some embodiments, the variant ICOSL polypeptide exhibits increased binding affinity to CD28, ICOS, and/or CTLA-4 compared to a wild-type or unmodified ICOSL polypeptide. In some embodiments, the variant

ICOSL polypeptide exhibits decreased binding affinity to CD28, ICOS, or CTLA-4 compared to a wild-type or unmodified ICOSL polypeptide.

**[0184]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from M10V, M10I, V11E, S13G, E16V, S18R, A20V, S25G, F27S, F27C, N30D, Y33del, Q37R, K42E, Y47H, T43A, N52H, N52D, N52Q, N52S, N52Y, N52K, S54A, S54P, N57D, N57Y, R61S, R61C, Y62F, L67P, A71T, G72R, L74Q, R75Q, D77G, F78L, L80P, N84Q, D89G, E90A, K92R, F93L, H94E, H94D, L96F, L96I, V97A, L98F, S99G, Q100R, Q100K, Q100P, L102R, G103E, V107A, V107I, S109G, S109N, V110D, V110N, V110A, E111del, T113E, H115R, H115Q, V116A, A117T, N119Q, F120I, F120S, S121G, V122A, V122M, S126T, S126R, H129P, S130G, S132F, Q133H, E135K, F138L, T139S, C140D, C140del, S142F, I143V, I143T, N144D, Y146C, V151A, Y152C, Y152H, W153R, I154F, N155H, N155Q, K156M, D158G, L161P, L161M, L166Q, N168Q, F172S, L173S, M175T, T190S, T190A, S192G, V193M, N194D, C198R, N201S, L203P, L203F, N207Q, L208P, V210A, S212G, D217V, I218T, I218N, E220G, R221G, R221I, I224V, T225A, N227K or a conservative amino acid modification, e.g. substitution thereof. A conservative amino acid modification, e.g. substitution is any amino acid that falls in the same class of amino acids as the substituted amino acids, other than the wild-type or unmodified amino acid. The classes of amino acids are aliphatic (glycine, alanine, valine, leucine, and isoleucine), hydroxyl or sulfur-containing (serine, cysteine, threonine, and methionine), cyclic (proline), aromatic (phenylalanine, tyrosine, tryptophan), basic (histidine, lysine, and arginine), and acidic/amide (aspartate, glutamate, asparagine, and glutamine).

**[0185]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from M10V, M10I, V11E, S13G, E16V, S18R, A20V, S25G, F27S, F27C, N30D, Y33del, Q37R, K42E, T43A, Y47H, N52H, N52D, N52Q, N52S, N52Y, N52K, S54A, S54P, N57D, N57Y, R61S, R61C, Y62F, L67P, A71T, G72R, L74Q, R75Q, D77G, F78L, L80P, N84Q, D89G, E90A, K92R, F93L, H94E, H94D, L96F, L96I, V97A, L98F, S99G, Q100R, Q100K, Q100P, G103E, L102R, V107A, V107I, S109G, S109N, V110D, V110N, V110A, E111del, T113E, H115R, H115Q, V116A, A117T, N119Q, F120I, F120S, S121G, V122A, V122M, S126T, S126R, H129P, S130G, S132F, Q133H, E135K, F138L, T139S, C140D, C140del, S142F, I143V, I143T, N144D, Y146C, V151A, Y152C, Y152H, W153R, I154F, N155H, N155Q, K156M, D158G, L161P, L161M, L166Q, N168Q, F172S, L173S,

M175T, T190A, T190S, S192G, V193M, N194D, C198R, N201S, L203P, L203F, N207Q, L208P, V210A, S212G, D217V, I218T, I218N, E220G, R221G, R221I, I224V, T225A, N227K.

**[0186]** In some embodiments, the one or more amino acid modification, e.g. substitution is N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52S/Y146C/Y152C, N52H/C198R, N52H/C140D/T225A, N52H/C198R/T225A, N52H/K92R, N52H/S99G, N57Y/Q100P, N52S/G103E, N52S/S130G/Y152C, N52S/Y152C, N52S/C198R, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52S/T113E, N52D/S54P, N52K/L208P, N52S/Y152H, N52D/V151A, N52H/I143T, N52S/L80P, F120S/Y152H/N201S, N52S/R75Q/L203P, N52S/D158G, N52D/Q133H, N52S/N57Y/H94D/L96F/L98F/Q100R, N52S/N57Y/H94D/L96F/L98F/Q100R/G103E/F120S, N52H/F78L/Q100R, N52H/N57Y/Q100R/V110D, N52H/N57Y/R75Q/Q100R/V110D, N52H/N57Y/Q100R, N52H/N57Y/L74Q/Q100R/V110D, N52H/Q100R, N52H/S121G, A20V/N52H/N57Y/Q100R/S109G, N52H/N57Y/Q100P, N52H/N57Y/R61S/Q100R/V110D/L173S, N52H/N57Y/Q100R/V122A, N52H/N57Y/Q100R/F172S, N52H/N57Y, N52S/F120S, N52S/V97A, N52S/G72R, N52S/A71T/A117T, N52S/E220G, Y47H/N52S/V107A/F120S, N52H/N57Y/Q100R/V110D/S132F/M175T, E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R, Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G, N52H/N57Y/Q100R/V110D/C198R, N52H/N57Y/Q100R/V110D/V116A/L161M/F172S/S192G/C198R, F27S/N52H/N57Y/V110N, N52S/H94E/L96I/S109N/L166Q, S18R/N52S/F93L/I143V/R221G, A20T/N52D/Y146C/Q164L, V11E/N30D/N52H/N57Y/H94E/L96I/L98F/N194D/V210A/I218T, N52S/H94E/L96I/V122M, N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N, M10V/S18R/N30D/N52S/S126R/T139S/L203F, S25G/N30D/N52S/F120S/N227K, N30D/N52S/L67P/Q100K/D217G/R221K/T225S, N52H/N57Y/Q100R/V110D/A117T/T190S/C198R, N52H/N57Y/Q100R/V110D/F172S/C198R, S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R, N52H/N57Y/V110A/C198R/R221I, M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M, C198R, N52H/N57Y/R61C/Y62F/Q100R/V110N/F120S/C198R,

N52H/N57Y/Q100R/V110D/H115R/C198R,  
 N52H/N57Y/Q100R/V110D/N144D/F172S/C198R, N52S/H94E/L98F/Q100R, N52S/E90A,  
 N30D/K42E/N52S, N52S/F120S/I143V/I224V, N52H/ N57Y/Q100R/V110D/C198R/S212G,  
 N52H/N57Y/Q100R/C198R, N52S/N194D, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R,  
 N52S/S54P, T38P/N52S/N57D, N52H/C140del/T225A, N52H/F78L/Q100R/C198R,  
 N52H/N57Y/R75Q/Q100P/V110D, N52H/N57Y/L74Q/V110D/S192G, N52H/S121G/C198R,  
 N52S/F120S/N227K, N52S/A71T/A117T/T190A/C198R,  
 T43A/N52H/N57Y/L74Q/D89G/V110D/F172S, N52H/N57Y/Q100R/V110D/S132F/M175T,  
 N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G, Q100R, F138L/L203P,  
 N57Y/F138L/L203P, N57Y/Q100R/C198R, N57Y/F138L/L203P, Q100R/F138L, L203P,  
 N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/Q100R/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/F172S/C198R, N52H/N57Y/Q100R/H115R/I143V/F172S/C198R,  
 N52H/N57Y/Q100R/L102R/H115R/F172S/C198R, N52H/V122A/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/F172S/N194D, N52H/N57Y/H115R/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/H115R, N52H/N57Y/Q100R/H115R,  
 N52H/N57Y/Q100R/H115R/F172S/I224V, N52H/N57Y/Q100R/H115R/F172S,  
 N52H/N57Y/Q100R/F172S, N52H/Q100R/H115R/I143T/F172S,  
 N52H/N57Y/Q100P/H115R/F172S, N52Y/N57Y/Q100P/F172S,  
 E16V/N52H/N57Y/Q100R/V110D/H115R/C198R,  
 E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R, N52S/E90A/H115R,  
 N30D/K42E N52S/H115R, N30D/K42E/N52S/H115R/C198R/R221I,  
 N30D/K42E/N52S/H115R/C198R, N30D/K42E/N52S/H115R/F172S/N194D,  
 N52S/H115R/F120S/I143V/C198R, N52S/H115R/F172S/C198R, N52H/N57Y/Q100P/C198R,  
 N52H/N57Y/Q100P H115R/F172S/C198R, N52H/N57Y/Q100P/F172S/C198R,  
 N52H/N57Y/Q100P/H115R, N52H/N57Y/Q100P/H115R/C198R, N52H/Q100R/C198R,  
 N52H/Q100R/H115R/F172S, N52H/Q100R/F172S/C198R,  
 N52H/Q100R/H115Q/F172S/C198R, N52H/N57Y/Q100R /F172S/C198R, N52Q/N207Q,  
 N168Q/N207Q, N52Q/N168Q, N84Q/N207Q, N155Q/N207Q, N119Q/N168Q , N119Q/N207Q,  
 N119Q/N155Q, N52Q/N84Q, N52Q/N119Q, N84Q/N119Q, N52Q/N84Q/N168Q,  
 N52Q/N84Q/N207Q, N84Q/N155Q/N168Q, N84Q/N168Q/N207Q, N84Q/N155H/N207Q,  
 N155Q/N168Q/N207Q, N119Q/N155Q/N168Q, N119Q/N168Q/N207Q, N84Q/N119Q/N207Q,

N119Q/N155H/N207Q, N84Q/N119Q/N155Q, N52Q/N119Q/N155Q, N52H/N84Q/N119Q, N52H/N84Q, N52H/N84Q/N168Q, N52H/N84Q/N207Q, N52H/N84Q/N168Q/N207Q, N52Q/N84Q/N155Q, N52Q/N84Q/ N168Q, N52Q/N84Q/N155Q/N168Q, N52Q/N84Q/N119Q/N168Q, N84Q/N119Q/N155Q/N168Q, N84Q/N155Q/N168Q/N207Q, N84Q/N119Q/N155Q/N207Q, N52Q/N84Q/N119Q/N207Q, N52Q/N84Q/N119Q/N155Q, N52Q/N84Q/N119Q/N155Q/N207Q or, N84Q/N119Q/N155Q/N168Q/N207Q.

**[0187]** In some embodiments, the variant ICOSL polypeptide comprises any of the mutations listed in Table 1. Table 1 also provides exemplary sequences by reference to SEQ ID NO for the extracellular domain (ECD) or IgV domain of wild-type ICOSL or exemplary variant ICOSL polypeptides. As indicated, the exact locus or residues corresponding to a given domain can vary, such as depending on the methods used to identify or classify the domain. Also, in some cases, adjacent N- and/or C-terminal amino acids of a given domain (e.g. IgV) also can be included in a sequence of a variant IgSF polypeptide, such as to ensure proper folding of the domain when expressed. Thus, it is understood that the exemplification of the SEQ ID NOS in Table 1 is not to be construed as limiting. For example, the particular domain, such as the ECD domain, of a variant ICOSL polypeptide can be several amino acids longer or shorter, such as 1-10, e.g. 1, 2, 3, 4, 5, 6 or 7 amino acids longer or shorter, than the sequence of amino acids set forth in the respective SEQ ID NO.

**[0188]** In some embodiments, the variant ICOSL polypeptide comprises any of the mutations listed in Table 1. In some embodiments, the variant ICOSL polypeptide comprises any of the extracellular domain (ECD) sequences listed in Table 1 (i.e., any one of SEQ ID NOS: 109-142, 239, 280-325, 364-381, 387-424, 427-433, 435-470). In some embodiments, the variant ICOSL polypeptide comprises a polypeptide sequence that exhibits at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, such as at least 96% identity, 97% identity, 98% identity, or 99% identity to any of the extracellular domain (ECD) sequences listed in Table 1 (i.e., any one of SEQ ID NOS: 109-142, 239, 280-325, 364-381, 387-424, 427-433, 435-470) and contains the amino acid modification(s), e.g. substitution(s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of any of the extracellular domain (ECD) sequences listed in Table 1 (i.e., any one of SEQ ID NOS: 109-142, 239, 280-325, 364-381, 387-424, 427-433, 435-470) and contains the amino acid modification(s), e.g.

substitution (s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises any of the IgV sequences listed in Table 1 (i.e., any one of SEQ ID NOS: 197-199, 201-208, 210, 212, 240, 326-340, 382-386, 425-426, and 434). In some embodiments, the variant ICOSL polypeptide comprises a polypeptide sequence that exhibits at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, such as at least 96% identity, 97% identity, 98% identity, or 99% identity to any of the IgV sequences listed in Table 1 (i.e., any one of SEQ ID NOS: 197-199, 201-208, 210, 212, 240, 326-340, 382-386, 425-426, and 434) and contains the amino acid modification(s), e.g. substitution(s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of any of the IgV sequences listed in Table 1 (i.e., any one of SEQ ID NOS: 197-199, 201-208, 210, 212, 240, 326-340, 382-386, 425-426, and 434) and contains the amino acid substitution(s) not present in the wild-type or unmodified ICOSL. Mutations designated with an “X” indicate the designated position contains a Q or the wildtype residue set forth in the corresponding position of SEQ ID NO: 32.

| <b>TABLE 1: Exemplary variant ICOSL polypeptides</b> |                                  |                              |
|------------------------------------------------------|----------------------------------|------------------------------|
| <b>Mutation(s)</b>                                   | <b>ECD<br/>SEQ<br/>ID<br/>NO</b> | <b>IgV<br/>SEQ ID<br/>NO</b> |
| Wild-type                                            | 32                               | 196                          |
| N52S                                                 | 109                              | 197                          |
| N52H                                                 | 110                              | 198                          |
| N52D                                                 | 111                              | 199                          |
| N52Y/N57Y/F138L/L203P                                | 112                              |                              |
| N52H/N57Y/Q100P                                      | 113                              | 201                          |
| N52S/Y146C/Y152C                                     | 114                              |                              |
| N52H/C198R                                           | 115                              |                              |
| N52H/C140D/T225A                                     | 116                              |                              |
| N52H/C198R/T225A                                     | 117                              |                              |
| N52H/K92R                                            | 118                              | 202                          |
| N52H/S99G                                            | 119                              | 203                          |
| N52Y                                                 | 120                              | 204                          |
| N57Y                                                 | 121                              | 205                          |
| N57Y/Q100P                                           | 122                              | 206                          |
| N52S/S130G/Y152C                                     | 123                              |                              |

| <b>TABLE 1: Exemplary variant ICOSL polypeptides</b> |                                  |                              |
|------------------------------------------------------|----------------------------------|------------------------------|
| <b>Mutation(s)</b>                                   | <b>ECD<br/>SEQ<br/>ID<br/>NO</b> | <b>IgV<br/>SEQ ID<br/>NO</b> |
| N52S/Y152C                                           | 124                              |                              |
| N52S/C198R                                           | 125                              |                              |
| N52Y/N57Y/Y152C                                      | 126                              |                              |
| N52Y/N57Y/H129P/C198R                                | 127                              |                              |
| N52H/L161P/C198R                                     | 128                              |                              |
| N52S/T113E                                           | 129                              |                              |
| S54A                                                 | 130                              | 207                          |
| N52D/S54P                                            | 131                              | 208                          |
| N52K/L208P                                           | 132                              |                              |
| N52S/Y152H                                           | 133                              |                              |
| N52D/V151A                                           | 134                              |                              |
| N52H/I143T                                           | 135                              |                              |
| N52S/L80P                                            | 136                              | 210                          |
| F120S/Y152H/N201S                                    | 137                              |                              |
| N52S/R75Q/L203P                                      | 138                              |                              |
| N52S/D158G                                           | 139                              |                              |
| N52D/Q133H                                           | 140                              |                              |
| N52S/N57Y/H94D/L96F/L98F/Q100R                       | 141                              | 212                          |
| N52S/N57Y/H94D/L96F/L98F/Q100R/G103E/F120S           | 142                              |                              |
| N52S/G103E                                           | 239                              | 240                          |
| N52H/F78L/Q100R                                      | 280                              | 326                          |
| N52H/N57Y/Q100R/V110D                                | 281                              | 327                          |
| N52H/N57Y/R75Q/Q100R/V110D                           | 282                              | 328                          |
| N52H/N57Y/Q100R                                      | 283                              | 329                          |
| N52H/N57Y/L74Q/Q100R/V110D                           | 284                              | 330                          |
| N52H/Q100R                                           | 285                              | 331                          |
| N52H/S121G                                           | 286                              |                              |
| A20V/N52H/N57Y/Q100R/S109G                           | 287                              | 332                          |
| N52H/N57Y/Q100P                                      | 288                              | 333                          |
| N52H/N57Y/R61S/Q100R/V110D/L173S                     | 289                              |                              |
| N52H/N57Y/Q100R/V122A                                | 290                              |                              |
| N52H/N57Y/Q100R/F172S                                | 291                              |                              |
| N52H/N57Y                                            | 292                              | 334                          |
| N52S/F120S                                           | 293                              |                              |
| N52S/V97A                                            | 294                              | 335                          |
| N52S/G72R                                            | 295                              | 336                          |
| N52S/A71T/A117T                                      | 296                              |                              |
| N52S/E220G                                           | 297                              |                              |
| Y47H/N52S/V107A/F120S                                | 298                              |                              |

| <b>TABLE 1: Exemplary variant ICOSL polypeptides</b>         |                                  |                              |
|--------------------------------------------------------------|----------------------------------|------------------------------|
| <b>Mutation(s)</b>                                           | <b>ECD<br/>SEQ<br/>ID<br/>NO</b> | <b>IgV<br/>SEQ ID<br/>NO</b> |
| N52H/N57Y/Q100R/V110D/S132F/M175T                            | 299                              |                              |
| E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R           | 300                              |                              |
| Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G           | 301                              |                              |
| N52H/N57Y/Q100R/V110D/C198R                                  | 302                              |                              |
| N52H/N57Y/Q100R/V110D/V116A/L161M/F172S/S192G/C198R          | 303                              |                              |
| F27S/N52H/N57Y/V110N                                         | 304                              | 337                          |
| N52S/H94E/L96I/S109N/L166Q                                   | 305                              |                              |
| S18R/N52S/F93L/I143V/R221G                                   | 306                              |                              |
| A20T/N52D/Y146C/Q164L                                        | 307                              |                              |
| V11E/N30D/N52H/N57Y/H94E/L96I/L98F/N194D/V210A/I218T         | 308                              |                              |
| N52S/H94E/L96I/V122M                                         | 309                              |                              |
| N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N                  | 310                              |                              |
| M10V/S18R/N30D/N52S/S126R/T139S/L203F                        | 311                              |                              |
| S25G/N30D/N52S/F120S/N227K                                   | 312                              |                              |
| N30D/N52S/L67P/Q100K/D217G/R221K/T225S                       | 313                              |                              |
| N52H/N57Y/Q100R/V110D/A117T/T190S/C198R                      | 314                              |                              |
| N52H/N57Y/Q100R/V110D/F172S/C198R                            | 315                              |                              |
| S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R            | 316                              |                              |
| N52H/N57Y/V110A/C198R/R221I                                  | 317                              |                              |
| M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M/C198R | 318                              |                              |
| N52H/N57Y/R61C/Y62F/Q100R/V110N/F120S/C198R                  | 319                              |                              |
| N52H/N57Y/Q100R/V110D/H115R/C198R                            | 320                              |                              |
| N52H/N57Y/Q100R/V110D/N144D/F172S/C198R                      | 321                              |                              |
| N52S/H94E/L98F/Q100R                                         | 322                              | 338                          |
| N52S/E90A                                                    | 323                              | 339                          |
| N30D/K42E/N52S                                               | 324                              | 340                          |
| N52S/F120S/I143V/I224V                                       | 325                              |                              |
| N52H/ N57Y/Q100R/V110D/C198R/S212G                           | 364                              |                              |
| N52H/N57Y/Q100R/C198R                                        | 365                              |                              |
| N52S/N194D                                                   | 366                              |                              |
| N52H/N57Y/Q100R/L102R/V110D/H115R/C198R                      | 367                              |                              |
| N52S/S54P                                                    | 368                              | 382                          |
| T38P/N52S/N57D                                               | 369                              | 383                          |
| E111del                                                      | 370                              | 384                          |
| Y33del                                                       | 371                              | 385                          |
| N52H/C140del/T225A                                           | 372                              |                              |
| N52H/F78L/Q100R/C198R                                        | 373                              |                              |
| N52H/N57Y/R75Q/Q100P/V110D                                   | 374                              | 386                          |
| N52H/N57Y/L74Q/V110D/S192G                                   | 375                              |                              |

| <b>TABLE 1: Exemplary variant ICOSL polypeptides</b> |                                  |                              |
|------------------------------------------------------|----------------------------------|------------------------------|
| <b>Mutation(s)</b>                                   | <b>ECD<br/>SEQ<br/>ID<br/>NO</b> | <b>IgV<br/>SEQ ID<br/>NO</b> |
| N52H/S121G/C198R                                     | 376                              |                              |
| N52S/F120S/N227K                                     | 377                              |                              |
| N52S/A71T/A117T/T190A/C198R                          | 378                              |                              |
| T43A/N52H/N57Y/L74Q/D89G/V110D/F172S                 | 379                              |                              |
| N52H/N57Y/Q100R/V110D/S132F/M175T                    | 380                              |                              |
| N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G        | 381                              |                              |
| N84Q                                                 | 387                              | 425                          |
| N119Q                                                | 388                              |                              |
| N168Q                                                | 389                              |                              |
| N207Q                                                | 390                              |                              |
| N52Q/N207X                                           | 391                              |                              |
| N168X/N207X                                          | 392                              |                              |
| N52Q/N168Q                                           | 393                              |                              |
| N84Q/N207Q                                           | 394                              |                              |
| N155Q/N207Q                                          | 395                              |                              |
| N119Q/N168Q                                          | 396                              |                              |
| N119Q/N207Q                                          | 397                              |                              |
| N119Q/N155X                                          | 398                              |                              |
| N52Q/N84Q                                            | 399                              | 426                          |
| N52Q/N119Q                                           | 400                              |                              |
| N84Q/N119Q                                           | 401                              |                              |
| N52Q/N84Q/N168Q                                      | 402                              |                              |
| N52Q/N84Q/N207Q                                      | 403                              |                              |
| N84Q/N155Q/N168Q                                     | 404                              |                              |
| N84Q/N168Q/N207Q                                     | 405                              |                              |
| N84Q/N155H/N207Q                                     | 406                              |                              |
| N155Q/N168Q/N207Q                                    | 407                              |                              |
| N119Q N155Q/N168Q                                    | 408                              |                              |
| N119Q/N168Q/N207Q                                    | 409                              |                              |
| N84Q/N119Q/N207Q                                     | 410                              |                              |
| N119Q/N155H/N207Q                                    | 411                              |                              |
| N84Q/N119Q/N155Q                                     | 412                              |                              |
| N52Q/N119Q/N155Q                                     | 413                              |                              |
| N52H/N84Q/N119Q                                      | 414                              |                              |
| N52H/N84Q/N168X/N207X                                | 415                              |                              |
| N52Q/N84Q/N155X/N168X                                | 416                              |                              |
| N52Q/N84Q/N119Q/N168Q                                | 417                              |                              |
| N84Q/N119Q/N155Q/N168Q                               | 418                              |                              |
| N84Q/N155Q/N168Q/N207Q                               | 419                              |                              |

| <b>TABLE 1: Exemplary variant ICOSL polypeptides</b>     |                                  |                              |
|----------------------------------------------------------|----------------------------------|------------------------------|
| <b>Mutation(s)</b>                                       | <b>ECD<br/>SEQ<br/>ID<br/>NO</b> | <b>IgV<br/>SEQ ID<br/>NO</b> |
| N84Q/N119Q/N155Q/N207Q                                   | 420                              |                              |
| N52Q/N84Q/N119Q/N207Q                                    | 421                              |                              |
| N52Q/N84Q/N119Q/N155Q                                    | 422                              |                              |
| N52Q/N84Q/N119Q/N155Q/N207Q                              | 423                              |                              |
| N84Q/N119Q/N155Q/N168Q/N207Q                             | 424                              |                              |
| Q100R                                                    | 427                              | 434                          |
| F138L/L203P                                              | 428                              |                              |
| N52Y/F138L/L203P                                         | 429                              |                              |
| N57Y/Q100R/C198R                                         | 430                              |                              |
| N57Y/F138L/L203P                                         | 431                              |                              |
| Q100R/F138L                                              | 432                              |                              |
| L203P                                                    | 433                              |                              |
| N52H/N57Y/Q100R/H115R/C198R                              | 435                              |                              |
| N52H/N57Y/Q100R/F172S/C198R                              | 436                              |                              |
| N52H/N57Y/Q100R/H115R/F172S/C198R                        | 437                              |                              |
| N52H/N57Y/Q100R/H115R/I143V/F172S/C198R                  | 438                              |                              |
| N52H/N57Y/Q100R/L102R/H115R/F172S/C198R                  | 439                              |                              |
| N52H/V122A/F172S/C198R                                   | 440                              |                              |
| N52H/N57Y/Q100R/H115R/F172S/N194D                        | 441                              |                              |
| N52H/N57Y/H115R/F172S/C198R                              | 442                              |                              |
| N52H/N57Y/Q100R/H115R/C198R                              | 443                              |                              |
| N52H/N57Y/H115R                                          | 444                              |                              |
| N52H/N57Y/Q100R/H115R                                    | 445                              |                              |
| N52H/N57Y/Q100R/H115R/F172S/I224V                        | 446                              |                              |
| N52H/N57Y/Q100R/H115R/F172S                              | 447                              |                              |
| N52H/N57Y/Q100R/F172S                                    | 448                              |                              |
| N52H/Q100R/H115R/I143T/F172S                             | 449                              |                              |
| N52H/N57Y/Q100P/H115R/F172S                              | 450                              |                              |
| N52Y/N57Y/Q100P/F172S                                    | 451                              |                              |
| E16V/N52H/N57Y/Q100R/V110D/H115R/C198R                   | 452                              |                              |
| E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R | 453                              |                              |
| N52S/E90A/H115R                                          | 454                              |                              |
| N30D/K42E N52S/H115R                                     | 455                              |                              |
| N30D/K42E/N52S/H115R/C198R/R221I                         | 456                              |                              |
| N30D/K42E/N52S/H115R/C198R                               | 457                              |                              |
| N30D/K42E/N52S/H115R/F172S/N194D                         | 458                              |                              |
| N52S/H115R/F120S/I143V/C198R                             | 459                              |                              |
| N52S/H115R/F172S/C198R                                   | 460                              |                              |
| N52H/N57Y/Q100P/C198R                                    | 461                              |                              |

| <b>TABLE 1: Exemplary variant ICOSL polypeptides</b> |                                  |                              |
|------------------------------------------------------|----------------------------------|------------------------------|
| <b>Mutation(s)</b>                                   | <b>ECD<br/>SEQ<br/>ID<br/>NO</b> | <b>IgV<br/>SEQ ID<br/>NO</b> |
| N52H/N57Y/Q100P H115R/F172S/C198R                    | 462                              |                              |
| N52H/N57Y/Q100P/F172S/C198R                          | 463                              |                              |
| N52H/N57Y/Q100P/H115R                                | 464                              |                              |
| N52H/N57Y/Q100P/H115R/C198R                          | 465                              |                              |
| N52H/Q100R/C198R                                     | 466                              |                              |
| N52H/Q100R/H115R/F172S                               | 467                              |                              |
| N52H/Q100R/H115X/F172S/C198R                         | 468                              |                              |
| N52H/Q100R/H115R/F172S/C198R                         | 469                              |                              |
| N52H/N57Y/Q100R/F172S/C198R                          | 470                              |                              |

**[0189]** In some embodiments, the variant ICOSL polypeptide exhibits increased affinity for the ectodomain of CD28 compared to the wild-type or unmodified ICOSL polypeptide, such as comprising the sequence set forth in SEQ ID NO:32 or 196. In some embodiments, the ICOSL polypeptide exhibits increased affinity for the ectodomain of ICOS compared to the wild-type or unmodified ICOSL, such as comprising the sequence set forth in SEQ ID NO:32 or 196. In some embodiments, the ICOSL polypeptide exhibits increased affinity for the ectodomain of CD28 and the ectodomain of ICOS compared to the wild-type or unmodified ICOSL, such as comprising the sequence set forth in SEQ ID NO:32 or 196.

**[0190]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution corresponding to position(s) 52, 54 or 57. In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from N52H, N52D, N52Q, N52S, N52Y, N52K, S54A, S54P, or N57Y or a conservative amino acid modification, e.g. substitution thereof. In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from N52H, N52D, N52S, N52K or N57Y or a conservative amino acid modification, e.g. substitution thereof.

**[0191]** In some embodiments, the variant ICOSL polypeptide can contain one or more further amino acid modification, e.g. substitution in addition to an amino acid modification, e.g. substitution at a position corresponding to position 52, 54 or 57. In some embodiments, the one or more further amino acid modification, e.g. substitution is at a position corresponding to 10, 11,

13, 16, 18, 20, 25, 27, 30, 37, 42, 43, 47, 52, 54, 57, 61, 62, 67, 71, 72, 74, 75, 77, 78, 80, 84, 89, 90, 92, 93, 94, 96, 97, 98, 99, 100, 102, 103, 107, 109, 110, 113, 115, 116, 117, 119, 120, 121, 122, 126, 129, 130, 132, 133, 135, 138, 139, 140, 142, 143, 144, 146, 151, 152, 153, 154, 155, 156, 158, 161, 166, 168, 172, 173, 175, 190, 192, 193, 194, 198, 201, 203, 207, 208, 210, 212, 217, 218, 220, 221, 224, 225 or 227. In some embodiments, the variant ICOSL contains one or more further amino acid modification, e.g. substitution selected from M10V, M10I, V11E, S13G, E16V, S18R, A20V, S25G, F27S, F27C, N30D, Y33del, Q37R, K42E, T43A, Y47H, N52H, N52D, N52S, N52Y, N52K, N52Q, S54A, S54P, N57D, N57Y, R61S, R61C, Y62F, L67P, A71T, G72R, L74Q, R75Q, D77G, F78L, L80P, N84Q, D89G, E90A, K92R, F93L, H94E, H94D, L96F, L96I, V97A, L98F, S99G, Q100R, Q100K, Q100P, L102R, G103E, V107A, V107I, S109G, S109N, V110D, V110N, V110A, E111del, T113E, H115R, H115Q, V116A, A117T, N119Q, F120I, F120S, S121G, V122A, V122M, S126T, S126R, H129P, S130G, S132F, Q133H, E135K, F138L, T139S, , C140del, S142F, I143V, I143T, N144D, Y146C, V151A, Y152C, Y152H, W153R, I154F, K156M, D158G, L161P, L161M, L166Q, N168Q, F172S, L173S, M175T, T190A, T190S, S192G, V193M, N194D, C198R, N201S, L203P, L203F, N207Q, L208P, V210A, S212G, D217V, I218T, I218N, E220G, R221G, R221I, I224V, T225A, N227K, or a conservative amino acid substitution thereof.

**[0192]** In some embodiments of any one of the variant ICOSL polypeptides described above, the variant ICOSL polypeptide further comprises one or more amino acid deletions corresponding to positions 140 of SEQ ID NO: 32.

**[0193]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52S/Y146C/Y152C, N52H/C198R, N52H/C140del/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52S/C198R, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52S/T113E, N52S/S54P, N52K/L208P, N52S/Y152H, N52H/I143T, N52S/R75Q/L203P, N52S/D158G, N52D/Q133H, N52H/ N57Y/Q100R/V110D/C198R/S212G, N52H/N57Y/Q100R/C198R, N52S/N194D, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R, N52S/S54P, T38P/N52S/N57D, N52H/C140del/T225A, N52H/F78L/Q100R/C198R, N52H/N57Y/R75Q/Q100P/V110D, N52H/N57Y/L74Q/V110D/S192G, N52H/S121G/C198R, N52S/F120S/N227K, N52S/A71T/A117T/T190A/C198R, T43A/N52H/N57Y/L74Q/D89G/V110D/F172S, N52H/N57Y/Q100R/V110D/S132F/M175T,

N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G, N52Q/N207Q, N52Q/N168Q,  
 N52Q/N84Q, N52Q/N119Q, N52Q/N84Q/N168Q, N52Q/N84Q/N207Q,  
 N52Q/N119Q/N155Q, N52H/N84Q/N119Q, N52H/N84Q, N52H/N84Q/N168Q,  
 N52H/N84Q/N207Q, N52H/N84Q/N168Q/N207Q, N52Q/N84Q/N155Q, N52Q/N84Q/N168Q,  
 N52Q/N84Q/N155Q/N168Q, N52Q/N84Q/N119Q/N168Q, N52Q/N84Q/N119Q/N207Q,  
 N52Q/N84Q/N119Q/N155Q, N52Q/N84Q/N119Q/N155Q/N207Q, N52Y/F138L/L203P,  
 N57Y/Q100R/C198R, N57Y/F138L/L203P, N52H/N57Y/Q100R/H115R/C198R,  
 N52H/N57Y/Q100R/F172S/C198R, N52H/N57Y/Q100R/H115R/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/I143V/F172S/C198R,  
 N52H/N57Y/Q100R/L102R/H115R/F172S/C198R, N52H/V122A/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/F172S/N194D, N52H/N57Y/H115R/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/H115R, N52H/N57Y/Q100R/H115R,  
 N52H/N57Y/Q100R/H115R/F172S/I224V, N52H/N57Y/Q100R/H115R/F172S,  
 N52H/N57Y/Q100R/F172S, N52H/Q100R/H115R/I143T/F172S,  
 N52H/N57Y/Q100P/H115R/F172S, N52Y/N57Y/Q100P/F172S,  
 E16V/N52H/N57Y/Q100R/V110D/H115R/C198R,  
 E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R, N52S/E90A/H115R,  
 N30D/K42E/N52S/H115R, N30D/K42E/N52S/H115R/C198R/R221I,  
 N30D/K42E/N52S/H115R/C198R, N30D/K42E/N52S/H115R/F172S/N194D,  
 N52S/H115R/F120S/I143V/C198R, N52S/H115R/F172S/C198R, N52H/N57Y/Q100P/C198R,  
 N52H/N57Y/Q100P H115R/F172S/C198R, N52H/N57Y/Q100P/F172S/C198R,  
 N52H/N57Y/Q100P/H115R, N52H/N57Y/Q100P/H115R/C198R, N52H/Q100R/C198R,  
 N52H/Q100R/H115R/F172S, N52H/Q100R/ F172S/C198R,  
 N52H/Q100R/H115R/F172S/C198R, or N52H/N57Y/Q100R/F172S/C198R.

**[0194]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid  
 modification, e.g. substitution selected from N52H/N57Y/Q100R/C198R,  
 N52H/N57Y/Q100R/V122A, N52H/N57Y/Q100R/F172S, N52Y/N57Y/F138L/L203P,  
 V11E/N30D/N52H/N57Y/H94E/L96I/L98F/N194D/V210A/I218T, N52H/N57Y/Q100R/L102R  
 /V110D/H115R/C198R, N52H/N57Y/Q100R, N52H/Q100R,  
 N52H/N57Y/Q100R/V110D/C198R/S212G,  
 N52H/N57Y/Q100R/L102R/V110D/H115R/C198R,

E16V/N52H/N57Y/Q100R/V110D/H115R/V152C/K156M/C198R, N30D/K42E/N52S, N52S/F120S/I143V/I224V, N52S/E90A, N52H/N57Y/V110A/C198R/R221I, N52H/N57Y/Q100P, or N52S/N194D.

**[0195]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from N52H/N57Y/Q100R/F172S, N52H/Q100R, N52H/N57Y/Q100R/C198R. In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R, N52H/N57Y/Q100R, and N52H/N57Y/Q100P.

**[0196]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from N52H/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52H/K92R, N52H/C140del/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52K/L208P or N52H/I143T.

**[0197]** In some embodiments, the variant ICOSL polypeptide exhibits increased binding affinity for binding one of the ectodomains of CD28 or ICOS and exhibits decreased binding affinity for binding to the other of the ectodomains of CD28 or ICOS compared to the wild-type or unmodified ICOSL polypeptide, such as comprising the sequence set forth in SEQ ID NO:32 or 196.

**[0198]** In some embodiments, the variant ICOSL polypeptide exhibits increased binding affinity for ICOS and exhibits decreased binding affinity for CD28. In some embodiments, the one or more further amino acid substitution is at a position corresponding to 52, 57, 80 100, 130, 152, 161 or 198. In some embodiments, the variant ICOSL contains one or more amino acid substitutions selected from N52S, N52H, N52Y, N52H, N57Y, L80P, Q100P Q100R, Q100K, V110D, S130G, Y152C, L161P, L161M, C198R, R221G, or a conservative amino acid substitution thereof. In some embodiments, the variant ICOSL polypeptide has one or more amino acid substitutions selected from N57Y/Q100P, N52S/S130G/Y152C, N52S/Y152C, N52Y/N57Y/Y152C, N52H/L161P/C198R, N52H/L161P/C198R, N52S/L80P, A20V/N52H/N57Y/Q100R/S109G, N52H/N57Y/R61S/Q100R/V110D/L173S, N52H/N57Y/Q100R/V107I/V110D/S132F/I154F/C198R/R221G, Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G, N52H/N57Y/Q100R/V110D/C198R, F27S/N52H/N57Y/V110N,

S18R/N52S/F93L/I143V/R221G, A20T/N52D/Y146C/Q164L,  
N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N,  
N52H/N57Y/Q100R/V110D/F172S/C198R,  
S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R,  
M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M/C198R,.

**[0199]** In some embodiments, the variant ICOSL polypeptide exhibits increased binding affinity for CD28 and exhibits decreased binding affinity for ICOS. In some embodiments, the one or more amino acid substitution is at a position corresponding to 52, 75 or 203. In some embodiments, the variant ICOSL contains one or more amino acid substitution selected from N52S, R75Q, L203F, or L203P. In some embodiments, the variant ICOSL polypeptide has amino acid substitutions N52S/R75Q/L203P.

**[0200]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution in an unmodified ICOSL or specific binding fragment thereof corresponding to position(s) 16, 30, 42, 52, 57, 90, 100, 102, 110, 115, 120, 122, 138, 143, 152, 156, 172, 194, 198, 203, 221, or 224 with reference to numbering of SEQ ID NO:32. In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from E16V, N30D, K42E, N52H, N52Y, N52S, N57Y, E90A, Q100R, Q100P, L102R, V110D, H115R, F120S, V122A, F138L, I143V, I143T, H152C, K156M, F172S, N194D, C198R, L203P, R221I, or I224V. In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution in an unmodified ICOSL or specific binding fragment thereof corresponding to position(s) 115, 172, or 198 with reference to numbering of SEQ ID NO:32. In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from H115R, F172S or C198R. In some embodiments, the one or more amino acid modification, e.g. substitution is

N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/Q100R/F172S/C198R,  
N52H/N57Y/Q100R/H115R/F172S/C198R, N52H/N57Y/Q100R/H115R/I143V/F172S/C198R,  
N52H/N57Y/Q100R/L102R/H115R/F172S/C198R, N52H/V122A/F172S/C198R,  
N52H/N57Y/Q100R/H115R/F172S/N194D, N52H/N57Y/H115R/F172S/C198R,  
N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/H115R, N52H/N57Y/Q100R/H115R,  
N52H/N57Y/Q100R/H115R/F172S/I224V, N52H/N57Y/Q100R/H115R/F172S,  
N52H/N57Y/Q100R/F172S, N52H/Q100R/H115R/I143T/F172S,

N52H/N57Y/Q100P/H115R/F172S, N52Y/N57Y/Q100P/F172S, E16V/N52H/N57Y/Q100R/V110D/H115R/C198R, E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R, N52S/E90A/H115R, N30D/K42E N52S/H115R, N30D/K42E/N52S/H115R/C198R/R221I, N30D/K42E/N52S/H115R/C198R, N30D/K42E/N52S/H115R/F172S/N194D, N52S/H115R/F120S/I143V/C198R, N52S/H115R/F172S/C198R, N52H/N57Y/Q100P/C198R, N52H/N57Y/Q100P H115R/F172S/C198R, N52H/N57Y/Q100P/F172S/C198R, N52H/N57Y/Q100P/H115R, N52H/N57Y/Q100P/H115R/C198R, N52H/Q100R/C198R, N52H/Q100R/H115R/F172S, N52H/Q100R/F172S/C198R, N52H/Q100R/H115R/F172S/C198R or N52H/N57Y/Q100R /F172S/C198R. In some embodiments, the variant ICOSL polypeptides exhibit potentially enhanced protein solubility or enhanced protein expression ('solubility mutations') compared to the wild-type or unmodified ICOSL polypeptide.

**[0201]** In some embodiments, the variant ICOSL polypeptide comprises any of the extracellular domain (ECD) sequences set forth in SEQ ID NOS: 435-470. In some embodiments, the variant ICOSL polypeptide comprises a polypeptide sequence that exhibits at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, such as at least 96% identity, 97% identity, 98% identity, or 99% identity to any of the extracellular domain (ECD) set forth in SEQ ID NOS: 435-470 and contains the amino acid modification(s), e.g. substitution(s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of any of the extracellular domain (ECD) sequences set forth in SEQ ID NOS: 435-470 and contains the amino acid modification(s), e.g. substitution (s) not present in the wild-type or unmodified ICOSL.

**[0202]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution in an unmodified ICOSL or specific binding fragment thereof corresponding to position(s) 52, 57, 100, 138, 198, or 203 with reference to numbering of SEQ ID NO:32. In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from N52H, N52Y, N57Y, Q100R, Q100P, F138L, C198R, or L203P. In some embodiments, the one or more amino acid modification, e.g. substitution is Q100R, F138L/L203P, N52Y/F138L/L203P, N57Y/Q100R/C198R, N57Y/F138L/L203P, N52H, N57Y, N57Y/Q100P, Q100R/F138L, or L203P.

**[0203]** In some embodiments, the variant ICOSL polypeptide comprises any of the extracellular domain (ECD) sequences set forth in SEQ ID NOS: 427-433. In some embodiments, the variant ICOSL polypeptide comprises a polypeptide sequence that exhibits at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, such as at least 96% identity, 97% identity, 98% identity, or 99% identity to any of the extracellular domain (ECD) set forth in SEQ ID NOS: 427-433 and contains the amino acid modification(s), e.g. substitution(s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of any of the extracellular domain (ECD) sequences set forth in SEQ ID NOS: 427-433 and contains the amino acid modification(s), e.g. substitution (s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises the IgV sequence set forth in SEQ ID NO: 434. In some embodiments, the variant ICOSL polypeptide comprises a polypeptide sequence that exhibits at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, such as at least 96% identity, 97% identity, 98% identity, or 99% identity to the IgV sequence set forth in SEQ ID NO: 434 and contains the amino acid modification(s), e.g. substitution(s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of the IgV sequence set forth in SEQ ID NO: 434 and contains the amino acid substitution(s) not present in the wild-type or unmodified ICOSL.

**[0204]** In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution in an unmodified ICOSL or specific binding fragment thereof corresponding to position(s) 52, 84, 91, 119, 155, 168, 207 with reference to numbering of SEQ ID NO:32. In some embodiments, the variant ICOSL polypeptide has one or more amino acid modification, e.g. substitution selected from A91S, N52H, N52Q, N84Q, N119Q, N155H, N155Q, N168Q, N207Q. In some embodiments, the one or more amino acid modification, e.g. substitution is N84Q, N119Q, N168Q, N207Q, N52Q, N52Q/N207Q, N168Q/N207Q, N52Q/N168Q, N84Q/N207Q, N155Q/N207Q, N119Q/N168Q, N119Q/N207Q, N119Q/N155Q, N52Q/N84Q, N52Q/N119Q, N84Q/N119Q, N52Q/N84Q/N168Q, N52Q/N84Q/N207Q, N84Q/N155Q/N168Q, N84Q/N168Q/N207Q, N84Q/N155H/N207Q, N155Q/N168Q/N207Q, N119Q N155Q/N168Q, N119Q/N168Q/N207Q, N84Q/N119Q/N207Q, N119Q/N155H/N207Q,

N84Q/N119Q/N155Q, N52Q/N119Q/N155Q, N52H/N84Q/N119Q, N52H/N84Q, N52H/N84Q/N168Q, N52H/N84Q/N207Q, N52H/N84Q/N168Q/N207Q, N52Q/N84Q/N155Q, N52Q/N84Q/N168Q, N52Q/N84Q/N155Q/N168Q, N52Q/N84Q/N119Q/N168Q, N84Q/N119Q/N155Q/N168Q, N84Q/N155Q/N168Q/N207Q, N84Q/N119Q/N155Q/N207Q, N52Q/N84Q/N119Q/N207Q, N52Q/N84Q/N119Q/N155Q, N52Q/N84Q/N119Q/N155Q/N207Q, N84Q/N119Q/N155Q/N168Q/N207Q or A91S/N119Q/N168Q/N207Q. In some embodiments, the variant ICOSL polypeptides exhibit potentially reduced glycosylation compared to the wild-type or unmodified ICOSL polypeptide.

**[0205]** In some embodiments, the variant ICOSL polypeptide comprises any of the extracellular domain (ECD) sequences set forth in SEQ ID NOS: 387-424, 427-433, 435-470. In some embodiments, the variant ICOSL polypeptide comprises a polypeptide sequence that exhibits at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, such as at least 96% identity, 97% identity, 98% identity, or 99% identity to any of the extracellular domain (ECD) set forth in SEQ ID NOS: 387-424, 427-433, 435-470 and contains the amino acid modification(s), e.g. substitution(s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of any of the extracellular domain (ECD) sequences set forth in SEQ ID NOS: 387-424, 427-433, 435-470 and contains the amino acid modification(s), e.g. substitution (s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises any of the IgV sequences set forth in SEQ ID NOS: 425-426. In some embodiments, the variant ICOSL polypeptide comprises a polypeptide sequence that exhibits at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, such as at least 96% identity, 97% identity, 98% identity, or 99% identity to any of the IgV sequences set forth in SEQ ID NO: 425-426 and contains the amino acid modification(s), e.g. substitution(s) not present in the wild-type or unmodified ICOSL. In some embodiments, the variant ICOSL polypeptide comprises a specific binding fragment of any of the IgV sequences set forth in SEQ ID NO: 425-426 and contains the amino acid substitution(s) not present in the wild-type or unmodified ICOSL.

### **III. FORMATS OF VARIANT POLYPEPTIDES**

**[0206]** The immunomodulatory polypeptide comprising a variant ICOSL provided herein in which is contained a vIgD can be formatted in a variety of ways, including as a soluble protein, membrane bound protein, secreted protein, conjugate or fusion or for expression by an infectious agent. In some embodiments, the particular format can be chosen for the desired therapeutic application. In some cases, an immunomodulatory polypeptide comprising a variant ICOSL polypeptide is provided in a format to antagonize or block activity of its cognate binding partner, e.g. CD28. In some embodiments, antagonism of CD28 may be useful to treat inflammation or autoimmunity. In some cases, an immunomodulatory polypeptide comprising a variant ICOSL polypeptide is provided in a format to agonize or stimulate activity of its cognate binding partner, e.g. CD28. In some embodiments, agonism of CD28 may be useful for treating oncology indications. A skilled artisan can readily determine the activity of a particular format, such as for antagonizing or agonizing one or more specific cognate binding partner. Exemplary methods for assessing such activities are provided herein, including in the examples.

**[0207]** In some aspects, provided are immunomodulatory proteins comprising a vIgD of ICOSL in which such proteins are soluble, e.g. fused to an Fc chain. In some aspects, one or more additional IgSF domain, such as one or more additional vIgD, may be linked to a vIgD of ICOSL as provided herein (hereinafter called a “stack” or “stacked” immunomodulatory protein). In some embodiments, the modular format of the provided immunomodulatory proteins provides flexibility for engineering or generating immunomodulatory proteins for modulating activity of multiple counterstructures (multiple cognate binding partners). In some embodiments, such “stack” molecules can be provided in a soluble format or, in some cases, may be provided as membrane bound or secreted proteins. In some embodiments, a variant ICOSL immunomodulatory protein is provided as a conjugate in which is contained a vIgD of ICOSL linked, directly or indirectly, to a targeting agent or moiety, e.g. to an antibody or other binding molecules that specifically binds to a ligand, e.g. an antigen, for example, for targeting or localizing the vIgD to a specific environment or cell, such as when administered to a subject. In some embodiments, the targeting agent, e.g. antibody or other binding molecule, binds to a tumor antigen, thereby localizing the variant ICOSL containing the vIgD to the tumor microenvironment, for example, to modulate activity of tumor infiltrating lymphocytes (TILs) specific to the tumor microenvironment.

**[0208]** In some embodiments, provided immunomodulatory proteins are expressed in cells and provided as part of an engineered cellular therapy (ECT). In some embodiments, the variant ICOSL polypeptide is expressed in a cell, such as an immune cell (e.g. T cell or antigen presenting cell), in membrane-bound form, thereby providing a transmembrane immunomodulatory protein (hereinafter also called a “TIP”). In some embodiments, depending on the cognate binding partner recognized by the TIP, engineered cells expressing a TIP can agonize a cognate binding partner by providing a costimulatory signal, either positive to negative, to other engineered cells and/or to endogenous T cells. In some aspects, the variant ICOSL polypeptide is expressed in a cell, such as an immune cell (e.g. T cell or antigen presenting cell), in secretable form to thereby produce a secreted or soluble form of the variant ICOSL polypeptide (hereinafter also called a “SIP”), such as when the cells are administered to a subject. In some aspects, a SIP can antagonize a cognate binding partner in the environment (e.g. tumor microenvironment) in which it is secreted. In some embodiments, a variant ICOSL polypeptide is expressed in an infectious agent (e.g. viral or bacterial agent) which, upon administration to a subject, is able to infect a cell in vivo, such as an immune cell (e.g. T cell or antigen presenting cell) or tumor, for delivery or expression of the variant polypeptide as a TIP or a SIP in the cell.

**[0209]** In some embodiments, a soluble immunomodulatory polypeptide, such as a variant ICOSL containing a vIgD, can be encapsulated within a liposome which itself can be conjugated to any one of or any combination of the provided conjugates (e.g., a targeting moiety). In some embodiments, the soluble or membrane bound immunomodulatory polypeptides of the invention are deglycosylated. In more specific embodiments, the variant ICOSL sequence is deglycosylated. In even more specific embodiments, the IgV and/or IgC (e.g. IgC2) domain or domains of the variant ICOSL is deglycosylated.

**[0210]** Non-limiting examples of provided formats are described in FIGS. 13A-13C and further described below.

#### **A. Soluble Protein**

**[0211]** In some embodiments, the immunomodulatory protein containing a variant ICOSL polypeptide is a soluble protein. Those of skill will appreciate that cell surface proteins typically have an intracellular, transmembrane, and extracellular domain (ECD) and that a soluble form of

such proteins can be made using the extracellular domain or an immunologically active subsequence thereof. Thus, in some embodiments, the immunomodulatory protein containing a variant ICOSL polypeptide lacks a transmembrane domain or a portion of the transmembrane domain. In some embodiments, the immunomodulatory protein containing a variant ICOSL lacks the intracellular (cytoplasmic) domain or a portion of the intracellular domain. In some embodiments, the immunomodulatory protein containing the variant ICOSL polypeptide only contains the vIgD portion containing the ECD domain or a portion thereof containing an IgV domain and/or IgC (e.g. IgC2) domain or domains or specific binding fragments thereof containing the amino acid modification(s).

**[0212]** In some embodiments, an immunomodulatory polypeptide comprising a variant ICOSL can include one or more variant ICOSL polypeptides of the invention. In some embodiments a polypeptide of the invention will comprise exactly 1, 2, 3, 4, 5 variant ICOSL sequences. In some embodiments, at least two of the variant ICOSL sequences are identical variant ICOSL sequences.

**[0213]** In some embodiments, the provided immunomodulatory polypeptide comprises two or more vIgD sequences of ICOSL. Multiple variant ICOSL polypeptides within the polypeptide chain can be identical (i.e., the same species) to each other or be non-identical (i.e., different species) variant ICOSL sequences. In addition to single polypeptide chain embodiments, in some embodiments two, three, four, or more of the polypeptides of the invention can be covalently or non-covalently attached to each other. Thus, monomeric, dimeric, and higher order (e.g., 3, 4, 5, or more) multimeric proteins are provided herein. For example, in some embodiments exactly two polypeptides of the invention can be covalently or non-covalently attached to each other to form a dimer. In some embodiments, attachment is made via interchain cysteine disulfide bonds. Compositions comprising two or more polypeptides of the invention can be of an identical species or substantially identical species of polypeptide (e.g, a homodimer) or of non-identical species of polypeptides (e.g., a heterodimer). A composition having a plurality of linked polypeptides of the invention can, as noted above, have one or more identical or non-identical variant ICOSL polypeptides of the invention in each polypeptide chain.

**[0214]** In some embodiments, the immunomodulatory protein comprises a variant ICOSL polypeptide attached to an immunoglobulin Fc (yielding an “immunomodulatory Fc fusion,” such as a “ICOSL-Fc variant fusion,” also termed a ICOSL vIgD-Fc fusion). In some

embodiments, the attachment of the variant ICOSL polypeptide is at the N-terminus of the Fc. In some embodiments, the attachment of the variant ICOSL polypeptide is at the C-terminus of the Fc. In some embodiments, two or more ICOSL variant polypeptides (the same or different) are independently attached at the N-terminus and at the C-terminus.

**[0215]** In some embodiments, the Fc is murine or human Fc. In some embodiments, the Fc is a mammalian or human IgG1, IgG2, IgG3, or IgG4 Fc regions. In some embodiments, the Fc is derived from IgG1, such as human IgG1. In some embodiments, the Fc comprises the amino acid sequence set forth in SEQ ID NO: 226 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 226.

**[0216]** In some embodiments, the Fc region contains one more modifications to alter (e.g. reduce) one or more of its normal functions. In general, the Fc region is responsible for effector functions, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell cytotoxicity (ADCC), in addition to the antigen-binding capacity, which is the main function of immunoglobulins. Additionally, the FcRn sequence present in the Fc region plays the role of regulating the IgG level in serum by increasing the *in vivo* half-life by conjugation to an *in vivo* FcRn receptor. In some embodiments, such functions can be reduced or altered in an Fc for use with the provided Fc fusion proteins.

**[0217]** In some embodiments, one or more amino acid modifications may be introduced into the Fc region of a ICOSL-Fc variant fusion provided herein, thereby generating an Fc region variant. In some embodiments, the Fc region variant has decreased effector function. There are many examples of changes or mutations to Fc sequences that can alter effector function. For example, WO 00/42072, WO2006019447, WO2012125850, WO2015/107026, US2016/0017041 and Shields *et al. J Biol. Chem.* 9(2): 6591-6604 (2001) describe exemplary Fc variants with improved or diminished binding to FcRs. The contents of those publications are specifically incorporated herein by reference.

**[0218]** In some embodiments, the provided variant ICOSL-Fc fusions comprise an Fc region that exhibits reduced effector functions, which makes it a desirable candidate for applications in which the half-life of the ICOSL-Fc variant fusion *in vivo* is important yet certain effector functions (such as CDC and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC

activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the ICOSL-Fc variant fusion lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991). Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Pat. No. 5,500,362 (see, *e.g.* Hellstrom, I. *et al. Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I *et al.*, *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); U.S. Pat. No. 5,821,337 (see Bruggemann, M. *et al.*, *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assay methods may be employed (see, for example, ACTITM non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, Calif.; and CytoTox 96TM non-radioactive cytotoxicity assay (Promega, Madison, Wis.). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes *et al. Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the ICOSL-Fc variant fusion is unable to bind C1q and hence lacks CDC activity. See, *e.g.*, C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996); Cragg, M. S. *et al.*, *Blood* 101:1045-1052 (2003); and Cragg, M. S. and M. J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (see, *e.g.*, Petkova, S. B. *et al.*, *Int'l. Immunol.* 18(12):1759-1769 (2006)).

**[0219]** ICOSL-Fc variant fusions with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 by EU numbering (U.S. Pat. No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327 by EU numbering, including the so-called “DANA” Fc mutant with substitution of residues 265 and 297 to alanine (U.S. Pat. No. 7,332,581).

**[0220]** In some embodiments, the Fc region of ICOSL-Fc variant fusions has an Fc region in which any one or more of amino acids at positions 234, 235, 236, 237, 238, 239, 270, 297, 298,

325, and 329 (indicated by EU numbering) are substituted with different amino acids compared to the native Fc region. Such alterations of Fc region are not limited to the above-described alterations, and include, for example, alterations such as deglycosylated chains (N297A and N297Q), IgG1-N297G, IgG1-L234A/L235A, IgG1-L234A/L235E/G237A, IgG1-A325A/A330S/P331S, IgG1-C226S/C229S, IgG1-C226S/C229S/E233P/L234V/L235A, IgG1-E233P/L234V/L235A/G236del/ S267K, IgG1-L234F/L235E/P331S, IgG1-S267E/L328F, IgG2-V234A/G237A, IgG2-H268Q/V309L/A330S/A331S, IgG4-L235A/G237A/E318A, and IgG4-L236E described in Current Opinion in Biotechnology (2009) 20 (6), 685-691; alterations such as G236R/L328R, L235G/G236R, N325A/L328R, and N325LL328R described in WO 2008/092117; amino acid insertions at positions 233, 234, 235, and 237 (indicated by EU numbering); and alterations at the sites described in WO 2000/042072.

**[0221]** Certain Fc variants with improved or diminished binding to FcRs are described. (See, *e.g.*, U.S. Pat. No. 6,737,056; WO 2004/056312, WO2006019447 and Shields *et al.*, *J. Biol. Chem.* 9(2): 6591-6604 (2001).)

**[0222]** In some embodiments, there is provided a ICOSL-Fc variant fusion comprising a variant Fc region comprising one or more amino acid substitutions which increase half-life and/or improve binding to the neonatal Fc receptor (FcRn). Antibodies with increased half-lives and improved binding to FcRn are described in US2005/0014934A1 (Hinton *et al.*) or WO2015107026. Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434 by EU numbering, *e.g.*, substitution of Fc region residue 434 (U.S. Pat. No. 7,371,826).

**[0223]** In some embodiments, the Fc region of a ICOSL-Fc variant fusion comprises one or more amino acid substitution E356D and M358L. In some embodiments, the Fc region of a ICOSL-Fc variant fusion comprises one or more amino acid substitutions C220S, C226S, C229S. In some embodiments, the Fc region of a ICOSL variant fusion comprises one or more amino acid substitutions R292C and V302C. *See also* Duncan & Winter, *Nature* 322:738-40 (1988); U.S. Pat. No. 5,648,260; U.S. Pat. No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

**[0224]** In some embodiments, alterations are made in the Fc region that result in diminished C1q binding and/or Complement Dependent Cytotoxicity (CDC), *e.g.*, as described in U.S. Pat. No. 6,194,551, WO 99/51642, and Idusogie *et al.*, *J. Immunol.* 164: 4178-4184 (2000).

**[0225]** In some embodiments, there is provided a ICOSL-Fc variant fusion comprising a variant Fc region comprising one or more amino acid modifications, wherein the variant Fc region is derived from IgG1, such as human IgG1. In some embodiments, the variant Fc region is derived from the amino acid sequence set forth in SEQ ID NO: 226. In some embodiments, the Fc contains at least one amino acid substitution that is N82G by numbering of SEQ ID NO: 226 (corresponding to N297G by EU numbering). In some embodiments, the Fc further contains at least one amino acid substitution that is R77C or V87C by numbering of SEQ ID NO: 226 (corresponding to R292C or V302C by EU numbering). In some embodiments, the variant Fc region further comprises a C5S amino acid modification by numbering of SEQ ID NO: 226 (corresponding to C220S by EU numbering). For example, in some embodiments, the variant Fc region comprises the following amino acid modifications: N82G and one or more of the following amino acid modifications C5S, R77C or V87C with reference to SEQ ID NO:226.

**[0226]** In some embodiments, there is provided a ICOSL-Fc variant fusion comprising a variant Fc region in which the variant Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:474, 476, 477, 478 or 507 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 474, 476, 477, 478 or 507.

**[0227]** In some embodiments, the Fc is derived from IgG2, such as human IgG2. In some embodiments, the Fc comprises the amino acid sequence set forth in SEQ ID NO: 227 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 227.

**[0228]** In some embodiments, the Fc comprises the amino acid sequence set forth in SEQ ID NO: 505 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 505. In some embodiments, the IgG4 Fc is a stabilized Fc in which the CH3 domain of human IgG4 is substituted with the CH3 domain of human IgG1 and which exhibits inhibited aggregate formation, an antibody in which the CH3 and CH2 domains of human IgG4 are substituted with the CH3 and CH2 domains of human IgG1, respectively, or an antibody in which arginine at

position 409 indicated in the EU index proposed by Kabat et al. of human IgG4 is substituted with lysine and which exhibits inhibited aggregate formation (see e.g. U.S. Patent No. 8,911,726). In some embodiments, the Fc is an IgG4 containing the S228P mutation, which has been shown to prevent recombination between a therapeutic antibody and an endogenous IgG4 by Fab-arm exchange (see e.g. Labrijin et al. (2009) Nat. Biotechnol., 27(8)767-71.) In some embodiments, the Fc comprises the amino acid sequence set forth in SEQ ID NO: 506 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 506.

**[0229]** In some embodiments, the variant ICOSL polypeptide is directly linked to the Fc sequence. In some embodiments, the variant ICOSL polypeptide is indirectly linked to the Fc sequence, such as via a linker. In some embodiments, one or more “peptide linkers” link the variant ICOSL polypeptide and the Fc domain. In some embodiments, a peptide linker can be a single amino acid residue or greater in length. In some embodiments, the peptide linker has at least one amino acid residue but is no more than 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residues in length. In some embodiments, the linker is (in one-letter amino acid code): GGGGS (“4GS”) or multimers of the 4GS linker, such as repeats of 2, 3, 4, or 5 4GS linkers.

**[0230]** In some embodiments, the variant ICOSL-Fc fusion protein is a dimer formed by two variant ICOSL Fc polypeptides linked to an Fc domain. In some specific embodiments, identical or substantially identical species (allowing for 3 or fewer N-terminus or C-terminus amino acid sequence differences) of ICOSL-Fc variant fusion polypeptides will be dimerized to create a homodimer. In some embodiments, the dimer is a homodimer in which the two variant ICOSL Fc polypeptides are the same. Alternatively, different species of ICOSL-Fc variant fusion polypeptides can be dimerized to yield a heterodimer. Thus, in some embodiments, the dimer is a heterodimer in which the two variant ICOSL Fc polypeptides are different.

**[0231]** Also provided are nucleic acid molecules encoding the variant ICOSL-Fc fusion protein. In some embodiments, for production of an Fc fusion protein, a nucleic acid molecule encoding a variant ICOSL-Fc fusion protein is inserted into an appropriate expression vector. The resulting variant ICOSL-Fc fusion protein can be expressed in host cells transformed with the expression where assembly between Fc domains occurs by interchain disulfide bonds formed between the Fc moieties to yield dimeric, such as divalent, variant ICOSL-Fc fusion proteins.

**[0232]** The resulting Fc fusion proteins can be easily purified by affinity chromatography over Protein A or Protein G columns. For the generation of heterodimers, additional steps for purification can be necessary. For example, where two nucleic acids encoding different variant ICOSL polypeptides are transformed into cells, the formation of heterodimers must be biochemically achieved since variant ICOSL molecules carrying the Fc-domain will be expressed as disulfide-linked homodimers as well. Thus, homodimers can be reduced under conditions that favor the disruption of interchain disulfides, but do no effect intra-chain disulfides. In some cases, different variant-ICOSL Fc monomers are mixed in equimolar amounts and oxidized to form a mixture of homo- and heterodimers. The components of this mixture are separated by chromatographic techniques. Alternatively, the formation of this type of heterodimer can be biased by genetically engineering and expressing Fc fusion molecules that contain a variant ICOSL polypeptide using knob-into-hole methods described below.

### **B. Stack Molecules with Additional IgSF Domains**

**[0233]** In some embodiments, the immunomodulatory proteins can contain any of the variant ICOSL polypeptides provided herein linked, directly or indirectly, to one or more other immunoglobulin superfamily (IgSF) domain (“stacked” immunomodulatory protein construct and also called a “Type II” immunomodulatory protein). In some aspects, this can create unique multi-domain immunomodulatory proteins that bind two or more, such as three or more, cognate binding partners, thereby providing a multi-targeting modulation of the immune synapse.

**[0234]** In some embodiments, an immunomodulatory protein comprises a combination (a “non-wild-type combination”) and/or arrangement (a “non-wild type arrangement” or “non-wild-type permutation”) of a variant ICOSL domain with one or more other affinity modified and/or non-affinity modified IgSF domain sequences of another IgSF family member (e.g. a mammalian IgSF family member) that are not found in wild-type IgSF family members. In some embodiments, the immunomodulatory protein contains 2, 3, 4, 5 or 6 immunoglobulin superfamily (IgSF) domains, where at least one of the IgSF domain is a variant ICOSL IgSF domain (vIgD of ICOSL) according to the provided description.

**[0235]** In some embodiments, the sequences of the additional IgSF domains can be a modified IgSF domain that contains one or more amino acid modifications, e.g. substitutions, compared to a wildtype or unmodified IgSF domain. In some embodiments, the IgSF domain

can be non-affinity modified (e.g., wild-type) or have been affinity modified. In some embodiments, the unmodified or wild-type IgSF domain can be from mouse, rat, cynomolgus monkey, or human origin, or combinations thereof. In some embodiments, the additional IgSF domains can be an IgSF domain of an IgSF family member set forth in Table 2. In some embodiments, the additional IgSF domain can be an affinity-modified IgSF domain containing one or more amino acid modifications, e.g. substitutions, compared to an IgSF domain contained in an IgSF family member set forth in Table 2.

**[0236]** In some embodiments, the additional IgSF domain is an affinity or non-affinity modified IgSF domain contained in an IgSF family member of a family selected from Signal-Regulatory Protein (SIRP) Family, Triggering Receptor Expressed On Myeloid Cells Like (TREM) Family, Carcinoembryonic Antigen-related Cell Adhesion Molecule (CEACAM) Family, Sialic Acid Binding Ig-Like Lectin (SIGLEC) Family, Butyrophilin Family, B7 family, CD28 family, V-set and Immunoglobulin Domain Containing (VSIG) family, V-set transmembrane Domain (VSTM) family, Major Histocompatibility Complex (MHC) family, Signaling lymphocytic activation molecule (SLAM) family, Leukocyte immunoglobulin-like receptor (LIR), Nectin (Nec) family, Nectin-like (NECL) family, Poliovirus receptor related (PVR) family, Natural cytotoxicity triggering receptor (NCR) family, T cell immunoglobulin and mucin (TIM) family or Killer-cell immunoglobulin-like receptors (KIR) family. In some embodiments, the additional IgSF domains are independently derived from an IgSF protein selected from the group consisting of CD80(B7-1), CD86(B7-2), CD274 (PD-L1, B7-H1), PDCD1LG2(PD-L2, CD273), ICOSLG(B7RP1, CD275, ICOSL, B7-H2), CD276(B7-H3), VTCN1(B7-H4), CD28, CTLA4, PDCD1(PD-1), ICOS, BTLA(CD272), CD4, CD8A(CD8-alpha), CD8B(CD8-beta), LAG3, HAVCR2(TIM-3), CEACAM1, TIGIT, PVR(CD155), PVRL2(CD112), CD226, CD2, CD160, CD200, CD200R1(CD200R), and NC R3 (NKP30).

**[0237]** The first column of Table 2 provides the name and, optionally, the name of some possible synonyms for that particular IgSF member. The second column provides the protein identifier of the UniProtKB database, a publicly available database accessible via the internet at [uniprot.org](http://uniprot.org) or, in some cases, the GenBank Number. The Universal Protein Resource (UniProt) is a comprehensive resource for protein sequence and annotation data. The UniProt databases include the UniProt Knowledgebase (UniProtKB). UniProt is a collaboration between the European Bioinformatics Institute (EMBL-EBI), the SIB Swiss Institute of Bioinformatics and

the Protein Information Resource (PIR) and supported mainly by a grant from the U.S. National Institutes of Health (NIH). GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences (Nucleic Acids Research, 2013 Jan;41(D1):D36-42). The third column provides the region where the indicated IgSF domain is located. The region is specified as a range where the domain is inclusive of the residues defining the range. Column 3 also indicates the IgSF domain class for the specified IgSF region. Column 4 provides the region where the indicated additional domains are located (signal peptide, S; extracellular domain, E; transmembrane domain, T; cytoplasmic domain, C). It is understood that description of domains can vary depending on the methods used to identify or classify the domain, and may be identified differently from different sources. The description of residues corresponding to a domain in Table 2 is for exemplification only and can be several amino acids (such as one, two, three or four) longer or shorter. Column 5 indicates for some of the listed IgSF members, some of its cognate cell surface binding partners.

| <b>TABLE 2. IgSF members according to the present disclosure.</b> |                                     |                                                                                                                                                          |                                                     |                                              |                                                    |                |               |
|-------------------------------------------------------------------|-------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------|----------------------------------------------|----------------------------------------------------|----------------|---------------|
| <b>IgSF Member (Synonyms)</b>                                     | <b>UniProtKB Protein Identifier</b> | <b>IgSF Region &amp; Domain Class</b>                                                                                                                    | <b>Other Domains</b>                                | <b>Cognate Cell Surface Binding Partners</b> | <b>IgSF Member Amino Acid Sequence (SEQ ID NO)</b> |                |               |
|                                                                   |                                     |                                                                                                                                                          |                                                     |                                              | <b>Precursor (mature residues)</b>                 | <b>Mature</b>  | <b>ECD</b>    |
| CD80 (B7-1)                                                       | NP_005182.1<br><br>P33681           | 35-135, 35-138 or 37-138<br><br>IgV,<br>145-230 or<br>154-232 IgC<br> | S: 1-34,<br>E: 35-242,<br>T: 243-263,<br>C: 264-288 | CD28, CTLA4, PD-L1                           | SEQ ID NO: 1<br>(35-288)                           | SEQ ID NO: 253 | SEQ ID NO: 28 |
| CD86 (B7-2)                                                       | P42081.2                            | 33-131 IgV,<br>150-225 IgC2<br>                                       | S: 1-23,<br>E: 24-247,<br>T: 248-268,<br>C: 269-329 | CD28, CTLA4                                  | SEQ ID NO: 2<br>(24-329)                           | SEQ ID NO: 254 | SEQ ID NO: 29 |
| CD274 (PD-L1, B7-H1)                                              | Q9NZQ7.1                            | 24-130 IgV,<br>133-225 IgC2<br>                                       | S: 1-18,<br>E: 19-238,<br>T: 239-259,<br>C: 260-290 | PD-1, B7-1                                   | SEQ ID NO: 3<br>(19-290)                           | SEQ ID NO: 255 | SEQ ID NO: 30 |

| <b>TABLE 2. IgSF members according to the present disclosure.</b> |                                     |                                                             |                                                     |                                              |                                                    |                |               |
|-------------------------------------------------------------------|-------------------------------------|-------------------------------------------------------------|-----------------------------------------------------|----------------------------------------------|----------------------------------------------------|----------------|---------------|
| <b>IgSF Member<br/>(Synonyms)</b>                                 | <b>UniProtKB Protein Identifier</b> | <b>IgSF Region &amp; Domain Class</b>                       | <b>Other Domains</b>                                | <b>Cognate Cell Surface Binding Partners</b> | <b>IgSF Member Amino Acid Sequence (SEQ ID NO)</b> |                |               |
|                                                                   |                                     |                                                             |                                                     |                                              | <b>Precursor (mature residues)</b>                 | <b>Mature</b>  | <b>ECD</b>    |
| PDCD1L<br>G2<br>(PD-L2, CD273)                                    | Q9BQ51.2                            | 21-118 IgV,<br>122-203 IgC2                                 | S: 1-19,<br>E: 20-220,<br>T: 221-241,<br>C: 242-273 | PD-1, RGMb                                   | SEQ ID NO: 4<br>(20-273)                           | SEQ ID NO: 256 | SEQ ID NO: 31 |
| ICOSLG<br>(B7RP1, CD275, ICOSL, B7-H2)                            | O75144.2                            | 19-129 IgV,<br>141-227 IgC2                                 | S: 1-18,<br>E: 19-256,<br>T: 257-277,<br>C: 278-302 | ICOS, CD28, CTLA4                            | SEQ ID NO: 5<br>(19-302)                           | SEQ ID NO: 257 | SEQ ID NO: 32 |
| CD276<br>(B7-H3)                                                  | Q5ZPR3.1                            | 29-139 IgV,<br>145-238 IgC2,<br>243-357 IgV,<br>367-453 IgC | S: 1-28,<br>E: 29-466,<br>T: 467-487,<br>C: 488-534 |                                              | SEQ ID NO: 6<br>(29-534)                           | SEQ ID NO: 258 | SEQ ID NO: 33 |
| VTCN1<br>(B7-H4)                                                  | Q7Z7D3.1                            | 35-146 IgV,<br>153-241 IgV                                  | S: 1-24,<br>E: 25-259,<br>T: 260-280,<br>C: 281-282 |                                              | SEQ ID NO: 7<br>(25-282)                           | SEQ ID NO: 259 | SEQ ID NO: 34 |
| CD28                                                              | P10747.1                            | 28-137 IgV                                                  | S: 1-18,<br>E: 19-152,<br>T: 153-179,<br>C: 180-220 | B7-1, B7-2, B7RP1                            | SEQ ID NO: 8<br>(19-220)                           | SEQ ID NO: 260 | SEQ ID NO: 35 |
| CTLA4                                                             | P16410.3                            | 39-140 IgV                                                  | S: 1-35,<br>E: 36-161,<br>T: 162-182,<br>C: 183-223 | B7-1, B7-2, B7RP1                            | SEQ ID NO: 9<br>(36-223)                           | SEQ ID NO: 261 | SEQ ID NO: 36 |

| <b>TABLE 2. IgSF members according to the present disclosure.</b> |                                     |                                                               |                                                     |                                              |                                                    |                |               |
|-------------------------------------------------------------------|-------------------------------------|---------------------------------------------------------------|-----------------------------------------------------|----------------------------------------------|----------------------------------------------------|----------------|---------------|
| <b>IgSF Member<br/>(Synonyms)</b>                                 | <b>UniProtKB Protein Identifier</b> | <b>IgSF Region &amp; Domain Class</b>                         | <b>Other Domains</b>                                | <b>Cognate Cell Surface Binding Partners</b> | <b>IgSF Member Amino Acid Sequence (SEQ ID NO)</b> |                |               |
|                                                                   |                                     |                                                               |                                                     |                                              | <b>Precursor (mature residues)</b>                 | <b>Mature</b>  | <b>ECD</b>    |
| PDCD1 (PD-1)                                                      | Q15116.3                            | 35-145 IgV                                                    | S: 1-20,<br>E: 21-170,<br>T: 171-191,<br>C: 192-288 | PD-L1, PD-L2                                 | SEQ ID NO: 10<br>(21-288)                          | SEQ ID NO: 262 | SEQ ID NO: 37 |
| ICOS                                                              | Q9Y6W8.1                            | 30-132 IgV                                                    | S: 1-20,<br>E: 21-140,<br>T: 141-161,<br>C: 162-199 | B7RP1                                        | SEQ ID NO: 11<br>(21-199)                          | SEQ ID NO: 263 | SEQ ID NO: 38 |
| BTLA (CD272)                                                      | Q7Z6A9.3                            | 31-132 IgV                                                    | S: 1-30,<br>E: 31-157,<br>T: 158-178,<br>C: 179-289 | HVEM                                         | SEQ ID NO: 12<br>(31-289)                          | SEQ ID NO: 264 | SEQ ID NO: 39 |
| CD4                                                               | P01730.1                            | 26-125 IgV,<br>126-203 IgC2,<br>204-317 IgC2,<br>317-389 IgC2 | S: 1-25,<br>E: 26-396,<br>T: 397-418,<br>C: 419-458 | MHC class II                                 | SEQ ID NO: 13<br>(26-458)                          | SEQ ID NO: 265 | SEQ ID NO: 40 |
| CD8A (CD8-alpha)                                                  | P01732.1                            | 22-135 IgV                                                    | S: 1-21, E: 22-182, T: 183-203,<br>C: 204-235       | MHC class I                                  | SEQ ID NO: 14<br>(22-235)                          | SEQ ID NO: 266 | SEQ ID NO: 41 |
| CD8B (CD8-beta)                                                   | P10966.1                            | 22-132 IgV                                                    | S: 1-21,<br>E: 22-170,<br>T: 171-191,<br>C: 192-210 | MHC class I                                  | SEQ ID NO: 15<br>(22-210)                          | SEQ ID NO: 267 | SEQ ID NO: 42 |

| <b>TABLE 2. IgSF members according to the present disclosure.</b> |                                     |                                                                                                                                                    |                                                     |                                                          |                                                    |                   |                  |
|-------------------------------------------------------------------|-------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------|----------------------------------------------------------|----------------------------------------------------|-------------------|------------------|
| <b>IgSF Member (Synonyms)</b>                                     | <b>UniProtKB Protein Identifier</b> | <b>IgSF Region &amp; Domain Class</b>                                                                                                              | <b>Other Domains</b>                                | <b>Cognate Cell Surface Binding Partners</b>             | <b>IgSF Member Amino Acid Sequence (SEQ ID NO)</b> |                   |                  |
|                                                                   |                                     |                                                                                                                                                    |                                                     |                                                          | <b>Precursor (mature residues)</b>                 | <b>Mature</b>     | <b>ECD</b>       |
| LAG3                                                              | P18627.5                            | 37-167 IgV,<br>168-252 IgC2,<br>265-343 IgC2,<br>349-419 IgC2<br> | S: 1-28,<br>E: 29-450,<br>T: 451-471,<br>C: 472-525 | MHC class II                                             | SEQ ID NO:<br>16<br>(29-525)                       | SEQ ID<br>NO: 268 | SEQ ID<br>NO: 43 |
| HAVCR2<br>(TIM-3)                                                 | Q8TDQ0.3                            | 22-124 IgV                                                                                                                                         | S: 1-21,<br>E: 22-202,<br>T: 203-223,<br>C: 224-301 | CEACAM-1,<br>phosphatidylserine,<br>Galectin-9,<br>HMGB1 | SEQ ID NO:<br>17<br>(22-301)                       | SEQ ID<br>NO: 269 | SEQ ID<br>NO: 44 |
| CEACAM<br>1                                                       | P13688.2                            | 35-142 IgV,<br>145-232 IgC2,<br>237-317 IgC2,<br>323-413 IgC                                                                                       | S: 1-34,<br>E: 35-428,<br>T: 429-452,<br>C: 453-526 | TIM-3                                                    | SEQ ID NO:<br>18<br>(35-526)                       | SEQ ID<br>NO: 270 | SEQ ID<br>NO: 45 |
| TIGIT                                                             | Q495A1.1                            | 22-124 IgV                                                                                                                                         | S: 1-21,<br>E: 22-141,<br>T: 142-162,<br>C: 163-244 | CD155, CD112                                             | SEQ ID NO:<br>19<br>(22-244)                       | SEQ ID<br>NO: 271 | SEQ ID<br>NO: 46 |
| PVR<br>(CD155)                                                    | P15151.2                            | 24-139 IgV,<br>145-237 IgC2,<br>244-328 IgC2<br>                | S: 1-20,<br>E: 21-343,<br>T: 344-367,<br>C: 368-417 | TIGIT, CD226,<br>CD96,<br>poliovirus                     | SEQ ID NO:<br>20<br>(21-417)                       | SEQ ID<br>NO: 272 | SEQ ID<br>NO: 47 |
| PVRL2<br>(CD112)                                                  | Q92692.1                            | 32-156 IgV,<br>162-256 IgC2,<br>261-345 IgC2                                                                                                       | S: 1-31,<br>E: 32-360,<br>T: 361-381,<br>C: 382-538 | TIGIT, CD226,<br>CD112R                                  | SEQ ID NO:<br>21<br>(32-538)                       | SEQ ID<br>NO: 273 | SEQ ID<br>NO: 48 |

| <b>TABLE 2. IgSF members according to the present disclosure.</b> |                                     |                                       |                                                     |                                              |                                                    |                   |                   |
|-------------------------------------------------------------------|-------------------------------------|---------------------------------------|-----------------------------------------------------|----------------------------------------------|----------------------------------------------------|-------------------|-------------------|
| <b>IgSF Member (Synonyms)</b>                                     | <b>UniProtKB Protein Identifier</b> | <b>IgSF Region &amp; Domain Class</b> | <b>Other Domains</b>                                | <b>Cognate Cell Surface Binding Partners</b> | <b>IgSF Member Amino Acid Sequence (SEQ ID NO)</b> |                   |                   |
|                                                                   |                                     |                                       |                                                     |                                              | <b>Precursor (mature residues)</b>                 | <b>Mature</b>     | <b>ECD</b>        |
| CD226                                                             | Q15762.2                            | 19-126 IgC2,<br>135-239 IgC2          | S: 1-18,<br>E: 19-254,<br>T: 255-275,<br>C: 276-336 | CD155, CD112                                 | SEQ ID NO:<br>22<br>(19-336)                       | SEQ ID<br>NO: 274 | SEQ ID<br>NO: 49  |
| CD2                                                               | P06729.2                            | 25-128 IgV,<br>129-209 IgC2           | S: 1-24,<br>E: 25-209,<br>T: 210-235,<br>C: 236-351 | CD58                                         | SEQ ID NO:<br>23<br>(25-351)                       | SEQ ID<br>NO: 275 | SEQ ID<br>NO: 50  |
| CD160                                                             | O95971.1                            | 27-122 IgV                            | S: 1-26<br>E: 27-122                                | HVEM, MHC family of proteins                 | SEQ ID NO:<br>24<br>(27-159)                       | SEQ ID<br>NO: 276 | SEQ ID<br>NO: 51  |
| CD200                                                             | P41217.4                            | 31-141 IgV,<br>142-232 IgC2           | S: 1-30,<br>E: 31-232,<br>T: 233-259,<br>C: 260-278 | CD200R                                       | SEQ ID NO:<br>25<br>(31-278)                       | SEQ ID<br>NO: 277 | SEQ ID<br>NO: 52  |
| CD200R1 (CD200R)                                                  | Q8TD46.2                            | 53-139 IgV,<br>140-228 IgC2           | S: 1-28,<br>E: 29-243,<br>T: 244-264,<br>C: 265-325 | CD200                                        | SEQ ID NO:<br>26<br>(29-325)                       | SEQ ID<br>NO: 278 | SEQ ID<br>NO: 53  |
| NCR3 (NKp30)                                                      | O14931.1                            | 19-126 IgC-like                       | S: 1-18,<br>E: 19-135,<br>T: 136-156,<br>C: 157-201 | B7-H6                                        | SEQ ID NO:27<br>(19-201)                           | SEQ ID<br>NO: 279 | SEQ ID<br>NO: 54  |
| VSIG8                                                             | Q5VU13                              | 22-141 IgV 1<br>146-257<br>IgV 2      | S: 1-21<br>E: 22-263<br>T: 264-284<br>C: 285-414    | VISTA                                        | SEQ ID NO:<br>341<br>(22-414)                      | SEQ ID<br>NO: 342 | SEQ ID<br>NO: 343 |

**[0238]** In some embodiments, the provided immunomodulatory proteins, in addition to containing a variant ICOSL polypeptide, also contains at least 2, 3, 4, 5 or 6 additional immunoglobulin superfamily (IgSF) domains, such as an IgD domain of an IgSF family member set forth in Table 2. In some embodiments, the provided immunomodulatory proteins contain at least one additional IgSF domain (e.g. a second IgSF domain) in which at least one additional or second IgSF domain is an IgSF domain set forth in a wild-type or unmodified IgSF domain or a specific binding fragment thereof contained in the sequence of amino acids set forth in any of SEQ ID NOS: 1-27 and 341. In some embodiments, the wild-type or unmodified IgSF domain is an IgV domain or an IgC domain, such as an IgC1 or IgC2 domain.

**[0239]** In some embodiments, the provided immunomodulatory proteins, in addition to containing a variant ICOSL polypeptide, also contains at least one additional IgSF domain (e.g. a second IgSF domain) that is a vIgD that contains one or more amino acid modifications (e.g. substitution, deletion or mutation) compared to an IgSF domain in a wild-type or unmodified IgSF domain, such as an IgSF domain in an IgSF family member set forth in Table 2. In some embodiments, the additional or second affinity-modified IgSF domain comprises at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a wild-type or unmodified IgSF domain or a specific binding fragment thereof contained in the sequence of amino acids set forth in any of SEQ ID NOS: 1-27 and 341. In some embodiments, the wild-type or unmodified IgSF domain is an IgV domain or an IgC domain, such as an IgC1 or IgC2 domain. In some embodiments, the additional or second IgSF domain is an affinity-modified IgV domain or IgC domain.

**[0240]** In some embodiments, the provided immunomodulatory protein contains at least one additional or second IgSF domain that is a vIgD that contains one or more amino acid substitutions compared to an IgSF domain (e.g. IgV) of a wild-type or unmodified IgSF domain other than ICOSL.

**[0241]** In some embodiments, the additional or second IgSF domain contains one or more amino acid substitutions compared to an IgSF domain in a wild-type or unmodified IgSF domain, such as an IgSF domain in an IgSF family member set forth in Table 2. In some embodiments, the additional or second affinity-modified IgSF domain comprises at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a wild-type or unmodified IgSF domain or a specific binding fragment thereof contained in the

sequence of amino acids set forth in any of SEQ ID NOS: 1-27. In some embodiments, the wild-type or unmodified IgSF domain is an IgV domain or an IgC domain, such as an IgC1 or IgC2 domain. In some embodiments, the additional or second IgSF domain is an affinity-modified IgV domain or IgC domain. Tables 3-5 provide exemplary polypeptides containing one or more affinity-modified IgSF domains that can be used in stack constructs provided herein.

**[0242]** In some embodiments, the one or more additional IgSF domain (e.g. second IgSF) domain is an IgSF domain (e.g. IgV) of another IgSF family member that binds or recognizes a tumor antigen. In such embodiments, the IgSF family member serves as a tumor-localizing moiety, thereby bringing the vIgD of ICOSL in close proximity to immune cells in the tumor microenvironment. In some embodiments, the additional IgSF domain (e.g. second IgSF) domain is an IgSF domain of Nkp30, which binds or recognizes B7-H6 expressed on a tumor cell. In some embodiments, the at least one additional (e.g. second) IgSF domain, e.g. Nkp30, is a vIgD that contains one or more amino acid modifications (e.g. substitutions, deletions or additions). In some embodiments, the one or more amino acid modifications increase binding affinity and/or selectivity to B7-H6 compared to unmodified IgSF domain, e.g. Nkp30, such as by at least or at least about 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold.

| <b>TABLE 3: Exemplary variant CD80 polypeptides</b> |                      |                      |
|-----------------------------------------------------|----------------------|----------------------|
| <b>Mutation(s)</b>                                  | <b>ECD SEQ ID NO</b> | <b>IgV SEQ ID NO</b> |
| Wild-type                                           | 28                   | 152                  |
| L70Q/A91G                                           | 55                   | 153                  |
| L70Q/A91G/T130A                                     | 56                   |                      |
| L70Q/A91G/I118A/T120S/T130A                         | 57                   |                      |
| V4M/L70Q/A91G/T120S/T130A                           | 58                   | 154                  |
| L70Q/A91G/T120S/T130A                               | 59                   |                      |
| V20L/L70Q/A91S/T120S/T130A                          | 60                   | 155                  |
| S44P/L70Q/A91G/T130A                                | 61                   | 156                  |
| L70Q/A91G/E117G/T120S/T130A                         | 62                   |                      |

|                                                                                            |    |     |
|--------------------------------------------------------------------------------------------|----|-----|
| A91G/T120S/T130A                                                                           | 63 | 157 |
| L70R/A91G/T120S/T130A                                                                      | 64 | 158 |
| L70Q/E81A/A91G/T120S/I127T/T130A                                                           | 65 | 159 |
| L70Q/Y87N/A91G/T130A                                                                       | 66 | 160 |
| T28S/L70Q/A91G/E95K/T120S/T130A                                                            | 67 | 161 |
| N63S/L70Q/A91G/T120S/T130A                                                                 | 68 | 162 |
| K36E/I67T/L70Q/A91G/T120S/T130A/N152T                                                      | 69 | 163 |
| E52G/L70Q/A91G/T120S/T130A                                                                 | 70 | 164 |
| K37E/F59S/L70Q/A91G/T120S/T130A                                                            | 71 | 165 |
| A91G/S103P                                                                                 | 72 |     |
| K89E/T130A                                                                                 | 73 | 166 |
| A91G                                                                                       | 74 |     |
| D60V/A91G/T120S/T130A                                                                      | 75 | 167 |
| K54M/A91G/T120S                                                                            | 76 | 168 |
| M38T/L70Q/E77G/A91G/T120S/T130A/N152T                                                      | 77 | 169 |
| R29H/E52G/L70R/E88G/A91G/T130A                                                             | 78 | 170 |
| Y31H/T41G/L70Q/A91G/T120S/T130A                                                            | 79 | 171 |
| V68A/T110A                                                                                 | 80 | 172 |
| S66H/D90G/T110A/F116L                                                                      | 81 | 173 |
| R29H/E52G/T120S/T130A                                                                      | 82 | 174 |
| A91G/L102S                                                                                 | 83 |     |
| I67T/L70Q/A91G/T120S                                                                       | 84 | 175 |
| L70Q/A91G/T110A/T120S/T130A                                                                | 85 |     |
| M38V/T41D/M43I/W50G/D76G/V83A/K89E/T120S/T130A                                             | 86 | 176 |
| V22A/L70Q/S121P                                                                            | 87 | 177 |
| A12V/S15F/Y31H/T41G/T130A/P137L/N152T                                                      | 88 | 178 |
| I67F/L70R/E88G/A91G/T120S/T130A                                                            | 89 | 179 |
| E24G/L25P/L70Q/T120S                                                                       | 90 | 180 |
| A91G/F92L/F108L/T120S                                                                      | 91 | 181 |
| R29D/Y31L/Q33H/K36G/M38I/T41A/M43R/M47T/E81V/L85R/K89N/A91<br>T/F92P/K93V/R94L/I118T/N149S | 92 | 182 |
| R29D/Y31L/Q33H/K36G/M38I/T41A/M43R/M47T/E81V/L85R/K89N/A91                                 | 93 |     |

|                                                                                                           |     |          |
|-----------------------------------------------------------------------------------------------------------|-----|----------|
| T/F92P/K93V/R94L/N144S/N149S                                                                              |     |          |
| R29D/Y31L/Q33H/K36G/M38I/T41A/M42T/M43R/M47T/E81V/L85R/K89<br>N/A91T/F92P/K93V/R94L/L148S/N149S           | 94  | 183      |
| E24G/R29D/Y31L/Q33H/K36G/M38I/T41A/M43R/M47T/F59L/E81V/L85<br>R/K89N/A91T/F92P/K93V/R94L/H96R/N149S/C182S | 95  | 184      |
| R29D/Y31L/Q33H/K36G/M38I/T41A/M43R/M47T/E81V/L85R/K89N/A91<br>T/F92P/K93V/R94L/N149S                      | 96  |          |
| R29V/M43Q/E81R/L85I/K89R/D90L/A91E/F92N/K93Q/R94G                                                         | 97  | 185      |
| T41I/A91G                                                                                                 | 98  | 186      |
| K89R/D90K/A91G/F92Y/K93R/N122S/N177S                                                                      | 99  | 187      |
| K89R/D90K/A91G/F92Y/K93R                                                                                  | 100 |          |
| K36G/K37Q/M38I/F59L/E81V/L85R/K89N/A91T/F92P/K93V/R94L/E99G/<br>T130A/N149S                               | 101 | 188      |
| E88D/K89R/D90K/A91G/F92Y/K93R                                                                             | 102 | 189, 543 |
| K36G/K37Q/M38I/L40M                                                                                       | 103 | 190      |
| K36G                                                                                                      | 104 | 191      |
| R29H/Y31H/T41G/Y87N/E88G/K89E/D90N/A91G/P109S                                                             | 105 | 192      |
| A12T/H18L/M43V/F59L/E77K/P109S/I118T                                                                      | 106 | 193      |
| R29V/Y31F/K36G/M38L/M43Q/E81R/V83I/L85I/K89R/D90L/A91E/F92N/<br>K93Q/R94G                                 | 107 | 194      |
| V68M/L70P/L72P/K86E                                                                                       | 108 | 195      |
| L70Q/A91G/N144D                                                                                           | 508 |          |
| L70Q/A91G/I118A/T120S/T130A/K169E                                                                         | 509 |          |
| V4M/L70Q/A91G/I118V/T120S/T130A/K169E                                                                     | 510 |          |
| L70Q/A91G/I118V/T120S/T130A/K169E                                                                         | 511 |          |
| L70Q/A91G/I118V/T120S/T130A                                                                               | 512 |          |
| V20L/L70Q/A91S/I118V/T120S/T130A                                                                          | 513 |          |
| L70Q/A91G/E117G/I118V/T120S/T130A                                                                         | 514 |          |
| A91G/I118V/T120S/T130A                                                                                    | 515 |          |
| L70R/A91G/I118V/T120S/T130A/T199S                                                                         | 516 |          |
| L70Q/E81A/A91G/I118V/T120S/I127T/T130A                                                                    | 517 |          |
| T28S/L70Q/A91G/E95K/I118V/T120S/I126V/T130A/K169E                                                         | 518 |          |
| N63S/L70Q/A91G/S114T/I118V/T120S/T130A                                                                    | 519 |          |

|                                                                                  |     |          |
|----------------------------------------------------------------------------------|-----|----------|
| K36E/I67T/L70Q/A91G/I118V/T120S/T130A/N152T                                      | 520 |          |
| E52G/L70Q/A91G/D107N/I118V/T120S/T130A/K169E                                     | 521 |          |
| K37E/F59S/L70Q/A91G/I118V/T120S/T130A/K185E                                      | 522 |          |
| D60V/A91G/I118V/T120S/T130AK169E                                                 | 523 |          |
| K54M/L70Q/A91G/Y164H/T120S                                                       | 524 |          |
| M38T/L70Q/E77G/A91G/I118V/T120S/T130A/N152T                                      | 525 |          |
| Y31H/T41G/M43L/L70Q/A91G/I118V/T120S/I126V/T130A                                 | 526 |          |
| LS656H/D90G/T110A/F116L                                                          | 527 |          |
| R29H/E52G/D90N/I118V/T120S/T130A                                                 | 528 |          |
| R29H/E52G/D90N/I118V/T120S/T130A                                                 | 529 |          |
| I67T/L70Q/A91G/I118V/T120S                                                       | 530 |          |
| L70Q/A91G/T110A/I118V/T120S/T130A                                                | 531 |          |
| M38V/T41D/M43I/W50G/D76G/V83A/K89E/I118V/T120S/I126V/T130A                       | 532 |          |
| A12V/S15F/Y31H/M38L/T41G/M43L/D90N/T130A/P137L/N149D/N152T                       | 533 |          |
| I67F/L70R/E88G/A91G/I118V/T120S/T130A                                            | 534 |          |
| E24G/L25P/L70Q/A91G/I118V/T120S/N152T                                            | 535 |          |
| A91G/F92L/F108L/I118V/T120S                                                      | 536 |          |
| E88D/K89R/D90K/A91G/F92Y/K93R/N122S/N177S                                        | 537 |          |
| K36G/K37Q/M38I/L40M/F59L/E81V/L85R/K89N/A91T/F92P/K93V/R94L/<br>E99G/T130A/N149S | 539 |          |
| K36G/L40M                                                                        | 540 | 542, 544 |

**TABLE 4: Exemplary variant NKp30 polypeptides**

| <b>Mutation(s)</b>  | <b>ECD<br/>SEQ ID<br/>NO</b> | <b>IgC-like<br/>domain<br/>SEQ ID<br/>NO</b> |
|---------------------|------------------------------|----------------------------------------------|
| Wild-type           | 54                           | 214                                          |
| L30V/A60V/S64P/S86G | 143                          | 215                                          |
| L30V                | 144                          | 216                                          |
| A60V                | 145                          | 217                                          |

|      |     |     |
|------|-----|-----|
| S64P | 146 | 218 |
| S86G | 147 | 219 |

| <b>TABLE 5: Exemplary variant CD86 polypeptides</b> |                              |                              |
|-----------------------------------------------------|------------------------------|------------------------------|
| <b>Mutation(s)</b>                                  | <b>ECD<br/>SEQ ID<br/>NO</b> | <b>IgV<br/>SEQ ID<br/>NO</b> |
| Wild-type                                           | 29                           | 220                          |
| Q35H/H90L/Q102H                                     | 148                          | 221                          |
| Q35H                                                | 149                          | 222                          |
| H90L                                                | 150                          | 223                          |
| Q102H                                               | 151                          | 224                          |

**[0243]** The number of such non-affinity modified or affinity modified IgSF domains present in a “stacked” immunomodulatory protein construct (whether non-wild type combinations or non-wild type arrangements) is at least 2, 3, 4, or 5 and in some embodiments exactly 2, 3, 4, or 5 IgSF domains (whereby determination of the number of affinity modified IgSF domains disregards any non-specific binding fractional sequences thereof and/or substantially immunologically inactive fractional sequences thereof).

**[0244]** In some embodiments of a stacked immunomodulatory protein provided herein, the number of IgSF domains is at least 2 wherein the number of affinity modified and the number of non-affinity modified IgSF domains is each independently at least: 0, 1, 2, 3, 4, 5, or 6. Thus, the number of affinity modified IgSF domains and the number of non-affinity modified IgSF domains, respectively, (affinity modified IgSF domain: non-affinity modified IgSF domain), can be exactly or at least: 2:0 (affinity modified: wild-type), 0:2, 2:1, 1:2, 2:2, 2:3, 3:2, 2:4, 4:2, 1:1, 1:3, 3:1, 1:4, 4:1, 1:5, or 5:1.

**[0245]** In some embodiments of a stacked immunomodulatory protein, at least two of the non-affinity modified and/or affinity modified IgSF domains are identical IgSF domains.

**[0246]** In some embodiments, a stacked immunomodulatory protein provided herein comprises at least two affinity modified and/or non-affinity modified IgSF domains from a single IgSF member but in a non-wild-type arrangement (alternatively, “permutation”). One illustrative example of a non-wild type arrangement or permutation is an immunomodulatory protein comprising a non-wild-type order of affinity modified and/or non-affinity modified IgSF domain sequences relative to those found in the wild-type ICOSL whose IgSF domain sequences served as the source of the variant IgSF domains as provided herein. Thus, in one example, the immunomodulatory protein can comprise an IgV proximal and an IgC distal to the transmembrane domain albeit in a non-affinity modified and/or affinity modified form. The presence, in an immunomodulatory protein provided herein, of both non-wild-type combinations and non-wild-type arrangements of non-affinity modified and/or affinity modified IgSF domains is also within the scope of the provided subject matter.

**[0247]** In some embodiments of a stacked immunomodulatory protein, the non-affinity modified and/or affinity modified IgSF domains are non-identical (i.e., different) IgSF domains. Non-identical affinity modified IgSF domains specifically bind, under specific binding conditions, different cognate binding partners and are “non-identical” irrespective of whether or not the wild-type or unmodified IgSF domains from which they are engineered was the same. Thus, for example, a non-wild-type combination of at least two non-identical IgSF domains in an immunomodulatory protein can comprise at least one IgSF domain sequence whose origin is from and unique to one ICOSL, and at least one of a second IgSF domain sequence whose origin is from and unique to another IgSF family member that is not ICOSL, wherein the IgSF domains of the immunomodulatory protein are in non-affinity modified and/or affinity modified form. However, in alternative embodiments, the two non-identical IgSF domains originate from the same IgSF domain sequence but at least one is affinity modified such that they specifically bind to different cognate binding partners.

**[0248]** A plurality of non-affinity modified and/or affinity modified IgSF domains in a stacked immunomodulatory protein polypeptide chain need not be covalently linked directly to one another. In some embodiments, an intervening span of one or more amino acid residues indirectly covalently bonds the non-affinity modified and/or affinity modified IgSF domains to each other. The linkage can be via the N-terminal to C-terminal residues.

**[0249]** In some embodiments, the linkage can be made via side chains of amino acid residues that are not located at the N-terminus or C-terminus of the non-affinity modified and/or affinity modified IgSF domain. Thus, linkages can be made via terminal or internal amino acid residues or combinations thereof.

**[0250]** In some embodiments, the two or more IgSF domain, including a vIgD of ICOSL and one or more additional IgSF domain (e.g. second variant IgSF domain) from another IgSF family member, are covalently or non-covalently linked. In some embodiments, the two or more IgSF domains are linked directly or indirectly, such as via a linker. In some embodiments, an intervening span of one or more amino acid residues indirectly covalently bonds IgSF domains to each other. The linkage can be via the N-terminal to C-terminal residues. In some embodiments, the linkage can be made via side chains of amino acid residues that are not located at the N-terminus or C-terminus of the IgSF domain(s). Thus, linkages can be made via terminal or internal amino acid residues or combinations thereof.

**[0251]** In some embodiments, one or more “peptide linkers” link the vIgD of ICOSL and an additional IgSF domain (e.g. second variant IgSF domain). In some embodiments, a peptide linker can be a single amino acid residue or greater in length. In some embodiments, the peptide linker has at least one amino acid residue but is no more than 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residues in length. In some embodiments, the linker is (in one-letter amino acid code): GGGGS (“4GS”) or multimers of the 4GS linker, such as repeats of 2, 3, 4, or 5 4GS linkers. In some embodiments, the peptide linker is (GGGGS)<sub>2</sub> or (GGGGS)<sub>3</sub>. In some embodiments, the linker also can include a series of alanine residues alone or in addition to another peptide linker (such as a 4GS linker or multimer thereof). In some embodiments, the number of alanine residues in each series is: 2, 3, 4, 5, or 6 alanines.

**[0252]** In some embodiments, the non-affinity modified and/or affinity modified IgSF domains are linked by “wild-type peptide linkers” inserted at the N-terminus and/or C-terminus of the first and/or second non-affinity modified and/or affinity modified IgSF domains. In some embodiments, there is present a leading peptide linker inserted at the N-terminus of the first IgSF domain and/or a first trailing sequence inserted at the C-terminus of the first non-affinity modified and/or affinity modified IgSF domain. In some embodiments, there is present a second leading peptide linker inserted at the N-terminus of the second IgSF domain and/or a second trailing sequence inserted at the C-terminus of the second non-affinity modified and/or affinity

modified IgSF domain. When the first and second non-affinity modified and/or affinity modified IgSF domains are derived from the same parental protein and are connected in the same orientation, wild-type peptide linkers between the first and second non-affinity modified and/or affinity modified IgSF domains are not duplicated. For example, when the first trailing wild-type peptide linker and the second leading wild-type peptide linker are the same, the Type II immunomodulatory protein does not comprise either the first trailing wild-type peptide linker or the second leading wild-type peptide linker.

**[0253]** In some embodiments, the Type II immunomodulatory protein comprises a first leading wild-type peptide linker inserted at the N-terminus of the first non-affinity modified and/or affinity modified IgSF domain, wherein the first leading wild-type peptide linker comprises at least 5 (such as at least about any of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more) consecutive amino acids from the intervening sequence in the wild-type protein from which the first non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately preceding domain (such as a signal peptide or an IgSF domain). In some embodiments, the first leading wild-type peptide linker comprises the entire intervening sequence in the wild-type protein from which the first non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately preceding domain (such as a signal peptide or an IgSF domain).

**[0254]** In some embodiments, the Type II immunomodulatory protein further comprises a first trailing wild-type peptide linker inserted at the C-terminus of the first non-affinity modified and/or affinity modified IgSF domain, wherein the first trailing wild-type peptide linker comprises at least 5 (such as at least about any of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more) consecutive amino acids from the intervening sequence in the wild-type protein from which the first non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately following domain (such as an IgSF domain or a transmembrane domain). In some embodiments, the first trailing wild-type peptide linker comprises the entire intervening sequence in the wild-type protein from which the first non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately following domain (such as an IgSF domain or a transmembrane domain).

**[0255]** In some embodiments, the Type II immunomodulatory protein further comprises a second leading wild-type peptide linker inserted at the N-terminus of the second non-affinity modified and/or affinity modified IgSF domain, wherein the second leading wild-type peptide linker comprises at least 5 (such as at least about any of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more) consecutive amino acids from the intervening sequence in the wild-type protein from which the second non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately preceding domain (such as a signal peptide or an IgSF domain). In some embodiments, the second leading wild-type peptide linker comprises the entire intervening sequence in the wild-type protein from which the second non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately preceding domain (such as a signal peptide or an IgSF domain).

**[0256]** In some embodiments, the Type II immunomodulatory protein further comprises a second trailing wild-type peptide linker inserted at the C-terminus of the second non-affinity modified and/or affinity modified IgSF domain, wherein the second trailing wild-type peptide linker comprises at least 5 (such as at least about any of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more) consecutive amino acids from the intervening sequence in the wild-type protein from which the second non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately following domain (such as an IgSF domain or a transmembrane domain). In some embodiments, the second trailing wild-type peptide linker comprises the entire intervening sequence in the wild-type protein from which the second non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately following domain (such as an IgSF domain or a transmembrane domain).

**[0257]** Exemplary of a leading sequence and trailing sequence for a Type II protein containing a CD80 IgSF domain is set forth in SEQ ID NO:231 and SEQ ID NO:232. Exemplary of a leading sequence and trailing sequence for a Type II protein containing an ICOSL IgSF domain is set forth in SEQ ID NO: 233 and 234. Exemplary of a leading sequence and a trailing sequence for a Type II protein containing an CD86 IgSF domain is set forth in any of SEQ ID NOS: 236-238. Exemplary of a wild-type linker sequence for a Type II protein containing an NKp30 IgSF domain is set forth in SEQ ID NO:235.

**[0258]** In some embodiments, the two or more IgSF domain, including a vIgD of ICOSL and one or more additional IgSF domain (e.g. second variant IgSF domain) from another IgSF family member, are linked or attached to an Fc to form a dimeric multi-domain stack immunomodulatory protein. In some embodiments, the variant ICOSL polypeptide and second IgSF domain are independently linked, directly or indirectly, to the N- or C-terminus of an Fc subunit. In some embodiments, the variant ICOSL polypeptide and second IgSF domain are linked, directly or indirectly, and one of the variant ICOSL or second IgSF domain is also linked, directly or indirectly, to the N- or C-terminus of an Fc subunit. In some embodiments, linkage to the Fc is via a peptide linker, e.g. a peptide linker, such as described above. In some embodiments, linkage between the variant ICOSL and second IgSF domain is via a peptide linker, e.g. a peptide linker, such as described above. In some embodiments, the vIgD of ICOSL, the one or more additional IgSF domains, and the Fc domain can be linked together in any of numerous configurations as depicted in FIG. 16. Exemplary configurations are described in the Examples.

**[0259]** In some embodiments, the stacked immunomodulatory protein is a dimer formed by two stacked immunomodulatory Fc fusion polypeptides. Also provided are nucleic acid molecules encoding any of the stacked immunomodulatory proteins. In some embodiments, the dimeric multi-domain stack immunomodulatory protein can be produced in cells by expression, or in some cases co-expression, of stack immunomodulatory Fc fusion polypeptides, such as described above in accordance with generating dimeric Fc fusion proteins.

**[0260]** In some embodiments, the dimeric multi-domain stack immunomodulatory protein is divalent for each Fc subunit, monovalent for each subunit, or divalent for one subunit and tetravalent for the other.

**[0261]** In some embodiments, the dimeric multi-domain stack immunomodulatory protein is a homodimeric multi-domain stack Fc protein. In some embodiments, the dimeric multi-domain stack immunomodulatory protein comprises a first stack immunomodulatory Fc fusion polypeptide and a second stack immunomodulatory Fc fusion polypeptide in which the first and second polypeptide are the same. In some embodiments, the Fc portion of the polypeptide can be any Fc as described above.

**[0262]** In some embodiments, the multi-domain stack molecule is heterodimeric, comprising two different Fc polypeptides wherein at least one is an Fc polypeptide containing at least one

variant ICOSL polypeptide and/or at least one second IgSF domain (e.g. second variant IgSF domain). In some embodiments, the multi-domain stack molecule contains a first Fc polypeptide containing a variant ICOSL and a second IgSF domain and a second Fc polypeptide containing the variant ICOSL and the second IgSF domain. In some embodiments, the multi-domain stack molecule contains a first Fc polypeptide containing a variant ICOSL polypeptide and a second IgSF domain and a second Fc polypeptide that is not linked to either a variant ICOSL polypeptide or second IgSF domain.

**[0263]** In some embodiments, the multi-domain stack molecule contains a first Fc polypeptide containing 1, 2, 3, 4 or more variant ICOSL polypeptides and/or 1, 2, 3, 4 or more second IgSF domains, wherein the total number of IgSF domains in the first stack Fc polypeptide is greater than 2, 3, 4, 5, 6 or more. In one example of such an embodiment, the second stack Fc polypeptide contains 1, 2, 3, 4 or more variant ICOSL polypeptides and/or 1, 2, 3, 4 or more second IgSF domains, wherein the total number of IgSF domains in the second stack Fc polypeptide is greater than 2, 3, 4, 5, 6 or more. In another example of such an embodiment, the second Fc polypeptide is not linked to either a variant ICOSL polypeptide or second IgSF domain.

**[0264]** In some embodiments, the heterodimeric stack molecule contains a first stack immunomodulatory Fc fusion polypeptide and a second stack immunomodulatory Fc fusion polypeptide in which the first and second polypeptide are different. In some embodiments, a heterodimeric stack molecule contains a first Fc subunit containing a first variant ICOSL polypeptide and/or second IgSF domain (e.g. second variant IgSF domain) and a second Fc subunit containing the other of the first variant ICOSL polypeptide or the second IgSF domain. In some embodiments, the heterodimeric stack molecule contains a first stack immunomodulatory Fc fusion polypeptide and a second stack immunomodulatory Fc fusion polypeptide in which the first and second polypeptide are different. In some embodiments, a heterodimeric stack molecule contains a first Fc subunit containing a first variant ICOSL polypeptide and/or second IgSF domain (e.g. second variant IgSF domain) and a second Fc subunit containing both the first variant ICOSL polypeptide and second IgSF domain (e.g. second variant IgSF domain) but in a different orientation or configuration from the first Fc subunit.

**[0265]** In some embodiments, the Fc domain of one or both of the first and second stacked immunomodulatory Fc fusion polypeptide comprises a modification (e.g. substitution) such that the interface of the Fc molecule is modified to facilitate and/or promote heterodimerization. In some embodiments, modifications include introduction of a protuberance (knob) into a first Fc polypeptide and a cavity (hole) into a second Fc polypeptide such that the protuberance is positionable in the cavity to promote complexing of the first and second Fc-containing polypeptides. Amino acids targeted for replacement and/or modification to create protuberances or cavities in a polypeptide are typically interface amino acids that interact or contact with one or more amino acids in the interface of a second polypeptide.

**[0266]** In some embodiments, a first polypeptide that is modified to contain protuberance (hole) amino acids include replacement of a native or original amino acid with an amino acid that has at least one side chain which projects from the interface of the first polypeptide and is therefore positionable in a compensatory cavity (hole) in an adjacent interface of a second polypeptide. Most often, the replacement amino acid is one which has a larger side chain volume than the original amino acid residue. One of skill in the art knows how to determine and/or assess the properties of amino acid residues to identify those that are ideal replacement amino acids to create a protuberance. In some embodiments, the replacement residues for the formation of a protuberance are naturally occurring amino acid residues and include, for example, arginine (R), phenylalanine (F), tyrosine (Y), or tryptophan (W). In some examples, the original residue identified for replacement is an amino acid residue that has a small side chain such as, for example, alanine, asparagine, aspartic acid, glycine, serine, threonine, or valine.

**[0267]** In some embodiments, a second polypeptide that is modified to contain a cavity (hole) is one that includes replacement of a native or original amino acid with an amino acid that has at least one side chain that is recessed from the interface of the second polypeptide and thus is able to accommodate a corresponding protuberance from the interface of a first polypeptide. Most often, the replacement amino acid is one which has a smaller side chain volume than the original amino acid residue. One of skill in the art knows how to determine and/or assess the properties of amino acid residues to identify those that are ideal replacement residues for the formation of a cavity. Generally, the replacement residues for the formation of a cavity are naturally occurring amino acids and include, for example, alanine (A), serine (S), threonine (T) and valine (V). In some examples, the original amino acid identified for replacement is an amino

acid that has a large side chain such as, for example, tyrosine, arginine, phenylalanine, or typtophan.

**[0268]** The CH3 interface of human IgG1, for example, involves sixteen residues on each domain located on four anti-parallel  $\beta$ -strands which buries 1090 Å<sup>2</sup> from each surface (see e.g., Deisenhofer et al. (1981) *Biochemistry*, 20:2361-2370; Miller et al., (1990) *J Mol. Biol.*, 216, 965-973; Ridgway et al., (1996) *Prot. Engin.*, 9: 617-621; U.S. Pat. No. 5,731,168). Modifications of a CH3 domain to create protuberances or cavities are described, for example, in U.S. Pat. No. 5,731,168; International Patent Applications WO98/50431 and WO 2005/063816; and Ridgway et al., (1996) *Prot. Engin.*, 9: 617-621. In some examples, modifications of a CH3 domain to create protuberances or cavities are typically targeted to residues located on the two central anti-parallel  $\beta$ -strands. The aim is to minimize the risk that the protuberances which are created can be accommodated by protruding into the surrounding solvent rather than being accommodated by a compensatory cavity in the partner CH3 domain.

**[0269]** In some embodiments, the heterodimeric molecule contains a T366W mutation in the CH3 domain of the “knobs chain” and T366S, L368A, Y407V mutations in the CH3 domain of the “hole chain”. In some cases, an additional interchain disulfide bridge between the CH3 domains can also be used (Merchant, A. M., et al., *Nature Biotech.* 16 (1998) 677-681) e.g. by introducing a Y349C mutation into the CH3 domain of the “knobs” or “hole” chain and a E356C mutation or a S354C mutation into the CH3 domain of the other chain. In some embodiments, the heterodimeric molecule contains S354C, T366W mutations in one of the two CH3 domains and Y349C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. In some embodiments, the heterodimeric molecule comprises E356C, T366W mutations in one of the two CH3 domains and Y349C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. In some embodiments, the heterodimeric molecule comprises Y349C, T366W mutations in one of the two CH3 domains and E356C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. In some embodiments, the heterodimeric molecule comprises Y349C, T366W mutations in one of the two CH3 domains and S354C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. Examples of other knobs-in-holes technologies are known in the art, e.g. as described by EP 1 870 459 A1.

[0270] In some embodiments, the Fc subunits of the heterodimeric molecule additionally can contain one or more other Fc mutation, such as any described above. In some embodiments, the heterodimer molecule contains an Fc subunit with a mutation that reduces effector function.

[0271] In some embodiments, an Fc variant containing CH3 protuberance/cavity modifications can be joined to a stacked immunomodulatory polypeptide anywhere, but typically via its N- or C-terminus, to the N- or C-terminus of a first and/or second stacked immunomodulatory polypeptide, such as to form a fusion polypeptide. The linkage can be direct or indirect via a linker. Typically, a knob and hole molecule is generated by co-expression of a first stacked immunomodulatory polypeptide linked to an Fc variant containing CH3 protuberance modification(s) with a second stacked immunomodulatory polypeptide linked to an Fc variant containing CH3 cavity modification(s).

### **C. Conjugates and Fusions of Variant Polypeptides and Immunomodulatory Proteins**

[0272] In some embodiments, the variant polypeptides provided herein, which are immunomodulatory proteins comprising variants of an Ig domain of the IgSF family (vIgD), can be conjugated with or fused with a moiety, such as an effector moiety, such as another protein, directly or indirectly, to form a conjugate ("IgSF conjugate"). In some embodiments, the attachment can be covalent or non-covalent, e.g., via a biotin-streptavidin non-covalent interaction. In some embodiments of a ICOSL-Fc variant fusion, any one or combination of any two or more of the foregoing conjugates can be attached to the Fc or to the variant CD80 polypeptide or to both

[0273] In some embodiments, the moiety can be a targeting moiety, a small molecule drug (non-polypeptide drug of less than 500 daltons molar mass), a toxin, a cytostatic agent, a cytotoxic agent, an immunosuppressive agent, a radioactive agent suitable for diagnostic purposes, a radioactive metal ion for therapeutic purposes, a prodrug-activating enzyme, an agent that increases biological half-life, or a diagnostic or detectable agent.

[0274] In some embodiments the effector moiety is a therapeutic agent, such as a cancer therapeutic agent, which is either cytotoxic, cytostatic or otherwise provides some therapeutic benefit. In some embodiments, the effector moiety is a targeting moiety or agent, such as an agent that targets a cell surface antigen, e.g., an antigen on the surface of a tumor cell. In some

embodiments, the effector moiety is a label, which can generate a detectable signal, either directly or indirectly. In some embodiments, the effector moiety is a toxin. In some embodiments, the effector moiety is a protein, peptide, nucleic acid, small molecule or nanoparticle.

**[0275]** In some embodiments, 1, 2, 3, 4, 5 or more effector moieties, which can be the same or different, are conjugated, linked or fused to the variant polypeptide or protein to form an IgSF conjugate. In some embodiments, such effector moieties can be attached to the variant polypeptide or immunomodulatory protein using various molecular biological or chemical conjugation and linkage methods known in the art and described below. In some embodiments, linkers such as peptide linkers, cleavable linkers, non-cleavable linkers or linkers that aid in the conjugation reaction, can be used to link or conjugate the effector moieties to the variant polypeptide or immunomodulatory protein.

**[0276]** In some embodiments, the IgSF conjugate comprises the following components: (protein or polypeptide),  $(L)_q$  and (effector moiety) $_m$ , wherein the protein or polypeptide is any of the described variant polypeptides or immunomodulatory proteins capable of binding one or more cognate counter structure ligands as described; L is a linker for linking the protein or polypeptide to the moiety; m is at least 1; q is 0 or more; and the resulting IgSF conjugate binds to the one or more counter structure ligands. In particular embodiments, m is 1 to 4 and q is 0 to 8.

**[0277]** In some embodiments, there is provided an IgSF conjugate comprising a variant polypeptide or immunomodulatory protein provided herein conjugated with a targeting agent that binds to a cell surface molecule, for example, for targeted delivery of the variant polypeptide or immunomodulatory protein to a specific cell. In some embodiments, the targeting agent is a molecule(s) that has the ability to localize and bind to a molecule present on a normal cell/tissue and/or tumor cell/tumor in a subject. In other words, IgSF conjugates comprising a targeting agent can bind to a ligand (directly or indirectly), which is present on a cell, such as a tumor cell. The targeting agents of the invention contemplated for use include antibodies, polypeptides, peptides, aptamers, other ligands, or any combination thereof, that can bind a component of a target cell or molecule.

**[0278]** In some embodiments, the targeting agent binds a tumor cell(s) or can bind in the vicinity of a tumor cell(s) (e.g., tumor vasculature or tumor microenvironment) following

administration to the subject. The targeting agent may bind to a receptor or ligand on the surface of the cancer cell. In another aspect of the invention, a targeting agent is selected which is specific for a noncancerous cells or tissue. For example, a targeting agent can be specific for a molecule present normally on a particular cell or tissue. Furthermore, in some embodiments, the same molecule can be present on normal and cancer cells. Various cellular components and molecules are known. For example, if a targeting agent is specific for EGFR, the resulting IgSF conjugate can target cancer cells expressing EGFR as well as normal skin epidermal cells expressing EGFR. Therefore, in some embodiments, an IgSF conjugate of the invention can operate by two separate mechanisms (targeting cancer and non-cancer cells).

**[0279]** In various aspects of the invention disclosed herein an IgSF conjugate of the invention comprises a targeting agent which can bind/target a cellular component, such as a tumor antigen, a bacterial antigen, a viral antigen, a mycoplasma antigen, a fungal antigen, a prion antigen, an antigen from a parasite. In some aspects, a cellular component, antigen or molecule can each be used to mean, a desired target for a targeting agent. For example, in various embodiments, a targeting agent is specific for or binds to a component, which includes but is not limited to, epidermal growth factor receptor (EGFR, ErbB-1, HER1), ErbB-2 (HER2/neu), ErbB-3/HER3, ErbB-4/HER4, EGFR ligand family; insulin-like growth factor receptor (IGFR) family, IGF-binding proteins (IGFBPs), IGFR ligand family; platelet derived growth factor receptor (PDGFR) family, PDGFR ligand family; fibroblast growth factor receptor (FGFR) family, FGFR ligand family, vascular endothelial growth factor receptor (VEGFR) family, VEGF family; HGF receptor family; TRK receptor family; ephrin (EPH) receptor family; AXL receptor family; leukocyte tyrosine kinase (LTK) receptor family; TIE receptor family, angiopoietin 1,2; receptor tyrosine kinase-like orphan receptor (ROR) receptor family, e.g. ROR1; CD171 (L1CAM); B7-H6 (NCR3LG1); PD-L1, tumor glycosylation antigen, e.g. sTn or Tn, such as sTn Ag of MUC1; LHR (LHCGR); phosphatidylserine, discoidin domain receptor (DDR) family; RET receptor family; KLG receptor family; RYK receptor family; MuSK receptor family; Transforming growth factor- $\alpha$  (TGF- $\alpha$ ) receptors, TGF- $\beta$ ; Cytokine receptors, Class I (hematopoietin family) and Class II (interferon/IL-10 family) receptors, tumor necrosis factor (TNF) receptor superfamily (TNFRSF), death receptor family; cancer-testis (CT) antigens, lineage-specific antigens, differentiation antigens, alpha-actinin-4, ARTCl, breakpoint cluster region-Abelson (Bcr-abl) fusion products, B-RAF, caspase-5 (CASP-5), caspase-8 (CASP-8),  $\beta$ -catenin

(CTNBN1), cell division cycle 27 (CDC27), cyclin-dependent kinase 4 (CDK4), CDKN2A, COA-I, dek-can fusion protein, EFTUD-2, Elongation factor 2 (ELF2), Ets variant gene 6/acute myeloid leukemia 1 gene ETS (ETC6-AML1) fusion protein, fibronectin (FN), e.g. the extradomain A (EDA) of fibronectin, GPNMB, low density lipid receptor/GDP-L fucose:  $\beta$ -Dgalactose 2- $\alpha$ -L-fucosyltransferase (LDLR/FUT) fusion protein, HLA-A2. arginine to isoleucine exchange at residue 170 of the  $\alpha$ -helix of the  $\alpha$ 2-domain in the HLA-A2 gene (HLA-A\*201-R170I), HLA-A1 1, heat shock protein 70-2 mutated (HSP70-2M), K1AA0205, MART2, melanoma ubiquitous mutated 1, 2, 3 (MUM-I, 2, 3), prostatic acid phosphatase (PAP), neo-PAP, Myosin class I, NFYC, OGT, OS-9, pml-RAR $\alpha$  fusion protein, PRDX5, PTPRK, K-ras (KRAS2), N-ras (NRAS), HRAS, RBAF600, SIRT2, SNRPD1, SYT-SSX1 or -SSX2 fusion protein, Triosephosphate Isomerase, BAGE, BAGK- 1, BAGE-2,3,4,5, GAGE-1,2,3,4,5,6,7,8, GnT-V (aberrant N-acetyl glucosaminyl transferase V, MGAT5), HERV-K-MEL, KK-LC, KM-HN-I, LAGE, LAGE-I, CTL-recognized antigen on melanoma (CAMEL), MAGE-A1 (MAGE-I), MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-3, MAGE-B1, MAGE-B2, MAGE-B5, MAGE-B6, MAGE- C1, MAGE-C2, mucin 1 (MUC1), MART-1/Melan-A (MLANA), gp100, gp100/Pmel17 (SILV), tyrosinase (TYR), TRP-I, HAGE, NA-88, NY-ESO-I, NY-ESO-I/LAGE-2, SAGE, Spl7, SSX-1,2,3,4, TRP2-INT2, carcino-embryonic antigen (CEA), Kallikrein 4, mammaglobin-A, OAI, prostate specific antigen (PSA), TRP- 1/ gp75, TRP-2, adipophilin, interferon inducible protein absent in melanoma 2 (AIM-2), BING-4, CPSF, cyclin D1, epithelial cell adhesion molecule (Ep-CAM), EphA3, fibroblast growth factor-5 (FGF-5), glycoprotein 250 (gp250), EGFR (ERBB1), HER-2/neu (ERBB2), interleukin 13 receptor  $\alpha$ 2 chain (IL13R $\alpha$ 2), IL-6 receptor, intestinal carboxyl esterase (iCE), alpha-feto protein (AFP), M-CSF, mdm-2, MUC1, p53 (TP53), PBF, PRAME, PSMA, RAGE-I, RNF43, RU2AS, SOX10, STEAP1, survivin (BIRC5), human telomerase reverse transcriptase (hTERT), telomerase, Wilms' tumor gene (WT1), SYCP1, BRDT, SPANX, XAGE, ADAM2, PAGE-5, LIPI, CTAGE-I, CSAGE, MMAL, CAGE, BORIS, HOM-TES-85, AF15q14, HCA661, LDHC, MORC, SGY-I, SPO1 1, TPX1, NY-SAR-35, FTHL17, NXF2, TDRD1, TEX15, FATE, TPTE, immunoglobulin idiotypes, Bence-Jones protein, estrogen receptors (ER), androgen receptors (AR), CD40, CD30, CD20, CD 19, CD33, cancer antigen 72-4 (CA 72-4), cancer antigen 15-3 (CA 15-3), cancer antigen 27- 29 (CA 27-29), cancer antigen 125 (CA 125), cancer antigen 19-9 (CA 19-9),  $\beta$ -human chorionic

gonadotropin,  $\beta$ -2 microglobulin, squamous cell carcinoma antigen, neuron-specific enolase, heat shock protein gp96, GM2, sargramostim, CTLA-4, 707 alanine proline (707-AP), adenocarcinoma antigen recognized by T cells 4 (ART-4), carcinoembryogenic antigen peptide-1 (CAP-I), calcium-activated chloride channel-2 (CLCA2), cyclophilin B (Cyp-B), human signet ring tumor-2 (HST-2), Human papilloma virus (HPV) proteins (HPV-E6, HPV-E7, major or minor capsid antigens, others), Epstein-Barr virus (EBV) proteins (EBV latent membrane proteins - LMPI, LMP2; others), Hepatitis B or C virus proteins, and HIV proteins.

**[0280]** In some embodiments, an IgSF conjugate, through its targeting agent, will bind a cellular component of a tumor cell, tumor vasculature or tumor microenvironment, thereby promoting killing of targeted cells via modulation of the immune response, (e.g., by activation of co-stimulatory molecules or inhibition of negative regulatory molecules of immune cell activation), inhibition of survival signals (e.g., growth factor or cytokine or hormone receptor antagonists), activation of death signals, and/or immune-mediated cytotoxicity, such as through antibody dependent cellular cytotoxicity. Such IgSF conjugates can function through several mechanisms to prevent, reduce or eliminate tumor cells, such as to facilitate delivery of conjugated effector moieties to the tumor target, such as through receptor-mediated endocytosis of the IgSF conjugate; or such conjugates can recruit, bind, and/or activate immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells, T cells, B cells). Moreover, in some instances one or more of the foregoing pathways may operate upon administration of one or more IgSF conjugates of the invention.

**[0281]** In some embodiments, an IgSF conjugate, through its targeting agent, will be localized to, such as bind to, a cellular component of a tumor cell, tumor vasculature or tumor microenvironment, thereby modulating cells of the immune response in the vicinity of the tumor. In some embodiments, the targeting agent facilitates delivery of the conjugated IgSF (e.g. vIgD) to the tumor target, such as to interact with its cognate binding partner to alter signaling of immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells, T cells, B cells) bearing the cognate binding partner. In some embodiments, localized delivery agonizes or stimulates the costimulatory receptor.

**[0282]** In some embodiments, the targeting agent is an immunoglobulin. As used herein, the term “immunoglobulin” includes natural or artificial mono- or polyvalent antibodies including,

but not limited to, polyclonal, monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, single chain Fv (scFv); anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. The term “antibody,” as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, e.g., molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2) or subclass of immunoglobulin molecule.

**[0283]** In some embodiments, an IgSF conjugate, through its antibody targeting moiety, will bind a cellular component of a tumor cell, tumor vasculature or tumor microenvironment, thereby promoting apoptosis of targeted cells via modulation of the immune response, (e.g., by activation of co-stimulatory molecules or inhibition of negative regulatory molecules of immune cell activation), inhibition of survival signals (e.g., growth factor or cytokine or hormone receptor antagonists), activation of death signals, and/or immune-mediated cytotoxicity, such as through antibody dependent cellular cytotoxicity. Such IgSF conjugates can function through several mechanisms to prevent, reduce or eliminate tumor cells, such as to facilitate delivery of conjugated effector moieties to the tumor target, such as through receptor-mediated endocytosis of the IgSF conjugate; or such conjugates can recruit, bind, and/or activate immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells, T cells, B cells).

**[0284]** In some embodiments, an IgSF conjugate, through its antibody targeting moiety, will bind a cellular component of a tumor cell, tumor vasculature or tumor microenvironment, thereby modulating the immune response (e.g., by activation of co-stimulatory molecules or inhibition of negative regulatory molecules of immune cell activation). In some embodiments, such conjugates can recognize, bind, and/or modulate (e.g. inhibit or activate) immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells, T cells, B cells).

**[0285]** Antibody targeting moieties of the invention include antibody fragments that include, but are not limited to, Fab, Fab' and F(ab')<sub>2</sub>, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region,

CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains. Also included in the invention are Fc fragments, antigen-Fc fusion proteins, and Fc-targeting moiety conjugates or fusion products (Fc-peptide, Fc-aptamer). The antibody targeting moieties of the invention may be from any animal origin including birds and mammals. In one aspect, the antibody targeting moieties are human, murine (e.g., mouse and rat), donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken. Further, such antibodies may be humanized versions of animal antibodies. The antibody targeting moieties of the invention may be monospecific, bispecific, trispecific, or of greater multispecificity.

**[0286]** In various embodiments, an antibody/targeting moiety recruits, binds, and/or activates immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells) via interactions between Fc (in antibodies) and Fc receptors (on immune cells) and via the conjugated variant polypeptides or immunomodulatory proteins provided herein. In some embodiments, an antibody/targeting moiety recognizes or binds a tumor agent via and localizes to the tumor cell the conjugated variant polypeptides or immunomodulatory proteins provided herein to facilitate modulation of immune cells in the vicinity of the tumor.

**[0287]** Examples of antibodies which can be incorporated into IgSF conjugates include but are not limited to antibodies such as Cetuximab (IMC-C225; Erbitux®), Trastuzumab (Herceptin®), Rituximab (Rituxan®; MabThera®), Bevacizumab (Avastin®), Alemtuzumab (Campath®; Campath-1H®; Mabcampath®), Panitumumab (ABX-EGF; Vectibix®), Ranibizumab (Lucentis®), Ibiritumomab, Ibiritumomab tiuxetan, (Zevalin®), Tositumomab, Iodine I 131 Tositumomab (BEXXAR®), Catumaxomab (Removab®), Gemtuzumab, Gemtuzumab ozogamicine (Mylotarg®), Abatacept (CTLA4-Ig; Orencia®), Belatacept (L104EA29YIg; LEA29Y; LEA), Ipilimumab (MDX-010; MDX-101), Tremelimumab (ticilimumab; CP-675,206), PRS-010, PRS-050, Aflibercept (VEGF Trap, AVE005), Volociximab (M200), F200, MORAb-009, SS1P (CAT-5001), Cixutumumab (IMC-A12), Matuzumab (EMD72000), Nimotuzumab (h-R3), Zalutumumab (HuMax-EGFR), Necitumumab IMC-11F8, mAb806 / ch806, Sym004, mAb-425, Panorex @ (17-1A) (murine monoclonal antibody); Panorex @ (17-1A) (chimeric murine monoclonal antibody); IDEC- Y2B8 (murine, anti- CD20 MAb) ; BEC2 (anti-idiotypic MAb, mimics the GD epitope) (with BCG); Oncolym (Lym-1 monoclonal antibody); SMART MI95 Ab, humanized 13' I LYM-I (Oncolym), Ovarex

(B43.13, anti-idiotypic mouse MAb); MDX-210 (humanized anti-HER-2 bispecific antibody); 3622W94 MAb that binds to EGP40 (17-1A) pancarcinoma antigen on adenocarcinomas; Anti-VEGF, Zenapax (SMART Anti-Tac (IL-2 receptor); SMART MI95 Ab, humanized Ab, humanized); MDX-210 (humanized anti- HER-2 bispecific antibody); MDX-447 (humanized anti-EGF receptor bispecific antibody); NovoMAb-G2 (pancarcinoma specific Ab); TNT (chimeric MAb to histone antigens); TNT (chimeric MAb to histone antigens); Gliomab-H (Monoclonal s - Humanized Abs); GNI-250 Mab; EMD-72000 (chimeric-EGF antagonist); LymphoCide (humanized LL2 antibody); and MDX-260 bispecific, targets GD-2, ANA Ab, SMART IDIO Ab, SMART ABL 364 Ab or ImmuRAIT-CEA. As illustrated by the foregoing list, it is conventional to make antibodies to a particular target epitope.

**[0288]** In some embodiments, the antibody targeting moiety is a full length antibody, or antigen-binding fragment thereof, containing an Fc domain. In some embodiments, the variant polypeptide or immunomodulatory protein is conjugated to the Fc portion of the antibody targeting moiety, such as by conjugation to the N-terminus of the Fc portion of the antibody.

**[0289]** In some embodiments, the vIgD is linked, directly or indirectly, to the N- or C-terminus of the light and/or heavy chain of the antibody. In some embodiments, linkage can be via a peptide linker, such as any described above. Various configurations can be constructed. FIG. 10A-10C depict exemplary configurations. In some embodiments, the antibody conjugate can be produced by co-expression of the heavy and light chain of the antibody in a cell.

**[0290]** In one aspect of the invention, the targeting agent is an aptamer molecule. For example, in some embodiments, the aptamer is comprised of nucleic acids that function as a targeting agent. In various embodiments, an IgSF conjugate of the invention comprises an aptamer that is specific for a molecule on a tumor cell, tumor vasculature, and/or a tumor microenvironment. In some embodiments, the aptamer itself can comprise a biologically active sequence, in addition to the targeting module (sequence), wherein the biologically active sequence can induce an immune response to the target cell. In other words, such an aptamer molecule is a dual use agent. In some embodiments, an IgSF conjugate of the invention comprises conjugation of an aptamer to an antibody, wherein the aptamer and the antibody are specific for binding to separate molecules on a tumor cell, tumor vasculature, tumor microenvironment, and/or immune cells.

**[0291]** The term “aptamer” includes DNA, RNA or peptides that are selected based on specific binding properties to a particular molecule. For example, an aptamer(s) can be selected for binding a particular gene or gene product in a tumor cell, tumor vasculature, tumor microenvironment, and/or an immune cell, as disclosed herein, where selection is made by methods known in the art and familiar to one of skill in the art.

**[0292]** In some aspects of the invention the targeting agent is a peptide. For example, the variant polypeptides or immunomodulatory proteins provided herein can be conjugated to a peptide which can bind with a component of a cancer or tumor cells. Therefore, such IgSF conjugates of the invention comprise peptide targeting agents which binds to a cellular component of a tumor cell, tumor vasculature, and/or a component of a tumor microenvironment. In some embodiments, targeting agent peptides can be an antagonist or agonist of an integrin. Integrins, which comprise an alpha and a beta subunit, include numerous types well known to a skilled artisan.

**[0293]** In one embodiment, the targeting agent is Vv $\beta$ 3. Integrin Vv $\beta$ 3 is expressed on a variety of cells and has been shown to mediate several biologically relevant processes, including adhesion of osteoclasts to bone matrix, migration of vascular smooth muscle cells, and angiogenesis. Suitable targeting molecules for integrins include RGD peptides or peptidomimetics as well as non-RGD peptides or peptidomimetics (see, e.g., U.S. Pat. Nos. 5,767,071 and 5,780,426) for other integrins such as V4. $\beta$ i (VLA-4), V4-P7 (see, e.g., U.S. Pat. No. 6,365,619; Chang et al, Bioorganic & Medicinal Chem Lett, 12:159-163 (2002); Lin et al., Bioorganic & Medicinal Chem Lett, 12:133-136 (2002)), and the like.

**[0294]** In some embodiments, there is provided an IgSF conjugate comprising a variant polypeptide or immunomodulatory protein provided herein conjugated with a therapeutic agent. In some embodiments, the therapeutic agent includes, for example, daunomycin, doxorubicin, methotrexate, and vindesine (Rowland et al., Cancer Immunol. Immunother. 21:183-187, 1986). In some embodiments, the therapeutic agent has an intracellular activity. In some embodiments, the IgSF conjugate is internalized and the therapeutic agent is a cytotoxin that blocks the protein synthesis of the cell, therein leading to cell death. In some embodiments, the therapeutic agent is a cytotoxin comprising a polypeptide having ribosome-inactivating activity including, for example, gelonin, bouganin, saporin, ricin, ricin A chain, bryodin, diphtheria toxin, restrictocin, Pseudomonas exotoxin A and variants thereof. In some embodiments, where the therapeutic

agent is a cytotoxin comprising a polypeptide having a ribosome-inactivating activity, the IgSF conjugate must be internalized upon binding to the target cell in order for the protein to be cytotoxic to the cells.

**[0295]** In some embodiments, there is provided an IgSF conjugate comprising a variant polypeptide or immunomodulatory protein provided herein conjugated with a toxin. In some embodiments, the toxin includes, for example, bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al., *J.Nat. Cancer Inst.* 92(19):1573-1581 (2000); Mandler et al., *Bioorganic & Med. Chem. Letters* 10:1025- 1028 (2000); Mandler et al., *Bioconjugate Chem.* 13:786-791 (2002)), maytansinoids (EP 1391213; Liu et al., *Proc. Natl. Acad. Sci. USA* 93:8618-8623 (1996)), and calicheamicin (Lode et al., *Cancer Res.* 58:2928 (1998); Hinman et al., *Cancer Res.* 53:3336-3342 (1993)). The toxins may exert their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition.

**[0296]** In some embodiments, there is provided an IgSF conjugate comprising a variant polypeptide or immunomodulatory protein provided herein conjugated with a label, which can generate a detectable signal, indirectly or directly. These IgSF conjugates can be used for research or diagnostic applications, such as for the in vivo detection of cancer. The label is preferably capable of producing, either directly or indirectly, a detectable signal. For example, the label may be radio-opaque or a radioisotope, such as <sup>3</sup>H, <sup>14</sup>C, <sup>32</sup>P, <sup>35</sup>S, <sup>123</sup>I, <sup>125</sup>I, <sup>131</sup>I; a fluorescent (fluorophore) or chemiluminescent (chromophore) compound, such as fluorescein isothiocyanate, rhodamine or luciferin; an enzyme, such as alkaline phosphatase,  $\beta$ -galactosidase or horseradish peroxidase; an imaging agent; or a metal ion. In some embodiments, the label is a radioactive atom for scintigraphic studies, for example <sup>99</sup>Tc or <sup>123</sup>I, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, MRI), such as zirconium-89, iodine-123, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron. Zirconium-89 may be complexed to various metal chelating agents and conjugated to antibodies, e.g., for PET imaging (WO 2011/056983). In some embodiments, the IgSF conjugate is detectable indirectly. For example, a secondary antibody that is specific for the IgSF conjugate and contains a detectable label can be used to detect the IgSF conjugate.

**[0297]** The IgSF conjugates may be prepared using any methods known in the art. See, e.g., WO 2009/067800, WO 2011/133886, and U.S. Patent Application Publication No. 2014322129, incorporated by reference herein in their entirety.

**[0298]** The variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be “attached to” the effector moiety by any means by which the variant polypeptides or immunomodulatory proteins can be associated with, or linked to, the effector moiety. For example, the variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be attached to the effector moiety by chemical or recombinant means. Chemical means for preparing fusions or conjugates are known in the art and can be used to prepare the IgSF conjugate. The method used to conjugate the variant polypeptides or immunomodulatory proteins and effector moiety must be capable of joining the variant polypeptides or immunomodulatory proteins with the effector moiety without interfering with the ability of the variant polypeptides or immunomodulatory proteins to bind to their one or more counter structure ligands.

**[0299]** The variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be linked indirectly to the effector moiety. For example, the variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be directly linked to a liposome containing the effector moiety of one of several types. The effector moiety(s) and/or the variant polypeptides or immunomodulatory proteins may also be bound to a solid surface.

**[0300]** In some embodiments, the variant polypeptides or immunomodulatory proteins of an IgSF conjugate and the effector moiety are both proteins and can be conjugated using techniques well known in the art. There are several hundred crosslinkers available that can conjugate two proteins. (See for example “Chemistry of Protein Conjugation and Crosslinking,” 1991, Shans Wong, CRC Press, Ann Arbor). The crosslinker is generally chosen based on the reactive functional groups available or inserted on the variant polypeptides or immunomodulatory proteins and/or effector moiety. In addition, if there are no reactive groups, a photoactivatable crosslinker can be used. In certain instances, it may be desirable to include a spacer between the variant polypeptides or immunomodulatory proteins and the effector moiety. Crosslinking agents known to the art include the homobifunctional agents: glutaraldehyde, dimethyladipimide and Bis(diazobenzidine) and the heterobifunctional agents: m Maleimidobenzoyl-N-Hydroxysuccinimide and Sulfo-m Maleimidobenzoyl-N-Hydroxysuccinimide.

**[0301]** In some embodiments, the variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be engineered with specific residues for chemical attachment of the effector moiety. Specific residues used for chemical attachment of molecule known to the art include lysine and cysteine. The crosslinker is chosen based on the reactive functional groups inserted on the variant polypeptides or immunomodulatory proteins, and available on the effector moiety.

**[0302]** An IgSF conjugate may also be prepared using recombinant DNA techniques. In such a case a DNA sequence encoding the variant polypeptides or immunomodulatory proteins is fused to a DNA sequence encoding the effector moiety, resulting in a chimeric DNA molecule. The chimeric DNA sequence is transfected into a host cell that expresses the fusion protein. The fusion protein can be recovered from the cell culture and purified using techniques known in the art.

**[0303]** Examples of attaching an effector moiety, which is a label, to the variant polypeptides or immunomodulatory proteins include the methods described in Hunter, et al., *Nature* 144:945 (1962); David, et al., *Biochemistry* 13:1014 (1974); Pain, et al., *J. Immunol. Meth.* 40:219 (1981); Nygren, J. *Histochem. and Cytochem.* 30:407 (1982); Wensel and Meares, *Radioimmunoimaging And Radioimmunotherapy*, Elsevier, N.Y. (1983); and Colcher et al., "Use Of Monoclonal Antibodies As Radiopharmaceuticals For The Localization Of Human Carcinoma Xenografts In Athymic Mice", *Meth. Enzymol.*, 121:802-16 (1986).

**[0304]** The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as <sup>99</sup>Tc or <sup>123</sup>I, <sup>186</sup>Re, <sup>188</sup>Re and <sup>111</sup>In can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al., *Biochem. Biophys. Res. Commun.* 80:49-57 (1978)) can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal, CRC Press 1989) describes other methods in detail.

**[0305]** Conjugates of the variant polypeptides or immunomodulatory proteins and a cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes

(such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)- ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene tnaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See, e.g., WO94/11026. The linker may be a “cleavable linker” facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Research 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

**[0306]** The IgSF conjugates of the invention expressly contemplate, but are not limited to, drug conjugates prepared with cross-linker reagents: BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL, U.S.A). See pages 467-498, 2003-2004 Applications Handbook and Catalog.

#### **D. Transmembrane and Secretable Immunomodulatory Proteins and Engineered Cells**

**[0307]** Provided herein are engineered cells which express the immunomodulatory variant ICOSL polypeptides (alternatively, “engineered cells”). In some embodiments, the expressed immunomodulatory variant ICOSL polypeptide is a transmembrane proteins and is surface expressed. In some embodiments, the expressed immunomodulatory variant ICOSL polypeptide is expressed and secreted from the cell.

##### **1. Transmembrane Immunomodulatory Proteins**

**[0308]** In some embodiments, an immunomodulatory polypeptide comprising a variant ICOSL can be a membrane bound protein. As described in more detail below, the immunomodulatory polypeptide can be a transmembrane immunomodulatory polypeptide comprising a variant ICOSL in which is contained: an ectodomain containing at least one affinity modified IgSF domain (IgV or IgC), a transmembrane domain and, optionally, a cytoplasmic

domain. In some embodiments, the transmembrane immunomodulatory protein can be expressed on the surface of an immune cell, such as a mammalian cell, including on the surface of a lymphocyte (e.g. T cell or NK cell) or antigen presenting cell. In some embodiments, the transmembrane immunomodulatory protein is expressed on the surface of a mammalian T-cell, including such T-cells as: a T helper cell, a cytotoxic T-cell (alternatively, cytotoxic T lymphocyte or CTL), a natural killer T-cell, a regulatory T-cell, a memory T-cell, or a gamma delta T-cell. In some embodiments, the mammalian cell is an antigen presenting cell (APC). Typically, but not exclusively, the ectodomain (alternatively, “extracellular domain”) of comprises the one or more amino acid variations (e.g. amino acid substitutions) of the variant ICOSL of the invention. Thus, for example, in some embodiments a transmembrane protein will comprise an ectodomain that comprises one or more amino acid substitutions of a variant ICOSL of the invention.

**[0309]** In some embodiments, the engineered cells express variant ICOSL polypeptides that are transmembrane immunomodulatory polypeptides (TIPs) that can be a membrane protein such as a transmembrane protein. In typical embodiments, the ectodomain of a membrane protein comprises an extracellular domain or IgSF domain thereof of a variant ICOSL provided herein in which is contained one or more amino acid substitutions in at least one IgSF domain as described. The transmembrane immunomodulatory proteins provided herein further contain a transmembrane domain linked to the ectodomain. In some embodiments, the transmembrane domain results in an encoded protein for cell surface expression on a cell. In some embodiments, the transmembrane domain is linked directly to the ectodomain. In some embodiments, the transmembrane domain is linked indirectly to the ectodomain via one or more linkers or spacers. In some embodiments, the transmembrane domain contains predominantly hydrophobic amino acid residues, such as leucine and valine.

**[0310]** In some embodiments, a full length transmembrane anchor domain can be used to ensure that the TIPs will be expressed on the surface of the engineered cell, such as engineered T cell. Conveniently, this could be from a particular native protein that is being affinity modified (e.g. ICOSL or other native IgSF protein), and simply fused to the sequence of the first membrane proximal domain in a similar fashion as the native IgSF protein (e.g. ICOSL). In some embodiments, the transmembrane immunomodulatory protein comprises a transmembrane domain of the corresponding wild-type or unmodified IgSF member, such as a transmembrane

domain contained in the sequence of amino acids set forth in SEQ ID NO:5 (Table 2). In some embodiments, the membrane bound form comprises a transmembrane domain of the corresponding wild-type or unmodified polypeptide, such as corresponding to residues 257-277 of SEQ ID NO:5.

**[0311]** In some embodiments, the transmembrane domain is a non-native transmembrane domain that is not the transmembrane domain of native ICOSL. In some embodiments, the transmembrane domain is derived from a transmembrane domain from another non- ICOSL family member polypeptide that is a membrane-bound or is a transmembrane protein. In some embodiments, a transmembrane anchor domain from another protein on T cells can be used. In some embodiments, the transmembrane domain is derived from CD8. In some embodiments, the transmembrane domain can further contain an extracellular portion of CD8 that serves as a spacer domain. An exemplary CD8 derived transmembrane domain is set forth in SEQ ID NO: 246 or 483 or a portion thereof containing the CD8 transmembrane domain. In some embodiments, the transmembrane domain is a synthetic transmembrane domain.

**[0312]** In some embodiments, the transmembrane immunomodulatory protein further contains an endodomain, such as a cytoplasmic signaling domain, linked to the transmembrane domain. In some embodiments, the cytoplasmic signaling domain induces cell signaling. In some embodiments, the endodomain of the transmembrane immunomodulatory protein comprises the cytoplasmic domain of the corresponding wild-type or unmodified polypeptide, such as a cytoplasmic domain contained in the sequence of amino acids set forth in SEQ ID NO:5 (see Table 2).

**[0313]** In some embodiments, a provided transmembrane immunomodulatory protein that is or comprises a variant ICOSL comprises a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO: 257 and contains an ectodomain comprising at least one affinity-modified ICOSL IgSF domain as described and a transmembrane domain. In some embodiments, the transmembrane immunomodulatory protein contains any one or more amino acid substitutions in an IgSF domain (e.g. IgV domain) as described, including any set forth in Table 1. In some embodiments, the transmembrane immunomodulatory protein can further comprise a cytoplasmic domain as described. In some embodiments, the transmembrane immunomodulatory protein can further contain a signal peptide. In some embodiments, the

signal peptide is the native signal peptide of wild-type IgSF member, such as contained in the sequence of amino acids set forth in SEQ ID NO:5 (see e.g. Table 2).

**[0314]** In some embodiments, provided are transmembrane immunomodulatory proteins comprising the amino acid substitutions E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R, N52H/N57Y/Q100R, or N52H/N57Y/Q100P. In some embodiments, the provided transmembrane immunomodulatory protein is or comprises a variant ICOSL comprising the sequence of amino acids set forth in SEQ ID NO:257, but in which is contained amino substitutions E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R, N52H/N57Y/Q100R, or N52H/N57Y/Q100P at corresponding positions in SEQ ID NO:257, or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO: 257 and contains the amino acid substitutions E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R, N52H/N57Y/Q100R, or N52H/N57Y/Q100P.

**[0315]** In some embodiments, provided are transmembrane immunomodulatory proteins comprising the sequence of amino acids set forth in SEQ ID NOS: 496 or 497 (each containing the amino acid substitution N52D), SEQ ID NOS: 498 or 499 (each containing the amino acid substitutions N52H/N57Y/Q100P), SEQ ID NOS: 500 or 501 (each containing the amino acid substitutions E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R) or SEQ ID NOS: 502 or 503 (each containing the amino acid substitutions N52H/N57Y/Q100R), or a sequence of amino acids that comprises at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 495-503 and that contains the indicated amino acid substitutions. In some embodiments, when expressed in an engineered cell, such transmembrane immunomodulatory proteins are expressed on the surface of the cell.

**[0316]** Also provided is a nucleic acid molecule encoding such transmembrane immunomodulatory proteins. In some embodiments, a nucleic acid molecule encoding a transmembrane immunomodulatory protein comprises a nucleotide sequence that encodes a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NOS: 257 and contains an ectodomain comprising at least one affinity-modified IgSF domain as described, a

transmembrane domain and, optionally, a cytoplasmic domain. In some embodiments, the nucleic acid molecule can further comprise a sequence of nucleotides encoding a signal peptide. In some embodiments, the signal peptide is the native signal peptide of the corresponding wild-type IgSF member (see e.g. Table 2).

**[0317]** Exemplary of a transmembrane immunomodulatory protein is a ICOSL TIP comprising i) the sequence of amino acids set forth in SEQ ID NO:383 or ii) a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:243 and that comprises the affinity-modified domain contained in SEQ ID NO:243 or the amino acid substitutions therein. Also provided is i) a sequence of nucleotides set forth in SEQ ID NO:244, ii) a sequence that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO: 244 and that encodes a TIP that comprises the affinity-modified domain of SEQ ID NO:243, or iii) a sequence of i) or ii) having degenerate codons.

**[0318]** In some embodiments, provided are CAR-related transmembrane immunomodulatory proteins in which the endodomain of a transmembrane immunomodulatory protein comprises a cytoplasmic signaling domain that comprises at least one ITAM (immunoreceptor tyrosine-based activation motif)-containing signaling domain. ITAM is a conserved motif found in a number of protein signaling domains involved in signal transduction of immune cells, including in the CD3-zeta chain (“CD3-z”) involved in T-cell receptor signal transduction. In some embodiments, the endodomain comprises at CD3-zeta signaling domain. In some embodiments, the CD3-zeta signaling domain comprises the sequence of amino acids set forth in SEQ ID NO: 243 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% to SEQ ID NO:247 and retains the activity of T cell signaling. In some embodiments, the endodomain of a CAR-related transmembrane immunomodulatory protein can further comprise a costimulatory signaling domain to further modulate immunomodulatory responses of the T-cell. In some embodiments, the costimulatory signaling domain is CD28, ICOS, 41BB or OX40. In some embodiments, the costimulatory signaling domain is a derived from CD28 or 4-1BB and comprises the sequence of amino acids set forth in any of SEQ ID NOS: 484-487 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% to SEQ ID NO:484-487 and retains the activity of T cell costimulatory signaling. In some embodiments, the

provided CAR-related transmembrane immunomodulatory proteins have features of CARs to stimulate T cell signaling upon binding of an affinity modified IgSF domain to a cognate binding partner or counter structure. In some embodiments, upon specific binding by the affinity-modified IgSF domain to its counter structure can lead to changes in the immunological activity of the T-cell activity as reflected by changes in cytotoxicity, proliferation or cytokine production.

**[0319]** In some embodiments, the transmembrane immunomodulatory protein does not contain an endodomain capable of mediating cytoplasmic signaling. In some embodiments, the transmembrane immunomodulatory protein lacks the signal transduction mechanism of the wild-type or unmodified polypeptide and therefore does not itself induce cell signaling. In some embodiments, the transmembrane immunomodulatory protein lacks an intracellular (cytoplasmic) domain or a portion of the intracellular domain of the corresponding wild-type or unmodified polypeptide, such as a cytoplasmic signaling domain contained in the sequence of amino acids set forth in SEQ ID NO:5 (see Table 2). In some embodiments, the transmembrane immunomodulatory protein does not contain an ITIM (immunoreceptor tyrosine-based inhibition motif), such as contained in certain inhibitory receptors, including inhibitory receptors of the IgSF family (e.g. PD-1 or TIGIT). Thus, in some embodiments, the transmembrane immunomodulatory protein only contains the ectodomain and the transmembrane domain, such as any as described.

## **2. Secreted Immunomodulatory Proteins and Engineered Cells**

**[0320]** In some embodiments, the ICOSL variant immunomodulatory polypeptide containing any one or more of the amino acid mutations as described herein, is secretable, such as when expressed from a cell. Such a variant ICOSL immunomodulatory protein does not comprise a transmembrane domain. In some embodiments, the variant ICOSL immunomodulatory protein is not conjugated to a half-life extending moiety (such as an Fc domain or a multimerization domain). In some embodiments, the variant ICOSL immunomodulatory protein comprises a signal peptide, e.g. an antibody signal peptide or other efficient signal sequence to get domains outside of cell. When the immunomodulatory protein comprises a signal peptide and is expressed by an engineered cell, the signal peptide causes the immunomodulatory protein to be secreted by the engineered cell. Generally, the signal peptide, or a portion of the signal peptide, is cleaved from the immunomodulatory protein with secretion. The immunomodulatory protein

can be encoded by a nucleic acid (which can be part of an expression vector). In some embodiments, the immunomodulatory protein is expressed and secreted by a cell (such as an immune cell, for example a primary immune cell).

**[0321]** Thus, in some embodiments, there are provided variant ICOSL immunomodulatory proteins that further comprise a signal peptide. In some embodiments, provided herein is a nucleic acid molecule encoding the variant ICOSL immunomodulatory protein operably connected to a secretion sequence encoding the signal peptide.

**[0322]** A signal peptide is a sequence on the N-terminus of an immunomodulatory protein that signals secretion of the immunomodulatory protein from a cell. In some embodiments, the signal peptide is about 5 to about 40 amino acids in length (such as about 5 to about 7, about 7 to about 10, about 10 to about 15, about 15 to about 20, about 20 to about 25, or about 25 to about 30, about 30 to about 35, or about 35 to about 40 amino acids in length).

**[0323]** In some embodiments, the signal peptide is a native signal peptide from the corresponding wild-type ICOSL (see Table 6). In some embodiments, the signal peptide is a non-native signal peptide. For example, in some embodiments, the non-native signal peptide is a mutant native signal peptide from the corresponding wild-type ICOSL, and can include one or more (such as 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more) substitutions insertions or deletions. In some embodiments, the non-native signal peptide is a signal peptide or mutant thereof of a family member from the same IgSF family as the wild-type IgSF family member. In some embodiments, the non-native signal peptide is a signal peptide or mutant thereof from an IgSF family member from a different IgSF family than the wild-type IgSF family member. In some embodiments, the signal peptide is a signal peptide or mutant thereof from a non-IgSF protein family, such as a signal peptide from an immunoglobulin (such as IgG heavy chain or IgG-kappa light chain), a cytokine (such as interleukin-2 (IL-2), or CD33), a serum albumin protein (e.g. HSA or albumin), a human azurocidin preprotein signal sequence, a luciferase, a trypsinogen (e.g. chumotrypsinogen or trypsinogen) or other signal peptide able to efficiently secrete a protein from a cell. Exemplary signal peptides include any described in the Table 6.

| <b>TABLE 6. Exemplary Signal Peptides</b> |                       |                         |
|-------------------------------------------|-----------------------|-------------------------|
| <b>SEQ ID NO</b>                          | <b>Signal Peptide</b> | <b>Peptide Sequence</b> |
| SEQ ID NO: 346                            | HSA signal peptide    | MKWVTFISLLFLFSSAYS      |
| SEQ ID NO: 347                            | Ig kappa light chain  | MDMRAPAGIFGFLVLFPGYRS   |

|                |                                             |                            |
|----------------|---------------------------------------------|----------------------------|
| SEQ ID NO: 348 | human azurocidin preprotein signal sequence | MTRLTVLALLAGLLASSRA        |
| SEQ ID NO: 349 | IgG heavy chain signal peptide              | MELGLSWIFLLAILKGVQC        |
| SEQ ID NO: 350 | IgG heavy chain signal peptide              | MELGLRWVFLVAILEGVQC        |
| SEQ ID NO: 351 | IgG heavy chain signal peptide              | MKHLWFFLLLVAAPRWVLS        |
| SEQ ID NO: 352 | IgG heavy chain signal peptide              | MDWTWRILFLVAAATGAHS        |
| SEQ ID NO: 353 | IgG heavy chain signal peptide              | MDWTWRFLFVVAATGVQS         |
| SEQ ID NO: 354 | IgG heavy chain signal peptide              | MEFGLSWLFLVAILKGVQC        |
| SEQ ID NO: 355 | IgG heavy chain signal peptide              | MEFGLSWVFLVALFRGVQC        |
| SEQ ID NO: 356 | IgG heavy chain signal peptide              | MDLLHKNMKHLWFFLLLVAAPRWVLS |
| SEQ ID NO: 357 | IgG Kappa light chain signal sequence       | MDMRVPAQLLGLLLLWLSGARC     |
| SEQ ID NO: 358 | IgG Kappa light chain signal sequence       | MKYLLPTAAAGLLLLAAQPAMA     |
| SEQ ID NO: 359 | Gaussia luciferase                          | MGVKVLFALICIAVAEA          |
| SEQ ID NO: 360 | Human albumin                               | MKWVTFISLLFLFSSAYS         |
| SEQ ID NO: 361 | Human chymotrypsinogen                      | MAFLWLLSCWALLGTTFG         |
| SEQ ID NO: 362 | Human interleukin-2                         | MQLLSIALILALV              |
| SEQ ID NO: 363 | Human trypsinogen-2                         | MNLLLILTFVAAAVA            |

**[0324]** In some embodiments of a secretable variant ICOSL immunomodulatory protein, the immunomodulatory protein comprises a signal peptide when expressed, and the signal peptide (or a portion thereof) is cleaved from the immunomodulatory protein upon secretion.

**[0325]** In some embodiments, the engineered cells expresses a variant ICOSL polypeptides that are secreted from the cell. In some embodiments, such a variant ICOSL polypeptide is encoded by a nucleic acid molecule encoding an immunomodulatory protein under the operable control of a signal sequence for secretion. In some embodiments, the encoded immunomodulatory protein is secreted when expressed from a cell. In some embodiments, the immunomodulatory protein encoded by the nucleic acid molecule does not comprise a transmembrane domain. In some embodiments, the immunomodulatory protein encoded by the nucleic acid molecule does not comprise a half-life extending moiety (such as an Fc domain or a multimerization domain). In some embodiments, the immunomodulatory protein encoded by the nucleic acid molecule comprises a signal peptide. In some embodiments, a nucleic acid of the invention further comprises nucleotide sequence that encodes a secretory or signal peptide operably linked to the nucleic acid encoding the immunomodulatory protein, thereby allowing for secretion of the immunomodulatory protein

### 3. Cells and Engineering Cells

**[0326]** Provided herein are engineered cells expressing any of the provided immunomodulatory polypeptide. In some embodiments, the engineered cells express on their surface any of the provided transmembrane immunomodulatory polypeptides. In some embodiments, the engineered cells express and are capable of or are able to secrete the immunomodulatory protein from the cells under conditions suitable for secretion of the protein. In some embodiments, the immunomodulatory protein is expressed on a lymphocyte such as a tumor infiltrating lymphocyte (TIL), T-cell or NK cell, or on a myeloid cell. In some embodiments, the engineered cells are antigen presenting cells (APCs). In some embodiments, the engineered cells are engineered mammalian T-cells or engineered mammalian antigen presenting cells (APCs). In some embodiments, the engineered T-cells or APCs are human or murine cells.

**[0327]** In some embodiments, engineered T-cells include, but are not limited to, T helper cell, cytotoxic T-cell (alternatively, cytotoxic T lymphocyte or CTL), natural killer T-cell, regulatory T-cell, memory T-cell, or gamma delta T-cell. In some embodiments, the engineered T cells are CD4+ or CD8+. In addition to the signal of the MHC, engineered T-cells also require a co-stimulatory signal which in some embodiments is provided by a variant ICOSL transmembrane immunomodulatory polypeptide expressed in membrane bound form as discussed previously.

**[0328]** In some embodiments, the engineered APCs include, for example, MHC II expressing APCs such as macrophages, B cells, and dendritic cells, as well as artificial APCs (aAPCs) including both cellular and acellular (e.g., biodegradable polymeric microparticles) aAPCs. Artificial APCs (aAPCs) are synthetic versions of APCs that can act in a similar manner to APCs in that they present antigens to T-cells as well as activate them. Antigen presentation is performed by the MHC (Class I or Class II). In some embodiments, in engineered APCs such as aAPCs, the antigen that is loaded onto the MHC is, in some embodiments, a tumor specific antigen or a tumor associated antigen. The antigen loaded onto the MHC is recognized by a T-cell receptor (TCR) of a T cell, which, in some cases, can express ICOS, CD28, or other molecule recognized by the variant ICOSL polypeptides provided herein. Materials which can be used to engineer an aAPC include: poly (glycolic acid), poly(lactic-co-glycolic acid), iron-oxide, liposomes, lipid bilayers, sepharose, and polystyrene.

**[0329]** In some embodiments, an immunomodulatory protein, such as a transmembrane immunomodulatory protein or a secretable immunomodulatory protein, provided herein is co-expressed or engineered into a cell that expresses an antigen-binding receptor, such as a recombinant receptor, such as a chimeric antigen receptor (CAR) or T cell receptor (TCR). In some embodiments, the engineered cell, such as an engineered T cell, recognizes a desired antigen associated with cancer, inflammatory and autoimmune disorders, or a viral infection. In specific embodiments, the antigen-binding receptor contains an antigen-binding moiety that specifically binds a tumor specific antigen or a tumor associated antigen. In some embodiments, the engineered T-cell is a CAR (chimeric antigen receptor) T-cell that contains an antigen-binding domain (e.g. scFv) that specifically binds to an antigen, such as a tumor specific antigen or tumor associated antigen. In some embodiments, the antigen-binding domain (e.g. scFv) is specific for CD19. Exemplary of a CAR is an anti-CD19 CAR, such as a CAR containing an anti-CD19 scFv set forth in SEQ ID NO:482 or SEQ ID NO:245. In some embodiments, the TIP protein is expressed in an engineered T-cell receptor cell or and engineered chimeric antigen receptor cell. In such embodiments, the engineered cell co-expresses the TIP and the CAR or TCR.

**[0330]** In some embodiments, the CAR further contains a spacer or hinge, a transmembrane domain, and an intracellular signaling domain (endodomain) comprising an ITAM signaling domain, such as a CD3zeta signaling domain. In some embodiments, the CAR further includes a costimulatory signaling domain. In some embodiments, the spacer or hinge is present between the antigen-binding domain and the transmembrane domain, such as is between the antigen-binding domain and plasma membrane when expressed on a cell. In some embodiments, the spacer or hinge is derived from IgG subclass (such as IgG1 and IgG4, IgD or CD8 (see e.g., Qin et al. (2017) J. Hematol. Oncol., 10:68). In some embodiments, the spacer or hinge is derived from IgG1.

**[0331]** In some embodiments, the spacer and transmembrane domain are the hinge and transmembrane domain derived from CD8, such as set forth in SEQ ID NO: 246 or 483 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:246 or 483. In some embodiments, the endodomain comprises a CD3-zeta signaling domain. In some embodiments, the CD3-zeta signaling domain comprises the sequence of amino acids set forth in SEQ ID NO:

243 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% to SEQ ID NO:247 and retains the activity of T cell signaling. In some embodiments, the endodomain of a CAR-related transmembrane immunomodulatory protein can further comprise a costimulatory signaling domain to further modulate immunomodulatory responses of the T-cell. In some embodiments, the costimulatory signaling domain is CD28, ICOS, 41BB or OX40. In some embodiments, the costimulatory signaling domain is a derived from CD28 or 4-1BB and comprises the sequence of amino acids set forth in any of SEQ ID NOS: 484-487 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% to SEQ ID NO:484-487 and retains the activity of T cell costimulatory signaling.

**[0332]** In another embodiment, the engineered T-cell possesses a TCR, including a recombinant or engineered TCR. In some embodiments, the TCR can be a native TCR. Those of skill in the art will recognize that generally native mammalian T-cell receptors comprise an alpha and a beta chain (or a gamma and a delta chain) involved in antigen specific recognition and binding. In some embodiments, the TCR is an engineered TCR that is modified. In some embodiments, the TCR of an engineered T-cell specifically binds to a tumor associated or tumor specific antigen presented by an APC.

**[0333]** In some embodiments, the immunomodulatory polypeptides, such as transmembrane immunomodulatory polypeptides or secretable immunomodulatory polypeptides, can be incorporated into engineered cells, such as engineered T cells or engineered APCs, by a variety of strategies such as those employed for recombinant host cells. A variety of methods to introduce a DNA construct into primary T cells are known in the art. In some embodiments, viral transduction or plasmid electroporation are employed. In typical embodiments, the nucleic acid molecule encoding the immunomodulatory protein, or the expression vector, comprises a signal peptide that localizes the expressed transmembrane immunomodulatory proteins to the cellular membrane or for secretion. In some embodiments, a nucleic acid encoding a transmembrane immunomodulatory proteins of the invention is sub-cloned into a viral vector, such as a retroviral vector, which allows expression in the host mammalian cell. The expression vector can be introduced into a mammalian host cell and, under host cell culture conditions, the immunomodulatory protein is expressed on the surface or is secreted.

**[0334]** In an exemplary example, primary T-cells can be purified ex vivo (CD4 cells or CD8 cells or both) and stimulated with an activation protocol consisting of various TCR/CD28 agonists, such as anti-CD3/anti-CD28 coated beads. After a 2 or 3 day activation process, a recombinant expression vector containing an immunomodulatory polypeptide can be stably introduced into the primary T cells through art standard lentiviral or retroviral transduction protocols or plasmid electroporation strategies. Cells can be monitored for immunomodulatory polypeptide expression by, for example, flow cytometry using anti-epitope tag or antibodies that cross-react with native parental molecule and polypeptides comprising variant ICOSL. T-cells that express the immunomodulatory polypeptide can be enriched through sorting with anti-epitope tag antibodies or enriched for high or low expression depending on the application.

**[0335]** Upon immunomodulatory polypeptide expression the engineered T-cell can be assayed for appropriate function by a variety of means. The engineered CAR or TCR co-expression can be validated to show that this part of the engineered T cell was not significantly impacted by the expression of the immunomodulatory protein. Once validated, standard in vitro cytotoxicity, proliferation, or cytokine assays (e.g., IFN-gamma expression) can be used to assess the function of engineered T-cells. Exemplary standard endpoints are percent lysis of the tumor line, proliferation of the engineered T-cell, or IFN-gamma protein expression in culture supernatants. An engineered construct which results in statistically significant increased lysis of tumor line, increased proliferation of the engineered T-cell, or increased IFN-gamma expression over the control construct can be selected for. Additionally, non-engineered, such as native primary or endogenous T-cells could also be incorporated into the same in vitro assay to measure the ability of the immunomodulatory polypeptide construct expressed on the engineered cells, such as engineered T-cells, to modulate activity, including, in some cases, to activate and generate effector function in bystander, native T-cells. Increased expression of activation markers such as CD69, CD44, or CD62L could be monitored on endogenous T cells, and increased proliferation and/or cytokine production could indicate desired activity of the immunomodulatory protein expressed on the engineered T cells.

**[0336]** In some embodiments, the similar assays can be used to compare the function of engineered T cells containing the CAR or TCR alone to those containing the CAR or TCR and a TIP construct. Typically, these in vitro assays are performed by plating various ratios of the engineered T cell and a “tumor” cell line containing the cognate CAR or TCR antigen together in

culture. Standard endpoints are percent lysis of the tumor line, proliferation of the engineered T cell, or IFN-gamma production in culture supernatants. An engineered immunomodulatory protein which resulted in statistically significant increased lysis of tumor line, increased proliferation of the engineered T cell, or increased IFN-gamma production over the same TCR or CAR construct alone can be selected for. Engineered human T cells can be analyzed in immunocompromised mice, like the NSG strain, which lacks mouse T, NK and B cells. Engineered human T cells in which the CAR or TCR binds a target counter-structure on the xenograft and is co-expressed with the TIP affinity modified IgSF domain can be adoptively transferred in vivo at different cell numbers and ratios compared to the xenograft. For example, engraftment of CD19+ leukemia tumor lines containing a luciferase/GFP vector can be monitored through bioluminescence or ex vivo by flow cytometry. In a common embodiment, the xenograft is introduced into the murine model, followed by the engineered T cells several days later. Engineered T cells containing the immunomodulatory protein can be assayed for increased survival, tumor clearance, or expanded engineered T cells numbers relative to engineered T cells containing the CAR or TCR alone. As in the in vitro assay, endogenous, native (i.e., non-engineered) human T cells could be co-adoptively transferred to look for successful epitope spreading in that population, resulting in better survival or tumor clearance.

#### **E. Infectious Agents Expressing Variant Polypeptides and Immunomodulatory Proteins**

[0337] Also provided are infectious agents that contain nucleic acids encoding any of the variant polypeptides, such as ICOSL vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins described herein. In some embodiments, such infectious agents can deliver the nucleic acids encoding the variant immunomodulatory polypeptides described herein, such as ICOSL vIgD polypeptides, to a target cell in a subject, e.g., immune cell and/or antigen-presenting cell (APC) or tumor cell in a subject. Also provided are nucleic acids contained in such infectious agents, and/or nucleic acids for generation or modification of such infectious agents, such as vectors and/or plasmids, and compositions containing such infectious agents.

[0338] In some embodiments, the infectious agent is a microorganism or a microbe. In some embodiments, the infectious agent is a virus or a bacterium. In some embodiments, the infectious agent is a virus. In some embodiments, the infectious agent is a bacterium. In some

embodiments, such infectious agents can deliver nucleic acid sequences encoding any of the variant polypeptides, such as ICOSL vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins, described herein. Thus, in some embodiments, the cell in a subject that is infected or contacted by the infectious agents can be rendered to express on the cell surface or secrete, the variant immunomodulatory polypeptides. In some embodiments, the infectious agent can also deliver one or more other therapeutics or nucleic acids encoding other therapeutics to the cell and/or to an environment within the subject. In some embodiments, other therapeutics that can be delivered by the infectious agents include cytokines or other immunomodulatory molecules.

**[0339]** In some embodiments, the infectious agent, e.g., virus or bacteria, contains nucleic acid sequences that encode any of the variant polypeptides, such as ICOSL vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins, described herein, and by virtue of contact and/or infection of a cell in the subject, the cell expresses the variant polypeptides, such as ICOSL vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins, encoded by the nucleic acid sequences contained in the infectious agent. In some embodiments, the infectious agent can be administered to the subject. In some embodiments, the infectious agent can be introduced to cells from the subject *ex vivo*.

**[0340]** In some embodiments, the variant polypeptides, such as ICOSL vIgD polypeptides, including transmembrane immunomodulatory proteins, expressed by the cell infected by the infectious agent is a transmembrane protein and is surface expressed. In some embodiments, the variant polypeptides, such as ICOSL vIgD polypeptides, including secretable immunomodulatory proteins, expressed by the cell infected by the infectious agent is expressed and secreted from the cell. The transmembrane immunomodulatory protein or secreted immunomodulatory protein can be any described herein.

**[0341]** In some embodiments, the cells in the subject that are targeted by the infectious agent include a tumor cell, an immune cell, and/or an antigen-presenting cell (APC). In some embodiments, the infectious agent targets a cell in the tumor microenvironment (TME). In some embodiments, the infectious agent delivers the nucleic acids encoding the variant polypeptides, such as ICOSL vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins, to an appropriate cell (for example, an APC, such as a cell that displays a peptide/MHC complex on its cell surface, such as a dendritic cell) or tissue (e.g., lymphoid tissue) that will

induce and/or augment the desired effect, e.g., immunomodulation and/or a specific cell-mediated immune response, e.g., CD4 and/or CD8 T cell response, which CD8 T cell response may include a cytotoxic T cell (CTL) response. In some embodiments, the infectious agent targets an APC, such as a dendritic cell (DC). In some embodiments, the nucleic acid molecule delivered by the infectious agents described herein include appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequences encoding the variant immunomodulatory polypeptides, in a particular target cell, e.g., regulatory elements such as promoters.

**[0342]** In some embodiments, the infectious agent that contains nucleic acid sequences encoding the immunomodulatory polypeptides can also contain nucleic acid sequences that encode one or more additional gene products, e.g., cytokines, prodrug converting enzymes, cytotoxins and/or detectable gene products. For example, in some embodiments, the infectious agent is an oncolytic virus and the virus can include nucleic acid sequences encoding additional therapeutic gene products (see, e.g., Kirn et al., (2009) *Nat Rev Cancer* 9:64-71; Garcia-Aragoncillo et al., (2010) *Curr Opin Mol Ther* 12:403-411; see U.S. Pat. Nos. 7,588,767, 7,588,771, 7,662,398 and 7,754,221 and U.S. Pat. Publ. Nos. 2007/0202572, 2007/0212727, 2010/0062016, 2009/0098529, 2009/0053244, 2009/0155287, 2009/0117034, 2010/0233078, 2009/0162288, 2010/0196325, 2009/0136917 and 2011/0064650. In some embodiments, the additional gene product can be a therapeutic gene product that can result in death of the target cell (e.g., tumor cell) or gene products that can augment or boost or regulate an immune response (e.g., cytokine). Exemplary gene products also include among an anticancer agent, an anti-metastatic agent, an antiangiogenic agent, an immunomodulatory molecule, an immune checkpoint inhibitor, an antibody, a cytokine, a growth factor, an antigen, a cytotoxic gene product, a pro-apoptotic gene product, an anti-apoptotic gene product, a cell matrix degradative gene, genes for tissue regeneration and reprogramming human somatic cells to pluripotency, and other genes described herein or known to one of skill in the art. In some embodiments, the additional gene product is Granulocyte-macrophage colony-stimulating factor (GM-CSF).

## **1. Viruses**

**[0343]** In some embodiments, the infectious agent is a virus. In some embodiments, the infectious agent is an oncolytic virus, or a virus that targets particular cells, e.g., immune cells.

In some embodiments, the infectious agent targets a tumor cell and/or cancer cell in the subject. In some embodiments, the infectious agent targets an immune cell or an antigen-presenting cell (APC).

**[0344]** In some embodiments, the infectious agent is an oncolytic virus. Oncolytic viruses are viruses that accumulate in tumor cells and replicate in tumor cells. By virtue of replication in the tumor cells, and optional delivery of nucleic acids encoding variant ICOSL polypeptides or immunomodulatory polypeptides described herein, tumor cells are lysed, and the tumor shrinks and can be eliminated. Oncolytic viruses can also have a broad host and cell type range. For example, oncolytic viruses can accumulate in immunoprivileged cells or immunoprivileged tissues, including tumors and/or metastases, and also including wounded tissues and cells, thus allowing the delivery and expression of nucleic acids encoding the variant immunomodulatory polypeptides described herein in a broad range of cell types. Oncolytic viruses can also replicate in a tumor cell specific manner, resulting in tumor cell lysis and efficient tumor regression.

**[0345]** Exemplary oncolytic viruses include adenoviruses, adeno-associated viruses, herpes viruses, Herpes Simplex Virus, Vesticular Stomatitis virus, Reovirus, Newcastle Disease virus, parvovirus, measles virus, vesicular stomatitis virus (VSV), Coxsackie virus and Vaccinia virus. In some embodiments, oncolytic viruses can specifically colonize solid tumors, while not infecting other organs, and can be used as an infectious agent to deliver the nucleic acids encoding the variant immunomodulatory polypeptides described herein to such solid tumors.

**[0346]** Oncolytic viruses for use in delivering the nucleic acids encoding variant ICOSL polypeptides or immunomodulatory polypeptides described herein, can be any of those known to one of skill in the art and include, for example, vesicular stomatitis virus, see, e.g., U.S. Pat. Nos. 7,731,974, 7,153,510, 6,653,103 and U.S. Pat. Pub. Nos. 2010/0178684, 2010/0172877, 2010/0113567, 2007/0098743, 20050260601, 20050220818 and EP Pat. Nos. 1385466, 1606411 and 1520175; herpes simplex virus, see, e.g., U.S. Pat. Nos. 7,897,146, 7,731,952, 7,550,296, 7,537,924, 6,723,316, 6,428,968 and U.S. Pat. Pub. Nos., 2014/0154216, 2011/0177032, 2011/0158948, 2010/0092515, 2009/0274728, 2009/0285860, 2009/0215147, 2009/0010889, 2007/0110720, 2006/0039894, 2004/0009604, 2004/0063094, International Patent Pub. Nos., WO 2007/052029, WO 1999/038955; retroviruses, see, e.g., U.S. Pat. Nos. 6,689,871, 6,635,472, 5,851,529, 5,716,826, 5,716,613 and U.S. Pat. Pub. No. 20110212530; vaccinia viruses, see, e.g., 2016/0339066, and adeno-associated viruses, see, e.g., U.S. Pat. Nos. 8,007,780, 7,968,340,

7,943,374, 7,906,111, 7,927,585, 7,811,814, 7,662,627, 7,241,447, 7,238,526, 7,172,893, 7,033,826, 7,001,765, 6,897,045, and 6,632,670.

**[0347]** Oncolytic viruses also include viruses that have been genetically altered to attenuate their virulence, to improve their safety profile, enhance their tumor specificity, and they have also been equipped with additional genes, for example cytotoxins, cytokines, prodrug converting enzymes to improve the overall efficacy of the viruses (see, e.g., Kirn et al., (2009) *Nat Rev Cancer* 9:64-71; Garcia-Aragoncillo et al., (2010) *Curr Opin Mol Ther* 12:403-411; see U.S. Pat. Nos. 7,588,767, 7,588,771, 7,662,398 and 7,754,221 and U.S. Pat. Publ. Nos. 2007/0202572, 2007/0212727, 2010/0062016, 2009/0098529, 2009/0053244, 2009/0155287, 2009/0117034, 2010/0233078, 2009/0162288, 2010/0196325, 2009/0136917 and 2011/0064650). In some embodiments, the oncolytic viruses can be those that have been modified so that they selectively replicate in cancerous cells, and, thus, are oncolytic. For example, the oncolytic virus is an adenovirus that has been engineered to have modified tropism for tumor therapy and also as gene therapy vectors. Exemplary of such is ONYX-015, H101 and Ad5 $\Delta$ CR (Hallden and Portella (2012) *Expert Opin Ther Targets*, 16:945-58) and TNFerade (McLoughlin et al. (2005) *Ann. Surg. Oncol.*, 12:825-30), or a conditionally replicative adenovirus Oncorine®.

**[0348]** In some embodiments, the infectious agent is a modified herpes simplex virus. In some embodiments, the infectious agent is a modified version of Talimogene laherparepvec (also known as T-Vec, Imlygic or OncoVex GM-CSF), that is modified to contain nucleic acids encoding any of the variant ICOSL polypeptides or immunomodulatory polypeptides described herein. In some embodiments, the infectious agent is a modified herpes simplex virus that is described, e.g., in WO 2007/052029, WO 1999/038955, US 2004/0063094, US 2014/0154216, or, variants thereof.

**[0349]** In some embodiments, the infectious agent is a virus that targets a particular type of cells in a subject that is administered the virus, e.g., a virus that targets immune cells or antigen-presenting cells (APCs). Dendritic cells (DCs) are essential APCs for the initiation and control of immune responses. DCs can capture and process antigens, migrate from the periphery to a lymphoid organ, and present the antigens to resting T cells in a major histocompatibility complex (MHC)-restricted fashion. In some embodiments, the infectious agent is a virus that specifically can target DCs to deliver nucleic acids encoding the variant ICOSL polypeptide or immunomodulatory polypeptides for expression in DCs. In some embodiments, the virus is a

lentivirus or a variant or derivative thereof, such as an integration-deficient lentiviral vector . In some embodiments, the virus is a lentivirus that is pseudotyped to efficiently bind to and productively infect cells expressing the cell surface marker dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), such as DCs. In some embodiments, the virus is a lentivirus pseudotyped with a Sindbis virus E2 glycoprotein or modified form thereof, such as those described in WO 2013/149167. In some embodiments, the virus allows for delivery and expression of a sequence of interest (e.g., a nucleic acid encoding any of the variant ICOSL polypeptides or immunomodulatory polypeptides described herein) to a DC. In some embodiments, the virus includes those described in WO 2008/011636, US 2011/0064763, Tareen et al. (2014) Mol. Ther., 22:575-587, or variants thereof. Exemplary of a dendritic cell-tropic vector platform is ZVex™.

## **2. Bacteria**

**[0350]** In some embodiments, the infectious agent is a bacterium. For example, in some embodiments, the bacteria can deliver nucleic acids encoding any of the variant immunomodulatory polypeptides described herein, e.g., variant ICOSL polypeptide or immunomodulatory polypeptide, to a target cell in the subject, such as a tumor cell, an immune cell, an antigen-presenting cell and/or a phagocytic cell. In some embodiments, the bacterium can be preferentially targeted to a specific environment within a subject, such as a tumor microenvironment (TME), for expression and/or secretion of the variant immunomodulatory polypeptides and/or to target specific cells in the environment for expression of the variant immunomodulatory polypeptides.

**[0351]** In some embodiments, the bacterium delivers the nucleic acids to the cells via bacterial-mediated transfer of plasmid DNA to mammalian cells (also referred to as “bactofection”). For example, in some embodiments, delivery of genetic material is achieved through entry of the entire bacterium into target cells. In some embodiments, spontaneous or induced bacterial lysis can lead to the release of plasmid for subsequent eukaryotic cell expression. In some embodiments, the bacterium can deliver nucleic acids to non-phagocytic mammalian cells (e.g., tumor cells) and/or to phagocytic cells, e.g., certain immune cells and/or APCs. In some embodiments, the nucleic acids delivered by the bacterium can be transferred to the nucleus of the cell in the subject for expression. In some embodiments, the nucleic acids also

include appropriate nucleic acid sequences necessary for the expression of the operably linked sequences encoding the variant immunomodulatory polypeptides in a particular host cell, e.g., regulatory elements such as promoters or enhancers. In some embodiments, the infectious agent that is a bacterium can deliver nucleic acids encoding the immunomodulatory proteins in the form of an RNA, such as a pre-made translation-competent RNA delivered to the cytoplasm of the target cell for translation by the target cell's machinery.

**[0352]** In some embodiments, the bacterium can replicate and lyse the target cells, e.g., tumor cells. In some embodiments, the bacterium can contain and/or release nucleic acid sequences and/or gene products in the cytoplasm of the target cells, thereby killing the target cell, e.g., tumor cell. In some embodiments, the infectious agent is bacterium that can replicate specifically in a particular environment in the subject, e.g., tumor microenvironment (TME). For example, in some embodiments, the bacterium can replicate specifically in anaerobic or hypoxic microenvironments. In some embodiments, conditions or factors present in particular environments, e.g., aspartate, serine, citrate, ribose or galactose produced by cells in the TME, can act as chemoattractants to attract the bacterium to the environment. In some embodiments, the bacterium can express and/or secrete the immunomodulatory proteins described herein in the environment, e.g., TME.

**[0353]** In some embodiments, the infectious agent is a bacterium that is a *Listeria* sp., a *Bifidobacterium* sp., an *Escherichia* sp., a *Clostridium* sp., a *Salmonella* sp., a *Shigella* sp., a *Vibrio* sp. or a *Yersinia* sp. In some embodiments, the bacterium is selected from among one or more of *Listeria monocytogenes*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Escherichia coli*, *Vibrio cholera*, *Clostridium perfringens*, *Clostridium butyricum*, *Clostridium novyi*, *Clostridium acetobutylicum*, *Bifidobacterium infantis*, *Bifidobacterium longum* and *Bifidobacterium adolescentis*. In some embodiments, the bacterium is an engineered bacterium. In some embodiments, the bacterium is an engineered bacterium such as those described in, e.g., Seow and Wood (2009) *Molecular Therapy* 17(5):767–777; Baban et al. (2010) *Bioengineered Bugs* 1:6, 385-394; Patyar et al. (2010) *J Biomed Sci* 17:21; Tangney et al. (2010) *Bioengineered Bugs* 1:4, 284-287; van Pijkeren et al. (2010) *Hum Gene Ther.* 21(4):405-416; WO 2012/149364; WO 2014/198002; US 9103831; US 9453227; US 2014/0186401; US 2004/0146488; US 2011/0293705; US 2015/0359909 and EP 3020816. The bacterium can be modified to deliver nucleic acid sequences encoding any of the variant immunomodulatory polypeptides, conjugates

and/or fusions provided herein, and/or to express such variant immunomodulatory polypeptides in the subject.

#### **F. Nucleic acids, Vectors and Methods for Producing the Polypeptides or Cells**

**[0354]** Provided herein are isolated or recombinant nucleic acids collectively referred to as “nucleic acids” which encode any of the various provided embodiments of the variant ICOSL polypeptides or immunomodulatory polypeptides provided herein. In some embodiments, nucleic acids provided herein, including all described below, are useful in recombinant production (e.g., expression) of variant ICOSL polypeptides or immunomodulatory polypeptides provided herein. In some embodiments, nucleic acids provided herein, including all described below, are useful in expression of variant ICOSL polypeptides or immunomodulatory polypeptides provided herein in cells, such as in engineered cells, e.g. immune cells, or infectious agent cells. The nucleic acids provided herein can be in the form of RNA or in the form of DNA, and include mRNA, cRNA, recombinant or synthetic RNA and DNA, and cDNA. The nucleic acids provided herein are typically DNA molecules, and usually double-stranded DNA molecules. However, single-stranded DNA, single-stranded RNA, double-stranded RNA, and hybrid DNA/RNA nucleic acids or combinations thereof comprising any of the nucleotide sequences of the invention also are provided.

**[0355]** Also provided herein are recombinant expression vectors and recombinant host cells useful in producing the variant ICOSL polypeptides or immunomodulatory polypeptides provided herein.

**[0356]** Also provided herein are engineered cells, such as engineered immune cells, containing any of the provided nucleic acid molecules or any of the variant ICOSL polypeptides or immunomodulatory polypeptides, such as any of the transmembrane immunomodulatory polypeptides or secretable immunomodulatory polypeptides.

**[0357]** Also provided herein are infectious agents, such as bacterial or viral cells, containing any of the provided nucleic acid molecules or any of the variant ICOSL polypeptides or immunomodulatory polypeptides, such as any of the transmembrane immunomodulatory polypeptides or secretable immunomodulatory polypeptides.

**[0358]** In any of the above provided embodiments, the nucleic acids encoding the variant polypeptides or immunomodulatory polypeptides provided herein can be introduced into cells using recombinant DNA and cloning techniques. To do so, a recombinant DNA molecule encoding a immunomodulatory polypeptide is prepared. Methods of preparing such DNA molecules are well known in the art. For instance, sequences coding for the peptides could be excised from DNA using suitable restriction enzymes. Alternatively, the DNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidite method. Also, a combination of these techniques could be used. In some instances, a recombinant or synthetic nucleic acid may be generated through polymerase chain reaction (PCR). In some embodiments, a DNA insert can be generated encoding one or more variant ICOSL polypeptides containing at least one affinity-modified IgSF domain and, in some embodiments, a signal peptide, a transmembrane domain and/or an endodomain in accord with the provided description. This DNA insert can be cloned into an appropriate transduction/transfection vector as is known to those of skill in the art. Also provided are expression vectors containing the nucleic acid molecules.

**[0359]** In some embodiments, the expression vectors are capable of expressing the immunomodulatory proteins in an appropriate cell under conditions suited to expression of the protein. In some aspects, nucleic acid molecule or an expression vector comprises the DNA molecule that encodes the immunomodulatory protein operatively linked to appropriate expression control sequences. Methods of effecting this operative linking, either before or after the DNA molecule is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation.

**[0360]** In some embodiments, expression of the immunomodulatory protein is controlled by a promoter or enhancer to control or regulate expression. The promoter is operably linked to the portion of the nucleic acid molecule encoding the variant polypeptide or immunomodulatory protein. In some embodiments, the promoter is a constitutively active promoter (such as a tissue-specific constitutively active promoter or other constitutive promoter). In some embodiments, the promoter is an inducible promoter, which may be responsive to an inducing agent (such as a T cell activation signal).

**[0361]** In some embodiments, a constitutive promoter is operatively linked to the nucleic acid molecule encoding the variant polypeptide or immunomodulatory protein. Exemplary constitutive promoters include the Simian vacuolating virus 40 (SV40) promoter, the cytomegalovirus (CMV) promoter, the ubiquitin C (UbC) promoter, and the EF-1 alpha (EF1a) promoter. In some embodiments, the constitutive promoter is tissue specific. For example, in some embodiments, the promoter allows for constitutive expression of the immunomodulatory protein in specific tissues, such as immune cells, lymphocytes, or T cells. Exemplary tissue-specific promoters are described in U.S. Patent No. 5,998,205, including, for example, a fetoprotein, DF3, tyrosinase, CEA, surfactant protein, and ErbB2 promoters.

**[0362]** In some embodiments, an inducible promoter is operatively linked to the nucleic acid molecule encoding the variant polypeptide or immunomodulatory protein such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription. For example, the promoter can be a regulated promoter and transcription factor expression system, such as the published tetracycline-regulated systems or other regulatable systems (see, e.g. published International PCT Appl. No. WO 01/30843), to allow regulated expression of the encoded polypeptide. An exemplary regulatable promoter system is the Tet-On (and Tet-Off) system available, for example, from Clontech (Palo Alto, CA). This promoter system allows the regulated expression of the transgene controlled by tetracycline or tetracycline derivatives, such as doxycycline. Other regulatable promoter systems are known (see e.g., published U.S. Application No. 2002-0168714, entitled “Regulation of Gene Expression Using Single-Chain, Monomeric, Ligand Dependent Polypeptide Switches,” which describes gene switches that contain ligand binding domains and transcriptional regulating domains, such as those from hormone receptors).

**[0363]** In some embodiments, the promoter is responsive to an element responsive to T-cell activation signaling. Solely by way of example, in some embodiments, an engineered T cell comprises an expression vector encoding the immunomodulatory protein and a promoter operatively linked to control expression of the immunomodulatory protein. The engineered T cell can be activated, for example by signaling through an engineered T cell receptor (TCR) or a chimeric antigen receptor (CAR), and thereby triggering expression and secretion of the immunomodulatory protein through the responsive promoter.

**[0364]** In some embodiments, an inducible promoter is operatively linked to the nucleic acid molecule encoding the immunomodulatory protein such that the immunomodulatory protein is expressed in response to a nuclear factor of activated T-cells (NFAT) or nuclear factor kappa-light-chain enhancer of activated B cells (NF- $\kappa$ B). For example, in some embodiments, the inducible promoter comprises a binding site for NFAT or NF- $\kappa$ B. For example, in some embodiments, the promoter is an NFAT or NF- $\kappa$ B promoter or a functional variant thereof. Thus, in some embodiments, the nucleic acids make it possible to control the expression of immunomodulatory protein while also reducing or eliminating the toxicity of the immunomodulatory protein. In particular, engineered immune cells comprising the nucleic acids of the invention express and secrete the immunomodulatory protein only when the cell (e.g., a T-cell receptor (TCR) or a chimeric antigen receptor (CAR) expressed by the cell) is specifically stimulated by an antigen and/or the cell (e.g., the calcium signaling pathway of the cell) is non-specifically stimulated by, e.g., phorbol myristate acetate (PMA)/Ionomycin. Accordingly, the expression and, in some cases, secretion, of immunomodulatory protein can be controlled to occur only when and where it is needed (e.g., in the presence of an infectious disease-causing agent, cancer, or at a tumor site), which can decrease or avoid undesired immunomodulatory protein interactions.

**[0365]** In some embodiments, the nucleic acid encoding an immunomodulatory protein described herein comprises a suitable nucleotide sequence that encodes a NFAT promoter, NF- $\kappa$ B promoter, or a functional variant thereof. “NFAT promoter” as used herein means one or more NFAT responsive elements linked to a minimal promoter. “NF- $\kappa$ B promoter” refers to one or more NF- $\kappa$ B responsive elements linked to a minimal promoter. In some embodiments, the minimal promoter of a gene is a minimal human IL-2 promoter or a CMV promoter. The NFAT responsive elements may comprise, e.g., NFAT1, NFAT2, NFAT3, and/or NFAT4 responsive elements. The NFAT promoter, NF- $\kappa$ B promoter, or a functional variant thereof may comprise any number of binding motifs, e.g., at least two, at least three, at least four, at least five, or at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, or up to twelve binding motifs.

**[0366]** The resulting recombinant expression vector having the DNA molecule thereon is used to transform an appropriate host. This transformation can be performed using methods well known in the art. In some embodiments, a nucleic acid provided herein further comprises

nucleotide sequence that encodes a secretory or signal peptide operably linked to the nucleic acid encoding an immunomodulatory polypeptide such that a resultant soluble immunomodulatory polypeptide is recovered from the culture medium, host cell, or host cell periplasm. In other embodiments, the appropriate expression control signals are chosen to allow for membrane expression of an immunomodulatory polypeptide. Furthermore, commercially available kits as well as contract manufacturing companies can also be utilized to make engineered cells or recombinant host cells provided herein.

**[0367]** In some embodiments, the resulting expression vector having the DNA molecule thereon is used to transform, such as transduce, an appropriate cell. The introduction can be performed using methods well known in the art. Exemplary methods include those for transfer of nucleic acids encoding the receptors, including via viral, e.g., retroviral or lentiviral, transduction, transposons, and electroporation. In some embodiments, the expression vector is a viral vector. In some embodiments, the nucleic acid is transferred into cells by lentiviral or retroviral transduction methods.

**[0368]** Any of a large number of publicly available and well-known mammalian host cells, including mammalian T-cells or APCs, can be used in the preparing the polypeptides or engineered cells. The selection of a cell is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all cells can be equally effective for the expression of a particular DNA sequence.

**[0369]** In some embodiments, the host cells can be a variety of eukaryotic cells, such as in yeast cells, or with mammalian cells such as Chinese hamster ovary (CHO) or HEK293 cells. Host cells can also be prokaryotic cells, such as with *E. coli*. The transformed recombinant host is cultured under polypeptide expressing conditions, and then purified to obtain a soluble protein. Recombinant host cells can be cultured under conventional fermentation conditions so that the desired polypeptides are expressed. Such fermentation conditions are well known in the art. Finally, the polypeptides provided herein can be recovered and purified from recombinant cell cultures by any of a number of methods well known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography,

phosphocellulose chromatography, hydrophobic interaction chromatography, and affinity chromatography. Protein refolding steps can be used, as desired, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed in the final purification steps.

**[0370]** In some embodiments, the cell is an immune cell, such as any described above in connection with preparing engineered cells. In some embodiments, such engineered cells are primary cells. In some embodiments, the engineered cells are autologous to the subject. In some embodiment, the engineered cells are allogeneic to the subject. In some embodiments, the engineered cells are obtained from a subject, such as by leukapheresis, and transformed ex vivo for expression of the immunomodulatory polypeptide, e.g. transmembrane immunomodulatory polypeptide or secretable immunomodulatory polypeptide.

**[0371]** Also provided are nucleic acids encoding any of the variant immunomodulatory polypeptides contained in infectious agents described herein. In some embodiments, the infectious agents deliver the nucleic acids to a cell in the subject, and/or permit expression of the encoded variant polypeptides in the cell. Also provided are nucleic acids that are used to generate, produce or modify such infectious agents. For example, in some embodiments, provided are vectors and/or plasmids that contain nucleic acids encoding the variant immunomodulatory polypeptides, for generation of the infectious agents, delivery to the cells in a subject and/or expression of the variant immunomodulatory polypeptides in the cells in the subject.

**[0372]** In some embodiments, the provided nucleic acids are recombinant viral or bacterial vectors containing nucleic acid sequences encoding the variant immunomodulatory polypeptides. In some embodiments, the recombinant vectors can be used to produce an infectious agent that contains nucleic acid sequences encoding the variant immunomodulatory polypeptides and/or to be delivered to a target cell in the subject for expression by the target cell. In some embodiments, the recombinant vector is an expression vector. In some embodiments, the recombinant vector includes appropriate sequences necessary for generation and/or production of the infectious agent and expression in the target cell.

**[0373]** In some embodiments, the recombinant vector is a plasmid or cosmid. Plasmid or cosmid containing nucleic acid sequences encoding the variant immunomodulatory polypeptides, as described herein, is readily constructed using standard techniques well known in

the art. For generation of the infectious agent, the vector or genome can be constructed in a plasmid form that can then be transfected into a packaging or producer cell line or a host bacterium. The recombinant vectors can be generated using any of the recombinant techniques known in the art. In some embodiments, the vectors can include a prokaryotic origin of replication and/or a gene whose expression confers a detectable or selectable marker such as a drug resistance for propagation and/or selection in prokaryotic systems.

**[0374]** In some embodiments, the recombinant vector is a viral vector. Exemplary recombinant viral vectors include a lentiviral vector genome, poxvirus vector genome, vaccinia virus vector genome, adenovirus vector genome, adenovirus-associated virus vector genome, herpes virus vector genome, and alpha virus vector genome. Viral vectors can be live, attenuated, replication conditional or replication deficient, non-pathogenic (defective), replication competent viral vector, and/or is modified to express a heterologous gene product, e.g., the variant immunomodulatory polypeptides provided herein. Vectors for generation of viruses also can be modified to alter attenuation of the virus, which includes any method of increasing or decreasing the transcriptional or translational load.

**[0375]** Exemplary viral vectors that can be used include modified vaccinia virus vectors (see, e.g., Guerra et al., *J. Virol.* 80:985-98 (2006); Tartaglia et al., *AIDS Research and Human Retroviruses* 8: 1445-47 (1992); Gheradi et al., *J. Gen. Virol.* 86:2925-36 (2005); Mayr et al., *Infection* 3:6-14 (1975); Hu et al., *J. Virol.* 75: 10300-308 (2001); U.S. Patent Nos. 5,698,530, 6,998,252, 5,443,964, 7,247,615 and 7,368,116); adenovirus vector or adenovirus-associated virus vectors (see., e.g., Molin et al., *J. Virol.* 72:8358-61 (1998); Narumi et al., *Am J. Respir. Cell Mol. Biol.* 19:936-41 (1998); Mercier et al., *Proc. Natl. Acad. Sci. USA* 101:6188-93 (2004); U.S. Patent Nos. 6,143,290; 6,596,535; 6,855,317; 6,936,257; 7,125,717; 7,378,087; 7,550,296); retroviral vectors including those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), ecotropic retroviruses, simian immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and combinations (see, e.g., Buchscher et al., *J. Virol.* 66:2731-39 (1992); Johann et al., *J. Virol.* 66: 1635-40 (1992); Sommerfelt et al., *Virology* 176:58-59 (1990); Wilson et al., *J. Virol.* 63:2374-78 (1989) ; Miller et al., *J. Virol.* 65:2220-24 (1991); Miller et al., *Mol. Cell Biol.* 10:4239 (1990) ; Kolberg, *NIH Res.* 4:43 1992; Cornetta et al., *Hum. Gene Ther.* 2:215 (1991)); lentiviral vectors including those based upon Human Immunodeficiency Virus (HIV-1), HIV-2, feline immunodeficiency virus (FIV), equine

infectious anemia virus, Simian Immunodeficiency Virus (SIV), and maedi/visna virus (see, e.g., Pfeifer et al., *Annu. Rev. Genomics Hum. Genet.* 2: 177-211 (2001); Zufferey et al., *J. Virol.* 72: 9873, 1998; Miyoshi et al., *J. Virol.* 72:8150, 1998; Philpott and Thrasher, *Human Gene Therapy* 18:483, 2007; Engelman et al., *J. Virol.* 69: 2729, 1995; Nightingale et al., *Mol. Therapy*, 13: 1121, 2006; Brown et al., *J. Virol.* 73:9011 (1999); WO 2009/076524; WO 2012/141984; WO 2016/011083; McWilliams et al., *J. Virol.* 77: 11150, 2003; Powell et al., *J. Virol.* 70:5288, 1996) or any, variants thereof, and/or vectors that can be used to generate any of the viruses described above. In some embodiments, the recombinant vector can include regulatory sequences, such as promoter or enhancer sequences, that can regulate the expression of the viral genome, such as in the case for RNA viruses, in the packaging cell line (see, e.g., U.S. Patent Nos. 5,385,839 and 5,168,062).

**[0376]** In some embodiments, the recombinant vector is an expression vector, e.g., an expression vector that permits expression of the encoded gene product when delivered into the target cell, e.g., a cell in the subject, e.g., a tumor cell, an immune cell and/or an APC. In some embodiments, the recombinant expression vectors contained in the infectious agent are capable of expressing the immunomodulatory proteins in the target cell in the subject, under conditions suited to expression of the protein.

**[0377]** In some aspects, nucleic acids or an expression vector comprises a nucleic acid sequence that encodes the immunomodulatory protein operatively linked to appropriate expression control sequences. Methods of affecting this operative linking, either before or after the nucleic acid sequence encoding the immunomodulatory protein is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation. The promoter can be operably linked to the portion of the nucleic acid sequence encoding the immunomodulatory protein. In some embodiments, the promoter is a constitutively active promoter in the target cell (such as a tissue-specific constitutively active promoter or other constitutive promoter). For example, the recombinant expression vector may also include, lymphoid tissue-specific transcriptional regulatory elements (TRE) such as a B lymphocyte, T lymphocyte, or dendritic cell specific TRE. Lymphoid tissue specific TRE are known in the art (see, e.g., Thompson et al., *Mol. Cell. Biol.* 12:1043-53 (1992); Todd et al., *J. Exp. Med.* 177:1663-74 (1993); Penix et al., *J. Exp. Med.*

178:1483-96 (1993)). In some embodiments, the promotor is an inducible promotor, which may be responsive to an inducing agent (such as a T cell activation signal). In some embodiments, nucleic acids delivered to the target cell in the subject, e.g., tumor cell, immune cell and/or APC, can be operably linked to any of the regulatory elements described above.

**[0378]** In some embodiments, the vector is a bacterial vector, e.g., a bacterial plasmid or cosmid. In some embodiments, the bacterial vector is delivered to the target cell, e.g., tumor cells, immune cells and/or APCs, via bacterial-mediated transfer of plasmid DNA to mammalian cells (also referred to as “bactofection”). In some embodiments, the delivered bacterial vector also contains appropriate expression control sequences for expression in the target cells, such as a promoter sequence and/or enhancer sequences, or any regulatory or control sequences described above. In some embodiments, the bacterial vector contains appropriate expression control sequences for expression and/or secretion of the encoded variant polypeptides in the infectious agent, e.g., the bacterium.

**[0379]** In some embodiments, polypeptides provided herein can also be made by synthetic methods. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides. For example, well known solid phase synthesis techniques include the use of protecting groups, linkers, and solid phase supports, as well as specific protection and deprotection reaction conditions, linker cleavage conditions, use of scavengers, and other aspects of solid phase peptide synthesis. Peptides can then be assembled into the polypeptides as provided herein.

#### **IV. METHODS OF ASSESSING ACTIVITY IMMUNE MODULATION OF VARIANT ICOSL POLYPEPTIDES AND IMMUNOMODULATORY PROTEINS**

**[0380]** In some embodiments, the variant ICOSL polypeptides provided herein (e.g. full-length and/or specific binding fragments or conjugates, stack constructs or fusion thereof) exhibit immunomodulatory activity to modulate T cell activation. In some embodiments, ICOSL polypeptides modulate IFN-gamma expression in a primary T cell assay relative to a wild-type or unmodified ICOSL control. In some cases, modulation of IFN-gamma expression can increase or decrease IFN-gamma expression relative to the control. Assays to determine specific binding and IFN-gamma expression are well-known in the art and include the MLR (mixed lymphocyte

reaction) assays measuring interferon-gamma cytokine levels in culture supernatants (Wang et al., Cancer Immunol Res. 2014 Sep; 2(9):846-56), SEB (staphylococcal enterotoxin B) T cell stimulation assay (Wang et al., Cancer Immunol Res. 2014 Sep; 2(9):846-56), and anti-CD3 T cell stimulation assays (Li and Kurlander, J Transl Med. 2010; 8: 104).

**[0381]** In some embodiments, a variant ICOSL polypeptide can in some embodiments increase or, in alternative embodiments, decrease IFN-gamma (interferon-gamma) expression in a primary T-cell assay relative to a wild-type ICOSL control. In some embodiments of the provided polypeptides containing a soluble variant ICOSL sequence, the polypeptide can increase IFN-gamma expression and, in alternative embodiments, decrease IFN-gamma expression in a primary T-cell assay relative to a wild-type ICOSL control. In some embodiments of the provided polypeptides containing multiple variant ICOSL sequences, the polypeptide can increase IFN-gamma expression and, in alternative embodiments, decrease IFN-gamma expression in a primary T-cell assay relative to a wild-type ICOSL control.

**[0382]** Those of skill will recognize that the format of the primary T-cell assay used to determine an increase in IFN-gamma expression can differ from that employed to assay for a decrease in IFN-gamma expression. In assaying for the ability of a variant ICOSL to decrease IFN-gamma expression in a primary T-cell assay, a Mixed Lymphocyte Reaction (MLR) assay can be used as described in Example 6. In some cases, a soluble form of a variant ICOSL can be employed to determine the ability of the variant ICOSL to antagonize and thereby decrease the IFN-gamma expression in a MLR as likewise described in Example 6.

**[0383]** Alternatively, in assaying for the ability of a variant ICOSL to increase IFN-gamma expression in a primary T-cell assay, a co-immobilization assay can be used as described in Example 6. In a co-immobilization assay, a TCR signal, provided in some embodiments by anti-CD3 antibody, is used in conjunction with a co-immobilized variant ICOSL to determine the ability to increase IFN-gamma expression relative to an ICOSL control. In some cases, a soluble form of a variant ICOSL that is multimerized to a degree to provide multivalent binding can be employed to determine the ability of the variant ICOSL to agonize and thereby increase the IFN-gamma expression in a MLR as likewise described in Example 6.

**[0384]** Use of proper controls is known to those of skill in the art, however, in the aforementioned embodiments, the control typically involves use of the unmodified ICOSL, such as a wild-type of native ICOSL isoform from the same mammalian species from which the

variant ICOSL was derived or developed. Irrespective of whether the binding affinity to either one or both of ICOS and CD28 is increased or decreased, a variant ICOSL in some embodiments will increase IFN-gamma expression and, in alternative embodiments, decrease IFN-gamma expression in a primary T-cell assay relative to a wild-type ICOSL control.

**[0385]** In some embodiments, a variant ICOSL increases IFN-gamma expression (i.e., protein expression) relative to a wild-type or unmodified ICOSL control by at least: 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or higher. In other embodiments, a variant ICOSL decreases IFN-gamma expression (i.e. protein expression) relative to a wild-type or unmodified ICOSL control by at least: 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or higher. In some embodiments, the wild-type ICOSL control is murine ICOSL, such as would typically be used for a variant ICOSL altered in sequence from that of a wild-type murine ICOSL sequence. In some embodiments, the wild-type ICOSL control is human ICOSL, such as would typically be used for a variant ICOSL altered in sequence from that of a wild-type human ICOSL sequence such as an ICOSL sequence comprising the sequence of amino acids of SEQ ID NO:32 or SEQ ID NO:196.

## **V. PHARMACEUTICAL FORMULATIONS**

**[0386]** Provided herein are compositions containing any of the variant ICOSL polypeptides, immomodulatory proteins, conjugates, engineered cells or infectious agents described herein. The pharmaceutical composition can further comprise a pharmaceutically acceptable excipient. For example, the pharmaceutical composition can contain one or more excipients for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption, or penetration of the composition. In some aspects, a skilled artisan understands that a pharmaceutical composition containing cells may differ from a pharmaceutical composition containing a protein.

**[0387]** In some embodiments, the pharmaceutical composition is a solid, such as a powder, capsule, or tablet. For example, the components of the pharmaceutical composition can be lyophilized. In some embodiments, the solid pharmaceutical composition is reconstituted or dissolved in a liquid prior to administration.

**[0388]** In some embodiments, the pharmaceutical composition is a liquid, for example variant ICOSL polypeptides dissolved in an aqueous solution (such as physiological saline or

Ringer's solution). In some embodiments, the pH of the pharmaceutical composition is between about 4.0 and about 8.5 (such as between about 4.0 and about 5.0, between about 4.5 and about 5.5, between about 5.0 and about 6.0, between about 5.5 and about 6.5, between about 6.0 and about 7.0, between about 6.5 and about 7.5, between about 7.0 and about 8.0, or between about 7.5 and about 8.5).

**[0389]** In some embodiments, the pharmaceutical composition comprises a pharmaceutically-acceptable excipient, for example a filler, binder, coating, preservative, lubricant, flavoring agent, sweetening agent, coloring agent, a solvent, a buffering agent, a chelating agent, or stabilizer. Examples of pharmaceutically-acceptable fillers include cellulose, dibasic calcium phosphate, calcium carbonate, microcrystalline cellulose, sucrose, lactose, glucose, mannitol, sorbitol, maltol, pregelatinized starch, corn starch, or potato starch. Examples of pharmaceutically-acceptable binders include polyvinylpyrrolidone, starch, lactose, xylitol, sorbitol, maltitol, gelatin, sucrose, polyethylene glycol, methyl cellulose, or cellulose. Examples of pharmaceutically-acceptable coatings include hydroxypropyl methylcellulose (HPMC), shellac, corn protein zein, or gelatin. Examples of pharmaceutically-acceptable disintegrants include polyvinylpyrrolidone, carboxymethyl cellulose, or sodium starch glycolate. Examples of pharmaceutically-acceptable lubricants include polyethylene glycol, magnesium stearate, or stearic acid. Examples of pharmaceutically-acceptable preservatives include methyl parabens, ethyl parabens, propyl paraben, benzoic acid, or sorbic acid. Examples of pharmaceutically-acceptable sweetening agents include sucrose, saccharine, aspartame, or sorbitol. Examples of pharmaceutically-acceptable buffering agents include carbonates, citrates, gluconates, acetates, phosphates, or tartrates.

**[0390]** In some embodiments, the pharmaceutical composition further comprises an agent for the controlled or sustained release of the product, such as injectable microspheres, bio-erodible particles, polymeric compounds (polylactic acid, polyglycolic acid), beads, or liposomes.

**[0391]** In some embodiments, the pharmaceutical composition is sterile. Sterilization may be accomplished by filtration through sterile filtration membranes or radiation. Where the composition is lyophilized, sterilization using this method may be conducted either prior to or following lyophilization and reconstitution. The composition for parenteral administration may be stored in lyophilized form or in solution. In addition, parenteral compositions generally are

placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

**[0392]** In some embodiments, provided are pharmaceutical compositions containing the transmembrane immunomodulatory proteins, including engineered cells expressing such transmembrane immunomodulatory proteins. In some embodiments, the pharmaceutical compositions and formulations include one or more optional pharmaceutically acceptable carrier or excipient. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present invention are preferably formulated for intravenous administration.

**[0393]** In some embodiments, the pharmaceutical composition contains infectious agents containing nucleic acid sequences encoding the immunomodulatory variant polypeptides. In some embodiments, the pharmaceutical composition contains a dose of infectious agents suitable for administration to a subject that is suitable for treatment. In some embodiments, the pharmaceutical composition contains an infectious agent that is a virus, at a single or multiple dosage amount, of between about  $1 \times 10^5$  and about  $1 \times 10^{12}$  plaque-forming units (pfu),  $1 \times 10^6$  and  $1 \times 10^{10}$  pfu, or  $1 \times 10^7$  and  $1 \times 10^{10}$  pfu, each inclusive, such as at least or at least about or at about  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$ ,  $1 \times 10^9$ ,  $2 \times 10^9$ ,  $3 \times 10^9$ ,  $4 \times 10^9$ ,  $5 \times 10^9$  pfu or about  $1 \times 10^{10}$  pfu. In some embodiments, the pharmaceutical composition can contain a virus concentration of from or from about  $10^5$  to about  $10^{10}$  pfu/mL, for example,  $5 \times 10^6$  to  $5 \times 10^9$  or  $1 \times 10^7$  to  $1 \times 10^9$  pfu/mL, such as at least or at least about or at about  $10^6$  pfu/mL,  $10^7$  pfu/mL,  $10^8$  pfu/mL or  $10^9$  pfu/mL. In some embodiments, the pharmaceutical composition contains an infectious agent that is a bacterium, at a single or multiple dosage amount, of between about between or between about  $1 \times 10^3$  and about  $1 \times 10^9$  colony-forming units (cfu),  $1 \times 10^4$  and  $1 \times 10^9$  cfu, or  $1 \times 10^5$  and  $1 \times 10^7$  cfu, each inclusive, such as at least or at least about or at about  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$  or  $1 \times 10^9$  cfu. In some embodiments, the pharmaceutical composition can contain a bacterial concentration of from or from about  $10^3$  to about  $10^8$  cfu/mL, for example,  $5 \times 10^5$  to  $5 \times 10^7$  or  $1 \times 10^6$  to  $1 \times 10^7$  cfu/mL, such as at least or at least about or at about  $10^5$  cfu/mL,  $10^6$  cfu/mL,  $10^7$  cfu/mL or  $10^8$  cfu/mL.

**[0394]** Such a formulation may, for example, be in a form suitable for intravenous infusion. A pharmaceutically acceptable carrier may be a pharmaceutically acceptable material, composition, or vehicle that is involved in carrying or transporting cells of interest from one tissue, organ, or portion of the body to another tissue, organ, or portion of the body. For example, the carrier may be a liquid or solid filler, diluent, excipient, solvent, or encapsulating material, or some combination thereof. Each component of the carrier must be “pharmaceutically acceptable” in that it must be compatible with the other ingredients of the formulation. It also must be suitable for contact with any tissue, organ, or portion of the body that it may encounter, meaning that it must not carry a risk of toxicity, irritation, allergic response, immunogenicity, or any other complication that excessively outweighs its therapeutic benefits.

**[0395]** In some embodiments, the pharmaceutical composition is administered to a subject. Generally, dosages and routes of administration of the pharmaceutical composition are determined according to the size and condition of the subject, according to standard pharmaceutical practice. For example, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models such as mice, rats, rabbits, dogs, pigs, or monkeys. An animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. The exact dosage will be determined in light of factors related to the subject requiring treatment. Dosage and administration are adjusted to provide sufficient levels of the active compound or to maintain the desired effect. Factors that may be taken into account include the severity of the disease state, the general health of the subject, the age, weight, and gender of the subject, time and frequency of administration, drug combination(s), reaction sensitivities, and response to therapy.

**[0396]** Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or biweekly depending on the half-life and clearance rate of the particular formulation. The frequency of dosing will depend upon the pharmacokinetic parameters of the molecule in the formulation used. Typically, a composition is administered until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, or as multiple doses (at the same or different concentrations/dosages) over time, or as a continuous infusion. Further refinement of the appropriate dosage is routinely made. Appropriate dosages may be ascertained through use of appropriate dose-response data. A

number of biomarkers or physiological markers for therapeutic effect can be monitored including T cell activation or proliferation, cytokine synthesis or production (e.g., production of TNF- $\alpha$ , IFN- $\gamma$ , IL-2), induction of various activation markers (e.g., CD25, IL-2 receptor), inflammation, joint swelling or tenderness, serum level of C-reactive protein, anti-collagen antibody production, and/or T cell-dependent antibody response(s).

**[0397]** In some embodiments, the pharmaceutical composition is administered to a subject through any route, including orally, transdermally, by inhalation, intravenously, intra-arterially, intramuscularly, direct application to a wound site, application to a surgical site, intraperitoneally, by suppository, subcutaneously, intradermally, transcutaneously, by nebulization, intrapleurally, intraventricularly, intra-articularly, intraocularly, or intraspinally.

**[0398]** In some embodiments, the dosage of the pharmaceutical composition is a single dose or a repeated dose. In some embodiments, the doses are given to a subject once per day, twice per day, three times per day, or four or more times per day. In some embodiments, about 1 or more (such as about 2 or more, about 3 or more, about 4 or more, about 5 or more, about 6 or more, or about 7 or more) doses are given in a week. In some embodiments, multiple doses are given over the course of days, weeks, months, or years. In some embodiments, a course of treatment is about 1 or more doses (such as about 2 or more doses, about 3 or more doses, about 4 or more doses, about 5 or more doses, about 7 or more doses, about 10 or more doses, about 15 or more doses, about 25 or more doses, about 40 or more doses, about 50 or more doses, or about 100 or more doses).

**[0399]** In some embodiments, an administered dose of the pharmaceutical composition is about 1  $\mu$ g of protein per kg subject body mass or more (such as about 2  $\mu$ g of protein per kg subject body mass or more, about 5  $\mu$ g of protein per kg subject body mass or more, about 10  $\mu$ g of protein per kg subject body mass or more, about 25  $\mu$ g of protein per kg subject body mass or more, about 50  $\mu$ g of protein per kg subject body mass or more, about 100  $\mu$ g of protein per kg subject body mass or more, about 250  $\mu$ g of protein per kg subject body mass or more, about 500  $\mu$ g of protein per kg subject body mass or more, about 1 mg of protein per kg subject body mass or more, about 2 mg of protein per kg subject body mass or more, or about 5 mg of protein per kg subject body mass or more).

**[0400]** In some embodiments, a therapeutic amount of a cell composition is administered. Typically, precise amount of the compositions of the present invention to be administered can be

determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising engineered cells, e.g. T cells, as described herein may be administered at a dosage of  $10^4$  to  $10^9$  cells/kg body weight, such as  $10^5$  to  $10^6$  cells/kg body weight, including all integer values within those ranges. Engineered cell compositions, such as T cell compositions, may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, e.g., Rosenberg et al, New Eng. J. of Med. 319: 1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

**[0401]** A variety of means are known for determining if administration of a therapeutic composition of the invention sufficiently modulates immunological activity by eliminating, sequestering, or inactivating immune cells mediating or capable of mediating an undesired immune response; inducing, generating, or turning on immune cells that mediate or are capable of mediating a protective immune response; changing the physical or functional properties of immune cells; or a combination of these effects. Examples of measurements of the modulation of immunological activity include, but are not limited to, examination of the presence or absence of immune cell populations (using flow cytometry, immunohistochemistry, histology, electron microscopy, polymerase chain reaction (PCR)); measurement of the functional capacity of immune cells including ability or resistance to proliferate or divide in response to a signal (such as using T-cell proliferation assays and pepsin analysis based on  $^3\text{H}$ -thymidine incorporation following stimulation with anti-CD3 antibody, anti-T-cell receptor antibody, anti-CD28 antibody, calcium ionophores, PMA (phorbol 12-myristate 13-acetate) antigen presenting cells loaded with a peptide or protein antigen; B cell proliferation assays); measurement of the ability to kill or lyse other cells (such as cytotoxic T cell assays); measurements of the cytokines, chemokines, cell surface molecules, antibodies and other products of the cells (e.g., by flow cytometry, enzyme-linked immunosorbent assays, Western blot analysis, protein microarray analysis, immunoprecipitation analysis); measurement of biochemical markers of activation of immune cells or signaling pathways within immune cells (e.g., Western blot and immunoprecipitation analysis of tyrosine, serine or threonine phosphorylation, polypeptide cleavage, and formation or dissociation of protein complexes; protein array analysis; DNA transcriptional, profiling using

DNA arrays or subtractive hybridization); measurements of cell death by apoptosis, necrosis, or other mechanisms (e.g., annexin V staining, TUNEL assays, gel electrophoresis to measure DNA laddering, histology; fluorogenic caspase assays, Western blot analysis of caspase substrates); measurement of the genes, proteins, and other molecules produced by immune cells (e.g., Northern blot analysis, polymerase chain reaction, DNA microarrays, protein microarrays, 2-dimensional gel electrophoresis, Western blot analysis, enzyme linked immunosorbent assays, flow cytometry); and measurement of clinical symptoms or outcomes such as improvement of autoimmune, neurodegenerative, and other diseases involving self-proteins or self-polypeptides (clinical scores, requirements for use of additional therapies, functional status, imaging studies) for example, by measuring relapse rate or disease severity (using clinical scores known to the ordinarily skilled artisan) in the case of multiple sclerosis, measuring blood glucose in the case of type I diabetes, or joint inflammation in the case of rheumatoid arthritis.

## **VI. ARTICLES OF MANUFACTURE AND KITS**

**[0402]** Also provided herein are articles of manufacture comprising the pharmaceutical compositions described herein in suitable packaging. Suitable packaging for compositions (such as ophthalmic compositions) described herein are known in the art, and include, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

**[0403]** Further provided are kits comprising the pharmaceutical compositions (or articles of manufacture) described herein, which may further comprise instruction(s) on methods of using the composition, such as uses described herein. The kits described herein may also include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein.

## **VII. THERAPEUTIC APPLICATIONS**

**[0404]** The pharmaceutical compositions described herein (including pharmaceutical composition comprising the variant ICOSL polypeptides, the immunomodulatory proteins, the conjugates, the engineered cells and infectious agents described herein) can be used in a variety

of therapeutic applications, such as the treatment of a disease. For example, in some embodiments the pharmaceutical composition is used to treat inflammatory or autoimmune disorders, cancer, organ transplantation, viral infections, and/or bacterial infections in a mammal. The pharmaceutical composition can modulate (e.g. increase or decrease) an immune response to treat the disease. In some embodiments, the provided methods are applicable to therapeutic administration of variant ICOSL polypeptides, the immunomodulatory proteins, the conjugates, the engineered cells and infectious agents described herein. It is within the level of a skilled artisan, in view of the provided disclosure, to choose a format for the indication depending on the type of modulation of the immune response, e.g. increase or decrease that is desired.

**[0405]** In some embodiments, a pharmaceutical composition provided herein that stimulates the immune response is administered, which can be useful, for example, in the treatment of cancer, viral infections, or bacterial infections. In some embodiments, the pharmaceutical composition contains a variant ICOSL polypeptide in a format that exhibits agonist activity of its cognate binding partner CD28 or ICOS and/or that stimulates or initiates costimulatory signaling via CD28 or ICOS. Exemplary formats of an ICOSL polypeptide for use in connection with such therapeutic applications include, for example, an immunomodulatory protein or “stack” of a variant ICOSL polypeptide and an IgSF domain or variant thereof that binds to a tumor antigen (e.g. Nkp30 or affinity-modified variant) (also called a “tumor-localizing IgSF domain), a conjugate containing a variant ICOSL polypeptide linked to a tumor-targeting moiety (also called a tumor-localizing moiety), an engineered cell expressing a transmembrane immunomodulatory protein or an infectious agent comprising a nucleic acid molecule encoding a transmembrane immunomodulatory protein, such as for expression of the transmembrane immunomodulatory protein in an infected cell (e.g. tumor cell or APC, e.g. dendritic cell).

**[0406]** In some embodiments, the pharmaceutical composition can be used to inhibit growth of mammalian cancer cells (such as human cancer cells). A method of treating cancer can include administering an effective amount of any of the pharmaceutical compositions described herein to a subject with cancer. The effective amount of the pharmaceutical composition can be administered to inhibit, halt, or reverse progression of cancers.

**[0407]** The cancers that can be treated by the pharmaceutical compositions and the treatment methods described herein include, but are not limited to, melanoma, bladder cancer, hematological malignancies (leukemia, lymphoma, myeloma), liver cancer, brain cancer, renal

cancer, breast cancer, pancreatic cancer (adenocarcinoma), colorectal cancer, lung cancer (small cell lung cancer and non-small-cell lung cancer), spleen cancer, cancer of the thymus or blood cells (i.e., leukemia), prostate cancer, testicular cancer, ovarian cancer, uterine cancer, gastric carcinoma, a musculoskeletal cancer, a head and neck cancer, a gastrointestinal cancer, a germ cell cancer, or an endocrine and neuroendocrine cancer . In some embodiments, the cancer is Ewing's sarcoma. In some embodiments, the cancer is selected a from melanoma, lung cancer, bladder cancer, and a hematological malignancy. In some embodiments, the cancer is a lymphoma, lymphoid leukemia, myeloid leukemia, cervical cancer, neuroblastoma, or multiple myeloma.

**[0408]** Human cancer cells can be treated in vivo, or ex vivo. In ex vivo treatment of a human patient, tissue or fluids containing cancer cells are treated outside the body and then the tissue or fluids are reintroduced back into the patient. In some embodiments, the cancer is treated in a human patient in vivo by administration of the therapeutic composition into the patient.

**[0409]** In some embodiments, the pharmaceutical composition is administered as a monotherapy (i.e., as a single agent) or as a combination therapy (i.e., in combination with one or more additional anticancer agents, such as a chemotherapeutic drug, a cancer vaccine, or an immune checkpoint inhibitor. In some embodiments, the pharmaceutical composition can also be administered with radiation therapy.

**[0410]** In some embodiments, the pharmaceutical composition suppresses an immune response, which can be useful in the treatment of inflammatory or autoimmune disorders, or organ transplantation. In some embodiments, the pharmaceutical composition contains a variant ICOSL polypeptide in a format that exhibits antagonist activity of its cognate binding partner CD28 or ICOS and/or that blocks or inhibits costimulatory signaling via CD28 or ICOS. Exemplary formats of an ICOSL polypeptide for use in connection with such therapeutic applications include, for example, a variant ICOSL polypeptide that is soluble (e.g. variant ICOSL-Fc fusion protein), an immunomodulatory protein or “stack” of a variant ICOSL polypeptide and another IgSF domain, including soluble forms thereof that are Fc fusions, an engineered cell expressing a secretable immunomodulatory protein, or an infectious agent comprising a nucleic acid molecule encoding a secretable immunomodulatory protein, such as for expression and secretion of the secretable immunomodulatory protein in an infected cell (e.g. tumor cell or APC, e.g. dendritic cell).

**[0411]** In some embodiments, the inflammatory or autoimmune disorder is Antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, a vasculitis, an autoimmune skin disease, transplantation, a Rheumatic disease, an inflammatory gastrointestinal disease, an inflammatory eye disease, an inflammatory neurological disease, an inflammatory pulmonary disease, an inflammatory endocrine disease, or an autoimmune hematological disease.

**[0412]** In some embodiments, the inflammatory and autoimmune disorders that can be treated by the pharmaceutical composition described herein is Addison's Disease, allergies, alopecia areata, Alzheimer's, antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, ankylosing spondylitis, antiphospholipid syndrome (Hughes Syndrome), asthma, atherosclerosis, rheumatoid arthritis, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune inner ear disease, autoimmune lymphoproliferative syndrome, autoimmune myocarditis, autoimmune oophoritis, autoimmune orchitis, azoospermia, Behcet's Disease, Berger's Disease, bullous pemphigoid, cardiomyopathy, cardiovascular disease, celiac Sprue/coeliac disease, chronic fatigue immune dysfunction syndrome (CFIDS), chronic idiopathic polyneuritis, chronic inflammatory demyelinating, polyradical neuropathy (CIPD), chronic relapsing polyneuropathy (Guillain-Barré syndrome), Churg-Strauss Syndrome (CSS), cicatricial pemphigoid, cold agglutinin disease (CAD), COPD (chronic obstructive pulmonary disease), CREST syndrome, Crohn's disease, dermatitis, herpetiformis, dermatomyositis, diabetes, discoid lupus, eczema, epidermolysis bullosa acquisita, essential mixed cryoglobulinemia, Evan's Syndrome, exophthalmos, fibromyalgia, Goodpasture's Syndrome, Graves' Disease, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, immunoproliferative disease or disorder, inflammatory bowel disease (IBD), interstitial lung disease, juvenile arthritis, juvenile idiopathic arthritis (JIA), Kawasaki's Disease, Lambert-Eaton Myasthenic Syndrome, lichen planus, lupus nephritis, lymphocytic laryngitis, Ménière's Disease, Miller Fish Syndrome/acute disseminated encephalomyeloradiculopathy, mixed connective tissue disease, multiple sclerosis (MS), muscular rheumatism, myalgic encephalomyelitis (ME), myasthenia gravis, ocular inflammation, pemphigus foliaceus, pemphigus vulgaris, pernicious anaemia, polyarteritis nodosa, polychondritis, polyglandular syndromes (Whitaker's syndrome), polymyalgia rheumatica, polymyositis, primary agammaglobulinemia, primary biliary cirrhosis/autoimmune cholangiopathy, psoriasis, psoriatic arthritis, Raynaud's Phenomenon, Reiter's

Syndrome/reactive arthritis, restenosis, rheumatic fever, rheumatic disease, sarcoidosis, Schmidt's syndrome, scleroderma, Sjörger's Syndrome, stiff-man syndrome, systemic lupus erythematosus (SLE), systemic scleroderma, Takayasu arteritis, temporal arteritis/giant cell arteritis, thyroiditis, Type 1 diabetes, ulcerative colitis, uveitis, vasculitis, vitiligo, interstitial bowel disease or Wegener's Granulomatosis. In some embodiments, the inflammatory or autoimmune disorder is selected from interstitial bowel disease, transplant, Crohn's disease, ulcerative colitis, multiple sclerosis, asthma, rheumatoid arthritis, and psoriasis.

**[0413]** In some embodiments, the pharmaceutical composition is administered to modulate an autoimmune condition. For example, suppressing an immune response can be beneficial in methods for inhibiting rejection of a tissue, cell, or organ transplant from a donor by a recipient. Accordingly, in some embodiments, the pharmaceutical compositions described herein are used to limit or prevent graft-related or transplant related diseases or disorders, such as graft versus host disease (GVHD). In some embodiments, the pharmaceutical compositions are used to suppress autoimmune rejection of transplanted or grafted bone marrow, organs, skin, muscle, neurons, islets, or parenchymal cells.

**[0414]** Pharmaceutical compositions comprising engineered cells and the methods described herein can be used in adoptive cell transfer applications. In some embodiments, cell compositions comprising engineered cells can be used in associated methods to, for example, modulate immunological activity in an immunotherapy approach to the treatment of, for example, a mammalian cancer or, in other embodiments the treatment of autoimmune disorders. The methods employed generally comprise a method of contacting a TIP of the present invention with a mammalian cell under conditions that are permissive to specific binding of the affinity modified IgSF domain and modulation of the immunological activity of the mammalian cell. In some embodiments, immune cells (such as tumor infiltrating lymphocytes (TILs), T-cells (including CD8+ or CD4+ T-cells), or APCs) are engineered to express as a membrane protein and/or as a soluble variant ICOSL polypeptide, immunomodulatory protein, or conjugate as described herein. The engineered cells can then contact a mammalian cell, such as an APC, a second lymphocyte or tumor cell in which modulation of immunological activity is desired and under conditions that are permissive of specific binding of the affinity modified IgSF domain to a counter-structure on the mammalian cell such that immunological activity can be modulated in the mammalian cell. Cells can be contacted in vivo or ex vivo.

**[0415]** In some embodiments, the engineered cells are autologous cells. In other embodiments, the cells are allogeneic. In some embodiments, the cells are autologous engineered cells reinfused into the mammal from which it was isolated. In some embodiments, the cells are allogenic engineered cells infused into the mammal. In some embodiments, the cells are harvested from a patient's blood or tumor, engineered to express a polypeptide (such as the variant ICOSL polypeptide, immunomodulatory protein, or conjugate as described herein), expanded in an in vitro culture system (for example, by stimulating the cells), and reinfused into the patient to mediate tumor destruction. In some embodiments, the methods are conducted by adoptive cell transfer wherein cells expressing the TIP (e.g., a T-cell) are infused back into the patient. In some embodiments, the therapeutic compositions and methods of the invention are used in the treatment of a mammalian patient of cancers such as lymphoma, lymphoid leukemia, myeloid leukemia, cervical cancer, neuroblastoma, or multiple myeloma.

## **VIII. EXEMPLARY EMBODIMENTS**

**[0416]** Among the provided embodiments are:

1. A variant ICOS Ligand (ICOSL) polypeptide, comprising an IgV domain or specific binding fragment thereof, an IgC domain or a specific binding fragment thereof, or both, wherein the variant ICOSL polypeptide comprises one or more amino acid modifications in an unmodified ICOSL or a specific binding fragment thereof corresponding to position(s) selected from 10, 11, 13, 16, 18, 20, 25, 27, 30, 33, 37, 38, 42, 43, 47, 52, 54, 57, 61, 62, 67, 71, 72, 74, 75, 77, 78, 80, 84, 89, 90, 92, 93, 94, 96, 97, 98, 99, 100, 102, 103, 107, 109, 110, 111, 113, 115, 116, 117, 119, 120, 121, 122, 126, 129, 130, 132, 133, 135, 138, 139, 140, 142, 143, 144, 146, 148, 151, 152, 153, 154, 155, 156, 158, 161, 164, 166, 168, 172, 173, 175, 190, 192, 193, 194, 198, 201, 203, 207, 208, 210, 212, 217, 218, 220, 221, 224, 225, or 227 with reference to SEQ ID NO:32.
2. The variant ICOSL polypeptide of embodiment 1, wherein the unmodified ICOSL is a mammalian ICOSL or a specific binding fragment thereof.
3. The variant ICOSL polypeptide of embodiment 2, wherein the unmodified ICOSL is a human ICOSL or a specific binding fragment thereof.
4. The variant ICOSL polypeptide of any one of embodiments 1-3, wherein the unmodified ICOSL comprises (i) the sequence of amino acids set forth in SEQ ID NO:32, (ii) a

sequence of amino acids that has at least 95% sequence identity to SEQ ID NO:32; or (iii) a portion thereof comprising an IgV domain or IgC domain or specific binding fragments thereof or both.

5. The variant ICOSL polypeptide of any one of embodiments 1-4, wherein:  
the specific binding fragment of the IgV domain or IgC domain has a length of at least 50, 60, 70, 80, 90, 100, 110 or more amino acids; or

the specific binding fragment of the IgV domain comprises a length that is at least 80% of the length of the IgV domain set forth as amino acids 19-129 of SEQ ID NO:5 and/or the specific binding fragment of the IgC domain comprises a length that is at least 80% of the length of the IgC domain set forth as amino acids 141-227 of SEQ ID NO:5.

6. The variant ICOSL polypeptide of any one of embodiments 1-5, wherein the variant ICOSL comprises up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid modifications, optionally amino acid substitutions, insertions and/or deletions.

7. The variant ICOSL of any of embodiments 1-6, wherein the variant ICOSL comprises a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:32 or a specific binding fragment thereof.

8. The variant ICOSL polypeptide of any of embodiments 1-7, wherein the variant ICOSL polypeptide exhibits altered binding to the ectodomain of ICOS, CD28 or CTLA-4 compared to the unmodified ICOSL.

9. The variant ICOSL polypeptide of any of embodiments 1-8, wherein the variant ICOSL polypeptide exhibits altered binding to the ectodomain of ICOS or CD28 compared to the unmodified ICOSL.

10. The variant ICOSL polypeptide of embodiment 8 or embodiment 9, wherein the altered binding is altered binding affinity and/or altered binding selectivity.

11. The variant ICOSL polypeptide of any of embodiments 1-10, wherein the one or more amino acid modifications are selected from M10V, M10I, V11E, S13G, E16V, S18R, A20V, S25G, F27S, F27C, N30D, Y33del, Q37R, K42E, T43A, Y47H, N52H, N52D, N52Q, N52S, N52Y, N52K, S54A, S54P, N57D, N57Y, R61S, R61C, Y62F, L67P, A71T, G72R, L74Q, R75Q, D77G, F78L, L80P, N84Q, E90A, K92R, F93L, H94E, H94D, L96F, L96I, V97A, L98F, S99G, Q100R, Q100K, Q100P, L102R, G103E, V107A, V107I, S109G, S109N, V110D,

V110N, V110A, E111del, T113E, H115Q, H115R, V116A, A117T, N119Q, F120I, F120S, S121G, V122A, V122M, S126T, S126R, H129P, S130G, S132F, Q133H, E135K, F138L, T139S, C140del, S142F, I143V, I143T, N144D, Y146C, V151A, Y152C, Y152H, W153R, I154F, N155H, N155Q, K156M, D158G, L161P, L161M, L166Q, N168Q, F172S, L173S, M175T, T190A, T190S, S192G, V193M, N194D, C198R, N201S, L203P, L203F, N207Q, L208P, V210A, S212G, D217V, I218T, I218N, E220G, R221G, R221I, I224V, T225A, N227K, or a conservative amino acid substitution thereof.

12. The variant ICOSL polypeptide of any of embodiments 1-11, wherein the one or more amino acid modifications are selected from among N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52S/Y146C/Y152C, N52H/C198R, N52H/C140D/T225A, N52H/C198R/T225A, N52H/K92R, N52H/S99G, N57Y/Q100P, N52S/S130G/Y152C, N52S/Y152C, N52S/C198R, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52S/T113E, N52D/S54P, N52K/L208P, N52S/Y152H, N52D/V151A, N52H/I143T, N52S/L80P, F120S/Y152H/N201S, N52S/R75Q/L203P, N52S/D158G, N52D/Q133H, N52S/N57Y/H94D/L96F/L98F/Q100R, N52S/N57Y/H94D/L96F/L98F/Q100R/G103E/F120S, N52H/F78L/Q100R, N52H/N57Y/Q100R/V110D, N52H/N57Y/R75Q/Q100R/V110D, N52H/N57Y/Q100R, N52H/N57Y/L74Q/Q100R/V110D, N52H/Q100R, N52H/S121G, A20V/N52H/N57Y/Q100R/S109G, N52H/N57Y/R61S/Q100R/V110D/L173S, N52H/N57Y/Q100R/V122A, N52H/N57Y/Q100R/F172S, N52H/N57Y, N52S/F120S, N52S/V97A, N52S/G72R, N52S/A71T/A117T, N52S/E220G, Y47H/N52S/V107A/F120S, N52H/N57Y/Q100R/V110D/S132F/M175T, E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R, Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G, N52H/N57Y/Q100R/V110D/C198R, N52H/N57Y/Q100R/V110D/V116A/L161M/F172S/S192G/C198R, F27S/N52H/N57Y/V110N, N52S/H94E/L96I/S109N/L166Q, S18R/N52S/F93L/I143V/R221G, A20T/N52D/Y146C/Q164L, V11E/N30D/N52H/N57Y/H94E/L96I/L98F/N194D/V210A/I218T, N52S/H94E/L96I/V122M, N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N, M10V/S18R/N30D/N52S/S126R/T139S/L203F, S25G/N30D/N52S/F120S/N227K, N30D/N52S/L67P/Q100K/D217G/R221K/T225S,

N52H/N57Y/Q100R/V110D/A117T/T190S/C198R, N52H/N57Y/Q100R/V110D/F172S/C198R,  
 S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R,  
 N52H/N57Y/V110A/C198R/R221I,  
 M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M,C198R,  
 N52H/N57Y/R61C/Y62F/Q100R/V110N/F120S/C198R,  
 N52H/N57Y/Q100R/V110D/H115R/C198R,  
 N52H/N57Y/Q100R/V110D/N144D/F172S/C198R, N52S/H94E/L98F/Q100R, N52S/E90A,  
 N30D/K42E/N52S, N52S/F120S/I143V/I224V, N52H/ N57Y/Q100R/V110D/C198R/S212G,  
 N52H/N57Y/Q100R/C198R, N52S/N194D, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R,  
 N52H/ N57Y/Q100R/V110D/C198R/S212G, N52H/N57Y/Q100R/C198R, N52S/N194D,  
 N52H/N57Y/Q100R/L102R/V110D/H115R/C198R, N52S/S54P, T38P/N52S/N57D, E111del,  
 Y33del, N52H/C140del/T225A, N52H/F78L/Q100R/C198R,  
 N52H/N57Y/R75Q/Q100P/V110D, N52H/N57Y/L74Q/V110D/S192G, N52H/S121G/C198R,  
 N52S/F120S/N227K, N52S/A71T/A117T/T190A/C198R,  
 T43A/N52H/N57Y/L74Q/D89G/V110D/F172S, N52H/N57Y/Q100R/V110D/S132F/M175T,  
 N52D, N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G, N52Q/N207Q,  
 N168Q/N207Q, N52Q/N168Q, N84Q/N207Q, N155Q/N207Q, N119Q/N168Q, N119Q/N207Q,  
 N119Q/N155Q, N52Q/N84Q, N52Q/N119Q, N84Q/N119Q, N52Q/N84Q/N168Q,  
 N52Q/N84Q/N207Q, N84Q/N155Q/N168Q, N84Q/N168Q/N207Q, N84Q/N155H/N207Q,  
 N155Q/N168Q/N207Q, N119Q/N155Q/N168Q, N119Q/N168Q/N207Q, N84Q/N119Q/N207Q,  
 N119Q/N155H/N207Q, N84Q/N119Q/N155Q, N52Q/N119Q/N155Q, N52H/N84Q/N119Q,  
 N52H/N84Q, N52H/N84Q/N168Q, N52H/N84Q/N207Q, N52H/N84Q/N168Q/N207Q,  
 N52Q/N84Q/N155Q, N52Q/N84Q/N168Q, N52Q/N84Q/N155Q/N168Q,  
 N52Q/N84Q/N119Q/N168Q, N84Q/N119Q/N155Q/N168Q, N84Q/N155Q/N168Q/N207Q,  
 N84Q/N119Q/N155Q/N207Q, N52Q/N84Q/N119Q/N207Q, N52Q/N84Q/N119Q/N155Q,  
 N52Q/N84Q/N119Q/N155Q/N207Q, N84Q/N119Q/N155Q/N168Q/N207Q, F138L/L203P,  
 N52Y/F138L/L203P, N57Y/Q100R/C198R, N57Y/F138L/L203P, Q100R/F138L,  
 N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/Q100R/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/F172S/C198R, N52H/N57Y/Q100R/H115R/I143V/F172S/C198R,  
 N52H/N57Y/Q100R/L102R/H115R/F172S/C198R, N52H/V122A/F172S/C198R,  
 N52H/N57Y/Q100R/H115R/F172S/N194D, N52H/N57Y/H115R/F172S/C198R,

N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/H115R, N52H/N57Y/Q100R/H115R,  
 N52H/N57Y/Q100R/H115R/F172S/I224V, N52H/N57Y/Q100R/H115R/F172S,  
 N52H/N57Y/Q100R/F172S, N52H/Q100R/H115R/I143T/F172S,  
 N52H/N57Y/Q100P/H115R/F172S, N52Y/N57Y/Q100P/F172S,  
 E16V/N52H/N57Y/Q100R/V110D/H115R/C198R,  
 E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R, N52S/E90A/H115R,  
 N30D/K42E N52S/H115R, N30D/K42E/N52S/H115R/C198R/R221I,  
 N30D/K42E/N52S/H115R/C198R, N30D/K42E/N52S/H115R/F172S/N194D,  
 N52S/H115R/F120S/I143V/C198R, N52S/H115R/F172S/C198R, N52H/N57Y/Q100P/C198R,  
 N52H/N57Y/Q100P H115R/F172S/C198R, N52H/N57Y/Q100P/F172S/C198R,  
 N52H/N57Y/Q100P/H115R, N52H/N57Y/Q100P/H115R/C198R, N52H/Q100R/C198R,  
 N52H/Q100R/H115R/F172S, N52H/Q100R/ F172S/C198R,  
 N52H/Q100R/H115R/F172S/C198R, or N52H/N57Y/Q100R/F172S/C198R.

13. The variant ICOSL polypeptide of any of embodiments 1-12, wherein the one or more amino acid modifications correspond are at a position(s) selected from 52, 57, 100, 110, or 198.

14. The variant ICOSL polypeptide of any of embodiments 1-13, wherein the one or more amino acid modifications are selected from N52H, N52D, N52Q, N52S, N52K, S54A, S54P, N57Y, Q100P, Q100R, V110A, V110D, C198R, or a conservative amino acid substitution thereof.

15. The variant ICOSL polypeptide of any of embodiments 1-14, further comprising one or more amino acid modifications selected from V11E, E16V, N30D, K42E, N52H, N52S, N52Y, N57Y, E90A, H94E, L96I, L98F, Q100R, Q100P, V110A, V110D, H115R, F120S, V122A, F138L, I143V, K156M, K156R, F172S, N194D, C198R, L203P, V210A, R221I, I224V, or a conservative amino acid substitution thereof.

16. The variant ICOSL polypeptide of any of embodiments 1-14, wherein the one or more amino acid modifications are N52H/N57Y/Q100R/C198R, N52H/N57Y/Q100R/V122A, N52H/N57Y/Q100R/F172S, N52Y/N57Y/F138L/L203P, V11E/N30D/N52H/N57Y/H94E/L96I/L98F/N194D/V210A/I218T, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R, N52H/N57Y/Q100R, N52H/Q100R, N52H/N57Y/Q100R/V110D/C198R/S212G,

E16V/N52H/N57Y/Q100R/V110D/H115R/V152C/K156M/C198R, N30D/K42E/N52S, N52S/F120S/I143V/I224V, N52S/E90A, N52H/N57Y/V110A/C198R/R221I, N52H/N57Y/Q100P, or N52S/N194D.

17. The variant ICOSL polypeptide of any of embodiments 1-16, wherein the variant ICOSL polypeptide comprises the IgV domain or a specific fragment thereof and the IgC domain or a specific fragment thereof.

18. The variant ICOSL polypeptide of any of embodiments 1-17, comprising the sequence of amino acids set forth in any of SEQ ID NOS: 109-142, 239, 280-325, 364-381, 387-424, 427-433, 435-470 or a specific binding fragment thereof, or a sequence of amino acids that exhibits at least 95% sequence identity to any of SEQ ID NOS: 109-142, 239, 280-325, 364-381, 387-424, 427-433, 435-470 or a specific binding fragment thereof and that contains the one or more of the amino acid substitutions.

19. The variant ICOSL polypeptide of any of embodiments 1-18, wherein the variant ICOSL polypeptide comprises the IgV domain or a specific binding fragment thereof.

20. The variant ICOSL polypeptide of any of embodiments 1-19, wherein the IgV domain or specific binding fragment thereof is the only ICOSL portion of the variant ICOSL polypeptide.

21. The variant ICOSL polypeptide of any of embodiments 1-18, wherein the IgC domain or specific binding fragment thereof is the only ICOSL portion of the variant ICOSL polypeptide.

22. The variant ICOSL polypeptide of any of embodiments 1-20, comprising the sequence of amino acids set forth in any of SEQ ID NOS: 197-199, 201-208, 210, 212, 240, 326-340, 382-386, 425-426, and 434 or a specific binding fragment thereof, a sequence of amino acids that exhibits at least 95% sequence identity to any of SEQ ID NOS: 197-199, 201-208, 210, 212, 240, 326-340, 382-386, 425-426, and 434 or a specific binding fragment thereof and that contains the one or more of the amino acid substitutions.

23. The variant ICOSL polypeptide of any of embodiments 1-22, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS, CD28 or CTLA-4 with increased affinity compared to the unmodified ICOSL.

24. The variant ICOSL polypeptide of any of embodiments 1-23, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS or CD28 with increased affinity compared to the unmodified ICOSL.

25. The variant ICOSL polypeptide of any of embodiments 1-24, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS and the ectodomain of CD28 each with increased affinity compared to the unmodified ICOSL.

26. The variant ICOSL polypeptide of any of embodiments 1-24, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of CD28 with increased affinity compared to the unmodified ICOSL.

27. The variant ICOSL polypeptide of any of embodiments 1-26, wherein the increased affinity to the ectodomain of CD28 is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold or 60-fold compared to the unmodified ICOSL.

28. The variant ICOSL polypeptide of any of embodiments 1-24, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS with increased affinity compared to the unmodified ICOSL.

29. The variant ICOSL polypeptide of any of embodiments 1-25 and 28, wherein the increased affinity to the ectodomain of ICOS is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 40-fold, 50-fold, 60-fold or 70-fold compared to the unmodified ICOSL.

30. The variant ICOSL polypeptide of any of embodiments 1-29, wherein the one or more amino acid modifications correspond to position(s) selected from 52, 54 or 57.

31. The variant ICOSL polypeptide of any of embodiments 1-30, wherein the one or more amino acid modifications are selected from N52H, N52D, N52S, N52Y, N52K, S54A, S54P, N57Y or a conservative amino acid substitution thereof.

32. The variant ICOSL polypeptide of any of embodiments 1-31, wherein the one or more amino acid modifications correspond to position(s) selected from 52 or 57.

33. The variant ICOSL polypeptide of any of embodiments 1-31, wherein the one or more amino acid modifications are selected from N52H, N52D, N52S, N52K or N57Y.

34. The variant ICOSL polypeptide of any of embodiments 25-29 and embodiment 33, further comprising one or more amino acid modifications selected from N52H, N52D, N52S,

N52Y, N52K, S54A, S54P, N57Y, R75Q, L80P, K92R, S99G, H94D, L96F, L98F, S99G, Q100R, Q100P, G103E, T113E, F120S, H129P, S130G, Q133H, F138L, C140D, C140del, I143T, Y146C, V151A, Y152C, Y152H, D158G, L161P, C198R, N201S, L203P, L208P or T225A, or a conservative amino acid substitution thereof.

35. The variant ICOSL polypeptide of any of embodiments 1-34, wherein the one or more amino acid modifications are selected from among N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52S/Y146C/Y152C, N52H/C198R, N52H/C140del/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52S/C198R, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52S/T113E, N52S/S54P, N52K/L208P, N52S/Y152H, N52H/I143T, N52S/R75Q/L203P, N52S/D158G, N52D/Q133H, N52H/N57Y/Q100R/V110D/C198R/S212G, N52H/N57Y/Q100R/C198R, N52S/N194D, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R, N52S/S54P, T38P/N52S/N57D, N52H/C140del/T225A, N52H/F78L/Q100R/C198R, N52H/N57Y/R75Q/Q100P/V110D, N52H/N57Y/L74Q/V110D/S192G, N52H/S121G/C198R, N52S/F120S/N227K, N52S/A71T/A117T/T190A/C198R, T43A/N52H/N57Y/L74Q/D89G/V110D/F172S, N52H/N57Y/Q100R/V110D/S132F/M175T, N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G, N52Q/N207Q, N52Q/N168Q, N52Q/N84Q, N52Q/N119Q, N52Q/N84Q/N168Q, N52Q/N84Q/N207Q, N52Q/N119Q/N155Q, N52H/N84Q/N119Q, N52H/N84Q, N52H/N84Q/N168Q, N52H/N84Q/N207Q, N52H/N84Q/N168Q/N207Q, N52Q/N84Q/N155Q, N52Q/N84Q/N168Q, N52Q/N84Q/N155Q/N168Q, N52Q/N84Q/N119Q/N168Q, N52Q/N84Q/N119Q/N207Q, N52Q/N84Q/N119Q/N155Q, N52Q/N84Q/N119Q/N155Q/N207Q, N52Y/F138L/L203P, N57Y/Q100R/C198R, N57Y/F138L/L203P, N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/Q100R/F172S/C198R, N52H/N57Y/Q100R/H115R/F172S/C198R, N52H/N57Y/Q100R/H115R/I143V/F172S/C198R, N52H/N57Y/Q100R/L102R/H115R/F172S/C198R, N52H/V122A/F172S/C198R, N52H/N57Y/Q100R/H115R/F172S/N194D, N52H/N57Y/H115R/F172S/C198R, N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/H115R, N52H/N57Y/Q100R/H115R, N52H/N57Y/Q100R/H115R/F172S/I224V, N52H/N57Y/Q100R/H115R/F172S, N52H/N57Y/Q100R/F172S, N52H/Q100R/H115R/I143T/F172S, N52H/N57Y/Q100P/H115R/F172S, N52Y/N57Y/Q100P/F172S,

E16V/N52H/N57Y/Q100R/V110D/H115R/C198R,  
E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R, N52S/E90A/H115R,  
N30D/K42E/N52S/H115R, N30D/K42E/N52S/H115R/C198R/R221I,  
N30D/K42E/N52S/H115R/C198R, N30D/K42E/N52S/H115R/F172S/N194D,  
N52S/H115R/F120S/I143V/C198R, N52S/H115R/F172S/C198R, N52H/N57Y/Q100P/C198R,  
N52H/N57Y/Q100P H115R/F172S/C198R, N52H/N57Y/Q100P/F172S/C198R,  
N52H/N57Y/Q100P/H115R, N52H/N57Y/Q100P/H115R/C198R, N52H/Q100R/C198R,  
N52H/Q100R/H115R/F172S, N52H/Q100R/ F172S/C198R,  
N52H/Q100R/H115R/F172S/C198R, or N52H/N57Y/Q100R/F172S/C198R..

36. The variant ICOSL polypeptide of any of embodiments 1-35, wherein the one or more amino acid modifications are selected from N52H/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52H/K92R, N52H/C140del/T225A, N52H/C198R/T225A, N52H/K92R, N57Y/Q100P, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52K/L208P or N52H/I143T.

37. The variant ICOSL polypeptide of any of embodiments 1-34, wherein the one or more amino acid substitutions are selected from N57Y/Q100P, N52S/S130G/Y152C, N52S/Y152C, N52Y/N57Y/Y152C, N52H/L161P/C198R, N52H/L161P/C198R, N52S/L80P, A20V/N52H/N57Y/Q100R/S109G, N52H/N57Y/R61S/Q100R/V110D/L173S, N52H/N57Y/Q100R/V107I/V110D/S132F/I154F/C198R/R221G, Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G, N52H/N57Y/Q100R/V110D/C198R, F27S/N52H/N57Y/V110N, S18R/N52S/F93L/I143V/R221G, A20T/N52D/Y146C/Q164L, N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N, N52H/N57Y/Q100R/V110D/F172S/C198R, S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R, or M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M/C198R.

38. The variant ICOSL polypeptide of any of embodiments 1-37, wherein the variant polypeptide specifically binds to the ectodomain of ICOS, CD28 or CTLA4 with increased selectivity compared to the unmodified ICOSL.

39. The variant ICOSL polypeptide of embodiment 38, wherein the increased selectivity comprises a greater ratio of binding of the variant polypeptide for one cognate binding partner selected from among ICOS, CD28 and CTLA4 versus another of the cognate binding

partner compared to the ratio of binding of the unmodified ICOSL polypeptide for the one cognate binding partner versus the another of the cognate binding partner.

40. The variant ICOSL polypeptide of embodiment 39, wherein the ratio is greater by at least or at least about 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5-fold, 10-fold, 15-fold, 20-fold, 30-fold, 40-fold, 50-fold or more.

41. The variant ICOSL polypeptide of any of embodiments 1-40, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS or CD28 with increased affinity and specifically binds to the ectodomain of the other of ICOS or CD28 with decreased affinity compared to the unmodified ICOSL.

42. The variant ICOSL polypeptide of embodiment 41, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of ICOS with increased affinity and specifically binds to the ectodomain of CD28 with decreased affinity compared to the unmodified ICOSL.

43. The variant ICOSL polypeptide of embodiment 41, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of CD28 with increased affinity and specifically binds to the ectodomain of ICOS with decreased affinity compared to the unmodified ICOSL.

44. The variant ICOSL polypeptide of any of embodiments 1-43, comprising the amino acid substitutions N52S/R75Q/L203P or N30D/K42E/N52S.

45. The variant ICOSL polypeptide of any of embodiments 1-40, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of CTLA-4 with increased affinity compared to the unmodified ICOSL.

46. The variant ICOSL polypeptide of embodiment 45, wherein the increased affinity to the ectodomain of CTLA-4 is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 40-fold, 50-fold, 60-fold or 70-fold compared to the unmodified ICOSL.

47. The variant ICOSL polypeptide of any of embodiments 8-46, wherein the ICOS is a human ICOS.

48. The variant ICOSL polypeptide of any of embodiments 8-47, wherein the CD28 is a human CD28.

49. The variant ICOSL polypeptide of any of embodiments 1-48, wherein the binding is altered (increased or decreased) more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-

fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold compared to the unmodified ICOSL.

50. The variant ICOSL polypeptide of any of embodiments 1-49 that is a soluble protein.

51. The variant ICOSL polypeptide of any of embodiments 1-50 that is linked to a multimerization domain.

52. The variant ICOSL polypeptide of any of embodiments 1-51, wherein the variant ICOSL polypeptide is a multimeric polypeptide, optionally a dimeric polypeptide, comprising a first variant ICOSL polypeptide linked to a multimerization domain and a second variant ICOSL polypeptide linked to a multimerization domain.

53. The variant ICOSL polypeptide of embodiment 52, wherein the first variant ICOSL polypeptide and the second variant ICOSL polypeptide are the same or different.

54. The variant ICOSL polypeptide of any of embodiments 51-53, wherein the multimerization domain is an Fc domain or a variant thereof with reduced effector function.

55. The variant ICOSL polypeptide of any of embodiments 1-54 that is linked to a moiety that increases biological half-life of the polypeptide.

56. The variant ICOSL polypeptide of any of embodiments 1-55 that is linked to an Fc domain or a variant thereof with reduced effector function.

57. The variant ICOSL polypeptide of any of embodiments 54-56, wherein:  
the Fc domain is mammalian, optionally human; or  
the variant Fc domain comprises one or more amino acid modifications compared to an unmodified Fc domain that is mammalian, optionally human.

58. The variant ICOSL polypeptide of any one of embodiments 54, 56, and 57, wherein the Fc domain or variant thereof comprises the sequence of amino acids set forth in SEQ ID NO:226 or SEQ ID NO:227 or a sequence of amino acids that exhibits at least 85% sequence identity to SEQ ID NO:226 or SEQ ID NO:227.

59. The variant ICOSL polypeptide of any of embodiments 54 and 56-58, wherein the Fc domain comprises one or more amino acid modifications selected from among E233P, L234A, L234V, L235A, L235E, G236del, G237A, S267K, R292C, N297G and V302C, each by EU numbering.

60. The variant ICOSL polypeptide of any of embodiments 54 and 56-59, wherein the Fc domain comprises the amino acid modification C220S by EU numbering.

61. The variant ICOSL polypeptide of any of embodiments 54 and 56-60, wherein the Fc domain comprises the sequence of amino acids set forth in any of SEQ ID NOS:474, 476, 477, 478 or a sequence of amino acids that exhibits at least 85% sequence identity to any of SEQ ID NOS: 474, 476, 477, 478 and exhibits reduced effector function.

62. The variant ICOSL polypeptide of any of embodiments 51-61, wherein the variant ICOSL polypeptide is linked to the multimerization domain or Fc indirectly via a linker, optionally a G4S linker.

63. The variant ICOSL polypeptide of any of embodiments 1-49, that is a transmembrane immunomodulatory protein further comprising a transmembrane domain linked to the extracellular domain (ECD) or specific binding fragment thereof of the variant ICOSL polypeptide.

64. The variant ICOSL polypeptide of embodiment 63, wherein the transmembrane domain comprises the sequence of amino acids set forth as residues 257-277 of SEQ ID NO:5 or a functional variant thereof that exhibits at least 85% sequence identity to residues 257-277 of SEQ ID NO:5.

65. The variant ICOSL polypeptide of embodiment 63 or embodiment 64, further comprising a cytoplasmic signaling domain linked to the transmembrane domain.

66. The variant ICOSL polypeptide of embodiment 65, wherein the cytoplasmic signaling domain comprises the sequence of amino acids set forth as residues 278-302 of SEQ ID NO:5 or a functional variant thereof that exhibits at least 85% sequence identity to residues 278-302 of SEQ ID NO:5.

67. The variant ICOSL polypeptide of any of embodiments 63-66, comprising the sequence of amino acids set forth in any of SEQ ID NOS:494-503 or a sequence of amino acids that exhibits at least 85% sequence identity to any of SEQ ID NOS:494-503.

68. The variant ICOSL polypeptide of any of embodiments 1-67, wherein the variant ICOSL increases IFN-gamma (interferon-gamma) expression relative to the unmodified ICOSL in an in vitro primary T-cell assay.

69. The variant ICOSL polypeptide of any of embodiments 1-68, wherein the variant ICOSL decreases IFN-gamma (interferon-gamma) expression relative to the unmodified ICOSL in an in vitro primary T-cell assay.
70. The variant ICOSL polypeptide of any of embodiments 1-51 that is deglycosylated.
71. An immunomodulatory protein, comprising the variant ICOSL polypeptide of any of embodiments 1-70 linked to a second polypeptide comprising an immunoglobulin superfamily (IgSF) domain.
72. The immunomodulatory protein of embodiment 71, wherein the IgSF domain is affinity modified and exhibits altered binding to one or more of its cognate binding partner(s) compared to the unmodified or wild-type IgSF domain.
73. The immunomodulatory polypeptide of embodiment 72, wherein the IgSF domain exhibits increased binding to one or more of its cognate binding partner(s) compared to the unmodified or wild-type IgSF domain.
74. The immunomodulatory polypeptide of any one of embodiments 71-73, wherein the variant ICOSL polypeptide is a first ICOSL variant polypeptide and the IgSF domain of the second polypeptide is an IgSF domain from a second variant ICOSL polypeptide of any of embodiments 1-70, wherein the first and second ICOSL variant are the same or different.
75. The immunomodulatory protein of any one of embodiments 71-74, wherein the variant ICOSL polypeptide is capable of specifically binding to CD28 or ICOS and the IgSF domain of the second polypeptide is capable of binding to a cognate binding partner other than one specifically bound by the ICOSL variant polypeptide.
76. The immunomodulatory polypeptide of embodiment 75, wherein the IgSF domain is from a member of the B7 family.
77. The immunomodulatory polypeptide of any of embodiments 71-73 and 75, wherein the IgSF domain is a tumor-localizing moiety that binds to a ligand expressed on a tumor.
78. The immunomodulatory polypeptide of embodiment 77, wherein the ligand is B7H6.
79. The immunomodulatory polypeptide of embodiment 77 or embodiment 78, wherein the IgSF domain is from NKp30.

80. The immunomodulatory polypeptide of any of embodiments 71-79, wherein the IgSF domain is or comprises an IgV domain.
81. The immunomodulatory polypeptide of any of embodiments 71-80, wherein the variant ICOSL polypeptide is or comprise an IgV domain.
82. The immunomodulatory protein of any of embodiments 71-81, wherein the immunomodulatory protein comprises a multimerization domain linked to one or both of the variant ICOSL polypeptide or the second polypeptide comprising the IgSF domain.
83. The immunomodulatory protein of embodiment 82, wherein the multimerization domain is an Fc domain or a variant thereof with reduced effector function.
84. The immunomodulatory protein of any of embodiments 71-83 that is dimeric.
85. The immunomodulatory protein of embodiment 84 that is homodimeric.
86. The immunomodulatory protein of embodiment 84 that is heterodimeric.
87. A conjugate, comprising a variant ICOSL polypeptide of any of embodiments 1-70 or an immunomodulatory polypeptide of any of embodiments 71-86 linked to a moiety.
88. The conjugate of embodiment 87, wherein the moiety is a targeting moiety that specifically binds to a molecule on the surface of a cell.
89. The conjugate of embodiment 88, wherein the targeting moiety specifically binds to a molecule on the surface of an immune cell.
90. The conjugate of embodiment 89, wherein the immune cell is an antigen presenting cell or a lymphocyte.
91. The conjugate of embodiment 88, wherein the targeting moiety is a tumor-localizing moiety that binds to a molecule on the surface of a tumor.
92. The conjugate of any of embodiments 87-91, wherein the moiety is a protein, a peptide, nucleic acid, small molecule or nanoparticle.
93. The conjugate of any of embodiments 87-92, wherein the moiety is an antibody or antigen-binding fragment.
94. The conjugate of any of embodiments 87-93, wherein the conjugate is divalent, tetravalent, hexavalent or octavalent.
95. A nucleic acid molecule(s), encoding a variant ICOSL polypeptide of any of embodiments 1-70 or an immunomodulatory polypeptide of any of embodiments 71-86.
96. The nucleic acid molecule(s) of embodiment 95 that is synthetic nucleic acid.

97. The nucleic acid molecule(s) of embodiment 95 or embodiment 96 that is cDNA.
98. A vector, comprising the nucleic acid molecule(s) of any of embodiments 95-97.
99. The vector of embodiment 98 that is an expression vector.
100. The vector of embodiment 98 or embodiment 99, wherein the vector is a mammalian expression vector or a viral vector.
101. A cell, comprising the vector of embodiment 102 or embodiment 103.
102. The cell of embodiment 101 that is a mammalian cell.
103. The cell of embodiment 101 or embodiment 102 that is a human cell.
104. A method of producing a variant ICOSL polypeptide or an immunomodulatory protein, comprising introducing the nucleic acid molecule of any of embodiments 95-97 or vector of any of embodiments 98-100 into a host cell under conditions to express the protein in the cell.
105. The method of embodiment 104, further comprising isolating or purifying the variant ICOSL polypeptide or immunomodulatory protein from the cell.
106. A method of engineering a cell expressing a variant ICOSL variant polypeptide, comprising introducing a nucleic acid molecule encoding the variant ICOSL polypeptide of any of embodiments 1-70 or immunomodulatory polypeptide of any of embodiments 71-86 into a host cell under conditions in which the polypeptide is expressed in the cell.
107. An engineered cell, comprising the variant ICOSL polypeptide of any of embodiments 1-70, the immunomodulatory polypeptide of any of embodiments 71-86, the nucleic acid molecule of any of embodiments 95-97 or the vector of any of embodiments 98-100.
108. The engineered cell of embodiment 107, wherein the variant ICOSL polypeptide or immunomodulatory polypeptide comprises a signal peptide.
109. The engineered cell of embodiment 107 or embodiment 108, wherein the variant ICOSL polypeptide or immunomodulatory polypeptide does not comprise a transmembrane domain and/or is not expressed on the surface of the cell.
110. The engineered cell of any of embodiments 107-109, wherein the variant ICOSL polypeptide or immunomodulatory polypeptide is secreted from the engineered cell.
111. The engineered cell of embodiment 107 or embodiment 108, wherein the engineered cell comprises a variant ICOSL polypeptide that comprises a transmembrane domain and/or is the transmembrane immunomodulatory protein of any of embodiments 63-67.

112. The engineered cell of embodiment 107, embodiment 108 or embodiment 111, wherein the variant ICOSL polypeptide is expressed on the surface of the cell.
113. The engineered cell of embodiment 112, wherein the cell is an immune cell.
114. The engineered cell of embodiment 113, wherein the immune cell is an antigen presenting cell (APC) or a lymphocyte.
115. The engineered cell of any of embodiments 107-114 that is a primary cell.
116. The engineered cell of any of embodiments 107-115, wherein the cell is a mammalian cell.
117. The engineered cell of any of embodiments 107-116, wherein the cell is a human cell.
118. The engineered cell of any of embodiments 107-117, wherein the lymphocyte is a T cell.
119. The engineered cell of embodiment 114, wherein the APC is an artificial APC.
120. The engineered cell of any of embodiments 107-119, further comprising a chimeric antigen receptor (CAR) or an engineered T-cell receptor.
121. An infectious agent, comprising a nucleic acid molecule encoding a variant ICOSL polypeptide of any of embodiments 1-70 or an immunomodulatory polypeptide of any of embodiments 71-86.
122. The infectious agent of embodiment 121, wherein the encoded variant ICOSL polypeptide or immunomodulatory polypeptide does not comprise a transmembrane domain and/or is not expressed on the surface of a cell in which it is expressed.
123. The infectious agent of embodiment 121 or embodiment 122, wherein the encoded variant ICOSL polypeptide or immunomodulatory polypeptide is secreted from a cell in which it is expressed.
124. The infectious agent of embodiment 121, wherein the encoded variant ICOSL polypeptide comprises a transmembrane domain.
125. The engineered cell of embodiment 107, embodiment 108 or embodiment 111, wherein the encoded variant ICOSL polypeptide is expressed on the surface of a cell in which it is expressed.
126. The infectious agent of any of embodiments 121-125, wherein the infectious agent is a bacteria or a virus.

127. The infectious agent of embodiment 126, wherein the virus is an oncolytic virus.
128. The infectious agent of embodiment 127, wherein the oncolytic virus is an adenoviruses, adeno-associated viruses, herpes viruses, Herpes Simplex Virus, Vesticular Stomatic virus, Reovirus, Newcastle Disease virus, parvovirus, measles virus, vesticular stomatitis virus (VSV), Coxsackie virus or a Vaccinia virus.
129. The infectious agent of embodiment 126, wherein the virus specifically targets dendritic cells (DCs) and/or is dendritic cell-tropic.
130. The infectious agent of embodiment 129, wherein the virus is a lentiviral vector that is pseudotyped with a modified Sindbis virus envelope product.
131. The infectious agent of any of embodiments 121-130, further comprising a nucleic acid molecule encoding a further gene product that results in death of a target cell or that can augment or boost an immune response.
132. The infectious agent of embodiment 131, wherein the further gene product is selected from an anticancer agent, anti-metastatic agent, an antiangiogenic agent, an immunomodulatory molecule, an immune checkpoint inhibitor, an antibody, a cytokine, a growth factor, an antigen, a cytotoxic gene product, a pro-apoptotic gene product, an anti-apoptotic gene product, a cell matrix degradative gene, genes for tissue regeneration or a reprogramming human somatic cells to pluripotency.
133. A pharmaceutical composition, comprising the variant ICOSL polypeptide of any of embodiments 1-70, an immunomodulatory protein of any of embodiments 71-86, a conjugate of any of embodiments 87-94, an engineered cell of any of embodiments 107-120 or an infectious agent of any of embodiments 121-132.
134. The pharmaceutical composition of embodiment 133, comprising a pharmaceutically acceptable excipient.
135. The pharmaceutical composition of embodiment 123 or 134, wherein the pharmaceutical composition is sterile.
136. An article of manufacture comprising the pharmaceutical composition of any of embodiments 133-135 in a vial.
137. The article of manufacture of embodiment 136, wherein the vial is sealed.
138. A kit comprising the pharmaceutical composition of any of embodiments 133-135, and instructions for use.

139. A kit comprising the article of manufacture according to embodiment 136 and 137, and instructions for use.
140. A method of modulating an immune response in a subject, comprising administering the pharmaceutical composition of any of embodiments 133-135 to the subject.
141. A method of modulating an immune response in a subject, comprising administering the engineered cells of any of embodiments 107-120.
142. The method of embodiment 141, wherein the engineered cells are autologous to the subject.
143. The method of embodiment 141, wherein the engineered cells are allogenic to the subject.
144. The method of any of embodiments 140-143, wherein modulating the immune response treats a disease or condition in the subject.
145. The method of any of embodiments 140-144, wherein the immune response is increased.
146. The method of any of embodiments 140, 144 and 145, wherein an immunomodulatory protein or conjugate comprising a variant ICOSL polypeptide linked to a tumor-localizing moiety is administered to the subject.
147. The method of embodiment 146, wherein the tumor-localizing moiety is or comprises a binding molecule that recognizes a tumor antigen.
148. The method of embodiment 147, wherein the binding molecule comprises an antibody or an antigen-binding fragment thereof or comprises a wild-type IgSF domain or variant thereof.
149. The method of any of embodiments 140 and 144-148, wherein a pharmaceutical composition comprising the immunomodulatory protein of any of embodiments 77-86 or the conjugate of any of embodiments 87-94 is administered to the subject.
150. The method of any of embodiments 140-145, wherein an engineered cell comprising a variant ICOSL polypeptide that is a transmembrane immunomodulatory protein is administered to the subject and/or the engineered cell of 107, 108 and 111-120.
151. The method of any of embodiments 140, 144 and 145, wherein an infectious agent encoding a variant ICOSL polypeptide that is a transmembrane immunomodulatory protein is administered to the subject, optionally under conditions in which the infectious agent infects a

tumor cell or immune cell and the transmembrane immunomodulatory protein is expressed on the surface of the infected cell.

152. The method of any of embodiment 150 or embodiment 151, wherein the transmembrane immunomodulatory protein is of any of embodiments 63-70

153. The method of any of embodiments 140-152, wherein the disease or condition is a tumor or cancer.

154. The method of any one of embodiments 140-153, wherein the disease or condition is selected from melanoma, lung cancer, bladder cancer, a hematological malignancy, liver cancer, brain cancer, renal cancer, breast cancer, pancreatic cancer, colorectal cancer, spleen cancer, prostate cancer, testicular cancer, ovarian cancer, uterine cancer, gastric carcinoma, a musculoskeletal cancer, a head and neck cancer, a gastrointestinal cancer, a germ cell cancer, or an endocrine and neuroendocrine cancer.

155. The method of any of embodiments 140-144, wherein the immune response is decreased.

156. The method of any of embodiments 140, 144 and 155, wherein a variant ICOSL polypeptide or immunomodulatory protein that is soluble is administered to the subject.

157. The method of embodiment 156, wherein the soluble polypeptide or immunomodulatory protein is an Fc fusion protein.

158. The method of any of embodiments 140, 144 and 155-157, wherein a pharmaceutical composition comprising a variant ICOSL polypeptide of any of embodiments 1-62 and 68-70, or the immunomodulatory protein of any of embodiments 71-76 and 80-86 is administered to the subject.

159. The method of any of embodiments 140-144 and 155-157, wherein an engineered cell comprising a secretable variant ICOSL polypeptide is administered to the subject.

160. The method of any of embodiments 140-144, 155-157 and 159, wherein an engineered cell of any of embodiments 107-110 and 113-120 is administered to the subject.

161. The method of any of embodiments 140, 144 and 155-157 and 159, wherein an infectious agent encoding a variant ICOSL polypeptide that is a secretable immunomodulatory protein is administered to the subject, optionally under conditions in which the infectious agent infects a tumor cell or immune cell and the secretable immunomodulatory protein is secreted from the infected cell.

162. The method of any of embodiments 140-144 and 155-161, wherein the disease or condition is an inflammatory or autoimmune disease or condition.

163. The method of any of embodiments 140-144 and 155-162, wherein the disease or condition is an Antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, a vasculitis, an autoimmune skin disease, transplantation, a Rheumatic disease, an inflammatory gastrointestinal disease, an inflammatory eye disease, an inflammatory neurological disease, an inflammatory pulmonary disease, an inflammatory endocrine disease, or an autoimmune hematological disease.

164. The method of embodiment 162 or embodiment 163, wherein the disease or condition is selected from inflammatory bowel disease, transplant, Crohn's disease, ulcerative colitis, multiple sclerosis, asthma, rheumatoid arthritis, or psoriasis.

165. The variant ICOSL polypeptide of any of embodiments 1-70, wherein the amino acid modification is an amino acid substitution, insertion or deletion.

## **IX. EXAMPLES**

[0417] The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

### **EXAMPLE 1**

#### **Example 1 Generation of Mutant DNA Constructs of IgSF Domains**

[0418] Example 1 describes the generation of mutant DNA constructs of human ICOSL IgSF domains for translation and expression on the surface of yeast as yeast display libraries.

##### **A. Degenerate Libraries**

[0419] Constructs were generated based on a wildtype human ICOSL sequence of the extracellular domain (ECD) set forth in SEQ ID NO:32 (containing the ECD domain corresponding to residues 19-256 as set forth in UniProt Accession No. O75144.2) as follows:

DTQEKEVRAMVGSDVELSCACPEGSRFDLNDVYVYWQTSESKTVVTYHIPQNSSLEN  
VDSRYRNRALMSPAGMLRGDFSLRLFNVTQPDEQKFHCLVLSQSLGFQEVLSVEVTL

HVAANFSVPVVSAPHSPSQDELFTFTCTTSINGYPRPNVYWINKTDNSLLDQALQNDTV  
LNMRGLYDVVSVLRIARTPSVNIGCCIEENVLLQQNLTVGSQTGNDIGERDKITENPVS  
TGEKNAAT

**[0420]** For libraries that target specific residues for complete or partial randomization with degenerate codons, the coding DNA encoding SEQ ID NO:32 was ordered from Integrated DNA Technologies (Coralville, IA) as a set of overlapping oligonucleotides of up to 80 base pairs (bp) in length. To generate a library of diverse variants of the ECD, the oligonucleotides contained desired degenerate codons at desired amino acid positions. Degenerate codons were generated using an algorithm at the URL: [rosettadesign.med.unc.edu/SwiftLib/](http://rosettadesign.med.unc.edu/SwiftLib/).

**[0421]** In general, positions to mutate and degenerate codons were chosen from homology models (ICOSL) of the target-ligand pairs of interest to identify ligand contact residues as well as residues that are at the protein interaction interface. This analysis was performed using a structure viewer available at the URL: [spdbv.vital-it.ch](http://spdbv.vital-it.ch).

**[0422]** The next step in library design was the alignment of human, mouse, rat and monkey ICOSL sequences to identify conserved residues. Based on this analysis, conserved target residues were mutated with degenerate codons that only specified conservative amino acid changes plus the wild-type residue. Residues that were not conserved, were mutated more aggressively, but also including the wild-type residue. Degenerate codons that also encoded the wild-type residue were deployed to avoid excessive mutagenesis of target protein. For the same reason, only up to 20 positions were targeted for mutagenesis at a time. These residues were a combination of contact residues and non-contact interface residues.

**[0423]** The oligonucleotides were dissolved in sterile water, mixed in equimolar ratios, heated to 95°C for five minutes and slowly cooled to room temperature for annealing. ECD-specific oligonucleotide primers that anneal to the start and end of the ECDs, respectively, were then used to generate PCR product. ECD-specific oligonucleotides which overlap by 40-50bp with a modified version of pBYDS03 cloning vector (Life Technologies USA), beyond and including the BamH1 and Kpn1 cloning sites, were then used to amplify 100ng of PCR product from the prior step to generate a total of 5 µg of DNA. Both PCR's were by polymerase chain reaction (PCR) using OneTaq 2x PCR master mix (New England Biolabs, USA). The second PCR products were purified using a PCR purification kit (Qiagen, Germany) and resuspended in sterile deionized water.

**[0424]** To prepare for library insertion, a modified yeast display version of vector pBYDS03 was digested with BamH1 and Kpn1 restriction enzymes (New England Biolabs, USA) and the large vector fragment was gel-purified and dissolved in sterile, deionized water. Electroporation-ready DNA for the next step was generated by mixing 12 µg of library DNA with 4 µg of linearized vector in a total volume of 50 µl deionized and sterile water. An alternative way to generate targeted libraries, was to carry out site-directed mutagenesis (Multisite kit, Agilent, USA) of the target ECD with oligonucleotides containing degenerate codons. This approach was used to generate sublibraries that only target specific stretches of target protein for mutagenesis. In these cases, sublibraries were mixed before proceeding to the selection steps. In general, library sizes were in the range of 10E7 to 10E8 clones, except that sublibraries were only in the range of 10E4 to 10E5. Large libraries and sublibraries are generated for ICOSL.

#### **B. Random Libraries**

**[0425]** Random libraries were also constructed to identify variants of the ECD of ICOSL set forth in SEQ ID NO:32 (containing the ECD domain corresponding to residues 19-256 as set forth in UniProt Accession No. O75144.2 flanked by adjacent N- and C-terminal residues of the wildtype sequence). DNA encoding the wild-type ECD was cloned between the BamH1 and Kpn1 sites of modified yeast display vector pBYDS03 and then released using the same restriction enzymes. The released DNA was then mutagenized with the Genemorph II kit (Agilent, USA) so as to generate an average of three to five amino acid changes per library variant. Mutagenized DNA was then amplified by the two-step PCR and further processed as described above for targeted libraries.

### **EXAMPLE 2**

#### **Introduction of DNA Libraries into Yeast**

**[0426]** Example 2 describes the introduction of ICOSL DNA libraries into yeast.

**[0427]** To introduce degenerate and random library DNA into yeast, electroporation-competent cells of yeast strain BJ5464 (ATCC.org; ATCC number 208288) were prepared and electroporated on a Gene Pulser II (Biorad, USA) with the electroporation-ready DNA from the step above essentially as described (Colby, D.W. et al. 2004 *Methods Enzymology* 388, 348-358). The only exception is that transformed cells were grown in non-inducing minimal selective

SCD-Leu medium to accommodate the LEU2 selective marker carried by modified plasmid pBYDS03.

**[0428]** Library size was determined by plating dilutions of freshly recovered cells on SCD-Leu agar plates and then extrapolating library size from the number of single colonies from plating that generated at least 50 colonies per plate. The remainder of the electroporated culture was grown to saturation and cells from this culture were subcultured into the same medium once more to minimize the fraction of untransformed cells. To maintain library diversity, this subculturing step was carried out using an inoculum that contained at least 10x more cells than the calculated library size. Cells from the second saturated culture were resuspended in fresh medium containing sterile 25% (weight/volume) glycerol to a density of  $10^8$ /ml and frozen and stored at  $-80^{\circ}\text{C}$  (frozen library stock).

**[0429]** One liter of SCD-Leu media consists of 14.7 grams of sodium citrate, 4.29 grams of citric acid monohydrate, 20 grams of dextrose, 6.7 grams of Difco brand yeast nitrogen base, and 1.6 grams yeast synthetic drop-out media supplement without leucine. Media was filtered sterilized before use using a  $0.2\ \mu\text{m}$  vacuum filter device.

**[0430]** Library size was determined by plating dilutions of freshly recovered cells on SCD-Leu agar plates and then extrapolating library size from the number of single colonies from a plating that generate at least 50 colonies per plate.

**[0431]** To segregate plasmid from cells that contain two or more different library clones, a number of cells corresponding to 10 times the library size, were taken from the overnight SCD-Leu culture and subcultured 1/100 into fresh SCD-Leu medium and grown overnight. Cells from this overnight culture were resuspended in sterile 25% (weight/volume) glycerol to a density of  $10^8$ /ml and frozen and stored at  $-80^{\circ}\text{C}$  (frozen library stock).

### **EXAMPLE 3**

#### **Yeast Selection**

**[0432]** Example 3 describes the selection of yeast expressing affinity modified variants of ICOSL.

**[0433]** A number of cells equal to at least 10 times the library size were thawed from individual library stocks, suspended to  $0.1 \times 10^6$  cells/ml in non-inducing SCD-Leu medium, and grown overnight. The next day, a number of cells equal to 10 times the library size were

centrifuged at 2000 RPM for two minutes and resuspended to  $0.5 \times 10^6$  cells/ml in inducing SCDG-Leu media. One liter of the SCDG-Leu induction media consists of 5.4 grams  $\text{Na}_2\text{HPO}_4$ , 8.56 grams of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 20 grams galactose, 2.0 grams dextrose, 6.7 grams Difco yeast nitrogen base, and 1.6 grams of yeast synthetic drop out media supplement without leucine dissolved in water and sterilized through a  $0.22 \mu\text{m}$  membrane filter device. The culture was grown for two days at  $20^\circ\text{C}$  to induce expression of library proteins on the yeast cell surface.

**[0434]** Cells were processed with magnetic beads to reduce non-binders and enrich for all ICOSL variants with the ability to bind their exogenous recombinant counter-structure proteins. This was then followed by two to three rounds of flow cytometry sorting using exogenous counter-structure protein staining to enrich the fraction of yeast cells that displays improved binders. Magnetic bead enrichment and selections by flow cytometry are essentially as described in Keith D. Miller,<sup>1</sup> Noah B. Pefaur,<sup>2</sup> and Cheryl L. Baird<sup>1</sup> Current Protocols in Cytometry 4.7.1-4.7.30, July 2008.

**[0435]** With ICOSL libraries, target ligand proteins were sourced from R&D Systems (USA) as follows: human rCD28.Fc (i.e., recombinant CD28-Fc fusion protein), rCTLA4.Fc and rICOS.Fc. Magnetic streptavidin beads were obtained from New England Biolabs, USA. For biotinylation of counter-structure protein, biotinylation kit cat# 21955, Life Technologies, USA, was used. For two-color, flow cytometric sorting, a Becton Dickinson FACS Aria II sorter was used. ICOSL display levels were monitored with an anti-hemagglutinin antibody labeled with Alexafluor 488 (Life Technologies, USA). Ligand binding Fc fusion proteins rCD28.Fc, rCTLA4.Fc, or rICOS.Fc were detected with PE conjugated human Ig specific goat Fab (Jackson ImmunoResearch, USA). Doublet yeast were gated out using forward scatter (FSC) / side scatter (SSC) parameters, and sort gates were based upon higher ligand binding detected in FL4 that possessed more limited tag expression binding in FL1.

**[0436]** Yeast outputs from the flow cytometric sorts were assayed for higher specific binding affinity. Sort output yeast were expanded and re-induced to express the particular IgSF affinity modified domain variants they encode. This population then can be compared to the parental, wild-type yeast strain, or any other selected outputs, such as the bead output yeast population, by flow cytometry.

[0437] For ICOSL, the second sort outputs (F2) were compared to parental ICOSL yeast for binding of each rICOS.Fc, rCD28.Fc, and rCTLA4.Fc by double staining each population with anti-HA (hemagglutinin) tag expression and the anti-human Fc secondary to detect ligand binding.

[0438] In the case of ICOSL yeast variants selected for binding to ICOS, the F2 sort outputs gave Mean Fluorescence Intensity (MFI) values of 997, when stained with 5.6 nM rICOS.Fc, whereas the parental ICOSL strain MFI was measured at 397 when stained with the same concentration of rICOS.Fc. This represents a roughly three-fold improvement of the average binding in this F2 selected pool of clones, and it is predicted that individual clones from that pool will have much better improved MFI/affinity when individually tested.

[0439] In the case of ICOSL yeast variants selected for binding to CD28, the F2 sort outputs gave MFI values of 640 when stained with 100nM rCD28.Fc, whereas the parental ICOSL strain MFI was measured at 29 when stained with the same concentration of rCD28.Fc (22-fold improvement). In the case of ICOSL yeast variants selected for binding to CTLA4, the F2 sort outputs gave MFI values of 949 when stained with 100nM rCTLA4.Fc, whereas the parental ICOSL strain MFI was measured at 29 when stained with the same concentration of rCTLA4.Fc (32-fold improvement).

[0440] Importantly, the MFIs of all F2 outputs described above when measured with the anti-HA tag antibody on FL1 did not increase and sometimes went down compared to wild-type strains, indicating that increased binding was not a function of increased expression of the selected variants on the surface of yeast, and validated gating strategies of only selecting mid to low expressors with high ligand binding.

[0441] Selected variant ICOSL ECD domains were further formatted as fusion proteins and tested for binding and functional activity as described below.

#### **EXAMPLE 4**

##### **Reformatting Selection Outputs as Fc-Fusions and in Various Immunomodulatory Protein Types**

[0442] Example 4 describes reformatting of selection outputs as immunomodulatory proteins containing an affinity modified (variant) extracellular domain (ECD) of ICOSL fused to an Fc molecule (variant ECD-Fc fusion molecules).

**[0443]** Output cells from final flow cytometric ICOSL sorts were grown to terminal density in SCD-Leu medium. Plasmid DNA's from each output were isolated using a yeast plasmid DNA isolation kit (Zymoresearch, USA). For Fc fusions, PCR primers with added restriction sites suitable for cloning into the Fc fusion vector of choice were used to batch-amplify from the plasmid DNA preps the coding DNA's for the mutant target ECD's. After restriction digestion, the PCR products were ligated into an appropriate Fc fusion vector followed by chemical transformation into strain XL1 Blue E. Coli (Agilent, USA) or NEB5alpha (New England Biolabs) as directed by supplier. Exemplary of an Fc fusion vector is pFUSE-hIgG1-Fc2 (Invivogen, USA).

**[0444]** Dilutions of transformation reactions were plated on LB-agar containing 100 µg/ml carbenicillin (Teknova, USA) to generate single colonies. Up to 96 colonies from each transformation were then grown in 96 well plates to saturation overnight at 37°C in LB-broth (Teknova cat # L8112) and a small aliquot from each well was submitted for DNA sequencing of the ECD insert in order to identify the mutation(s) in all clones. Sample preparation for DNA sequencing was carried out using protocols provided by the service provider (Genewiz; South Plainfield, NJ). After removal of sample for DNA sequencing, glycerol was then added to the remaining cultures for a final glycerol content of 25% and plates were stored at -20°C for future use as master plates (see below). Alternatively, samples for DNA sequencing were generated by replica plating from grown liquid cultures to solid agar plates using a disposable 96 well replicator (VWR, USA). These plates were incubated overnight to generate growth patches and the plates were submitted to Genewiz as specified by Genewiz. In some instances, resequencing was performed to verify mutations.

**[0445]** After identification of clones of interest from analysis of Genewiz-generated DNA sequencing data, clones of interest were recovered from master plates and individually grown to density in 5 ml liquid LB-broth containing 100 µg/ml carbenicillin (Teknova, USA) and 2 ml of each culture were then used for preparation of approximately 10 µg of miniprep plasmid DNA of each clone using a standard kit such as the Pureyield kit (Promega). Identification of clones of interest generally involved the following steps. First, DNA sequence data files were downloaded from the Genewiz website. All sequences were then manually curated so that they start at the beginning of the ECD coding region. The curated sequences were then batch-translated using a suitable program available at the URL: [www.ebi.ac.uk/Tools/st/emboss\\_transeq/](http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The translated

sequences were then aligned using a suitable program available at the URL:  
[multalin.toulouse.inra.fr/multalin/multalin.html](http://multalin.toulouse.inra.fr/multalin/multalin.html).

**[0446]** Clones of interest were then identified using the following criteria: 1.) identical clone occurs at least two times in the alignment and 2.) a mutation occurs at least two times in the alignment and preferably in distinct clones. Clones that meet at least one of these criteria were clones that have been enriched by the sorting process due to improved binding.

**[0447]** To generate immunomodulatory proteins that are Fc fusion proteins containing an ECD of ICOSL with at least one affinity-modified domain (e.g. variant ICOSL ECD-Fc), the encoding nucleic acid molecule was generated to encode a protein designed as follows: signal peptide followed by variant (mutant) ECD followed by a linker of three alanines (AAA) followed by a human IgG1 Fc containing the mutation N82G with reference to wild-type human IgG1 Fc set forth in SEQ ID NO: 226 (corresponding to N297G by EU numbering). This exemplary Fc also contained stabilizing cysteine mutations R77C and V87C and replacement of the cysteine residue to a serine residue at position 5 (C5S), each with reference to wild-type human IgG1 Fc set forth in SEQ ID NO:226. (corresponding to R292C, V302C and C220S, respectively, by EU numbering). In some cases, the NotI cloning site which contributes to the AAA linker sequence was deleted to generate a direct fusion of the ICOSL ECD and the beginning of the Fc. Since the construct does not include any antibody light chains that can form a covalent bond with a cysteine, the human IgG1 Fc also contains replacement of the cysteine residues to a serine residue at position 5 (C5S) compared to the wild-type or unmodified Fc set forth in SEQ ID NO: 226.

## **EXAMPLE 5**

### **Expression and Purification of Fc-Fusions**

**[0448]** Example 5 describes the high throughput expression and purification of Fc-fusion proteins containing variant ECD ICOSL.

**[0449]** Recombinant variant Fc fusion proteins were produced with Expi293 expression system (Invitrogen, USA). 4µg of each plasmid DNA from the previous step was added to 200µl Opti-MEM (Invitrogen, USA) at the same time as 10.8µl ExpiFectamine was separately added to another 200µl Opti-MEM. After 5 minutes, the 200µl of plasmid DNA was mixed with the 200µl of ExpiFectamine and was further incubated for an additional 20 minutes before adding

this mixture to cells. Ten million Expi293 cells were dispensed into separate wells of a sterile 10ml, conical bottom, deep 24 well growth plate (Thomson Instrument Company, USA) in a volume 3.4ml Expi293 media (Invitrogen, USA). Plates were shaken for 5 days at 120 RPM in a mammalian cell culture incubator set to 95% humidity and 8% CO<sub>2</sub>. Following a 5 day incubation, cells were pelleted and culture supernatants were removed.

**[0450]** Protein was purified from supernatants using a high throughput 96 well Protein A purification kit using the manufacturer's protocol (Catalog number 45202, Life Technologies, USA). Resulting elution fractions were buffer exchanged into PBS using Zeba 96 well spin desalting plate (Catalog number 89807, Life Technologies, USA) using the manufacturer's protocol. Purified protein was quantitated using 280nm absorbance measured by Nanodrop instrument (Thermo Fisher Scientific, USA), and protein purity was assessed by loading 5 µg of protein on NUPAGE pre-cast, polyacrylamide gels (Life Technologies, USA) under denaturing and reducing conditions and subsequent gel electrophoresis. Proteins were visualized in gel using standard Coomassie staining.

## **EXAMPLE 6**

### **Assessment of Binding and Activity of Affinity-Matured IgSF Domain-Containing Molecules**

#### **A. Binding to Cell-Expressed Counter Structures**

**[0451]** This Example describes Fc-fusion binding studies to show specificity and affinity of ICOSL domain variant immunomodulatory proteins for cognate binding partners.

**[0452]** To produce cells expressing cognate binding partners, full-length mammalian surface expression constructs for each of human CD28 and ICOS were designed in pcDNA3.1 expression vector (Life Technologies) and sourced from Genscript, USA. Binding studies were carried out using the Expi293F transient transfection system (Life Technologies, USA) described above. The number of cells needed for the experiment was determined, and the appropriate 30 ml scale of transfection was performed using the manufacturer's suggested protocol. For each CD28, , ICOS or mock 30 ml transfection, 75 million Expi293F cells were incubated with 30 µg expression construct DNA and 1.5ml diluted ExpiFectamine 293 reagent for 48 hours, at which point cells were harvested for staining.

**[0453]** For staining by flow cytometry, 200,000 cells of appropriate transient transfection or negative control were plated in 96 well round bottom plates. Cells were spun down and resuspended in staining buffer (PBS (phosphate buffered saline), 1% BSA (bovine serum albumin), and 0.1% sodium azide) for 20 minutes to block non-specific binding. Afterwards, cells were centrifuged again and resuspended in staining buffer containing 100nM to 1nM variant immunomodulatory protein, depending on the experiment of each candidate CD80 variant Fc, ICOSL variant Fc, or stacked IgSF variant Fc fusion protein in 50 µl. Primary staining was performed on ice for 45 minutes, before washing cells in staining buffer twice. PE-conjugated anti-human Fc (Jackson ImmunoResearch, USA) was diluted 1:150 in 50 µl staining buffer and added to cells and incubated another 30 minutes on ice. Secondary antibody was washed out twice, cells were fixed in 4% formaldehyde/PBS, and samples were analyzed on FACScan flow cytometer (Becton Dickinson, USA) or a Hypercyt flow cytometer (Intellicyte, USA).

**[0454]** Mean Fluorescence Intensity (MFI) was calculated for each transfectant and negative parental line with Cell Quest Pro software (Becton Dickinson, USA) or a Hypercyt flow cytometer (Intellicyte, USA).

## **B. Bioactivity Characterization**

**[0455]** This Example further describes Fc-fusion variant protein bioactivity characterization in human primary T cell in vitro assays.

### **1. Mixed Lymphocyte Reaction (MLR)**

**[0456]** Soluble rICOSL.Fc bioactivity was tested in a human Mixed Lymphocyte Reaction (MLR). Human primary dendritic cells (DC) were generated by culturing monocytes isolated from PBMC (BenTech Bio, USA) in vitro for 7 days with 500U/ml rIL-4 (R&D Systems, USA) and 250U/ml rGM-CSF (R&D Systems, USA) in Ex-Vivo 15 media (Lonza, Switzerland). 10,000 matured DC and 100,000 purified allogeneic CD4+ T cells (BenTech Bio, USA) were co-cultured with ICOSL variant Fc fusion proteins and controls in 96 well round bottom plates in 200µl final volume of Ex-Vivo 15 media. On day 5, IFN-gamma secretion in culture supernatants was analyzed using the Human IFN-gamma DuoSet ELISA kit (R&D Systems, USA). Optical density was measured by VMax ELISA Microplate Reader (Molecular Devices, USA) and quantitated against titrated rIFN-gamma standard included in the IFN-gamma Duo-set kit (R&D Systems, USA). A second MLR protocol consisted of human primary dendritic cells

(DC) generated by culturing monocytes isolated from PBMC (BenTech Bio, USA) in vitro for 7 days with 50 ng/mL rIL-4 (R&D Systems, USA) and 80ng/mL rGM-CSF (R&D Systems, USA) in Ex-Vivo 15 media (Lonza, Switzerland). On days 3 and 5, half of the media was removed and replaced with fresh media containing 50 ng/mL rIL-4 and 80 ng/mL rGM-CSF. To fully induce DC maturation, lipopolysaccharide (LPS) (InvivoGen Corp., USA) was added at 100 ng/mL to the DC cultures on day 6 and cells were incubated for an additional 24 hours. Approximately, 10,000 matured DC and 100,000 purified allogeneic CD3+ T cells (BenTech Bio, USA) were co-cultured with ICOSL variant Fc fusion proteins and controls in 96 well round bottom plates in 200µl final volume of Ex-Vivo 15 media. On day 4 -5, IFN-gamma secretion in culture supernatants was analyzed using the Human IFN-gamma DuoSet ELISA kit (R&D Systems, USA). Optical density was measured on a BioTek Cytation Multimode Microplate Reader (BioTek Corp., USA) and quantitated against titrated rIFN-gamma standard included in the IFN-gamma Duo-set kit (R&D Systems, USA).

## **2. Anti-CD3 Coimmobilization Assay**

**[0457]** Costimulatory bioactivity of ICOSL fusion variants was determined in anti-CD3 coimmobilization assays. 1nM or 10 nM mouse anti-human CD3 (OKT3, Biolegends, USA) was diluted in PBS with 1nM to 80nM rICOSL.Fc variant proteins. This mixture was added to tissue culture treated flat bottom 96 well plates (Corning, USA) overnight to facilitate adherence of the stimulatory proteins to the wells of the plate. The next day, unbound protein was washed off the plates and 100,000 purified human pan T cells (BenTech Bio, US) or human T cell clone BC3 (Astarte Biologics, USA) were added to each well in a final volume of 200µl of Ex-Vivo 15 media (Lonza, Switzerland). In some instances, human pan T cells were labeled with 0.25 µM carboxyfluorescein succinimidyl ester (CFSE, ThermoFisher Scientific, USA). Cells were cultured 3 days before harvesting culture supernatants and measuring human IFN-gamma levels with DuoSet ELISA kit (R&D Systems, USA) as mentioned above. Cellular proliferation was determined by the percent of input cells that entered division as measured by CFSE dilution on cells stained with fluorescently-conjugated anti-CD4, anti-CD8 antibodies (BD, USA) or total T cells via flow cytometric analysis on an LSR II (BD, USA),

## C. Results

**[0458]** Results for the binding and activity studies for exemplary tested variants are shown in Table 7 which indicates exemplary IgSF domain amino acid substitutions (replacements) in the ECD of ICOSL selected in the screen for affinity-maturation against the respective cognate structures ICOS and CD28. In the Tables, the exemplary amino acid substitutions are designated by amino acid position number corresponding to the respective reference unmodified ECD sequence as follows. For example, the reference unmodified ECD sequence in Table 7 (WT ICOSL) is the unmodified ICOSL ECD sequence set forth in SEQ ID NO:32. The amino acid position is indicated in the middle, with the corresponding unmodified (e.g. wild-type) amino acid listed before the number and the identified variant amino acid substitution listed after the number. Column 2 sets forth the SEQ ID NO identifier for the variant ECD for each variant ECD-Fc fusion molecule.

**[0459]** Also shown is the binding activity as measured by the Mean Fluorescence Intensity (MFI) value for binding of each variant Fc-fusion molecule to cells transfected to express the cognate ligand and the ratio of the MFI compared to the binding of the corresponding unmodified ECD-Fc fusion molecule not containing the amino acid substitution(s) to the same cell-expressed counter structure ligand. The functional activity of the variant Fc-fusion molecules to modulate the activity of T cells also is shown based on the calculated levels of IFN-gamma in culture supernatants (pg/ml) generated either i) with the indicated variant ECD-Fc fusion molecule coimmobilized with anti-CD3 or ii) with the indicated variant ECD-Fc fusion molecule in an MLR assay. The Table also depicts the ratio of IFN-gamma produced by each variant ECD-Fc compared to the corresponding unmodified (wild-type) ECD-Fc in both functional assays.

**[0460]** As shown, the selections resulted in the identification of a number of ICOSL IgSF domain variants that were affinity-modified to exhibit increased binding for at least one, and in some cases more than one, cognate counter structure ligand. In addition, the results showed that affinity modification of the variant molecules also exhibited improved activities to both increase and/or decrease immunological activity depending on the format of the molecule. For example, coimmobilization of the ligand likely provides a multivalent interaction with the cell to cluster or increase the avidity to favor agonist activity and increase T cell activation compared to the unmodified (e.g. wildtype) ECD-Fc molecule not containing the amino acid replacement(s). However, when the molecule is provided as a bivalent Fc molecule in solution, the same IgSF

domain variants exhibited an antagonist activity to decrease T cell activation compared to the unmodified (e.g. wildtype) ECD-Fv molecule not containing the amino acid replacement(s).

| <b>Table 7: ICOSL variants selected against CD28 or ICOS. Molecule sequences, binding data, and costimulatory bioactivity data.</b> |                 |                           |                           |                                  |                                         |
|-------------------------------------------------------------------------------------------------------------------------------------|-----------------|---------------------------|---------------------------|----------------------------------|-----------------------------------------|
| ICOSL mutation(s)                                                                                                                   | SEQ ID NO (ECD) | Binding                   |                           | Coimmobilization with anti-CD3   | MLR                                     |
|                                                                                                                                     |                 | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | IFN-gamma pg/ml (parental ratio) | IFN-gamma levels pg/ml (parental ratio) |
| N52S                                                                                                                                | 109             | 1.33 (1.55)               | 162 (9.00)                | 1334 (1.93)                      | 300 (0.44)                              |
| N52H                                                                                                                                | 110             | 1.30 (1.51)               | 368 (20.44)               | 1268 (1.83)                      | 39 (0.06)                               |
| N52D                                                                                                                                | 111             | 1.59 (1.85)               | 130 (7.22)                | 1943 (2.80)                      | 190 (0.28)                              |
| N52Y/N57Y/F138L/L203P                                                                                                               | 112             | 1.02 (1.19)               | 398 (22.11)               | 510* (1.47*)                     | 18 (0.03)                               |
| N52H/N57Y/Q100P                                                                                                                     | 113             | 1.57 (1.83)               | 447 (24.83)               | 2199 (3.18)                      | 25 (0.04)                               |
| N52S/Y146C/Y152C                                                                                                                    | 114             | 1.26 (1.47)               | 39 (2.17)                 | 1647 (2.38)                      | 152 (0.22)                              |
| N52H/C198R                                                                                                                          | 115             | 1.16 (1.35)               | 363 (20.17)               | 744* (2.15*)                     | ND (ND)                                 |
| N52H/C140del/T225A                                                                                                                  | 372             | ND (ND)                   | 154 (8.56)                | 522* (1.51*)                     | ND (ND)                                 |
| N52H/C198R/T225A                                                                                                                    | 117             | 1.41 (1.64)               | 344 (19.11)               | 778* (2.25*)                     | 0 (0)                                   |
| N52H/K92R                                                                                                                           | 118             | 1.48 (1.72)               | 347 (19.28)               | 288* (0.83*)                     | 89 (0.13)                               |
| N52H/S99G                                                                                                                           | 119             | 0.09 (0.10)               | 29 (1.61)                 | 184* (0.53*)                     | 421 (0.61)                              |
| N52Y                                                                                                                                | 120             | 0.08 (0.09)               | 18 (1.00)                 | 184* (0.53*)                     | 568 (0.83)                              |
| N57Y                                                                                                                                | 121             | 1.40 (1.63)               | 101 (5.61)                | 580* (1.68*)                     | 176 (0.26)                              |
| N57Y/Q100P                                                                                                                          | 122             | 0.62 (0.72)               | 285 (15.83)               | 301* (0.87*)                     | 177 (0.26)                              |
| N52S/S130G/Y152C                                                                                                                    | 123             | 0.16 (0.19)               | 24 (1.33)                 | 266* (0.77*)                     | 1617 (2.35)                             |
| N52S/Y152C                                                                                                                          | 124             | 0.18 (0.21)               | 29 (1.61)                 | 238* (0.69*)                     | 363 (0.53)                              |
| N52S/C198R                                                                                                                          | 125             | 1.80 (2.09)               | 82 (4.56)                 | 1427 (2.06)                      | 201 (0.29)                              |

| <b>Table 7: ICOSL variants selected against CD28 or ICOS. Molecule sequences, binding data, and costimulatory bioactivity data.</b> |                        |                                  |                                  |                                         |                                                |
|-------------------------------------------------------------------------------------------------------------------------------------|------------------------|----------------------------------|----------------------------------|-----------------------------------------|------------------------------------------------|
| <b>ICOSL mutation(s)</b>                                                                                                            | <b>SEQ ID NO (ECD)</b> | <b>Binding</b>                   |                                  | <b>Coimmobilization with anti-CD3</b>   | <b>MLR</b>                                     |
|                                                                                                                                     |                        | <b>ICOS MFI (parental ratio)</b> | <b>CD28 MFI (parental ratio)</b> | <b>IFN-gamma pg/ml (parental ratio)</b> | <b>IFN-gamma levels pg/ml (parental ratio)</b> |
| N52Y/N57Y/Y152C                                                                                                                     | 126                    | 0.08<br>(0.09)                   | 56<br>(3.11)                     | 377*<br>(1.09*)                         | 439<br>(0.64)                                  |
| N52Y/N57Y/H129P/C198R                                                                                                               | 127                    | ND<br>(ND)                       | 449<br>(24.94)                   | 1192<br>(1.72)                          | ND<br>(ND)                                     |
| N52H/L161P/C198R                                                                                                                    | 128                    | 0.18<br>(0.21)                   | 343<br>(19.05)                   | 643*<br>(1.86*)                         | 447<br>(0.65)                                  |
| N52S/T113E                                                                                                                          | 129                    | 1.51<br>(1.76)                   | 54<br>(3.00)                     | 451*<br>(1.30*)                         | 345<br>(0.50)                                  |
| S54A                                                                                                                                | 130                    | 1.62<br>(1.88)                   | 48<br>(2.67)                     | 386*<br>(1.12*)                         | 771<br>(1.12)                                  |
| N52D/S54P                                                                                                                           | 368                    | 1.50<br>(1.74)                   | 38<br>(2.11)                     | 476*<br>(1.38*)                         | 227<br>(0.33)                                  |
| N52K/L208P                                                                                                                          | 132                    | 1.91<br>(2.22)                   | 291<br>(16.17)                   | 1509<br>(2.18)                          | 137<br>(0.20)                                  |
| N52S/Y152H                                                                                                                          | 133                    | 0.85<br>(0.99)                   | 68<br>(3.78)                     | 2158<br>(3.12)                          | 221<br>(0.32)                                  |
| N52D/V151A                                                                                                                          | 134                    | 0.90<br>(1.05)                   | 19<br>(1.06)                     | 341*<br>(0.99*)                         | 450<br>(0.66)                                  |
| N52H/I143T                                                                                                                          | 135                    | 1.83<br>(2.13)                   | 350<br>(19.44)                   | 2216<br>(3.20)                          | 112<br>(0.16)                                  |
| N52S/L80P                                                                                                                           | 136                    | 0.09<br>(0.10)                   | 22<br>(1.22)                     | 192*<br>(0.55*)                         | 340<br>(0.49)                                  |
| F120S/Y152H/ N201S                                                                                                                  | 137                    | 0.63<br>(0.73)                   | 16<br>(0.89)                     | 351*<br>(1.01*)                         | 712<br>(1.04)                                  |
| N52S/R75Q/L203P                                                                                                                     | 138                    | 1.71<br>(1.99)                   | 12<br>(0.67)                     | 1996<br>(2.88)                          | 136<br>(0.20)                                  |
| N52S/D158G                                                                                                                          | 139                    | 1.33<br>(1.55)                   | 39<br>(2.17)                     | 325*<br>(0.94*)                         | 277<br>(0.40)                                  |
| N52D/Q133H                                                                                                                          | 140                    | 1.53<br>(1.78)                   | 104<br>(5.78)                    | 365*<br>(1.05*)                         | 178<br>(0.26)                                  |
| WT ICOSL                                                                                                                            | 32                     | 0.86<br>(1.00)                   | 18<br>(1.00)                     | 692 / 346*<br>(1.00)                    | 687<br>(1.00)                                  |

\*: Parental ratio calculated using 346 pg/ml IFN-gamma for WT ICOSL

**[0461]** Binding assays were repeated substantially as described above, except that binding also was assessed against cells expressing full-length human CTLA4. ICOSL variant Fc fusion proteins also were further assessed in an anti-CD3 coimmobilization assay substantially as

described above. The results confirmed identification of a number of ICOSL IgSF domain variants that exhibited increased binding affinity for at least one, and in some cases more than one, cognate ligand. In addition, the results showed that affinity modification of the variant molecules also exhibited improved activities in the coimmobilization assay.

## **EXAMPLE 7**

### **Additional Affinity Modified IgSF Domains**

**[0462]** This examples describe the design, creation, and screening of additional affinity modified CD80 (B7-1), CD86 (B7-2) and NKp30 immunomodulatory proteins, which are other components of the immune synapse (IS) that have a demonstrated dual role in both immune activation and inhibition. These examples demonstrate that affinity modification of IgSF domains yields proteins that can act to both increase and decrease immunological activity. This work also describes the various combinations of those domains fused in pairs (i.e., stacked) with a variant affinity modified ICOSL to form a Type II immunomodulatory protein to achieve immunomodulatory activity.

**[0463]** Mutant DNA constructs of human CD80, CD86 and NKp30 IgSF domains for translation and expression as yeast display libraries were generated substantially as described in Example 1. For libraries that target specific residues of target protein for complete or partial randomization with degenerate codons, the coding DNA's for the extracellular domains (ECD) of human CD80 (SEQ ID NO:28), and NKp30 (SEQ ID NO:54) were ordered from Integrated DNA Technologies (Coralville, IA) as a set of overlapping oligonucleotides of up to 80 base pairs (bp) in length. Alternatively, residues were mutated by site-directed targeted mutagenesis substantially as described in Example 1. Alternatively, random libraries were constructed to identify variants of the ECD of CD80 (SEQ ID NO:28), CD86 (SEQ ID NO: 29) and NKp30 (SEQ ID NO:54) substantially as described in Example 1.

**[0464]** The targeted and random library DNA was introduced into yeast substantially as described in Example 2 to generate yeast libraries. The libraries were used to select yeast expressing affinity modified variants of CD80, CD86 and NKp30 substantially as described in Example 3. Cells were processed to reduce non-binders and to enrich for CD80, CD86 or NKp30 variants with the ability to bind their exogenous recombinant counter-structure proteins substantially as described in Example 3. For example, yeast displayed targeted or random CD80

libraries were selected against each of CD28, CTL-4, and PD-L1, separately. This was then followed by two to three rounds of flow cytometry sorting using exogenous counter-structure protein staining to enrich the fraction of yeast cells that displays improved binders. Magnetic bead enrichment and selections by flow cytometry are essentially as described in Keith D. Miller,<sup>1</sup> Noah B. Pefaur,<sup>2</sup> and Cheryl L. Baird<sup>1</sup> *Current Protocols in Cytometry* 4.7.1-4.7.30, July 2008.

**[0465]** With CD80, CD86 and NKp30 libraries, target ligand proteins were sourced from R&D Systems (USA) as follows: human rCD28.Fc (i.e., recombinant CD28-Fc fusion protein), rPDL1.Fc, rCTLA4.Fc, and rB7H6.Fc. Two-color flow cytometry was performed substantially as described in Example 3. Yeast outputs from the flow cytometric sorts were assayed for higher specific binding affinity. Sort output yeast were expanded and re-induced to express the particular IgSF affinity modified domain variants they encode. This population then can be compared to the parental, wild-type yeast strain, or any other selected outputs, such as the bead output yeast population, by flow cytometry.

**[0466]** In the case of NKp30 yeast variants selected for binding to B7-H6, the F2 sort outputs gave MFI values of 533 when stained with 16.6nM rB7H6.Fc, whereas the parental NKp30 strain MFI was measured at 90 when stained with the same concentration of rB7H6.Fc (6-fold improvement).

**[0467]** Among the NKp30 variants that were identified, was a variant that contained mutations L30V/A60V/S64P/S86G with reference to positions in the NKp30 extracellular domain corresponding to positions set forth in SEQ ID NO:54. Among the CD86 variants that were identified, was a variant that contained mutations Q35H/H90L/Q102H with reference to positions in the CD86 extracellular domain corresponding to positions set forth in SEQ ID NO:29. Among the CD80 variants that were identified, were variants set forth in Table 8 and described further below.

**[0468]** As with ICOSL, the MFIs of all F2 outputs described above when measured with the anti-HA tag antibody on FL1 did not increase and sometimes went down compared to wild-type strains, indicating that increased binding was not a function of increased expression of the selected variants on the surface of yeast, and validated gating strategies of only selecting mid to low expressors with high ligand binding.

**[0469]** Exemplary selection outputs were reformatted as immunomodulatory proteins containing an affinity modified (variant) extracellular domain (ECD) of CD80 fused to an Fc molecule (variant ECD-Fc fusion molecules) substantially as described in Example 4 and the Fc-fusion protein was expressed and purified substantially as described in Example 5.

**[0470]** Binding of exemplary CD80 Fc-fusion variants to cell-expressed counter structures was then assessed substantially as described in Example 6. To produce cells expressing cognate binding partners, full-length mammalian surface expression constructs for each of human CD28, CTLA4 and PD-L1 were produced substantially as described in Example 6. Binding studies and flow cytometry were carried out substantially as described in Example 6. In addition, the bioactivity of the Fc-fusion variant protein was characterized by either mixed lymphocyte reaction (MLR) or anti-CD3 coimmobilization assay substantially as described in Example 6.

**[0471]** Results for the binding and activity studies for exemplary tested variants are shown in Tables 8 and 9. In particular, Table 8 indicates exemplary IgSF domain amino acid substitutions (replacements) in the ECD of CD80 selected in the screen for affinity-maturation against the respective cognate structure CD28. Table 9 indicates exemplary IgSF domain amino acid substitutions (replacements) in the ECD of CD80 selected in the screen for affinity-maturation against the respective cognate structure PD-L1. As above, for each Table, the exemplary amino acid substitutions are designated by amino acid position number corresponding to the respective reference unmodified ECD sequence as follows. For example, the reference unmodified ECD sequence in Tables 8 and 9 is the unmodified CD80 ECD sequence set forth in SEQ ID NO:28. The amino acid position is indicated in the middle, with the corresponding unmodified (e.g. wild-type) amino acid listed before the number and the identified variant amino acid substitution listed after the number. Column 2 sets forth the SEQ ID NO identifier for the variant ECD for each variant ECD-Fc fusion molecule.

**[0472]** Also shown is the binding activity as measured by the Mean Fluorescence Intensity (MFI) value for binding of each variant Fc-fusion molecule to cells engineered to express the cognate counter structure ligand and the ratio of the MFI compared to the binding of the corresponding unmodified ECD-Fc fusion molecule not containing the amino acid substitution(s) to the same cell-expressed counter structure ligand. The functional activity of the variant Fc-fusion molecules to modulate the activity of T cells also is shown based on the calculated levels of IFN-gamma in culture supernatants (pg/ml) generated either i) with the indicated variant ECD-

Fc fusion molecule coimmobilized with anti-CD3 or ii) with the indicated variant ECD-Fc fusion molecule in an MLR assay. The Tables also depict the ratio of IFN-gamma produced by each variant ECD-Fc compared to the corresponding unmodified ECD-Fc in both functional assays.

**[0473]** As shown, the selections resulted in the identification of a number of CD80 IgSF domain variants that were affinity-modified to exhibit increased binding for at least one, and in some cases more than one, cognate counter structure ligand. In addition, the results showed that affinity modification of the variant molecules also exhibited improved activities to both increase and decrease immunological activity depending on the format of the molecule. For example, coimmobilization of the ligand likely provides a multivalent interaction with the cell to cluster or increase the avidity to favor agonist activity and increase T cell activation compared to the unmodified (e.g. wildtype) ECD-Fc molecule not containing the amino acid replacement(s). However, when the molecule is provided as a bivalent Fc molecule in solution, the same IgSF domain variants exhibited an antagonist activity to decrease T cell activation compared to the unmodified (e.g. wildtype) ECD-Fv molecule not containing the amino acid replacement(s).

| <b>Table 8: CD80 variants selected against CD28. Molecule sequences, binding data, and costimulatory bioactivity data.</b> |                 |                           |                             |                            |                                  |                                         |
|----------------------------------------------------------------------------------------------------------------------------|-----------------|---------------------------|-----------------------------|----------------------------|----------------------------------|-----------------------------------------|
| CD80 mutation(s)                                                                                                           | SEQ ID NO (ECD) | Binding                   |                             |                            | Coimmobilization with anti-CD3   | MLR                                     |
|                                                                                                                            |                 | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | PD-L1 MFI (parental ratio) | IFN-gamma pg/ml (parental ratio) | IFN-gamma levels pg/ml (parental ratio) |
| L70Q/A91G/N144D                                                                                                            | 508             | 125 (1.31)                | 283 (1.36)                  | 6 (0.08)                   | 93 (1.12)                        | 716 (0.83)                              |
| L70Q/A91G/T130A                                                                                                            | 56              | 96 (1.01)                 | 234 (1.13)                  | 7 (0.10)                   | 99 (1.19)                        | 752 (0.87)                              |
| L70Q/A91G/I118A/T120S/T130A/K169E                                                                                          | 59              | 123 (1.29)                | 226 (1.09)                  | 7 (0.10)                   | 86 (1.03)                        | 741 (0.86)                              |
| V4M/L70Q/A91G/I118V/T120S/T130A/K169E                                                                                      | 510             | 89 (0.94)                 | 263 (1.26)                  | 6 (0.09)                   | 139 (1.67)                       | 991 (1.14)                              |
| L70Q/A91G/I118V/T120S/T130A/K169E                                                                                          | 59              | 106 (1.12)                | 263 (1.26)                  | 6 (0.09)                   | 104 (1.25)                       | 741 (0.86)                              |
| V20L/L70Q/A91S/I118V/T120S/T130A                                                                                           | 513             | 105 (1.11)                | 200 (0.96)                  | 9 (0.13)                   | 195 (2.34)                       | 710 (0.82)                              |

| <b>Table 8: CD80 variants selected against CD28. Molecule sequences, binding data, and costimulatory bioactivity data.</b> |                    |                              |                                |                               |                                     |                                            |
|----------------------------------------------------------------------------------------------------------------------------|--------------------|------------------------------|--------------------------------|-------------------------------|-------------------------------------|--------------------------------------------|
| CD80 mutation(s)                                                                                                           | SEQ ID NO<br>(ECD) | Binding                      |                                |                               | Coimmobilization with anti-CD3      | MLR                                        |
|                                                                                                                            |                    | CD28 MFI<br>(parental ratio) | CTLA-4 MFI<br>(parental ratio) | PD-L1 MFI<br>(parental ratio) | IFN-gamma pg/ml<br>(parental ratio) | IFN-gamma levels pg/ml<br>(parental ratio) |
| S44P/L70Q/A91G/ T130A                                                                                                      | 61                 | 88<br>(0.92)                 | 134<br>(0.64)                  | 5<br>(0.07)                   | 142<br>(1.71)                       | 854<br>(0.99)                              |
| L70Q/A91G/E117G/ I118V/T120S/T130A                                                                                         | 514                | 120<br>(1.27)                | 193<br>(0.93)                  | 6<br>(0.08)                   | 98<br>(1.05)                        | 736<br>(0.85)                              |
| A91G/ I118V/T120S/T130A                                                                                                    | 515                | 84<br>(0.89)                 | 231<br>(1.11)                  | 44<br>(0.62)                  | 276<br>(3.33)                       | 714<br>(0.82)                              |
| L70R/A91G/ I118V/T120S/T130A/T199S                                                                                         | 516                | 125<br>(1.32)                | 227<br>(1.09)                  | 6<br>(0.09)                   | 105<br>(1.26)                       | 702<br>(0.81)                              |
| L70Q/E81A/A91G/ I118V/T120S/I127T/ T130A                                                                                   | 517                | 140<br>(1.48)                | 185<br>(0.89)                  | 18<br>(0.25)                  | 98<br>(1.18)                        | 772<br>(0.89)                              |
| L70Q/Y87N/A91G/ T130A                                                                                                      | 66                 | 108<br>(1.13)                | 181<br>(0.87)                  | 6<br>(0.08)                   | 136<br>(1.63)                       | 769<br>(0.89)                              |
| T28S/L70Q/A91G/I118V/ E95K/T120S/ I126V/T130A/K169E                                                                        | 518                | 32<br>(0.34)                 | 65<br>(0.31)                   | 6<br>(0.08)                   | 120<br>(1.44)                       | 834<br>(0.96)                              |
| N63S/L70Q/A91G/S114T/I118V /T120S/T130A                                                                                    | 519                | 124<br>(1.30)                | 165<br>(0.79)                  | 6<br>(0.08)                   | 116<br>(1.39)                       | 705<br>(0.81)                              |
| K36E/I67T/L70Q/A91G/ I118V/T120S/T130A/N152T                                                                               | 520                | 8<br>(0.09)                  | 21<br>(0.10)                   | 5<br>(0.08)                   | 53<br>(0.63)                        | 852<br>(0.98)                              |
| E52G/L70Q/A91G/ D107N/I118V/T120S/T130A K169E                                                                              | 521                | 113<br>(1.19)                | 245<br>(1.18)                  | 6<br>(0.08)                   | 94<br>(1.13)                        | 874<br>(1.01)                              |
| K37E/F59S/L70Q/ A91G/ I118V/T120S/ T130A/K185E                                                                             | 522                | 20<br>(0.21)                 | 74<br>(0.36)                   | 6<br>(0.08)                   | 109<br>(1.31)                       | 863<br>(1.00)                              |
| A91G/S103P                                                                                                                 | 72                 | 39<br>(0.41)                 | 56<br>(0.27)                   | 9<br>(0.13)                   | 124<br>(1.49)                       | 670<br>(0.77)                              |
| K89E/T130A                                                                                                                 | 73                 | 90<br>(0.95)                 | 148<br>(0.71)                  | 75<br>(1.07)                  | 204<br>(2.45)                       | 761<br>(0.88)                              |
| A91G                                                                                                                       | 74                 | 96<br>(1.01)                 | 200<br>(0.96)                  | 85<br>(1.21)                  | 220<br>(2.65)                       | 877<br>(1.01)                              |
| D60V/A91G/ I118V/T120S/ T130A/K169E                                                                                        | 523                | 111<br>(1.17)                | 222<br>(1.07)                  | 12<br>(0.18)                  | 120<br>(1.44)                       | 744<br>(0.86)                              |
| K54M/ L70Q/A91G/Y164H                                                                                                      | 524                | 68<br>(0.71)                 | 131<br>(0.63)                  | 5<br>(0.08)                   | 152<br>(1.83)                       | 685<br>(0.79)                              |
| M38T/L70Q/E77G/ A91G/ I118V/T120S/ T130A/N152T                                                                             | 525                | 61<br>(0.64)                 | 102<br>(0.49)                  | 5<br>(0.07)                   | 119<br>(1.43)                       | 796<br>(0.92)                              |
| R29H/E52G/L70R/ E88G/A91G/T130A                                                                                            | 78                 | 100<br>(1.05)                | 119<br>(0.57)                  | 5<br>(0.08)                   | 200<br>(2.41)                       | 740<br>(0.85)                              |

| CD80 mutation(s)                                              | SEQ ID NO<br>(ECD) | Binding                      |                                |                               | Coimmobilization with anti-CD3      | MLR                                        |
|---------------------------------------------------------------|--------------------|------------------------------|--------------------------------|-------------------------------|-------------------------------------|--------------------------------------------|
|                                                               |                    | CD28 MFI<br>(parental ratio) | CTLA-4 MFI<br>(parental ratio) | PD-L1 MFI<br>(parental ratio) | IFN-gamma pg/ml<br>(parental ratio) | IFN-gamma levels pg/ml<br>(parental ratio) |
| Y31H/T41G/ M43L/L70Q/ A91G/ I118V/T120S/I126V/T130A           | 526                | 85<br>(0.89)                 | 85<br>(0.41)                   | 6<br>(0.08)                   | 288<br>(3.47)                       | 782<br>(0.90)                              |
| V68A/T110A                                                    | 80                 | 103<br>(1.08)                | 233<br>(1.12)                  | 48<br>(0.68)                  | 163<br>(1.96)                       | 861<br>(0.99)                              |
| L65H/D90G/T110A/ F116L                                        | 527                | 33<br>(0.35)                 | 121<br>(0.58)                  | 11<br>(0.15)                  | 129<br>(1.55)                       | 758<br>(0.88)                              |
| R29H/E52G/ D90N/I118V/T120S/ T130A                            | 82                 | 66<br>(0.69)                 | 141<br>(0.68)                  | 11<br>(0.15)                  | 124<br>(1.49)                       | 800<br>(0.92)                              |
| A91G/L102S                                                    | 83                 | 6<br>(0.06)                  | 6<br>(0.03)                    | 5<br>(0.08)                   | 75<br>(0.90)                        | 698<br>(0.81)                              |
| I67T/L70Q/A91G/I118V T120S                                    | 530                | 98<br>(1.03)                 | 160<br>(0.77)                  | 5<br>(0.08)                   | 1751<br>(21.1)                      | 794<br>(0.92)                              |
| L70Q/A91G/T110A/ I118V/T120S/T130A                            | 531                | 8<br>(0.09)                  | 14<br>(0.07)                   | 5<br>(0.07)                   | 77<br>(0.93)                        | 656<br>(0.76)                              |
| M38V/T41D/M43I/ W50G/D76G/V83A/ K89E/ I118V/T120S/I126V/T130A | 532                | 5<br>(0.06)                  | 8<br>(0.04)                    | 8<br>(0.11)                   | 82<br>(0.99)                        | 671<br>(0.78)                              |
| V22A/L70Q/S121P                                               | 87                 | 5<br>(0.06)                  | 7<br>(0.04)                    | 5<br>(0.07)                   | 105<br>(1.27)                       | 976<br>(1.13)                              |
| A12V/S15F/Y31H/ M38L/ T41G/M43L/D90N/T130A/P137L/ N149D N152T | 533                | 6<br>(0.06)                  | 6<br>(0.03)                    | 5<br>(0.08)                   | 104<br>(1.25)                       | 711<br>(0.82)                              |
| I67F/L70R/E88G/ A91G/I118V/T120S/T130A                        | 534                | 5<br>(0.05)                  | 6<br>(0.03)                    | 6<br>(0.08)                   | 62<br>(0.74)                        | 1003<br>(1.16)                             |
| E24G/L25P/L70Q/A91G/I118V/T120S/N152T                         | 535                | 26<br>(0.27)                 | 38<br>(0.18)                   | 8<br>(0.11)                   | 101<br>(1.21)                       | 969<br>(1.12)                              |
| A91G/F92L/F108L/I118V/T120S                                   | 536                | 50<br>(0.53)                 | 128<br>(0.61)                  | 16<br>(0.11)                  | 59<br>(0.71)                        | 665<br>(0.77)                              |
| WT CD80                                                       | 28                 | 95<br>(1.00)                 | 208<br>(1.00)                  | 70<br>(1.00)                  | 83<br>(1.00)                        | 866<br>(1.00)                              |

| CD80 mutation(s) | SEQ ID NO | Binding | Coimmobilization with anti-CD3 | MLR |
|------------------|-----------|---------|--------------------------------|-----|
|------------------|-----------|---------|--------------------------------|-----|

|                                                                                                                 | (ECD) | CD28<br>MFI<br>(parental<br>ratio) | CTLA-4<br>MFI<br>(parental<br>ratio) | PD-L1<br>MFI<br>(parental<br>ratio) | IFN-gamma<br>pg/ml<br>(parental<br>ratio) | IFN-<br>gamma<br>levels<br>pg/ml<br>(parental<br>ratio) |
|-----------------------------------------------------------------------------------------------------------------|-------|------------------------------------|--------------------------------------|-------------------------------------|-------------------------------------------|---------------------------------------------------------|
| R29D/Y31L/Q33H/<br>K36G/M38I/ T41A/<br>M43R/M47T/E81V/<br>L85R/K89N/A91T/<br>F92P/K93V/ R94L/<br>I118T/N149S    | 92    | 1071<br>(0.08)                     | 1089<br>(0.02)                       | 37245<br>(2.09)                     | 387<br>(0.76)                             | 5028<br>(0.26)                                          |
| R29D/Y31L/Q33H/<br>K36G/M38I/T41A/<br>M43R/M47T/E81V/<br>L85R/K89N/A91T/<br>F92P/K93V/R94L/<br>N144S/N149S      | 93    | 1065<br>(0.08)                     | 956<br>(0.02)                        | 30713<br>(1.72)                     | 400<br>(0.79)                             | 7943<br>(0.41)                                          |
| R29D/Y31L/Q33H/<br>K36G/M38I/T41A/<br>M42T/M43R/M47T/<br>E81V/L85R/K89N/<br>A91T/F92P/K93V/<br>R94L/L148S/N149S | 94    | 926<br>(0.07)                      | 954<br>(0.02)                        | 47072<br>(2.64)                     | 464<br>(0.91)                             | 17387<br>(0.91)                                         |
| E24G/R29D/Y31L/<br>Q33H/K36G/M38I/<br>T41A/M43R/M47T/<br>F59L/E81V/L85R/<br>K89N/A91T/F92P/<br>K93V/R94L/H96R   | 95    | 1074<br>(0.08)                     | 1022<br>(0.02)                       | 1121<br>(0.06)                      | 406<br>(0.80)                             | 13146<br>(0.69)                                         |
| R29D/Y31L/Q33H/<br>K36G/M38I/T41A/<br>M43R/M47T/E81V/<br>L85R/K89N/A91T/<br>F92P/K93V/R94L/ N149S               | 96    | 1018<br>(0.08)                     | 974<br>(0.02)                        | 25434<br>(1.43)                     | 405<br>(0.80)                             | 24029<br>(1.25)                                         |
| R29V/M43Q/E81R/<br>L85I/K89R/D90L/<br>A91E/F92N/K93Q/ R94G                                                      | 97    | 1029<br>(0.08)                     | 996<br>(0.02)                        | 1575<br>(0.09)                      | 342<br>(0.67)                             | 11695<br>(0.61)                                         |
| T41I/A91G                                                                                                       | 98    | 17890<br>(1.35)                    | 50624<br>(1.01)                      | 12562<br>(0.70)                     | 433<br>(0.85)                             | 26052<br>(1.36)                                         |
| E88D/K89R/D90K/A91G/<br>F92Y/K93R/N122S/<br>N178S                                                               | 537   | 41687<br>(3.15)                    | 49429<br>(0.99)                      | 20140<br>(1.13)                     | 773<br>(1.52)                             | 6345<br>(0.33)                                          |
| E88D/K89R/D90K/A91G/<br>F92Y/K93R                                                                               | 538   | 51663<br>(3.91)                    | 72214<br>(1.44)                      | 26405<br>(1.48)                     | 1125<br>(2.21)                            | 9356<br>(0.49)                                          |
| K36G/K37Q/M38I/<br>L40M/F59L/E81V/L85R/<br>K89N/A91T/F92P/<br>K93V/R94L/E99G/<br>T130A/N149S                    | 539   | 1298<br>(0.10)                     | 1271<br>(0.03)                       | 3126<br>(0.18)                      | 507<br>(1.00)                             | 3095<br>(0.16)                                          |
| AE88D/K89R/D90K/<br>A91G/F92Y/K93R                                                                              | 102   | 31535<br>(2.38)                    | 50868<br>(1.02)                      | 29077<br>(1.63)                     | 944<br>(1.85)                             | 5922<br>(0.31)                                          |
| K36G/K37Q/M38I/ L40M                                                                                            | 103   | 1170                               | 1405                                 | 959                                 | 427                                       | 811                                                     |

| <b>Table 9: CD80 variants selected against PD-L1. Molecule sequences, binding data, and costimulatory bioactivity data.</b> |                    |                              |                                |                               |                                     |                                            |
|-----------------------------------------------------------------------------------------------------------------------------|--------------------|------------------------------|--------------------------------|-------------------------------|-------------------------------------|--------------------------------------------|
| CD80 mutation(s)                                                                                                            | SEQ ID NO<br>(ECD) | Binding                      |                                |                               | Coimmobilization with anti-CD3      | MLR                                        |
|                                                                                                                             |                    | CD28 MFI<br>(parental ratio) | CTLA-4 MFI<br>(parental ratio) | PD-L1 MFI<br>(parental ratio) | IFN-gamma pg/ml<br>(parental ratio) | IFN-gamma levels pg/ml<br>(parental ratio) |
|                                                                                                                             |                    | (0.09)                       | (0.03)                         | (0.05)                        | (0.84)                              | (0.04)                                     |
| K36G/L40M                                                                                                                   | 540                | 29766<br>(2.25)              | 58889<br>(1.18)                | 20143<br>(1.13)               | 699<br>(1.37)                       | 30558<br>(1.59)                            |
| WTCD80                                                                                                                      | 28                 | 13224<br>(1.00)              | 50101<br>(1.00)                | 17846<br>(1.00)               | 509<br>(1.00)                       | 19211<br>(1.00)                            |

### EXAMPLE 8

#### Generation and Assessment of Stacked Molecules Containing Different Affinity-Modified Domains

**[0474]** This Example describes further immunomodulatory proteins that were generated as stack constructs containing at least two different affinity modified domains from identified variant ICOSL polypeptides and one more additional variant CD80, CD86, ICOSL, and NKp30 molecules linked together and fused to an Fc.

**[0475]** Selected variant molecules described above that were affinity-modified for one or more counter structure ligand were used to generate “stack” molecule (i.e., Type II immunomodulatory protein) containing two or more affinity-modified IgSF domains. Stack constructs were obtained as geneblocks (Integrated DNA Technologies, Coralville, IA) that encode the stack in a format that enables its fusion to Fc by standard Gibson assembly using a Gibson assembly kit (New England Biolabs).

**[0476]** The encoding nucleic acid molecule of all stacks was generated to encode a protein designed as follows: Signal peptide, followed by the first variant IgV of interest, followed by a 15 amino acid linker which is composed of three GGGGS(G4S) motifs (SEQ ID NO:228), followed by the second IgV of interest, followed by two GGGGS linkers (SEQ ID NO: 229) followed by three alanines (AAA), followed by a human IgG1 Fc as described above. To

maximize the chance for correct folding of the IgV domains in each stack, the first IgV was preceded by all residues that normally occur in the wild-type protein between this IgV and the signal peptide (leading sequence). Similarly, the first IgV was followed by all residues that normally connect it in the wild-type protein to either the next Ig domain (typically an IgC domain) or if such a second IgV domain is absent, the residues that connect it to the transmembrane domain (trailing sequence). The same design principle was applied to the second IgV domain except that when both IgV domains were derived from same parental protein (e.g. a ICOSL IgV stacked with another ICOSL IgV), the linker between both was not duplicated.

**[0477]** Table 10 sets forth the design for exemplary stacked constructs. The exemplary stack molecules shown in Table 10 contains the Ig domains (e.g. IgV domain) as indicated and additionally trailing sequences as described above. In the Table, the following components are present in order: signal peptide (SP; SEQ ID NO:225), Ig domain 1 (e.g. Ig1), trailing sequence 1 (TS1), linker 1 (LR1; SEQ ID NO:228), Ig domain 2 (Ig2), trailing sequence 2 (TS2), linker 2 (LR2; SEQ ID NO:230) and Fc domain (SEQ ID NO:226 containing C5S/R77C/N82G/V87C amino acid substitution). In some cases, a leading sequence 1(LS1) is present between the signal peptide and IgV1 and in some cases a leading sequence 2 (LS2) is present between the linker and IgV2.

| <b>Table 10: Amino acid sequence (SEQ ID NO) of components of exemplary stacked constructs</b> |    |              |     |     |     |               |     |     |     |    |
|------------------------------------------------------------------------------------------------|----|--------------|-----|-----|-----|---------------|-----|-----|-----|----|
|                                                                                                | SP | First domain |     |     | LR1 | Second domain |     |     | LR2 | Fc |
|                                                                                                |    | LS1          | Ig1 | TS1 |     | LS2           | Ig2 | TS2 |     |    |
| Domain 1: NKp30<br>WT                                                                          | +  | -            | 214 | 235 | +   | -             | 196 | 233 | +   | +  |
| Domain 2: ICOSL<br>WT                                                                          |    |              |     |     |     |               |     |     |     |    |
| Domain 1: NKp30<br>L30V/A60V/S64P/<br>S86G                                                     | +  | -            | 215 | 235 | +   | -             | 212 | 233 | +   | +  |
| Domain 2: ICOSL<br>N52S/N57Y/H94D/<br>L96F/L98F/Q100R                                          |    |              |     |     |     |               |     |     |     |    |
| Domain 1: NKp30<br>L30V/A60V/S64P/                                                             | +  | -            | 215 | 235 | +   | -             | 199 | 233 | +   | +  |

| Table 10: Amino acid sequence (SEQ ID NO) of components of exemplary stacked constructs |    |              |     |     |     |               |     |     |     |    |
|-----------------------------------------------------------------------------------------|----|--------------|-----|-----|-----|---------------|-----|-----|-----|----|
|                                                                                         | SP | First domain |     |     | LR1 | Second domain |     |     | LR2 | Fc |
|                                                                                         |    | LS1          | Ig1 | TS1 |     | LS2           | Ig2 | TS2 |     |    |
| S86G)                                                                                   |    |              |     |     |     |               |     |     |     |    |
| Domain 2: ICOSL N52D                                                                    |    |              |     |     |     |               |     |     |     |    |
| Domain 1: NKp30 L30V/A60V/S64P/S86G                                                     | +  | -            | 215 | 235 | +   | -             | 201 | 233 | +   | +  |
| Domain 2: ICOSL N52H/N57Y/Q100P                                                         |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL WT                                                                      | +  | -            | 196 | 233 | +   | -             | 214 | 235 | +   | +  |
| Domain 2: Nkp30 WT                                                                      |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL N52D                                                                    |    |              |     |     |     |               |     |     |     |    |
| Domain 2: NKp30 L30V/A60V/S64P/S86G                                                     | +  | -            | 199 | 233 | +   | -             | 215 | 235 | +   | +  |
| Domain 1: ICOSL N52H/N57Y/Q100P                                                         |    |              |     |     |     |               |     |     |     |    |
| Domain 2: NKp30 L30V/A60V/S64P/S86G                                                     | +  | -            | 201 | 233 | +   | -             | 215 | 235 | +   | +  |
| Domain 1: CD80 WT                                                                       |    |              |     |     |     |               |     |     |     |    |
| Domain 2: ICOSL WT                                                                      | +  | -            | 152 | 471 | +   | -             | 196 | 233 | +   | +  |
| Domain 1: CD80 E88D/K89R/D90K/A91G/F92Y/K93R                                            |    |              |     |     |     |               |     |     |     |    |
| Domain 2: ICOSL N52S/N57Y/H94D/L96F/L98F/Q100R/G103E/F120S                              | +  | -            | 189 | 471 | +   | -             | 213 | 233 | +   | +  |
| Domain 1: CD80 A12T/H18L/M43V/F59L/E77K/P109S/I118T                                     |    |              |     |     |     |               |     |     |     |    |
| Domain 2: ICOSL N52S/N57Y/H94D/L96F/L98F/Q100R/G103E/F120S                              | +  | -            | 193 | 471 | +   | -             | 213 | 233 | +   | +  |
| Domain 1: CD80 A12T/H18L/M43V/F59L/E77K/P109S/                                          |    |              |     |     |     |               |     |     |     |    |
|                                                                                         | +  | -            | 193 | 471 | +   | -             | 199 | 233 | +   | +  |

| Table 10: Amino acid sequence (SEQ ID NO) of components of exemplary stacked constructs |    |              |     |     |     |               |     |     |     |    |
|-----------------------------------------------------------------------------------------|----|--------------|-----|-----|-----|---------------|-----|-----|-----|----|
|                                                                                         | SP | First domain |     |     | LR1 | Second domain |     |     | LR2 | Fc |
|                                                                                         |    | LS1          | Ig1 | TS1 |     | LS2           | Ig2 | TS2 |     |    |
| I118T                                                                                   |    |              |     |     |     |               |     |     |     |    |
| Domain 2: ICOSL N52D                                                                    |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD80 E88D/K89R/D90K/A91G/F92Y/K93R                                            | +  | -            | 189 | 471 | +   | -             | 201 | 233 | +   | +  |
| Domain 2: ICOSL N52H/N57Y/Q100P                                                         |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD80 A12T/H18L/M43V/F59L/E77K/P109S/I118T                                     | +  | -            | 193 | 471 | +   | -             | 201 | 233 | +   | +  |
| Domain 2: ICOSL N52H/N57Y/Q100P                                                         |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL WT                                                                      | +  | -            | 196 | 233 | +   | -             | 152 | 471 | +   | +  |
| Domain 2: CD80 WT                                                                       |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL N52S/N57Y/H94D/L96F/L98F/Q100R/G103E/F120S                              | +  | -            | 213 | 233 | +   | -             | 189 | 471 | +   | +  |
| Domain 2: CD80 E88D/K89R/D90K/A91G/F92Y/K93R                                            |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL N52S/N57Y/H94D/L96F/L98F/Q100R/G103E/F120S                              | +  | -            | 213 | 233 | +   | -             | 193 | 471 | +   | +  |
| Domain 2: CD80 A12T/H18L/M43V/F59L/E77K/P109S/I118T                                     |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL N52D                                                                    | +  | -            | 199 | 233 | +   | -             | 189 | 471 | +   | +  |
| Domain 2: CD80 E88D/K89R/D90K/A91G/F92Y/K93R                                            |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL N52D                                                                    | +  | -            | 199 | 233 | +   | -             | 193 | 471 | +   | +  |
| Domain 2: CD80 A12T/H18L/M43V/F59L/E77K/P109S/I118T                                     |    |              |     |     |     |               |     |     |     |    |

| Table 10: Amino acid sequence (SEQ ID NO) of components of exemplary stacked constructs |    |              |     |     |     |               |     |     |     |    |
|-----------------------------------------------------------------------------------------|----|--------------|-----|-----|-----|---------------|-----|-----|-----|----|
|                                                                                         | SP | First domain |     |     | LR1 | Second domain |     |     | LR2 | Fc |
|                                                                                         |    | LS1          | Ig1 | TS1 |     | LS2           | Ig2 | TS2 |     |    |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P                                                      | +  | -            | 201 | 233 | +   | -             | 189 | 471 | +   | +  |
| Domain 2: CD80<br>E88D/K89R/D90K/<br>A91G/F92Y/K93R                                     |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P                                                      | +  | -            | 201 | 233 | +   | -             | 193 | 471 | +   | +  |
| Domain 2: CD80<br>A12T/H18L/M43V/<br>F59L/E77K/P109S/<br>I118T                          |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD86<br>WT                                                                    | +  | 236          | 220 | 237 | +   | -             | 196 | 233 | +   | +  |
| Domain 2: ICOSL<br>WT                                                                   |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD80<br>R29H/Y31H/T41G/<br>Y87N/E88G/K89E/<br>D90N/A91G/P109S                 | +  | -            | 192 | 471 | +   | -             | 213 | 233 | +   | +  |
| Domain 2: ICOSL<br>N52S/N57Y/H94D/<br>L96F/L98F/Q100R/<br>G103E/F120S                   |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD80<br>I67T/L70Q/A91G/T<br>120S                                              | +  | -            | 175 | 471 | +   | -             | 213 | 233 | +   | +  |
| Domain 2: ICOSL<br>N52S/N57Y/H94D/<br>L96F/L98F/Q100R/<br>G103E/F120S                   |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD80<br>R29H/Y31H/T41G/<br>Y87N/E88G/K89E/<br>D90N/A91G/P109S                 | +  | -            | 192 | 471 | +   | -             | 199 | 233 | +   | +  |
| Domain 2: ICOSL<br>N52D                                                                 |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD80<br>I67T/L70Q/A91G/T<br>120S                                              | +  | -            | 175 | 471 | +   | -             | 199 | 233 | +   | +  |
| Domain 2: ICOSL<br>N52D                                                                 |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD80<br>R29H/Y31H/T41G/<br>Y87N/E88G/K89E/<br>D90N/A91G/P109S                 | +  | -            | 192 | 471 | +   | -             | 201 | 233 | +   | +  |

| Table 10: Amino acid sequence (SEQ ID NO) of components of exemplary stacked constructs                                                              |    |              |     |     |     |               |     |     |     |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------|----|--------------|-----|-----|-----|---------------|-----|-----|-----|----|
|                                                                                                                                                      | SP | First domain |     |     | LR1 | Second domain |     |     | LR2 | Fc |
|                                                                                                                                                      |    | LS1          | Ig1 | TS1 |     | LS2           | Ig2 | TS2 |     |    |
| Domain 2: ICOSL<br>N52H/N57Y/Q100P                                                                                                                   |    |              |     |     |     |               |     |     |     |    |
| Domain 1: CD80<br>I67T/L70Q/A91G/T<br>120S<br><br>Domain 2: ICOSL<br>N52H/N57Y/Q100P                                                                 | +  | -            | 175 | 471 | +   | -             | 201 | 233 | +   | +  |
| Domain 1: CD86<br>Q35H/H90L/Q102<br>H<br><br>Domain 2: ICOSL<br>N52S/N57Y/H94D/<br>L96F/L98F/Q100R/<br>G103E/F120S                                   | +  | 236          | 221 | 237 | +   | -             | 213 | 233 | +   | +  |
| Domain 1: CD86<br>Q35H/H90L/Q102<br>H<br><br>Domain 2: ICOSL<br>N52D                                                                                 | +  | 236          | 221 | 237 | +   | -             | 199 | 233 | +   | +  |
| Domain 1: CD86<br>Q35H/H90L/Q102<br>H<br><br>Domain 2: ICOSL<br>N52H/N57Y/Q100P                                                                      | +  | 236          | 221 | 237 | +   | -             | 201 | 233 | +   | +  |
| Domain 1: ICOSL<br>WT<br><br>Domain 2: CD86<br>WT                                                                                                    | +  | -            | 196 | 233 | +   | 236           | 220 | 237 | +   | +  |
| Domain 1: ICOSL<br>N52S/N57Y/H94D/<br>L96F/L98F/Q100R/<br>G103E/F120S<br><br>Domain 2: CD80<br>R29H/Y31H/T41G/<br>Y87N/E88G/K89E/<br>D90N/A91G/P109S | +  | -            | 213 | 233 | +   | -             | 192 | 471 | +   | +  |
| Domain 1: ICOSL<br>N52S/N57Y/H94D/<br>L96F/L98F/Q100R/<br>G103E/F120S<br><br>Domain 2: CD80<br>I67T/L70Q/A91G/T<br>120S                              | +  | -            | 213 | 233 | +   | -             | 175 | 471 | +   | +  |
| Domain 1: ICOSL                                                                                                                                      | +  | -            | 199 | 233 | +   | -             | 192 | 471 | +   | +  |

| Table 10: Amino acid sequence (SEQ ID NO) of components of exemplary stacked constructs |    |              |     |     |     |               |     |     |     |    |
|-----------------------------------------------------------------------------------------|----|--------------|-----|-----|-----|---------------|-----|-----|-----|----|
|                                                                                         | SP | First domain |     |     | LR1 | Second domain |     |     | LR2 | Fc |
|                                                                                         |    | LS1          | Ig1 | TS1 |     | LS2           | Ig2 | TS2 |     |    |
| N52D                                                                                    |    |              |     |     |     |               |     |     |     |    |
| Domain 2: CD80<br>R29H/Y31H/T41G/<br>Y87N/E88G/K89E/<br>D90N/A91G/P109S                 |    |              |     |     |     |               |     |     |     |    |
| Domain 1: ICOSL<br>N52D                                                                 |    |              |     |     |     |               |     |     |     |    |
| Domain 2: CD80<br>I67T/L70Q/A91G/T<br>120S                                              | +  | -            | 199 | 233 | +   | -             | 175 | 471 | +   | +  |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P                                                      |    |              |     |     |     |               |     |     |     |    |
| Domain 2: CD80<br>R29H/Y31H/T41G/<br>Y87N/E88G/K89E/<br>D90N/A91G/P109S                 | +  | -            | 201 | 233 | +   | -             | 192 | 471 | +   | +  |
| Domain 1: ICOSL<br>N52S/N57Y/H94D/<br>L96F/L98F/Q100R/<br>G103E/F120S                   |    |              |     |     |     |               |     |     |     |    |
| Domain 2: CD86<br>Q35H/H90L/Q102<br>H                                                   | +  | -            | 213 | 233 | +   | 236           | 221 | 237 | +   | +  |
| Domain 1: ICOSL<br>N52D                                                                 |    |              |     |     |     |               |     |     |     |    |
| Domain 2: CD86<br>Q35H/H90L/Q102<br>H                                                   | +  | -            | 199 | 233 | +   | 236           | 221 | 237 | +   | +  |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P                                                      |    |              |     |     |     |               |     |     |     |    |
| Domain 2: CD86<br>Q35H/H90L/Q102<br>H                                                   | +  | -            | 201 | 233 | +   | 236           | 221 | 237 | +   | +  |

**[0478]** High throughput expression and purification of the variant IgV-stacked-Fc fusion molecules containing various combinations of variant IgV domains from CD80, CD86, ICOSL or Nkp30 containing at least one affinity-modified IgV domain were generated substantially as described in Example 5. Binding of the variant IgV-stacked-Fc fusion molecules to respective counter structures and functional activity by anti-CD3 coimmobilization assay also were assessed substantially as described in Example 6. For example, costimulatory bioactivity of the stacked

IgSF Fc fusion proteins was determined in a similar immobilized anti-CD3 assay as above. In this case, 4nM of anti-CD3 (OKT3, Biolegend, USA) was coimmobilized with 4 nM to 120 nM of human rB7-H6.Fc (R&D Systems, USA) or human rPD-L1.Fc (R&D Systems, USA) overnight on tissue-culture treated 96-well plates (Corning, USA). The following day unbound protein was washed off with PBS and 100,000 purified pan T cells were added to each well in 100  $\mu$ l Ex-Vivo 15 media (Lonza, Switzerland). The stacked IgSF domains were subsequently added at concentrations ranging from 8 nM to 40 nM in a volume of 100  $\mu$ l for 200  $\mu$ l volume total. Cells were cultured 3 days before harvesting culture supernatants and measuring human IFN-gamma levels with DuoSet ELISA kit (R&D Systems, USA) as mentioned above.

**[0479]** The results are set forth in Tables 11-13. Specifically, Table 11 sets forth binding and functional activity results for variant IgV-stacked-Fc fusion molecules containing an NKp30 IgV domain and an ICOSL IgV domain. Table 12 and 13 sets forth binding and functional activity results for variant IgV-stacked-Fc fusion molecules containing a variant ICOSL IgV domain and a variant CD80 IgV or CD86 IgV domain.

**[0480]** For each of Tables 11-13, Column 1 indicates the structural organization and orientation of the stacked, affinity modified or wild-type (WT) domains beginning with the amino terminal (N terminal) domain, followed by the middle WT or affinity modified domain located before the C terminal human IgG1 Fc domains. Column 2 sets forth the SEQ ID NO identifier for the sequence of each IgV domain contained in a respective “stack” molecule. Column 3 shows the binding partners which the indicated affinity modified stacked domains from column 1 were selected against.

**[0481]** Also shown is the binding activity as measured by the Mean Fluorescence Intensity (MFI) value for binding of each stack molecule to cells transfected to express various counter structure ligands and the ratio of the MFI compared to the binding of the corresponding stack molecule containing unmodified IgV domains not containing the amino acid substitution(s) to the same cell-expressed counter structure ligand. The functional activity of the variant stack molecules to modulate the activity of T cells also is shown based on the calculated levels of IFN-gamma in culture supernatants (pg/ml) generated with the indicated variant stack molecule in solution and the appropriate ligand coimmobilized with anti-CD3 as described in Example 6. The Table also depicts the ratio of IFN-gamma produced by each variant stack molecule compared to the corresponding unmodified stack molecule in the coimmobilization assay.

**[0482]** As shown, the results showed that it was possible to generate stack molecules containing at least one variant IgSF domains that exhibited affinity-modified activity of increased binding for at least one cognate counter structure ligand compared to a corresponding stack molecule containing the respective unmodified (e.g. wild-type) IgV domain. In some cases, the stack molecule, either from one or a combination of both variant IgSF domains in the molecule, exhibited increased binding for more than one cognate counter structure ligand. The results also showed that the order of the IgV domains in the stacked molecules could, in some cases, alter the degree of improved binding activity. In some cases, functional T cell activity also was altered when assessed in the targeted coimmobilization assay.

| <b>TABLE 11: Stacked variant IgV Fc fusion proteins containing an NKp30 IgV domain and an ICOSL IgV domain</b> |                                 |                                           |                                                |                                                |                                                |                                                                                                    |
|----------------------------------------------------------------------------------------------------------------|---------------------------------|-------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|----------------------------------------------------------------------------------------------------|
| <b>Domain Structure</b><br>N terminal to C terminal:<br>domain 1/domain 2/Fc                                   | <b>SEQ ID NO</b><br>(Ig domain) | <b>Counter structure selected against</b> | <b>Binding Activity</b>                        |                                                |                                                | <b>Anti-CD3 coimmobilization assay</b><br><br>pg/ml IFN-gamma<br><br>(WT parental IFN-gamma ratio) |
|                                                                                                                |                                 |                                           | <b>B7H6 MFI</b><br><br>(WT parental MFI ratio) | <b>ICOS MFI</b><br><br>(WT parental MFI ratio) | <b>CD28 MFI</b><br><br>(WT parental MFI ratio) |                                                                                                    |
| Domain 1: NKp30 WT                                                                                             | 214                             | -                                         | 64538                                          | 26235                                          | 6337                                           | 235                                                                                                |
| Domain 2: ICOSL WT                                                                                             | 196                             |                                           | (1.00)                                         | (1.00)                                         | (1.00)                                         | (1.00)                                                                                             |
| Domain 1: NKp30 L30V/A60V/S64P/S86G                                                                            | 215                             | B7-H6                                     | 59684<br>(0.92)                                | 12762<br>(0.49)                                | 9775<br>(1.54)                                 | 214<br>(0.91)                                                                                      |
| Domain 2: ICOSL N52S N57Y H94D L96F L98F Q100R                                                                 | 212                             | ICOS-CD28                                 |                                                |                                                |                                                |                                                                                                    |
| Domain 1: NKp30 L30V/A60V/S64P/S86G                                                                            | 215                             | B7-H6                                     | 65470<br>(1.01)                                | 30272<br>(1.15)                                | 9505<br>(1.50)                                 | 219<br>(0.93)                                                                                      |
| Domain 2: ICOSL N52D                                                                                           | 199                             | ICOS-CD28                                 |                                                |                                                |                                                |                                                                                                    |
| Domain 1: NKp30 L30V/A60V/S64P/S86G                                                                            | 215                             | B7-H6                                     | 38153<br>(0.59)                                | 27903<br>(1.06)                                | 11300<br>(1.78)                                | 189<br>(0.80)                                                                                      |
| Domain 2: ICOSL N52H N57Y Q100P                                                                                | 201                             | ICOS-CD28                                 |                                                |                                                |                                                |                                                                                                    |
| Domain 1: ICOSL WT                                                                                             | 196                             | -                                         | 117853                                         | 70320                                          | 7916                                           | 231                                                                                                |
| Domain 2: Nkp30 WT                                                                                             | 214                             |                                           | (1.0)                                          | (1.0)                                          | (1.0)                                          | (1.0)                                                                                              |

|                                        |     |           |                  |                 |                 |               |
|----------------------------------------|-----|-----------|------------------|-----------------|-----------------|---------------|
| Domain 1:ICOSL<br>N52D                 | 199 | ICOS-CD28 | 100396<br>(0.85) | 83912<br>(1.19) | 20778<br>(2.62) | 228<br>(0.98) |
| Domain 2: NKp30<br>L30V/A60V/S64P/S86G | 215 | B7-H6     |                  |                 |                 |               |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P     | 201 | ICOS-CD28 | 82792<br>(0.70)  | 68874<br>(0.98) | 72269<br>(9.12) | 561<br>(2.43) |
| Domain 2: NKp30<br>L30V/A60V/S64P/S86G | 215 | B7-H6     |                  |                 |                 |               |

| <b>TABLE 12: Stacked variant IgV Fc fusion proteins containing a CD80 IgV domain and a ICOSL IgV domain</b> |                                |                                             |                                               |                                                   |                                               |                                                                                                           |
|-------------------------------------------------------------------------------------------------------------|--------------------------------|---------------------------------------------|-----------------------------------------------|---------------------------------------------------|-----------------------------------------------|-----------------------------------------------------------------------------------------------------------|
| Domain Structure<br>N terminal to C terminal:<br>domain 1/domain 2/Fc                                       | SEQ ID<br>NO<br>(Ig<br>domain) | Counter<br>structure<br>selected<br>against | Binding Activity                              |                                                   |                                               | Anti-CD3<br>coimmobilization<br>assay<br><br>pg/ml IFN-<br>gamma<br><br>(WT parental IFN-<br>gamma ratio) |
|                                                                                                             |                                |                                             | CD28 MFI<br><br>(WT<br>parental<br>MFI ratio) | PD-L1<br>MFI<br><br>(WT<br>parental<br>MFI ratio) | ICOS MFI<br><br>(WT<br>parental<br>MFI ratio) |                                                                                                           |
| Domain 1: CD80 WT                                                                                           | 152                            |                                             | 1230<br>(1.00)                                | 2657<br>(1.00)                                    | 11122<br>(1.00)                               | 69<br>(1.00)                                                                                              |
| Domain 2: ICOSL WT                                                                                          | 196                            |                                             |                                               |                                                   |                                               |                                                                                                           |
| Domain 1: CD80<br>E88D/K89R/D90K/A91G/<br>F92Y/K93R                                                         | 189                            | PD-L1                                       |                                               |                                                   |                                               |                                                                                                           |
| Domain 2: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120<br>S                                       | 213                            | ICOS/CD28                                   | 3383<br>(2.75)                                | 4515<br>(1.70)                                    | 5158<br>(0.46)                                | 90<br>(1.30)                                                                                              |
| Domain 1: CD80<br>A12T/H18L/M43V/F59L/<br>E77K/P109S/I118T                                                  | 193                            | PD-L1                                       |                                               |                                                   |                                               |                                                                                                           |
| Domain 2: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120<br>S                                       | 213                            | ICOS/CD28                                   | 2230<br>(1.81)                                | 2148<br>(0.81)                                    | 3860<br>(0.35)                                | 112<br>(1.62)                                                                                             |
| Domain 1: CD80<br>A12T/H18L/M43V/F59L/<br>E77K/P109S/I118T                                                  | 193                            | PD-L1                                       |                                               |                                                   |                                               |                                                                                                           |
| Domain 2: ICOSL<br>N52D                                                                                     | 199                            | ICOS/CD28                                   | 5665<br>(4.61)                                | 6446<br>(2.43)                                    | 15730<br>(1.41)                               | 126<br>(1.83)                                                                                             |
| Domain 1: CD80<br>E88D/K89R/D90K/A91G/<br>F92Y/K93R                                                         | 189                            | PD-L1                                       |                                               |                                                   |                                               |                                                                                                           |
| Domain 2: ICOSL<br>N52H/N57Y/Q100P                                                                          | 201                            | ICOS/CD28                                   | 6260<br>(5.09)                                | 4543<br>(1.71)                                    | 11995<br>(1.08)                               | 269<br>(3.90)                                                                                             |
| Domain 1: CD80<br>A12T/H18L/M43V/F59L/                                                                      | 193                            | PD-L1                                       | 3359<br>(2.73)                                | 3874<br>(1.46)                                    | 8541<br>(0.77)                                | 97<br>(1.41)                                                                                              |

|                                                                       |     |           |                |                |                 |               |
|-----------------------------------------------------------------------|-----|-----------|----------------|----------------|-----------------|---------------|
| E77K/P109S/I118T                                                      |     |           |                |                |                 |               |
| Domain 2: ICOSL<br>N52H/N57Y/Q100P                                    | 201 | ICOS/CD28 |                |                |                 |               |
| Domain 1: ICOSL WT                                                    | 196 |           | 3000<br>(1.00) | 2966<br>(1.00) | 14366<br>(1.00) | 101<br>(1.00) |
| Domain 2: CD80 WT                                                     | 152 |           |                |                |                 |               |
| Domain 1: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120<br>S | 213 | ICOS/CD28 |                |                |                 |               |
| Domain 2: CD80<br>E88D/K89R/D90K/A91G/<br>F92Y/K93R                   | 189 | PD-L1     | 3634<br>(1.21) | 4893<br>(1.65) | 6403<br>(0.45)  | 123<br>(1.22) |
| Domain 1: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120<br>S | 213 | ICOS/CD28 |                |                |                 |               |
| Domain 2: CD80<br>A12T/H18L/M43V/F59L/<br>E77K/P109S/I118T            | 193 | PD-L1     | 1095<br>(0.37) | 5929<br>(2.0)  | 7923<br>(0.55)  | 127<br>(1.26) |
| Domain 1: ICOSL<br>N52D                                               | 199 | ICOS/CD28 |                |                |                 |               |
| Domain 2: CD80<br>E88D/K89R/D90K/A91G/<br>F92Y/K93R                   | 189 | PD-L1     | 2023<br>(0.67) | 5093<br>(1.72) | 16987<br>(1.18) | 125<br>(1.24) |
| Domain 1: ICOSL<br>N52D                                               | 199 | ICOS/CD28 |                |                |                 |               |
| Domain 2: CD80<br>A12T/H18L/M43V/F59L/<br>E77K/P109S/I118T            | 193 | PD-L1     | 3441<br>(1.15) | 3414<br>(1.15) | 20889<br>(1.45) | 165<br>(1.63) |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P                                    | 201 | ICOS/CD28 |                |                |                 |               |
| Domain 2: CD80<br>E88D/K89R/D90K/A91G/<br>F92Y/K93R                   | 189 | PD-L1     | 7835<br>(2.61) | 6634<br>(2.24) | 20779<br>(1.45) | 95<br>(0.94)  |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P                                    | 201 | ICOS/CD28 |                |                |                 |               |
| Domain 2: CD80<br>A12T/H18L/M43V/F59L/<br>E77K/P109S/I118T            | 193 | PD-L1     | 8472<br>(2.82) | 3789<br>(1.28) | 13974<br>(0.97) | 106<br>(1.05) |

**TABLE 13: Stacked variant IgV Fc fusion proteins containing a CD80 or CD86 IgV domain and an ICOSL IgV domain**

| Domain Structure<br>N terminal to C terminal: | SEQ ID<br>NO<br>(Ig<br>domain) | Counter<br>structure<br>selected<br>against | Binding Activity           |                             | Functional<br>Activity<br>MLR IFN-gamma<br>pg/ml |
|-----------------------------------------------|--------------------------------|---------------------------------------------|----------------------------|-----------------------------|--------------------------------------------------|
|                                               |                                |                                             | PD-L1 MFI<br>(WT parental) | CTLA-4 MFI<br>(WT parental) |                                                  |

| domain 1/domain 2/Fc                                                    |     |           | MFI ratio)      | MFI ratio)      |                |
|-------------------------------------------------------------------------|-----|-----------|-----------------|-----------------|----------------|
| Domain 1: CD80 WT                                                       | 152 |           | 1230<br>(1.00)  | 11122<br>(1.00) | 1756<br>(1.00) |
| Domain 2: ICOSL WT                                                      | 196 |           |                 |                 |                |
| Domain 1: CD86 WT                                                       | 220 |           | 29343<br>(1.00) | 55193<br>(1.00) | 6305<br>(1.00) |
| Domain 2: ICOSL WT                                                      | 196 |           |                 |                 |                |
| Domain 1: CD80<br>R29H/Y31H/T41G/Y87N/<br>E88G/K89E/D90N/A91G/<br>P109S | 192 | CD28      |                 |                 |                |
| Domain 2: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120<br>S   | 213 | ICOS/CD28 | 2280<br>(1.85)  | 3181<br>(0.29)  | 2281<br>(1.30) |
| Domain 1: CD80<br>I67T/L70Q/A91G/T120S                                  | 175 | CD28      |                 |                 |                |
| Domain 2: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120<br>S   | 213 | ICOS/CD28 | 2309<br>(1.88)  | 26982<br>(2.43) | 1561<br>(0.89) |
| Domain 1: CD80<br>R29H/Y31H/T41G/Y87N/<br>E88G/K89E/D90N/A91G/<br>P109S | 192 | CD28      |                 |                 |                |
| Domain 2: ICOSL<br>N52D                                                 | 199 | ICOS/CD28 | 4285<br>(3.48)  | 22744<br>(2.04) | 1612<br>(0.92) |
| Domain 1: CD80<br>I67T/L70Q/A91G/T120S                                  | 175 | CD28      |                 |                 |                |
| Domain 2: ICOSL<br>N52D                                                 | 199 | ICOS/CD28 | 3024<br>(2.46)  | 16916<br>(1.52) | 3857<br>(2.20) |
| Domain 1: CD80<br>R29H/Y31H/T41G/Y87N/<br>E88G/K89E/D90N/A91G/<br>P109S | 192 | CD28      |                 |                 |                |
| Domain 2: ICOSL<br>N52H/N57Y/Q100P                                      | 201 | ICOS/CD28 | 6503<br>(5.29)  | 7240<br>(0.65)  | 6886<br>(3.92) |
| Domain 1: CD80<br>I67T/L70Q/A91G/T120S                                  | 175 | CD28      |                 |                 |                |
| Domain 2: ICOSL<br>N52H/N57Y/Q100P                                      | 201 | ICOS/CD28 | 3110<br>(2.53)  | 4848<br>(0.44)  | 3393<br>(1.93) |
| Domain 1: CD86<br>Q35H/H90L/Q102H                                       | 221 | CD28      |                 |                 |                |
| Domain 2: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120        | 213 | ICOS/CD28 | 11662<br>(0.40) | 21165<br>(0.38) | 880<br>(0.14)  |

|                                                                         |     |           |                 |                 |                 |
|-------------------------------------------------------------------------|-----|-----------|-----------------|-----------------|-----------------|
| S                                                                       |     |           |                 |                 |                 |
| Domain 1: CD86<br>Q35H/H90L/Q102H                                       | 221 | CD28      | 24230<br>(0.83) | 73287<br>(1.33) | 1110<br>(0.18)  |
| Domain 2: ICOSL<br>N52D                                                 | 199 | ICOS/CD28 |                 |                 |                 |
| Domain 1: CD86<br>Q35H/H90L/Q102H                                       | 221 | CD28      | 1962<br>(0.07)  | 1630<br>(0.03)  | 587<br>(0.09)   |
| Domain 2: ICOSL<br>N52H/N57Y/Q100P                                      | 201 | ICOS/CD28 |                 |                 |                 |
| Domain 1: ICOSL WT                                                      | 196 |           | 3000<br>(1.00)  | 14366<br>(1.00) | 4113<br>(1.00)  |
| Domain 2: CD80 WT                                                       | 152 |           |                 |                 |                 |
| Domain 1: ICOSL WT                                                      | 196 |           | 18005<br>(1.00) | 53602<br>(1.00) | 18393<br>(1.00) |
| Domain 2: CD86 WT                                                       | 220 |           |                 |                 |                 |
| Domain 1: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120<br>S   | 213 | ICOS/CD28 | 10426<br>(3.48) | 51286<br>(3.57) | 18680<br>(4.54) |
| Domain 2: CD80<br>R29H/Y31H/T41G/Y87N/<br>E88G/K89E/D90N/A91G/<br>P109S | 192 | CD28      |                 |                 |                 |
| Domain 1: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120<br>S   | 213 | ICOS/CD28 | 17751<br>(5.92) | 29790<br>(2.07) | 10637<br>(2.59) |
| Domain 2: CD80<br>I67T/L70Q/A91G/T120S                                  | 175 | CD28      |                 |                 |                 |
| Domain 1: ICOSL<br>N52D                                                 | 199 | ICOS/CD28 |                 |                 |                 |
| Domain 2: CD80<br>R29H/Y31H/T41G/Y87N/<br>E88G/K89E/D90N/A91G/<br>P109S | 192 | CD28      | 2788<br>(0.93)  | 25870<br>(1.80) | 6205<br>(1.51)  |
| Domain 1: ICOSL<br>N52D                                                 | 199 | ICOS/CD28 | 2522<br>(0.84)  | 13569<br>(0.94) | 5447<br>(1.32)  |
| Domain 2: CD80<br>I67T/L70Q/A91G/T120S                                  | 175 | CD28      |                 |                 |                 |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P                                      | 201 | ICOS/CD28 |                 |                 |                 |
| Domain 2: CD80<br>R29H/Y31H/T41G/Y87N/<br>E88G/K89E/D90N/A91G/<br>P109S | 192 | CD28      | 9701<br>(3.23)  | 9187<br>(0.64)  | 5690<br>(1.38)  |
| Domain 1: ICOSL<br>N52S/N57Y/H94D/L96F/<br>L98F/Q100R/G103E/F120        | 213 | ICOS/CD28 | 27050<br>(1.50) | 21257<br>(0.40) | 8131<br>(0.44)  |

|                                    |     |           |                 |                 |                 |
|------------------------------------|-----|-----------|-----------------|-----------------|-----------------|
| S                                  |     |           |                 |                 |                 |
| Domain 2: CD86<br>Q35H/H90L/Q102H  | 221 | CD28      |                 |                 |                 |
| Domain 1: ICOSL<br>N52D            | 199 | ICOS/CD28 |                 |                 |                 |
| Domain 2: CD86<br>Q35H/H90L/Q102H  | 221 | CD28      | 34803<br>(1.93) | 80210<br>(1.50) | 6747<br>(0.37)  |
| Domain 1: ICOSL<br>N52H/N57Y/Q100P | 201 | ICOS/CD28 |                 |                 |                 |
| Domain 2: CD86<br>Q35H/H90L/Q102H  | 221 | CD28      | 5948<br>(0.33)  | 4268<br>(0.08)  | 26219<br>(1.43) |

### EXAMPLE 9

#### Generation and Assessment of Engineered Cells Expressing a Transmembrane Immunomodulatory Protein

**[0483]** Engineered T cells were generated in which a transmembrane immunomodulatory protein (TIP) containing an extracellular domain (ECD) containing either a variant CD80 as described above or an ICOSL affinity-modified IgSF domain was co-expressed with a chimeric antigen receptor (CAR). The TIP also contained a transmembrane domain and a cytoplasmic domain of the corresponding wild-type CD80 or ICOSL transmembrane protein sequence. The immunomodulatory activity of the engineered cells was compared to cells that only expressed the CAR or cells that co-expressed the corresponding wild-type CD80 or ICOSL transmembrane protein with the CAR.

**[0484]** The exemplary CD80-TIP was a variant CD80 having an affinity-modified IgSF domain containing amino acid mutations in the IgV and IgC domains corresponding to I67T/L70Q/A91G/T120S with reference to positions in the CD80 extracellular domain set forth in SEQ ID NO:28 and a transmembrane and cytoplasmic domain corresponding to residues 243-288 of SEQ ID NO:1. The amino acid sequence of the exemplary CD80-TIP is set forth in SEQ ID NO:241 and is encoded by the sequence of nucleotides set forth in SEQ ID NO:242. The corresponding wild-type CD80 transmembrane protein had the sequence of amino acids set forth as amino acid residues 35-288 of SEQ ID NO:1 and encoded by the sequence of amino acids set forth in SEQ ID NO: 251.

**[0485]** The exemplary ICOSL-TIP was a variant ICOSL having an affinity-modified IgSF domain containing amino acid mutations in the IgV domain corresponding to N52H/I143T with reference to positions in the ICOSL extracellular domain set forth in SEQ ID NO:32 and a transmembrane and cytoplasmic domain corresponding to residues 257-302 of SEQ ID NO:5. The amino acid sequence of the exemplary ICOSL-TIP is set forth in SEQ ID NO:243 and is encoded by the sequence of nucleotides set forth in SEQ ID NO:244. The corresponding wild-type ICOSL transmembrane protein had the sequence of amino acids set forth as amino acid residues 19-302 of SEQ ID NO:5 and encoded by the sequence of amino acids set forth in SEQ ID NO: 252.

**[0486]** The TIP containing the affinity-modified domain or the wild-type transmembrane protein containing a corresponding non-affinity modified IgSF domain were co-expressed in T cells with a 1<sup>st</sup> generation chimeric antigen receptor (CAR) containing a CD3zeta intracellular signaling domain. The 1<sup>st</sup> generation CAR included an scFv specific for CD19 (SEQ ID NO:245), a hinge and transmembrane domain derived from CD8 (SEQ ID NO:246) and an intracellular signaling domain derived from CD3zeta (set forth in SEQ ID NO:47). The nucleotide sequence encoding the CD19 scFv – CD3zeta CAR is set forth in SEQ ID NO:248 and the amino acid sequence of the CD19 scFv – CD3zeta CAR is set forth in SEQ ID NO:479.

**[0487]** Nucleic acid molecules encoding the CAR alone or also encoding one of the exemplary TIPs or wild-type transmembrane proteins separated from the CAR by a self-cleaving T2A sequence (SEQ ID NO:250 and encoded by the sequence of nucleotides set forth in SEQ ID NO:249) were generated. Exemplary constructs contained nucleic acid sequences set forth in Table 14. As a control, a nucleic acid construct encoding a 2<sup>nd</sup> generation CAR additionally containing a CD28 costimulatory domain also was generated (CD19 scFv – CD28 – CD3zeta).

| <b>Table 14: Nucleic Acid Constructs</b> |                            |                                   |                            |
|------------------------------------------|----------------------------|-----------------------------------|----------------------------|
|                                          | <b>CAR<br/>(SEQ ID NO)</b> | <b>T2A Linker<br/>(SEQ ID NO)</b> | <b>TIP<br/>(SEQ ID NO)</b> |
| CD19 scFv – CD3zeta                      | +(248)                     | -                                 | -                          |
| CD19 scFv – CD3zeta – T2A – B7-1         | +(248)                     | +(249)                            | Wildtype CD80 (251)        |
| CD19 scFv – CD3zeta – T2A – B7-1_TIP     | +(248)                     | +(249)                            | CD80 TIP (242)             |
| CD19 scFv – CD3zeta – T2A – ICOSL        | +(248)                     | +(249)                            | Wildtype ICOSL (252)       |

| Table 14: Nucleic Acid Constructs        |                    |                           |                    |
|------------------------------------------|--------------------|---------------------------|--------------------|
|                                          | CAR<br>(SEQ ID NO) | T2A Linker<br>(SEQ ID NO) | TIP<br>(SEQ ID NO) |
| CD19 scFv – CD3zeta – T2A<br>– ICOSL_TIP | +<br>(248)         | +<br>(249)                | ICOSL TIP<br>(244) |

**[0488]** The nucleic acid molecules were individually cloned into a lentiviral vector, which was used to transduce T cells isolated from human PBMC samples obtained from three different healthy donors. Lentivirus particles containing the nucleic acid sequences were produced after co-transfection of HEK293 cells with the vectors and lentivirus packaging constructs. The lentivirus particles were collected from the culture medium by ultracentrifugation and titered by qRT-PCR. Human peripheral blood mononuclear cells (PBMC) were isolated from three normal blood donors using density sedimentation. The PBMC were cultured overnight with anti-CD3 and anti-CD28 antibodies and IL-2, then transduced with the lentivirus preparations at a multiplicity of infection of 5:1. The lentiviral vectors encoding the control 2<sup>nd</sup> generation CAR was only used to transduce cells from one donor.

**[0489]** After two weeks (14 days) of culture, the cells were analyzed for cytotoxicity following co-culture with target antigen-expressing cells using the Acea Real-Time Cell Analyzer (RTCA), which measures the impedance variations in the culture media of a 96-well microelectronic plate (E-plate), and shows the changes in cell number and morphology in a real-time plot. CD19-expressing HeLa target cells (HeLa-CD19) were seeded into a 96-well E-plate and the impedance of each monolayer was monitored for 24 hours using the RTCA system. The engineered T cells were added to the wells at an effector:target ratio of 10:1 and the wells were monitored for another 48 hours. The results were displayed and recorded as Cell Index (CI) value derived from the change in measured electrical impedance and were then ratio transformed by dividing the CI readouts of all wells at all time points over the CI value of individual wells at a same time (base-time) to obtain a normalized cell index value representing the percentage of the value at the base-time (see Zhang et al. “Introduction to the Data Analysis of the Roche xCELLigence® System with RTCA Package.” *Bioconductor*. May, 3, 2016, [bioconductor.org/packages/devel/bioc/vignettes/RTCA/inst/doc/aboutRTCA.pdf](http://bioconductor.org/packages/devel/bioc/vignettes/RTCA/inst/doc/aboutRTCA.pdf). Accessed

September 9, 2016). In this assay, a decrease in the impedance of a monolayer reflects killing of the target cells by the transduced cells.

**[0490]** The results showed that decreased impedance was observed in cells expressing the 1<sup>st</sup> generation CAR compared to non-transduced T cells, although the degree of decreased impedance for cells expressing the 1<sup>st</sup> generation CAR was less than cells expressing the 2<sup>nd</sup> generation CAR. The decreased impedance in cells expressing the 1<sup>st</sup> generation CAR continued generally for up to the first 8 hours of the assay, while only the 2<sup>nd</sup> generation CAR-expressing cells continued to decrease the impedance thereafter.

**[0491]** As shown in FIG. 1, in one donor, each of the cells co-expressing the TIP or corresponding wild-type transmembrane protein with the 1<sup>st</sup> generation CAR exhibited a greater decrease in impedance, indicating greater cytotoxic activity, compared to cells only expressing the 1<sup>st</sup> generation CAR. Further, the results showed that the cytotoxic activity was greater in CAR-expressing cells that co-expressed the CD80-TIP or ICOSL-TIP relative to CAR-expressing cells that co-expressed the corresponding wild-type CD80 or ICOSL transmembrane proteins containing a non-affinity modified IgSF domain. The observed results of these TIP-engineered cells showed that cytotoxic activity in cells co-expressing the CD80-TIP or ICOSL-TIP with the CAR exhibit increased activity to modulate the cytotoxic immune response of antigen-specific T cells, such as the CAR-expressing T cells.

**[0492]** In the other two donors, the cells expressing the CD80-TIP did not result in a greater decreased impedance compared to cells expressing the corresponding wild-type CD80 transmembrane protein. In one donor, there were not enough cells to transduce with the wild-type transmembrane protein construct, although in this donor the ICOS-L TIP gave the best cytotoxicity compared to the other constructs tested. In the other donor, the cells expressing the ICOS-L-TIP did not result in a greater decreased impedance compared to cells expressing the corresponding wild-type ICOS-L transmembrane protein. In the tested cells, all cells co-expressing either a CD80-TIP, ICOSL-TIP or corresponding wild type transmembrane protein with the CAR exhibited greater cytotoxic activity than cells only expressing the 1<sup>st</sup> generation CAR. The differences in the results observed among donors may be related to the differences in the T cells among the donors, differences in expression levels of the various engineered proteins on the surface of the cells, the particular conditions used in this exemplary assay for assessing

killing in cells (e.g. assessing Day 14 transduced cells, assessing a single effector:target cell ratio) or other factors.

## **EXAMPLE 10**

### **Assessment of Binding and Activity of ICOSL IgSF Domain Variants**

**[0493]** Additional ECD ICOSL variants were identified by the yeast selection method substantially as described above and were used to produce ECD-Fc fusion proteins as described in Example 5. Binding studies were performed to assess specificity and affinity of ICOSL domain variant immunomodulatory proteins for cognate binding partners substantially as described in Example 6.

#### **A. Binding and Functional Characterization**

**[0494]** Binding was assessed to cells expressed full-length cognate binding partners CD28, ICOS and CTLA-4 substantially as described in Example 6. Bioactivity of the ECD ICOSL variants also was assessed in an anti-CD3 coimmobilization assay or human Mixed Lymphocyte Reaction (MLR) substantially as described in Example 6, except that for the coimmobilization assay, costimulatory activity was determined by culture of human T cells with a mixture of 10 nM plate-bound anti-CD3 and 40nM ICOSL Fc variant proteins.

**[0495]** Table 15 depicts exemplary results for the additional ICOSL IgSF domain variants for binding to cell-expressed counter structures and bioactivity in the anti-CD3 coimmobilization assay or MLR assay. The exemplary amino acid substitutions depicted in Table 15 are designated by amino acid position number corresponding to the respective reference unmodified ICOSL ECD sequence set forth in SEQ ID NO:32. The amino acid position is indicated in the middle, with the corresponding unmodified (e.g. wild-type) amino acid listed before the number and the identified variant amino acid substitution listed after the number. Column 2 sets forth the SEQ ID NO identifier for the variant ECD for each variant ECD-Fc fusion molecule.

**[0496]** The results in Table 15 depict binding activity as measured by the Mean Fluorescence Intensity (MFI) value for binding of each variant Fc-fusion molecule to cells engineered to express the cognate counter structure ligand and the ratio of the MFI compared to the binding of the corresponding unmodified ECD-Fc fusion molecule not containing the amino acid substitution(s) to the same cell-expressed counter structure ligand. The functional activity of the

variant Fc-fusion molecules to modulate the activity of T cells also is shown based on the calculated levels of IFN-gamma in culture supernatants (pg/ml) generated either i) with the indicated variant ECD-Fc fusion molecule coimmobilized with anti-CD3 or ii) with the indicated variant ECD-Fc fusion molecule in an MLR assay. The Table also depicts the ratio of IFN-gamma produced by each variant ECD-Fc compared to the corresponding unmodified (parental) ECD-Fc in both functional assays.

**[0497]** The results show altered, including increased, binding affinity of affinity-modified ICOSL IgSF domain variants for at least one cognate counter structure ligand and/or improved immunological activity. Specifically, similar to the initial hits identified in Example 6, the selections resulted in the identification of a number of additional ICOSL IgSF domain variants that were affinity-modified to exhibit increased binding for at least one, and in some cases more than one, cognate counter structure ligand. In addition, the results showed that affinity modification of the variant molecules also exhibited improved activities to both increase and/or decrease immunological activity depending on the format of the molecule as described in Example 6.

| <b>Table 15: ICOSL variants: binding data and costimulatory bioactivity data.</b> |                        |                                       |                                       |                                         |                                                                         |                                             |
|-----------------------------------------------------------------------------------|------------------------|---------------------------------------|---------------------------------------|-----------------------------------------|-------------------------------------------------------------------------|---------------------------------------------|
| <b>ICOSL mutation(s)</b>                                                          | <b>SEQ ID NO (ECD)</b> | <b>ICOS tfxn MFI (parental ratio)</b> | <b>CD28 tfxn MFI (parental ratio)</b> | <b>CTLA-4 tfxn MFI (parental ratio)</b> | <b>Anti-CD3 IFN-gamma Coimmobilization Assay pg/ml (parental ratio)</b> | <b>MLR IFN-gamma pg/ml (parental ratio)</b> |
| N52H, F78L, Q100R, C198R                                                          | 373                    | 9568 (0.12)                           | 1966 (0.24)                           | 1454 (0.12)                             | 130 (0.31)                                                              | 5927 (1.84)                                 |
| N52H, N57Y, Q100R, V110D, C198R, S212G                                            | 364                    | 9418 (1.16)                           | 136665 (16.55)                        | 115352 (9.59)                           | 944 (2.21)                                                              | 821 (0.25)                                  |
| N52H, N57Y, R75Q, Q100P, V110D                                                    | 374                    | 5558 (0.07)                           | 7465 (0.90)                           | 4689 (0.39)                             | 122 (0.28)                                                              | 1136 (0.35)                                 |
| N52H, N57Y, Q100R, C198R                                                          | 365                    | 9148 (1.13)                           | 134923 (16.33)                        | 83241 (6.92)                            | 1060 (2.48)                                                             | 375 (0.12)                                  |
| N52H, N57Y, L74Q, V110D, S192G                                                    | 375                    | 9448 (1.17)                           | 128342 (15.54)                        | 123510 (10.26)                          | 1137 (2.66)                                                             | 889 (0.28)                                  |
| N52H, Q100R                                                                       | 285                    | 9478 (1.17)                           | 151977 (18.40)                        | 133929 (11.13)                          | 972 (2.28)                                                              | 794 (0.25)                                  |
| N52H, S121G, C198R                                                                | 376                    | 9128 (1.13)                           | 124732 (15.10)                        | 182607 (15.18)                          | 827 (1.94)                                                              | 1257 (0.39)                                 |
| A20V, N52H, N57Y, Q100R, S109G                                                    | 287                    | 5828 (0.72)                           | 76973 (9.32)                          | 73640 (6.12)                            | 447 (1.05)                                                              | 2283 (0.71)                                 |
| N52H, N57Y, Q100P, C198R                                                          | 461                    | 9548 (1.18)                           | 130676 (15.82)                        | 81966 (6.81)                            | 1125 (2.64)                                                             | 643 (0.20)                                  |

| <b>Table 15: ICOSL variants: binding data and costimulatory bioactivity data.</b> |                        |                                       |                                       |                                         |                                                                         |                                             |
|-----------------------------------------------------------------------------------|------------------------|---------------------------------------|---------------------------------------|-----------------------------------------|-------------------------------------------------------------------------|---------------------------------------------|
| <b>ICOSL mutation(s)</b>                                                          | <b>SEQ ID NO (ECD)</b> | <b>ICOS tfxn MFI (parental ratio)</b> | <b>CD28 tfxn MFI (parental ratio)</b> | <b>CTLA-4 tfxn MFI (parental ratio)</b> | <b>Anti-CD3 IFN-gamma Coimmobilization Assay pg/ml (parental ratio)</b> | <b>MLR IFN-gamma pg/ml (parental ratio)</b> |
| N52H, N57Y, R61S, Q100R, V110D, L173S                                             | 289                    | 1018 (0.13)                           | 9129 (1.11)                           | 5790 (0.48)                             | 109 (0.25)                                                              | 5094 (1.58)                                 |
| N52H, N57Y, Q100R, V122A                                                          | 290                    | 9978 (1.23)                           | 137372 (16.63)                        | 70764 (5.88)                            | 1316 (3.08)                                                             | 473 (0.15)                                  |
| N52H, N57Y, Q100R, F172S                                                          | 291                    | 1028 (1.27)                           | 135821 (16.44)                        | 73320 (6.09)                            | 1561 (3.66)                                                             | 486 (0.15)                                  |
| N52H, N57Y, Q100R                                                                 | 283                    | 9858 (1.22)                           | 140612 (17.02)                        | 75106 (6.24)                            | 1648 (3.86)                                                             | 778 (0.24)                                  |
| N52S, F120S, N227K                                                                | 377                    | 9438 (1.17)                           | 67796 (8.21)                          | 82370 (6.85)                            | 1157 (2.71)                                                             | 1626 (0.50)                                 |
| N52S, N194D                                                                       | 366                    | 9798 (1.21)                           | 59431 (7.19)                          | 74502 (6.19)                            | 1671 (3.91)                                                             | 1690 (0.52)                                 |
| N52S, V97A                                                                        | 294                    | 3138 (0.04)                           | 1733 (0.21)                           | 1541 (0.13)                             | 84 (0.20)                                                               | 3858 (1.20)                                 |
| N52S, F120S                                                                       | 293                    | 9068 (1.12)                           | 67233 (8.14)                          | 97880 (8.13)                            | 1178 (2.76)                                                             | 2814 (0.87)                                 |
| N52S, G72R                                                                        | 295                    | 9288 (1.15)                           | 51638 (6.25)                          | 62339 (5.18)                            | 1161 (2.72)                                                             | 2947 (0.91)                                 |
| N52S, A71T, A117T, T190A, C198R                                                   | 378                    | 8918 (1.10)                           | 44044 (5.33)                          | 56646 (4.71)                            | 1076 (2.52)                                                             | 4031 (1.25)                                 |
| N52S, E220G                                                                       | 297                    | 3878 (0.05)                           | 2047 (0.25)                           | 1796 (0.15)                             | 122 (0.29)                                                              | 1927 (0.60)                                 |
| Y47H, N52S, V107A, F120S                                                          | 298                    | 3268 (0.04)                           | 2562 (0.31)                           | 2104 (0.17)                             | 334 (0.78)                                                              | 4390 (1.36)                                 |
| WT ICOSL                                                                          | 32                     | 8088 (1.00)                           | 8260 (1.00)                           | 12033 (1.00)                            | 427 (1.00)                                                              | 3226 (1.00)                                 |
| T43A, N52H, N57Y, L74Q, D89G, V110D, F172S                                        | 379                    | 2821 (0.02)                           | 2180 (0.49)                           | 2051 (0.12)                             | 184 (0.75)                                                              |                                             |
| N52H, N57Y, Q100R, V107I, V110D, S132F, I154F, C198R, R221G                       | 381                    | 174586 (0.97)                         | 122383 (27.24)                        | 76202 (4.31)                            | 985 (4.01)                                                              | 1037 (0.36)                                 |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R                        | 300                    | 190765 (1.05)                         | 129070 (28.73)                        | 68488 (3.87)                            | 4288 (17.46)                                                            | 1225 (0.43)                                 |
| Q37R, N52H, N57Y, Q100R, V110N, S142F, C198R, D217V, R221G                        | 301                    | 148638 (0.82)                         | 91104 (20.28)                         | 13498 (0.76)                            | 62 (0.25)                                                               | 7643 (2.68)                                 |
| N52H, N57Y, Q100R, V110D, C198R                                                   | 302                    | 179194 (0.99)                         | 123312 (27.45)                        | 84136 (4.76)                            | 762 (3.10)                                                              | 1342 (0.47)                                 |
| N52H, N57Y, Q100R, V110D, V116A, L161M, F172S, S192G, C198R                       | 303                    | 5236 (0.03)                           | 4160 (0.93)                           | 3305 (0.19)                             | 49 (0.20)                                                               | 2039 (0.72)                                 |
| F27S, N52H, N57Y, V110N                                                           | 304                    | 20154 (0.11)                          | 8613 (1.92)                           | 3903 (0.22)                             | 83 (0.34)                                                               | 7522 (2.64)                                 |
| F27S, N52H, N57Y, V110N                                                           | 304                    | 5236 (0.03)                           | 4160 (0.93)                           | 2957 (0.17)                             | 40 (0.16)                                                               | -                                           |

| <b>Table 15: ICOSL variants: binding data and costimulatory bioactivity data.</b> |                        |                                       |                                       |                                         |                                                                         |                                             |
|-----------------------------------------------------------------------------------|------------------------|---------------------------------------|---------------------------------------|-----------------------------------------|-------------------------------------------------------------------------|---------------------------------------------|
| <b>ICOSL mutation(s)</b>                                                          | <b>SEQ ID NO (ECD)</b> | <b>ICOS tfxn MFI (parental ratio)</b> | <b>CD28 tfxn MFI (parental ratio)</b> | <b>CTLA-4 tfxn MFI (parental ratio)</b> | <b>Anti-CD3 IFN-gamma Coimmobilization Assay pg/ml (parental ratio)</b> | <b>MLR IFN-gamma pg/ml (parental ratio)</b> |
| N52S, H94E, L96I, S109N, L166Q,                                                   | 305                    | 198604 (1.10)                         | 100361 (22.34)                        | 102892 (5.82)                           | 1253 (5.10)                                                             | 5645 (1.98)                                 |
| S18R, N52S, F93L, I143V, R221G                                                    | 306                    | 154561 (0.85)                         | 7625 (1.70)                           | 4254 (0.24)                             | 203 (0.83)                                                              | 5239 (1.84)                                 |
| A20T, N52D, Y146C, Q164L                                                          | 307                    | 149661 (0.83)                         | 9073 (2.02)                           | 6901 (0.39)                             | 287 (1.17)                                                              | 4829 (1.69)                                 |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T                     | 308                    | 180016 (1.00)                         | 120230 (26.76)                        | 62809 (3.55)                            | 2218 (9.03)                                                             | 7283 (2.56)                                 |
| N52S, H94E, L96I, V122M                                                           | 309                    | 198717 (1.10)                         | 88901 (19.79)                         | 94231 (5.33)                            | 590 (2.40)                                                              | 618 (0.22)                                  |
| N52H, N57Y, H94E, L96I, F120I, S126T, W153R, I218N                                | 310                    | 87711 (0.48)                          | 42035 (9.36)                          | 31798 (1.80)                            | 67 (0.27)                                                               | 2500 (0.88)                                 |
| M10V, S18R, N30D, N52S, S126R, T139S, L203F                                       | 311                    | 180665 (1.00)                         | 64929 (14.45)                         | 48362 (2.73)                            | 1193 (4.86)                                                             | 13647 (4.79)                                |
| S25G, N30D, N52S, F120S, N227K                                                    | 312                    | 178834 (0.99)                         | 66127 (14.72)                         | 46631 (2.64)                            | 1246 (5.07)                                                             | 2202 (0.77)                                 |
| N30D, N52S, L67P, Q100K, D217G, R221K, T225S                                      | 313                    | 18630 (0.10)                          | 1986 (0.44)                           | 1940 (0.11)                             | 54 (0.22)                                                               | 2752 (0.97)                                 |
| WT ICOSL                                                                          | 32                     | 180900 (1.00)                         | 4493 (1.00)                           | 17685 (1.00)                            | 246 (1.00)                                                              | 2850 (1.00)                                 |
| N52H, N57Y, Q100R, V110D, A117T, T190S, C198R                                     | 314                    | 2831 (0.04)                           | 2881 (0.57)                           | 2464 (0.23)                             | 59 (0.08)                                                               | -                                           |
| N52H, N57Y, Q100R, V110D, F172S, C198R                                            | 315                    | 58478 (0.79)                          | 74031 (14.75)                         | 56850 (5.33)                            | 712 (0.96)                                                              | 1093 (0.23)                                 |
| S25G, F27C, N52H, N57Y, Q100R, V110D, E135K, L173S, C198R                         | 316                    | 22514 (0.30)                          | 21320 (4.25)                          | 20450 (1.92)                            | 353 (0.48)                                                              | 5765 (1.21)                                 |
| N52H, N57Y, V110A, C198R, R221I                                                   | 317                    | 84236 (1.14)                          | 81842 (16.31)                         | 121519 (11.39)                          | 4593 (6.18)                                                             | 1137 (0.24)                                 |
| M10I, S13G, N52H, N57Y, D77G, V110A, H129P, I143V, F172S, V193M, C198R            | 318                    | 6362 (0.09)                           | 6001 (1.20)                           | 4834 (0.45)                             | 141 (0.19)                                                              | 4326 (0.91)                                 |
| N52H, N57Y, R61C, Y62F, Q100R, V110N, F120S, C198R                                | 319                    | 4355 (0.06)                           | 4316 (0.86)                           | 3430 (0.32)                             | 110 (0.15)                                                              | 6854 (1.44)                                 |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                                     | 367                    | 96736 (1.31)                          | 77881 (15.52)                         | 148012 (13.88)                          | 8765 (11.79)                                                            | 630 (0.13)                                  |
| N52H, N57Y, Q100R, V110D, N144D, F172S, C198R                                     | 321                    | 67578 (0.91)                          | 64953 (12.94)                         | 95731 (8.98)                            | 1672 (2.52)                                                             | 1490 (0.31)                                 |
| N52S, H94E, L98F, Q100R,                                                          | 322                    | 80690 (1.09)                          | 78750                                 | 148160                                  | 3564 (4.80)                                                             | 1497 (0.32)                                 |

| Table 15: ICOSL variants: binding data and costimulatory bioactivity data. |                 |                                |                                |                                  |                                                                  |                                      |
|----------------------------------------------------------------------------|-----------------|--------------------------------|--------------------------------|----------------------------------|------------------------------------------------------------------|--------------------------------------|
| ICOSL mutation(s)                                                          | SEQ ID NO (ECD) | ICOS tfxn MFI (parental ratio) | CD28 tfxn MFI (parental ratio) | CTLA-4 tfxn MFI (parental ratio) | Anti-CD3 IFN-gamma Coimmobilization Assay pg/ml (parental ratio) | MLR IFN-gamma pg/ml (parental ratio) |
|                                                                            |                 |                                | (15.69)                        | (13.89)                          |                                                                  |                                      |
| N52S, E90A                                                                 | 323             | 108908 (1.47)                  | 31086 (6.19)                   | 108866 (10.21)                   | 4564 (6.14)                                                      | 3927 (0.83)                          |
| N30D, K42E, N52S                                                           | 324             | 85726 (1.16)                   | 4293 (0.86)                    | 10755 (1.01)                     | 5211 (7.01)                                                      | 5656 (1.19)                          |
| N52S, F120S, I143V, I224V                                                  | 325             | 90862 (1.23)                   | 28443 (5.67)                   | 105229 (9.87)                    | 4803 (6.46)                                                      | 4357 (0.92)                          |
| WT ICOSL                                                                   | 32              | 73964 (1.00)                   | 5018 (1.00)                    | 10665 (1.00)                     | 743 (1.00)                                                       | 4748 (1.00)                          |

## B. Cytokine Production in Anti-CD3 Costimulation Assays

**[0498]** Exemplary variant ECD ICOSL Fc-fusion molecules described above were further assessed for stimulation of cytokines IL-17 in the anti-CD3 costimulatory (coimmobilization) bioactivity assay described above. A mixture of 10 nM plate-bound anti-CD3 and 40nM ICOSL Fc variant proteins were cultured with human T cells. Supernatants were collected and IL-17 levels were determined by ELISA. The amount of IL-17 in culture supernatants (pg/ml) generated with the indicated variant ECD-Fc fusion molecule and corresponding unmodified (parental) ECD-Fc coimmobilized with anti-CD3 was measured. For comparison, also shown in this Table are the results for production of IFN-gamma in the same assay as depicted in Table 15 for the exemplary variants.

**[0499]** Results are shown in Table 16, which depict the pg/mL of IL-17 measured in the supernatant as well as the ratio (fold increase) of IL-17 produced by each variant ECD-Fc compared to the corresponding unmodified (wild-type) ECD-Fc. Similar results are shown for IFN-gamma. Also shown is the % of total IL-17 or IFN-gamma cytokine produced by cells. The results showed that affinity modification of the variant molecules exhibited altered functional T cell activity to increase IL-17 in addition to IFN-gamma in the costimulation assay.

| <b>Table 16: Costimulatory Bioreactivity Data for ICOSL IgSF Domain Variants</b> |                 |                |                 |               |                |                |                              |         |                       |
|----------------------------------------------------------------------------------|-----------------|----------------|-----------------|---------------|----------------|----------------|------------------------------|---------|-----------------------|
| ICOSL mutation(s)                                                                | SEQ ID NO (ECD) | IL-17A [pg/mL] | IL-17A Fold ↑WT | IFN-g [pg/mL] | IFN-g Fold ↑WT | Total Fold ↑WT | % of Total Cytokine Produced |         | % Total IL-17 + IFN-g |
|                                                                                  |                 |                |                 |               |                |                | % IL-17                      | % IFN-g |                       |
| N52H, N57Y, Q100R, C198R                                                         | 365             | 617            | 7.93            | 1060          | 2.48           | 10.42          | 5.51                         | 0.77    | 6.28                  |
| N52H, N57Y, Q100R, V122A                                                         | 290             | 647            | 8.33            | 1316          | 3.08           | 11.41          | 5.79                         | 0.96    | 6.75                  |
| N52H, N57Y, Q100R, F172S                                                         | 291             | 549            | 7.06            | 1561          | 3.66           | 10.72          | 4.91                         | 1.14    | 6.05                  |
| N52Y, N57Y, F138L, L203P                                                         | 112             | 90             | 1.05            | 1999          | 2.69           | 3.74           | 0.81                         | 2.91    | 3.72                  |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T                    | 308             | 319            | 3.16            | 2218          | 9.03           | 12.19          | 2.85                         | 3.23    | 6.08                  |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                                    | 367             | 510            | 5.90            | 8765          | 11.79          | 17.70          | 4.56                         | 12.78   | 17.33                 |
| N52H, N57Y, Q100R                                                                | 283             | 473            | 6.08            | 1648          | 3.86           | 9.94           | 4.23                         | 1.20    | 5.43                  |
| N52H, Q100R                                                                      | 285             | 358            | 4.60            | 972           | 7.01           | 11.62          | 3.20                         | 0.71    | 3.91                  |
| N52H, N57Y, Q100R, V110D, C198R, S212G                                           | 364             | 124            | 1.60            | 944           | 2.21           | 3.81           | 1.11                         | 0.69    | 1.80                  |
| N52H, N57Y, Q100P                                                                | 113             | 127            | 1.47            | 4922          | 6.62           | 8.09           | 1.14                         | 7.17    | 8.31                  |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R                       | 300             | 22             | 7.11            | 130           | 17.46          | 24.57          | 6.41                         | 6.25    | 12.66                 |
| N30D, K42E, N52S                                                                 | 324             | 349            | 4.04            | 5211          | 7.01           | 11.05          | 3.12                         | 7.60    | 10.71                 |
| N52S, F120S, I143V, I224V                                                        | 325             | 292            | 3.39            | 4803          | 6.46           | 9.85           | 2.61                         | 7.00    | 9.62                  |
| N52S, E90A                                                                       | 323             | 306            | 3.54            | 4564          | 6.14           | 9.68           | 2.73                         | 6.65    | 9.39                  |
| N52H, N57Y, V110A, C198R, R221I                                                  | 317             | 290            | 3.35            | 4593          | 6.18           | 9.53           | 2.59                         | 6.69    | 9.28                  |
| N52S, N194D                                                                      | 366             | 428            | 5.50            | 1671          | 3.90           | 9.4            | 1.52                         | 5.19    | 5.40                  |
| N52H, I143T                                                                      | 135             | 84             | -               | 1727          | -              | 3.30           | 0.75                         | 2.52    | 3.27                  |
| N52D                                                                             | 111             | 126            | -               | 1447          | -              | 3.41           | 1.13                         | 2.11    | 3.23                  |

## EXAMPLE 11

### Generation of Additional Engineered T cell Expressing a Transmembrane Immunomodulatory Protein and Assessment of Proliferation

**[0500]** This Example describes the generation of additional engineered T cells in which a transmembrane immunomodulatory protein (TIP) containing an extracellular domain (ECD) containing ICOSL affinity-modified IgSF domain was co-expressed with a chimeric antigen receptor (CAR). Specifically, the TIP was generated to include the ECD of exemplary variant ICOSL containing amino acid mutations N52D, N52H/N57Y/Q100P, E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R or N52H/N57Y/Q100R with reference to positions in the ICOSL extracellular domain set forth in SEQ ID NO:32. The TIP also contained a transmembrane domain and a cytoplasmic domain of the corresponding wild-type ICOSL transmembrane protein sequence corresponding to residues 257-302 of SEQ ID NO:5. The sequence of the TIP with and without its signal peptide are as follows: N52D (SEQ ID NO: 496 and 497); N52H/N57Y/Q100P (SEQ ID NO: 498 and 499); E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R (SEQ ID NO: 500 and 501) and N52H/N57Y/Q100R (SEQ ID NO: 502 and 503). For comparison, the full-length transmembrane wild-type ICOSL (amino acid residues 19-302 of SEQ ID NO:5) also was expressed in cells. The sequence of the wildtype TIP with and without its signal peptide is set forth in SEQ ID NO:494 and 495. The nucleic acid encoding the TIP also included a sequence encoding a green fluorescent protein (GFP) separated from the TIP by a self-cleaving T2A sequence.

**[0501]** The TIP containing the affinity-modified domain or the wild-type transmembrane protein containing a corresponding non-affinity modified IgSF domain were co-expressed in T cells with a chimeric antigen receptor (CAR). The nucleotide sequence encoding the CAR encodes, in order: a CD8 signal sequence (SEQ ID NO:481), an anti-CD19 scFv (SEQ ID NO:482), a hinge/transmembrane region derived from CD8 (SEQ ID NO:483), a costimulatory signaling domain derived from 4-1BB (SEQ ID NO:484), and a CD3zeta signaling domain (SEQ ID NO:247). The resulting anti-CD19 CAR has the sequence of amino acids set forth in SEQ ID NO:490. The nucleic acid encoding the CAR also included a sequence encoding a blue

fluorescent protein (BFP; SEQ ID NO:489) separated from the CAR by a self-cleaving T2A sequence (set forth in SEQ ID NO:488).

**[0502]** Viral vector constructs were separately generated into which was cloned either the nucleic acid molecule encoding the CAR alone or a nucleic acid molecule encoding one of the exemplary TIPs or wild-type ICOSL. The viral vector encoding the CAR and the viral vector encoding the TIP or wild-type ICOSL were co-transduced into T cells. For transduction, primary T cells were activated with anti-CD3 and anti-CD28 beads (Dyna) at 1:1 bead:cell ratio and incubated in the presence of 100IU/mL of IL-2 at 37°C for 2 days. T cells were then harvested and transduced with 400µL of CAR viral supernatant and 400µL of TIP viral supernatant in the presence of 8µg/mL of polybrene. The cells were spinoculated at 1000g for 30 minutes at 30°C. The cells were then transferred and incubated overnight at 37°C. After the incubation, cells were collected and viral supernatant was removed. The cells were resuspended with complete media and 50IU/mL of IL-2. Cells were expanded, replenished with IL-2 and media every two days for a total of 6 days. Beads were removed from the cells using a magnet and counted before being assessed in a proliferation assay. An exemplary expression profile of a TIP and CAR in an exemplary transduced T cells is shown in FIG. 2A.

**[0503]** To assess proliferation of CAR T cells and CAR-TIP T cells in response to antigen, cells were labeled with cell trace far red dye. CD19-expressing Nalm6 target cells were titrated starting from 1.5:1 target:T cell ratio and by 1:2 dilutions with 8-point dilution. Labeled CAR T cells or CAR-TIP T cells were added to Nalm6 cells and the culture was incubated for 4 days before cells were analyzed by flow cytometry. The supernatant was collected and further assessed in a cytokine release assay.

**[0504]** As shown in FIG. 2B, CAR<sup>+</sup> primary T cells proliferate in a dose dependent manner to CD19<sup>+</sup> NALM6 cells. Compared to a CAR only T cells, T cells coexpressing the CAR and either wild-type ICOSL or one of the exemplary ICOSL TIP exhibited enhanced proliferation compared to CAR only expressing T cells. Co-expression of a CAR and a TIP containing either the N52H/N57Y/Q100P, E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R or N52H/N57Y/Q100R variant ICOSLECD exhibited greater proliferation than T cells co-expressing the CAR and wildtype ICOSL, indicating that TIPs expressed on primary T cells provide an improved costimulatory signal to enhance T cell proliferation.

## EXAMPLE 12

### Purification and Assessment of Purified of ICOSL IgSF Domain Variants

**[0505]** A purification strategy was employed for exemplary candidate hits described in Example 6 and 10. Human cells derived from the 293 cell line (Expi293) were transiently transfected with expression construct and the ECD ICOSL Fc fusion molecule was expressed in the cells. The Fc fusion proteins were then purified from supernatants with Protein A by affinity chromatography (MabSelect SuRe). This initial purification step was then followed by a preparative size exclusion chromatography (SEC) step to further purify the proteins (Superdex200 16x60). Samples from both purification steps were retained and compared by analytic SEC. The concentration of the protein was determined after Protein A purification. The resulting purified proteins also were analyzed by analytic SEC on a high performance liquid chromatography (HPLC) to assess purity.

**[0506]** The percent main peak in the purified samples was determined and compared to protein purified in the initial Protein A step (% Main Peak Prot A pool) versus protein purified with Protein A followed by preparative SEC (% Main Peak SEC pool T=D0). As shown in Table 17, the additional SEC step substantially increased protein purity of purified proteins. To further assess stability of the proteins, proteins purified by preparative SEC were left at room temperature for 24 hours and then % Main Peak by HPLC (% Main Peak SEC pool T=D24) was assessed and compared to D0 sample. The change in % Main Peak at D0 versus D24 was determined (▲% Main Peak SEC pool). As shown in Table 17, most of the tested exemplary variant ECD ICOSL Fc fusion molecules exhibited little change in % Main Peak at this time, indicating minimal aggregation of the protein variants had occurred.

| <b>Table 17: Purification of ICOSL Protein Variants</b> |                        |                                  |                                |                                  |                                   |                              |
|---------------------------------------------------------|------------------------|----------------------------------|--------------------------------|----------------------------------|-----------------------------------|------------------------------|
| <b>ICOSL mutation(s)</b>                                | <b>SEQ ID NO (ECD)</b> | <b>Expi293 Prod. Prot A mg/L</b> | <b>% Main Peak Prot A pool</b> | <b>% Main Peak SEC pool T=D0</b> | <b>% Main Peak SEC pool T=D24</b> | <b>▲% Main Peak SEC pool</b> |
| N52S, N194D                                             | 366                    | 120                              | 87.9                           | 93.5                             | 92                                | 1.5                          |
| N52H, N57Y, Q100R, F172S                                | 291                    | 217                              | 86.9                           | 97.4                             | 95.6                              | 1.8                          |
| N52S, E90A                                              | 323                    | 128                              | 86.5                           | 89.5                             | 88.3                              | 1.2                          |
| N52H, Q100R                                             | 285                    | 176                              | 85.9                           | 97.5                             | 96.1                              | 1.4                          |
| N52H, N57Y, Q100R                                       | 283                    | 186                              | 85.1                           | 97.6                             | 95.7                              | 1.9                          |

| <b>Table 17: Purification of ICOSL Protein Variants</b>       |                        |                                  |                                |                                  |                                   |                               |
|---------------------------------------------------------------|------------------------|----------------------------------|--------------------------------|----------------------------------|-----------------------------------|-------------------------------|
| <b>ICOSL mutation(s)</b>                                      | <b>SEQ ID NO (ECD)</b> | <b>Expi293 Prod. Prot A mg/L</b> | <b>% Main Peak Prot A pool</b> | <b>% Main Peak SEC pool T=D0</b> | <b>% Main Peak SEC pool T=D24</b> | <b>▲ % Main Peak SEC pool</b> |
| N52S, F120S, I143V, I224V                                     | 325                    | 87                               | 83.2                           | 88.9                             | 88.3                              | 0.6                           |
| N52H, N57Y, Q100R, C198R                                      | 365                    | 204                              | 82.9                           | 95.8                             | 92.3                              | 3.5                           |
| N52H, N57Y, Q100P                                             | 113                    | 63                               | 80.5                           | 94.5                             | 88.5                              | 6                             |
| N30D, K42E, N52S                                              | 324                    | 81                               | 80                             | 95.4                             | 91.3                              | 4.1                           |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                 | 367                    | 141                              | 78.9                           | 96                               | 92.9                              | 3.1                           |
| N52H, N57Y, Q100R, V122A                                      | 290                    | 260                              | 77.6                           | 96.4                             | 95.2                              | 1.2                           |
| N52Y/N57Y/F138L/L203P                                         | 112                    | 40                               | 75.6                           | 96.8                             | 94.8                              | 2                             |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R    | 300                    | 60                               | 73.8                           | 97.1                             | 95.8                              | 1.3                           |
| N52H, N57Y, V110A, C198R, R221I                               | 317                    | 95                               | 65.4                           | 90.9                             | 86                                | 4.9                           |
| N52H, N57Y, Q100R, V110D, C198R, S212G                        | 364                    | 73                               | 50.6                           | 87.9                             | 78.6                              | 9.3                           |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T | 308                    | 58                               | -                              | -                                | -                                 | -                             |
| N52H, I143T                                                   | 135                    | 134                              | 93.2                           | 96                               | 92.7                              | 3.3                           |
| N52D                                                          | 111                    | 136                              | 90.4                           | 95.5                             | 93.3                              | 2.2                           |

### EXAMPLE 13

#### Assessment of Costimulatory Bioactivity of Purified ICOSL IgSF Domain Variant Hits

**[0507]** Exemplary ECD ICOSL Fc fusion molecules purified as described in Example 12 were assessed for bioactivity by MLR substantially as described in Example 6. A mixture of 10 nM or 40nM ICOSL Fc variant proteins was bound overnight to 96-well plates in the presence of 10nM anti-CD3. The plates were washed and 100,000 CFSE labelled pan T cells were added for 96 hours. Supernatants were collected, and IFN-gamma and IL-17 levels were measured by ELISA.

**[0508]** Results for the cytokine secretion induced by anti-CD3 costimulation with exemplary tested variants (10nM and 40nM ICOSL Fc) are shown in FIG. 3A and 3B, which indicates exemplary IgSF domain amino acid substitutions (replacements) in the ECD of ICOSL. The bar graphs in FIG. 3A and 3B depict the amount of secreted IFN-gamma and IL-17, respectively, by

ELISA in the supernatants (pg/mL). The level of cytokine release induced by anti-CD3 costimulation with the tested variants compared to the level induced by anti-CD3 costimulation with WT ICOSL is indicated by the horizontal line. The results showed that affinity modification of the variant molecules exhibited activity to modulate functional T cell activity, including to substantially increase IFN-gamma and IL-17 secretion in the costimulation assay. Increased immunological activity was observed with some variants.

#### **EXAMPLE 14**

##### **Assessment of Proliferation of Purified ICOSL IgSF Domain Variant Hits**

**[0509]** Exemplary variant ECD ICOSL Fc fusion molecules purified as described in Example 12 were assessed for ability to costimulate anti-CD3-induced proliferation of T cells. Primary T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE). A mixture of 10 nM or 40nM variant ECD ICOSL Fc or wild-type ECD ICOSL proteins were bound overnight to 96-well plates in the presence of 10nM anti-CD3, and then labeled T cells were added and incubated for 3 days. As a control, proliferation also was assessed in the presence of bound anti-CD3 and IgG or IgG alone. Cells were stained for CD4 or CD8 surface markers and proliferation of total T cells, CD4+ T cells or CD8+ T cells was determined by assessing CFSE dilution by flow cytometry.

**[0510]** The results are set forth in FIG. 4A and FIG. 4B for exemplary variants tested at 40nM and 10nM ICOSL, respectively. As shown in FIG. 4A, nearly all tested variant ECD ICOSL Fc fusion molecules induced proliferation greater than WT control. As shown in FIG. 4B, differences in proliferation were more apparent at 10 nM with certain variants providing maximal proliferation even at this lower concentration.

#### **EXAMPLE 15**

##### **Assessment of Binding and Activity of Purified ICOSL IgSF Domain Variant Hits**

**[0511]** Exemplary variant ECD ICOSL Fc fusion molecules purified as described in Example 12 were assessed for binding and functional activities using methods substantially as described in Example 6 or Example 10.

### **A. Flow Cytometric Binding Assays**

**[0512]** Human cells derived from the 293 cell line (Expi293) were transfected with CD28, CTLA-4, ICOS or mock transfected. Cells were then incubated with ECD ICOSL Fc fusion molecules or wild-type ECD ICOSL-Fc that were titrated from 100,000 pM to 46 pM, and binding was observed using a PE-conjugated anti-human Fc as described in Example 6. Binding was assessed by flow cytometry and mean fluorescence intensity (MFI) and percent (%) of cells positive for signal was determined using Cell Quest Pro software (Becton Dickinson, USA). The concentration of ICOSL-Fc that gave a half-maximal MFI response (MFI EC50) or % positive cells (% (+) EC50) was determined.

**[0513]** Table 18 sets forth the results. The ICOSL amino acid substitutions depicted in Table 18 are designated by amino acid position number corresponding to the respective reference unmodified ICOSL ECD sequence set forth in SEQ ID NO:32. For some values (e.g. WT binding to CD28) it was not possible to obtain an EC50, therefore 1000000 pM was arbitrarily picked for data formatting purposes. Similar to results obtained from previous binding assays as described in Example 10 above, altered binding affinity of variant ICOSL ECD-Fc fusion molecule for at least one cognate counter structure ligand was observed.

| Table 18: Flow Cytometric EC50s for ICOSL variants            |                 |                    |                      |                      |                        |                    |                      |
|---------------------------------------------------------------|-----------------|--------------------|----------------------|----------------------|------------------------|--------------------|----------------------|
| ICOSL mutation(s)                                             | SEQ ID NO (ECD) |                    |                      |                      |                        |                    |                      |
|                                                               |                 | CD28 MFI EC50 [pM] | CD28 % (+) EC50 [pM] | CTLA-4 MFI EC50 [pM] | CTLA-4 % (+) EC50 [pM] | ICOS MFI EC50 [pM] | ICOS % (+) EC50 [pM] |
| WT ICOSL                                                      | 32              | 1000000            | 1000000              | 1000000              | 1000000                | 10543              | 762                  |
| N52H, I143T                                                   | 135             | 19147              | 567                  | 20259                | 1891                   | 2666               | 286                  |
| N52H, N57Y, Q100R, C198R                                      | 365             | 950                | 159                  | 73548                | 422                    | 1032               | 179                  |
| N52H, N57Y, Q100R, V122A                                      | 290             | 29701              | 152                  | 1008                 | 293                    | 302                | 64                   |
| N52H, N57Y, Q100R, F172S                                      | 291             | 1006               | 231                  | 1332                 | 396                    | 779                | 130                  |
| N52Y/N57Y/F138L/L203P                                         | 112             | 7844               | 386                  | 7457                 | 994                    | 3104               | 408                  |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T | 308             | 5961               | 595                  | 6909                 | 1026                   | 5514               | 852                  |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                 | 367             | 1034               | 307                  | 23328                | 579                    | 3172               | 347                  |
| N52H, N57Y, Q100R                                             | 283             | 1665               | 238                  | 11002                | 533                    | 383                | 131                  |
| N52H, Q100R                                                   | 285             | 1305               | 274                  | 8593                 | 1997                   | 702                | 167                  |
| N52H, N57Y, Q100R, V110D, C198R, S212G                        | 364             | 4987               | 594                  | 30382                | 922                    | 50219              | 814                  |
| N52H, N57Y, Q100P                                             | 113             | 21137              | 402                  | 22651                | 758                    | 4090               | 320                  |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R    | 300             | 2508               | 387                  | 5399                 | 806                    | 2381               | 421                  |
| N30D, K42E, N52S                                              | 324             | -                  | 3683800              | 8593                 | 1997                   | 3251               | 558                  |
| N52S, F120S, I143V, I224V                                     | 325             | 902400             | 9060                 | 28126                | 2948                   | 4366               | 245                  |
| N52S, E90A                                                    | 323             | 1339700            | 31302                | 31419                | 5828                   | 5225               | 473                  |
| N52H, N57Y, V110A, C198R, R221I                               | 317             | 1809               | 426                  | 7201                 | 841                    | 1293               | 433                  |
| N52S, N194D                                                   | 366             | 944669             | 11876                | 1254880              | 5170                   | 473                | 206                  |
| N52D                                                          | 111             | 288617             | 17793                | 396841               | 3891                   | 2642               | 137                  |

## B. ForteBio Binding Assay

**[0514]** Protein-protein interactions between the receptors and ICOSL domain variant immunomodulatory proteins were further assessed using ForteBio binding assays. ICOS, CD28, and CTLA-4 receptors were loaded individually onto anti-human capture sensors (ForteBio Octet AHC) and wildtype unmodified ICOSL ECD-Fc fusion molecule, wildtype PD-L2 ED-Fc fusion molecule or variant ICOSL Fc-fusion molecules were bound to the receptors in 4 point titrations. Each titration was globally fit to calculate the association ( $k_{on}$ ) and dissociation ( $K_{dis}$ ) of each protein. Loading response of anti-human capture sensors of each receptor being tested with the

variant ICOSL ECD-Fc fusion molecule was determined. The dissociation constant (KD) was calculated and compared to wildtype to determine a fold improvement value (fold imp.).

**[0515]** Binding results to ICOS are set forth in Table 19, to CD28 are set forth in Table 20 and to CTLA-4 are set forth in Table 21. The exemplary amino acid substitutions depicted in Table 19-21 are designated by amino acid position number corresponding to the respective reference unmodified ICOSL ECD sequence set forth in SEQ ID NO:32.

| <b>Table 19: ICOS ForteBio Binding Assay</b>                  |                        |                 |               |                              |                              |                           |                  |
|---------------------------------------------------------------|------------------------|-----------------|---------------|------------------------------|------------------------------|---------------------------|------------------|
| <b>ICOSL mutation(s)</b>                                      | <b>SEQ ID NO (ECD)</b> | <b>Response</b> | <b>KD (M)</b> | <b>K<sub>on</sub> (1/Ms)</b> | <b>K<sub>dis</sub> (1/s)</b> | <b>Full R<sup>2</sup></b> | <b>Fold Imp.</b> |
| WT ICOSL                                                      | 32                     | 0.73            | 8.83E-10      | 1.78E+05                     | 1.58E-04                     | 0.9908                    | -                |
| N52H, I143T                                                   | 135                    | 0.87            | 3.32E-10      | 3.13E+05                     | 1.04E-04                     | 0.9683                    | 2.7              |
| N52H, N57Y, Q100R, C198R                                      | 365                    | 0.74            | 4.92E-10      | 3.85E+05                     | 1.89E-04                     | 0.9882                    | 1.8              |
| N52H, N57Y, Q100R, V122A                                      | 290                    | 0.67            | 4.72E-10      | 3.77E+05                     | 1.78E-04                     | 0.9775                    | 1.9              |
| N52H, N57Y, Q100R, F172S                                      | 291                    | 0.68            | 4.20E-10      | 4.34E+05                     | 1.82E-04                     | 0.9545                    | 2.1              |
| N52Y/N57Y/F138L/L203P                                         | 112                    | 0.64            | 7.69E-10      | 2.22E+05                     | 1.71E-04                     | 0.9782                    | 1.1              |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T | 308                    | 0.67            | 3.62E-10      | 3.55E+05                     | 1.29E-04                     | 0.9687                    | 2.4              |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                 | 367                    | 0.76            | 4.77E-10      | 3.29E+05                     | 1.57E-04                     | 0.9616                    | 1.9              |
| N52H, N57Y, Q100R                                             | 283                    | 0.74            | 3.69E-10      | 2.87E+05                     | 1.06E-04                     | 0.9817                    | 2.4              |
| N52H, Q100R                                                   | 285                    | 0.79            | 3.73E-10      | 4.45E+05                     | 1.66E-04                     | 0.968                     | 2.4              |
| N52H, N57Y, Q100R, V110D, C198R, S212G                        | 364                    | 0.60            | 1.29E-09      | 1.66E+05                     | 2.15E-04                     | 0.9846                    | 0.7              |
| N52H, N57Y, Q100P                                             | 113                    | 0.73            | 3.82E-10      | 3.71 E+05                    | 1.42E-04                     | 0.9729                    | 2.3              |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R    | 300                    | 0.75            | 5.43E-10      | 2.65E+05                     | 1.44E-04                     | 0.9848                    | 1.6              |
| N30D, K42E, N52S                                              | 324                    | 0.80            | 3.71E-10      | 4.48E+05                     | 1.66E-04                     | 0.9651                    | 2.4              |
| N52S, F120S, I143V, I224V                                     | 325                    | 0.80            | 3.11E-10      | 5.03E+05                     | 1.56E-04                     | 0.9673                    | 2.8              |
| N52S, E90A                                                    | 323                    | 0.88            | 3.40E-10      | 4.85E+05                     | 1.65E-04                     | 0.9792                    | 2.6              |
| N52H, N57Y, V110A, C198R, R221I                               | 317                    | 0.68            | 4.77E-10      | 3.15E+05                     | 1.50E-04                     | 0.976                     | 1.9              |
| N52S, N194D                                                   | 366                    | 0.88            | 3.37E-10      | 3.38E+05                     | 1.14E-04                     | 0.9723                    | 2.6              |
| N52D                                                          | 111                    | 0.87            | 3.38E-10      | 3.91E+ 05                    | 1.32E-04                     | 0.9792                    | 2.6              |
| Wildtype PD-L2 ED-Fc                                          | -                      | 0.03            |               |                              |                              |                           |                  |

| <b>Table 20: CD28 ForteBio Binding Assay</b>                  |                        |                 |               |                              |                              |                           |                  |
|---------------------------------------------------------------|------------------------|-----------------|---------------|------------------------------|------------------------------|---------------------------|------------------|
| <b>ICOSL mutation(s)</b>                                      | <b>SEQ ID NO (ECD)</b> | <b>Response</b> | <b>KD (M)</b> | <b>K<sub>on</sub> (1/Ms)</b> | <b>K<sub>dis</sub> (1/s)</b> | <b>Full R<sup>2</sup></b> | <b>Fold Imp.</b> |
| WT ICOSL                                                      | 32                     | 0.33            | 1.39E-08      | 6.69E+04                     | 9.29E-04                     | 0.9715                    | -                |
| N52H, I143T                                                   | 135                    | 0.95            | 5.25E-10      | 4.27E+05                     | 2.24E-04                     | 0.9877                    | 26.5             |
| N52H, N57Y, Q100R, C198R                                      | 365                    | 1.14            | 4.47E-10      | 4.12E+05                     | 1.84E-04                     | 0.9877                    | 31.0             |
| N52H, N57Y, Q100R, V122A                                      | 290                    | 1.04            | 3.90E-10      | 4.07E+05                     | 1.59E-04                     | 0.9878                    | 35.6             |
| N52H, N57Y, Q100R, F172S                                      | 291                    | 1.06            | 2.93E-10      | 4.26E+05                     | 1.25E-04                     | 0.9836                    | 47.3             |
| N52Y/N57Y/F138L/L203P                                         | 112                    | 0.86            | 7.83E-10      | 1.79E+05                     | 1.40E-04                     | 0.993                     | 17.7             |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T | 308                    | 0.92            | 5.53E-10      | 2.54E+05                     | 1.40E-04                     | 0.9906                    | 25.1             |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                 | 367                    | 1.10            | 3.66E-10      | 3.41 E+05                    | 1.25E-04                     | 0.986                     | 37.9             |
| N52H, N57Y, Q100R                                             | 283                    | 1.04            | 3.68E-10      | 3.72E+05                     | 1.37E-04                     | 0.983                     | 37.7             |
| N52H, Q100R                                                   | 285                    | 1.09            | 4.01E-10      | 5.0SE+05                     | 2.02E-04                     | 0.9938                    | 34.7             |
| N52H, N57Y, Q100R, V110D, C198R, S212G                        | 364                    | 0.94            | 8.96E-10      | 1.78E+05                     | 1.60E-04                     | 0.9961                    | 15.5             |
| N52H, N57Y, Q100P                                             | 113                    | 0.99            | 4.36E-10      | 3.29E+05                     | 1.43E-04                     | 0.9835                    | 31.8             |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R    | 300                    | 1.06            | 5.03E-10      | 3.06E+05                     | 1.54E-04                     | 0.9872                    | 27.6             |
| N30D, K42E, N52S                                              | 324                    | 0.54            | 1.95E-09      | 2.74E+05                     | 5.33E-04                     | 0.9772                    | 7.1              |
| N52S, F120S, I143V, I224V                                     | 325                    | 0.84            | 9.10E-10      | 4.51 E+05                    | 4.10E-04                     | 0.9742                    | 15.3             |
| N52S, E90A                                                    | 323                    | 0.94            | 9.69E-10      | 4.74E+05                     | 4.59E-04                     | 0.978                     | 14.3             |
| N52H, N57Y, V110A, C198R, R221I                               | 317                    | 0.94            | 5.63E-10      | 2.63E+05                     | 1.48E-04                     | 0.9781                    | 24.7             |
| N52S, N194D                                                   | 366                    | 0.82            | 1.04E-09      | 3.53E+05                     | 3.68E-04                     | 0.9887                    | 13.3             |
| N52D                                                          | 111                    | 0.86            | 1.16E-09      | 3.36E+05                     | 3.90E-04                     | 0.989                     | 11.9             |
| wildtype PD-L2 ED-Fc                                          | -                      | -0.04           |               |                              |                              |                           |                  |

| <b>Table 21: CTLA-4 ForteBio Binding Assay</b>                |                        |                 |               |                              |                              |                           |                  |
|---------------------------------------------------------------|------------------------|-----------------|---------------|------------------------------|------------------------------|---------------------------|------------------|
| <b>ICOSL mutation(s)</b>                                      | <b>SEQ ID NO (ECD)</b> | <b>Response</b> | <b>KD (M)</b> | <b>K<sub>on</sub> (1/Ms)</b> | <b>K<sub>dis</sub> (1/s)</b> | <b>Full R<sup>2</sup></b> | <b>Fold Imp.</b> |
| WT ICOSL                                                      | 32                     | 0.21            | 7.71E-08      | 1.92E+04                     | 1.48E-03                     | 0.8919                    | -                |
| N52H, I143T                                                   | 135                    | 0.96            | 6.78E-10      | 7.26E+05                     | 4.92E-04                     | 0.9641                    | 113.8            |
| N52H, N57Y, Q100R, C198R                                      | 365                    | 1.57            | 6.45E-10      | 4.79E+05                     | 3.09E-04                     | 0.9875                    | 119.6            |
| N52H, N57Y, Q100R, V122A                                      | 290                    | 1.43            | 5.76E-10      | 4.73E+05                     | 2.72E-04                     | 0.9926                    | 133.9            |
| N52H, N57Y, Q100R, F172S                                      | 291                    | 1.47            | 5.36E-10      | 5.13E+05                     | 2.75E-04                     | 0.9924                    | 144.0            |
| N52Y/N57Y/F138L/L203P                                         | 112                    | 1.33            | 8.33E-10      | 3.45E+05                     | 2.87E-04                     | 0.9943                    | 92.6             |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T | 308                    | 1.50            | 6.48E-10      | 3.12E+05                     | 2.02E-04                     | 0.9943                    | 119.0            |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                 | 367                    | 1.60            | 8.64E-10      | 4.79E+05                     | 4.14E-04                     | 0.9825                    | 89.2             |
| N52H, N57Y, Q100R                                             | 283                    | 1.65            | 7.19E-10      | 4.28E+05                     | 3.08E-04                     | 0.9895                    | 107.2            |
| N52H, Q100R                                                   | 285                    | 1.17            | 5.92E-10      | 8.37E+05                     | 4.96E-04                     | 0.9629                    | 130.3            |
| N52H, N57Y, Q100R, V110D, C198R, S212G                        | 364                    | 1.32            | 1.47E-09      | 2.34E+05                     | 3.44E-04                     | 0.9937                    | 52.6             |
| N52H, N57Y, Q100P                                             | 113                    | 1.51            | 6.47E-10      | 3.61 E+05                    | 2.33E-04                     | 0.9911                    | 119.2            |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R    | 300                    | 1.58            | 1.06E-09      | 4.24E+05                     | 4.49E-04                     | 0.9779                    | 72.8             |
| N30D, K42E, N52S                                              | 324                    | 0.42            | 2.81E-09      | 2.42E+05                     | 6.81E-04                     | 0.9676                    | 27.4             |
| N52S, F120S, I143V, I224V                                     | 325                    | 0.58            | 1.20E-09      | 3.10E+05                     | 3.72E-04                     | 0.9283                    | 64.3             |
| N52S, E90A                                                    | 323                    | 0.64            | 1.12E-09      | 3.28E+05                     | 3.68E-04                     | 0.9184                    | 68.7             |
| N52H, N57Y, V110A, C198R, R221I                               | 317                    | 1.44            | 1.07E-09      | 4.05E+05                     | 4.32E-04                     | 0.9811                    | 72.3             |
| N52S, N194D                                                   | 366                    | 0.59            | 2.52E-09      | 2.66E+05                     | 6.69E-04                     | 0.9643                    | 30.6             |
| N52D                                                          | 111                    | 0.62            | 1.52E-09      | 4.16E+05                     | 6.32E-04                     | 0.9234                    | 50.7             |
| wildtype PD-L2 ED-Fc                                          | -                      | 0.00            |               |                              |                              |                           |                  |

### C. Coimmobilization Assay

**[0516]** Costimulatory bioactivity of ICOSL fusion variants was determined in anti-CD3 coimmobilization assays substantially as described in Example 6. Approximately 0.37nM, 1.3nM or 10nM mouse anti-human CD3 (OKT3, Biolegends, USA) was diluted in PBS with 10nM or 40nM variant ICOSL ECD Fc vor wild-type ICOSL ECD-Fc. This mixture was added to tissue culture treated flat bottom 96 well plates overnight to facilitate adherence of the

stimulatory proteins to the wells of the plate. The next day, unbound protein was washed off the plates and 100,000 purified human pan T cells were added to each well. Cells were cultured 3 days before harvesting culture supernatants and measuring human IFN-gamma levels with an ELISA kit.

**[0517]** Table 22 sets forth the amount of IFN-gamma (pg/mL) produced by cells under the various conditions in the anti-CD3 coimmobilization assay. In the Table, the amino acid substitutions of exemplary variant ECD ICOSL-Fc fusions are designated by amino acid position number corresponding to the unmodified ICOSL ECD sequence set forth in SEQ ID NO: 32 and the corresponding SEQ ID NO identifier for the variant ECD for each variant ICOSL ECD-Fc fusion molecule is set forth. The ratio of IFN-gamma produced in the presence of each variant ICOSL ECD-Fc in the functional assay compared to in the presence of the corresponding unmodified (wildtype) ECD-Fc is shown (Fold ↑WT). As shown, costimulatory signaling of the variant ICOSL-ECD-Fc molecules was substantially greater compared to wildtype ICOSL.

| <b>Table 22: Assessment of IFN-gamma Responses in Co-stimulation Assay</b> |                 |                   |          |             |          |             |             |              |
|----------------------------------------------------------------------------|-----------------|-------------------|----------|-------------|----------|-------------|-------------|--------------|
| ICOSL mutation(s)                                                          | SEQ ID NO (ECD) | IFN-gamma [pg/mL] |          |             |          |             |             |              |
|                                                                            |                 | 40nM Ligand       |          | 10nM Ligand |          | 40nM Ligand |             |              |
|                                                                            |                 | 10mM OKT3         | Fold ↑WT | 10mM OKT3   | Fold ↑WT | 10mM OKT3   | 1.1 nM OKT3 | 0.37 nM OKT3 |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R                 | 300             | 14372             | 17.3     | 4903        | 29.9     | 8379.2      | 7422.8      | 2893.7       |
| N52H, N57Y, Q100R, V122A                                                   | 290             | 10640             | 12.8     | 6456        | 39.4     | 5636.2      | 4724.2      | 2246.3       |
| N52H, N57Y, Q100R, F172S                                                   | 291             | 10379             | 12.5     | 3741        | 22.8     | 3979.7      | 4067.7      | 1415.5       |
| N52H, N57Y, Q100R, C198R                                                   | 365             | 9590              | 11.5     | 4048        | 24.7     | 4215.8      | 2787.1      | 1072.4       |
| N52H, N57Y, Q100R                                                          | 283             | 9568              | 11.5     | 3270        | 19.9     | 4412.3      | 3862.0      | 1820.0       |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                              | 367             | 6939              | 8.4      | 3234        | 19.7     | 5495.2      | 4081.6      | 1442.8       |
| N52S, F120S, I143V, I224V                                                  | 325             | 6567              | 7.9      | 717         | 4.4      | 2145.4      | 2185.7      | 646.1        |
| N52S, N194D                                                                | 366             | 5690              | 6.8      | 272         | 1.7      | 2315.1      | 1485.0      | 1140.6       |

| <b>Table 22: Assessment of IFN-gamma Responses in Co-stimulation Assay</b> |                 |                   |          |             |          |             |             |              |
|----------------------------------------------------------------------------|-----------------|-------------------|----------|-------------|----------|-------------|-------------|--------------|
| ICOSL mutation(s)                                                          | SEQ ID NO (ECD) | IFN-gamma [pg/mL] |          |             |          |             |             |              |
|                                                                            |                 | 40nM Ligand       |          | 10nM Ligand |          | 40nM Ligand |             |              |
|                                                                            |                 | 10mM OKT3         | Fold ↑WT | 10mM OKT3   | Fold ↑WT | 10mM OKT3   | 1.1 nM OKT3 | 0.37 nM OKT3 |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T              | 308             | 5345              | 6.4      | 1152        | 7.0      | 2747.0      | 3383.4      | 1701.2       |
| N52S, E90A                                                                 | 323             | 5097              | 6.1      | 706         | 4.3      | 5019.8      | 3036.4      | 1482.4       |
| N52H, N57Y, V110A, C198R, R221I                                            | 317             | 4737              | 5.7      | 520         | 3.2      | 2501.5      | 1632.1      | 937.5        |
| N52H, Q100R                                                                | 285             | 4122              | 5.0      | 1466        | 8.9      | 5782.1      | 2861.4      | 967.5        |
| N30D, K42E, N52S                                                           | 324             | 4080              | 4.9      | 273         | 1.7      | 1336.8      | 1260.7      | 541.1        |
| N52H, N57Y, Q100P                                                          | 113             | 3344              | 4.0      | 229         | 1.4      | 2525.4      | 2439.5      | 1233.9       |
| N52H, N57Y, Q100R, V110D, C198R, S212G                                     | 364             | 3064              | 3.7      | 1471        | 9.0      | 2699.5      | 2629.9      | 678.2        |
| N52Y, N57Y, F138L, L203P                                                   | 112             | 2177              | 2.6      | 200         | 1.2      | 1889.5      | 1757.9      | 808.8        |
| N52H, I143T                                                                | 135             | 1906              | 2.3      | 138         | 0.8      | 1417.1      | 1367.9      | 275.2        |
| WT ICOSL                                                                   | 32              | 831               | 1.0      | 164         | 1.0      | 558.8       | 377.7       | 152.0        |
| N52D                                                                       | 111             | 88                | 0.1      | 231         | 1.4      | 1288.9      | 1737.9      | 289.0        |

#### **D. Mixed Lymphocyte Reaction for Assessment of Bioreactivity Suppression**

**[0518]** Modulation of T cell activity by fusion variants was determined in a mixed lymphocyte reaction (MLR) substantially as described in Example 6. Human monocytes were incubated for 6 days in the presence of IL-4 and GM-CSF and matured to dendritic cells with the additional of LPS for the final 24 hours.  $1 \times 10^4$  dendritic cells and  $1 \times 10^5$  human CFSE- labeled T cells were plated per well and incubated for 4 days in the presence of three different concentrations (40 nM, 13.3 nM or 4.4 nM) of wildtype or recombinant variant ICOSL ECD-Fc molecules diluted in PBS. The same concentrations of human IgG, PD-L2-Fc or Belatacept (CTLA4-Fc containing L104E and A29Y mutations) were used as controls. Supernatants were harvested and IFN-gamma responses were characterized by ELISA.

**[0519]** FIG. 5 depicts the IFN-gamma production under the various conditions. The levels of IFN-gamma produced by cells in the presence of wild-type ICOSL is set forth by the horizontal line. No suppression of IFN-gamma production was observed in the presence of negative control protein PD-L2-Fc. In contrast, most of the tested ICOSL variants exhibited some degree of inhibition of IFN-gamma production in the MLR. Certain variants exhibited substantial inhibition of IFN-gamma with very low to no detectable IFN-gamma produced in the cultures, even at the lowest concentration of 4.4 nM tested. The percent MLR suppression in the presence of 4.4nM of variant of variant ECD ICOSL-Fc is set forth in Table 23. In the Table, the negative values indicate an inflammatory effect in the assay.

**Table 23: Costimulatory bioactivity data for ICOSL in MLR**

| ICOSL mutation(s)                                             | SEQ ID NO (ECD) | % MLR Suppression (4.4nM) |
|---------------------------------------------------------------|-----------------|---------------------------|
| N52H, N57Y, Q100R, C198R                                      | 365             | 93.6                      |
| N52H, N57Y, Q100R, V122A                                      | 290             | 94.4                      |
| N52H, N57Y, Q100R, F172S                                      | 291             | 100.0                     |
| N52Y/N57Y/F138L/L203P                                         | 112             | 100.0                     |
| V11E, N30D, N52H, N57Y, H94E, L96I, L98F, N194D, V210A, I218T | 308             | 100.0                     |
| N52H, N57Y, Q100R, L102R, V110D, H115R, C198R                 | 367             | 100.0                     |
| N52H, N57Y, Q100R                                             | 283             | 98.2                      |
| N52H, Q100R                                                   | 285             | 97.5                      |
| N52H, N57Y, Q100R, V110D, C198R, S212G                        | 364             | 90.4                      |
| N52H, N57Y, Q100P                                             | 113             | 100.0                     |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, C198R    | 300             | 100.0                     |
| N30D, K42E, N52S                                              | 324             | -38.8                     |
| N52S, F120S, I143V, I224V                                     | 325             | -44.2                     |
| N52S, E90A                                                    | 323             | -30.4                     |
| N52H, N57Y, V110A, C198R, R221I                               | 317             | 100.0                     |
| N52S, N194D                                                   | 366             | -22.3                     |
| N52H, I143T                                                   | 135             | -78.0                     |
| N52D                                                          | 111             | 0.5                       |

#### **E. Assessment of Proliferation and Intracellular Cytokine Markers by Flow Cytometry**

**[0520]** Carboxyfluorescein succinimidyl ester (CFSE) labeled pan T cells from MLR studies as described above that had been incubated for 4 days in the presence of of wildtype or recombinant variant ICOSL ECD-Fc molecules were further tested for cytokine levels by

restimulation with phorbol myristate acetate (PMA)/Ionomycin for 6 hours in the presence of golgi inhibitor (Golgi/Block/Plug). T cells from the MLR study that had been incubated with human IgG, anti-CD28, anti-ICOSL, PD-L2-Fc, or Belatacept (CTLA4-Fc containing L104E and A29Y mutations) also were restimulated. T cells were stained for CD4 or CD8 surface markers, fixed, permeabilized, and intracellularly stained for various cytokines as set forth in Table 24 and 25.

**[0521]** The percent (%) of CD4+ and CD8+ T cells that were positive for specific intracellular cytokines are shown in Table 24, respectively. The results showed that a number of the variant ICOSL ECD-Fc molecules were able to suppress one or more cytokines, including, in some cases, a majority of cytokines. A total score and mean score are calculated to assess the sum effects of individual molecules tested over the parameters examined in this assay. Proliferation was also assessed and a percentage of cells that have divided as determined by CFSE dilution is also shown in Table 24 and 25. Among the provided results, the results show that certain variants show comparable or better activity than Belatacept, particularly from the CD8+ cells.

| <b>Table 24. Assessment of Proliferation and Intracellular Cytokine levels of CD4+ T cells</b> |        |              |        |         |         |        |        |         |          |             |            |
|------------------------------------------------------------------------------------------------|--------|--------------|--------|---------|---------|--------|--------|---------|----------|-------------|------------|
| Variant SEQ ID NO (ECD) or Protein                                                             | Prolif | % Cytokine + |        |         |         |        |        |         |          | Total Score | Mean Score |
|                                                                                                |        | % IFNg+      | % IL4+ | % IL21+ | % IL22+ | % TNF+ | % IL2+ | % IL10+ | % IL17A+ |             |            |
| 308                                                                                            | 3.0    | 9.9          | 3.0    | 1.0     | 1.3     | 34.9   | 31.3   | 0.1     | 0.2      | 41.0        | 4.6        |
| 300                                                                                            | 2.7    | 11.1         | 3.4    | 1.1     | 1.4     | 38.0   | 35.0   | 0.0     | 0.1      | 63.0        | 7.0        |
| 317                                                                                            | 2.9    | 10.9         | 3.3    | 1.1     | 1.5     | 37.3   | 34.1   | 0.1     | 0.2      | 65.0        | 7.2        |
| 291                                                                                            | 3.2    | 8.1          | 2.5    | 0.6     | 1.1     | 27.9   | 24.1   | 0.9     | 1.3      | 66.0        | 7.3        |
| 283                                                                                            | 3.3    | 9.2          | 3.0    | 0.8     | 1.4     | 31.1   | 26.3   | 0.8     | 1.3      | 70.0        | 7.8        |
| 364                                                                                            | 3.4    | 10.9         | 3.3    | 1.0     | 1.6     | 36.5   | 32.3   | 0.5     | 0.9      | 89.0        | 9.9        |
| 390                                                                                            | 3.6    | 9.5          | 3.1    | 0.9     | 1.5     | 33.8   | 29.4   | 0.8     | 1.4      | 92.0        | 10.2       |
| 367                                                                                            | 2.8    | 12.0         | 3.5    | 1.1     | 1.6     | 40.9   | 38.5   | 0.1     | 0.3      | 92.0        | 10.2       |
| CTLA-4-Ig: L104E, A29Y (Belatacept)                                                            | 10.7   | 10.5         | 2.7    | 2.4     | 2.0     | 24.5   | 19.6   | 0.6     | 1.4      | 99.0        | 11.0       |
| 112                                                                                            | 3.5    | 12.0         | 3.8    | 1.3     | 1.6     | 41.3   | 36.7   | 0.2     | 0.4      | 109.0       | 12.1       |
| 285                                                                                            | 4.4    | 9.8          | 3.2    | 1.2     | 1.7     | 32.7   | 29.3   | 0.9     | 1.4      | 114.0       | 12.7       |
| 113                                                                                            | 3.0    | 13.2         | 4.1    | 1.2     | 1.8     | 43.2   | 39.8   | 0.1     | 0.2      | 115.0       | 12.8       |
| 365                                                                                            | 3.6    | 11.3         | 3.9    | 1.0     | 2.0     | 39.1   | 34.6   | 0.8     | 1.3      | 118.0       | 13.1       |
| WT ICOSL                                                                                       | 12.7   | 16.7         | 5.8    | 4.9     | 4.7     | 36.2   | 29.2   | 0.2     | 0.4      | 127.0       | 14.1       |
| 113                                                                                            | 3.5    | 12.3         | 3.9    | 1.1     | 1.8     | 39.2   | 37.5   | 0.4     | 0.6      | 127.0       | 14.1       |

|            |      |      |     |     |     |      |      |     |     |       |      |
|------------|------|------|-----|-----|-----|------|------|-----|-----|-------|------|
| 366        | 10.9 | 16.0 | 7.2 | 4.9 | 5.4 | 40.6 | 33.0 | 0.1 | 0.2 | 135.0 | 15.0 |
| 321        | 10.6 | 16.7 | 6.0 | 4.3 | 5.0 | 41.3 | 37.3 | 0.2 | 0.3 | 146.0 | 16.2 |
| mlgG ctl   | 15.5 | 15.6 | 5.9 | 5.2 | 4.6 | 31.7 | 26.0 | 0.4 | 1.7 | 146.0 | 16.2 |
| PDL2       | 12.3 | 17.9 | 5.7 | 4.7 | 5.2 | 41.4 | 36.0 | 0.3 | 0.6 | 163.0 | 18.1 |
| 323        | 11.9 | 17.7 | 6.2 | 5.2 | 5.3 | 42.4 | 37.5 | 0.2 | 0.4 | 167.0 | 18.6 |
| WT ICOSL   | 12.8 | 17.4 | 6.3 | 5.4 | 5.6 | 38.9 | 32.1 | 0.3 | 0.6 | 167.0 | 18.6 |
| Anti-ICOSL | 15.5 | 16.3 | 6.5 | 5.2 | 5.5 | 35.1 | 29.1 | 0.7 | 2.0 | 168.0 | 18.7 |
| 135        | 12.6 | 17.4 | 6.5 | 5.4 | 4.9 | 44.3 | 37.2 | 0.4 | 0.5 | 179.0 | 19.9 |
| HuIgG      | 12.7 | 17.1 | 6.3 | 5.9 | 4.9 | 41.1 | 32.4 | 0.7 | 1.3 | 181.0 | 20.1 |
| Anti-CD28  | 88.2 | 42.9 | 5.0 | 5.8 | 5.4 | 43.5 | 25.5 | 0.4 | 1.4 | 183.0 | 20.3 |
| 325        | 12.7 | 18.7 | 6.5 | 5.4 | 5.6 | 44.2 | 40.0 | 0.1 | 0.3 | 186.0 | 20.7 |
| 111        | 13.7 | 18.3 | 6.8 | 5.9 | 6.1 | 42.1 | 35.2 | 0.3 | 0.5 | 194.0 | 21.6 |

| <b>Table 25. Assessment of Proliferation and Intracellular Cytokine levels of CD8+ T cells</b> |        |                  |        |         |         |        |        |         |          |             |            |
|------------------------------------------------------------------------------------------------|--------|------------------|--------|---------|---------|--------|--------|---------|----------|-------------|------------|
| Variant SEQ ID NO (ECD) or Protein                                                             | Prolif | % Cytokine +     |        |         |         |        |        |         |          | Total Score | Mean Score |
|                                                                                                |        | % IFN $\gamma$ + | % IL4+ | % IL21+ | % IL22+ | % TNF+ | % IL2+ | % IL10+ | % IL17A+ |             |            |
| 308                                                                                            | 4.2    | 8.4              | 1.6    | 2.2     | 47.2    | 7.6    | 8.0    | 0.2     | 0.1      | 67.0        | 7.4        |
| 300                                                                                            | 3.8    | 8.5              | 2.0    | 2.2     | 47.2    | 8.1    | 9.1    | 0.1     | 0.0      | 69.0        | 7.7        |
| 317                                                                                            | 3.9    | 8.8              | 2.0    | 1.7     | 45.6    | 8.4    | 9.0    | 0.1     | 0.1      | 64.0        | 7.1        |
| 291                                                                                            | 3.8    | 7.0              | 1.4    | 1.8     | 46.6    | 5.9    | 5.9    | 1.1     | 1.1      | 78.0        | 8.7        |
| 283                                                                                            | 4.3    | 8.4              | 1.9    | 1.7     | 50.2    | 7.1    | 6.6    | 1.1     | 1.1      | 98.0        | 10.9       |
| 364                                                                                            | 4.1    | 9.0              | 1.8    | 1.8     | 46.4    | 8.2    | 8.5    | 0.7     | 0.8      | 87.0        | 9.7        |
| 390                                                                                            | 4.1    | 7.9              | 1.8    | 1.8     | 49.8    | 7.3    | 7.2    | 1.0     | 1.2      | 93.0        | 10.3       |
| 367                                                                                            | 3.5    | 9.0              | 1.7    | 2.0     | 47.3    | 8.6    | 10.4   | 0.2     | 0.2      | 78.0        | 8.7        |
| CTLA-4-Ig: L104E, A29Y (Belatacept)                                                            | 12.3   | 14.5             | 2.0    | 3.2     | 38.1    | 11.2   | 8.8    | 0.9     | 1.6      | 121.0       | 13.4       |
| 112                                                                                            | 4.2    | 9.6              | 1.9    | 1.8     | 40.4    | 9.4    | 9.9    | 0.3     | 0.3      | 81.0        | 9.0        |
| 285                                                                                            | 5.4    | 9.5              | 2.0    | 2.7     | 44.2    | 7.8    | 8.4    | 1.2     | 1.3      | 112.0       | 12.4       |
| 113                                                                                            | 3.7    | 9.5              | 1.9    | 1.3     | 44.4    | 9.4    | 10.5   | 0.1     | 0.1      | 62.0        | 6.9        |
| 365                                                                                            | 4.1    | 9.6              | 2.3    | 1.8     | 46.8    | 9.0    | 9.3    | 0.9     | 1.0      | 122.0       | 13.6       |
| ICOSL                                                                                          | 17.2   | 22.3             | 4.9    | 6.4     | 46.4    | 22.0   | 15.7   | 0.4     | 0.7      | 181.0       | 20.1       |
| 113                                                                                            | 4.2    | 9.5              | 2.0    | 2.1     | 45.6    | 8.9    | 10.7   | 0.5     | 0.5      | 110.0       | 12.2       |
| 366                                                                                            | 14.5   | 19.4             | 5.6    | 4.8     | 48.4    | 19.2   | 13.8   | 0.1     | 0.2      | 142.0       | 15.8       |
| 321                                                                                            | 13.4   | 18.9             | 4.3    | 5.0     | 46.3    | 18.4   | 15.4   | 0.3     | 0.6      | 138.0       | 15.3       |
| mlgG ctl                                                                                       | 20.2   | 25.0             | 4.4    | 4.1     | 35.5    | 24.6   | 15.8   | 0.7     | 1.8      | 174.0       | 19.3       |
| PDL2                                                                                           | 15.6   | 21.2             | 4.1    | 4.8     | 44.1    | 20.7   | 15.6   | 0.5     | 0.8      | 147.0       | 16.3       |
| 323                                                                                            | 15.4   | 20.8             | 4.7    | 5.6     | 44.9    | 20.6   | 17.1   | 0.2     | 0.5      | 149.0       | 16.6       |
| WT ICOSL                                                                                       | 17.5   | 22.1             | 5.5    | 4.6     | 45.0    | 21.0   | 12.8   | 0.2     | 0.4      | 148.0       | 16.4       |
| Anti-ICOSL                                                                                     | 21.5   | 26.4             | 4.8    | 5.3     | 33.4    | 26.9   | 19.0   | 1.0     | 2.3      | 198.0       | 22.0       |
| 135                                                                                            | 17.2   | 22.2             | 4.7    | 6.7     | 39.4    | 22.5   | 18.1   | 0.8     | 0.9      | 178.0       | 19.8       |
| HuIgG                                                                                          | 15.9   | 21.5             | 4.4    | 6.4     | 41.6    | 21.4   | 15.1   | 1.4     | 1.7      | 179.0       | 19.9       |
| Anti-CD28                                                                                      | 60.6   | 44.3             | 3.5    | 2.1     | 38.6    | 32.5   | 16.0   | 1.2     | 1.6      | 182.0       | 20.2       |

|     |      |      |     |     |      |      |      |     |     |       |      |
|-----|------|------|-----|-----|------|------|------|-----|-----|-------|------|
| 325 | 16.5 | 22.0 | 4.9 | 5.4 | 44.0 | 21.9 | 18.5 | 0.2 | 0.6 | 161.0 | 17.9 |
| 111 | 17.7 | 22.8 | 5.3 | 6.1 | 45.4 | 22.9 | 16.7 | 0.4 | 0.6 | 183.0 | 20.3 |

## EXAMPLE 16

### Assessment of Cytokine Production in B-T cell Co-culture

[0522] B cells and CD4<sup>+</sup> T cells were purified from the same donor and labeled with CFSE and plated in 96 well plates in 1:1 cellular ratios at  $5 \times 10^4$  cells of each per well. Variant ICOSL ECD-Fc fusion molecules or Belatacept were added at a final concentration of 40nM per well. Cells were either unstimulated or incubated with 100 ng/ml of Staphylococcus enterotoxin B (SEB), 1 µg/ml of Pokeweed Mitogen (PWM) or both for 7 days at 37°C in a final volume of 200 µl/well.

[0523] Cells were harvested and surface stained for the following B and T cell lineage markers (IgM, IgD, CD38, CD138, CD27, CD19, CD4, CD3). Proliferation was assessed by flow cytometry and culture supernatants were analyzed for IL-5, IL-13 or IL-21 cytokines using a LEGENDplex human Th cytokine detection kit (Biolegend, USA).

[0524] As shown in FIG. 6A, the number of dividing B cells was reduced in B/T cell co-cultures when incubated in the presence of variant ICOSL ECD Fc-fusion molecules compared to no protein controls. The degree of antagonistic effect for the tested exemplary variants was similar to CTLA-4-Ig Belatacept (L104E, A29Y). Likewise, as shown in FIGS. 6B-6D, variant ICOSL ECD Fc-fusion molecules inhibited cytokine production in primary human B cell/T cell coculture *in vitro* compared to no protein controls as well as cultures containing wild-type ICOSL control. Compared to Belatacept, exemplary tested variant ICOSL ECD Fc-fusion molecules were more effective in blocking cytokine production in some cases.

## EXAMPLE 17

### Assessment of Survival and Disease Activity in Graft-Versus-Host-Disease (GvHD)

#### Model

[0525] Exemplary ICOSL variant ECD-Fc protein, were assessed for activity in a graft-versus-host-disease (GvHD) model. Female NGS mice (n = 10 per group) were irradiated (100 rad) and administered 10 mg of gamma globulin subcutaneously on Day -1. On Day 0, the mice

received 10 million human peripheral blood mononuclear cells (PBMCs) and intraperitoneal injection dosing of either 100µg of WT-ICOSL ECD Fc, a variant ICOSL ECD Fc molecule N52H/I143T (ECD set forth in SEQ ID NO: 135), a variant ICOSL ECD Fc molecule N52H/N57Y/Q100P (ECD set forth in SEQ ID NO: 113), 75µg of Belatacept (CTLA-4-Ig L104E/A29Y; U.S. Patent Application Publication Number US20160271218) or saline as control. On Day 15, engrafted human CD45+ cells were phenotyped by flow cytometry. After the study was terminated on Day 35, endpoint measurements of survival, body weight loss, and disease activity were evaluated.

**[0526]** FIG. 7A shows the survival of GVHD mice treated with saline, WT ICOSL-ECD Fc, the variant ICOSL ECD-Fc molecules, or Belatacept. A significant difference in the survival of mice administered variant ICOSL ECD- Fc N52H/N57Y/Q100P (ECD set forth in SEQ ID NO: 113) compared to mice administered saline or WT ICOSL ECD-Fc was observed ( $p < 0.0001$  by Mantel-Cox and Gehan-Breslow-Wilcoxon test). FIG. 7B shows similar differences between the body weight loss of mice treated with saline, WT ICOSL ECD- Fc, the variant ICOSL ECD- Fc molecules, or Belatacept over the course of the study.

**[0527]** A disease activity index (DAI) was determined three times a week during the study and evaluated the scoring of weight loss, posture, activity, appearance of the hair coat and skin of the mice. The grade of disease over the course of the study is shown in FIG. 7C. Treatment groups that received ICOSL Fc variant N52H/N57Y/Q100P (ECD set forth SEQ ID NO: 113) or Belatacept showed significantly improved DAI scores. The percentage of human T cells in the peripheral blood on day 14 of the study was also assessed by flow cytometry. Measurements were averaged by treatment group and error bars represent standard error of the mean (SEM). FIG. 7D shows the percent of live CD3+/CD4+ or CD3+/CD8+ cells in the blood. Treatment groups that received variant ICOSL ECD Fc with N52H/N57Y/Q100P (ECD set forth SEQ ID NO: 113) or Belatacept showed significantly different levels of CD4+ T cells compared to saline treatment group ( $p = 0.008$  and  $0.006$ , respectively, by unpaired t-test). This study demonstrates the therapeutic effect of the variant ICOSL Fc variant protein on human primary T cells and GVHD during *in vivo* modeling.

## EXAMPLE 18

### Assessment of Activation by Stacked Molecules

[0528] Stacked variant IgV Fc fusion proteins containing an NKp30 IgV domain as a localizing domain (designated “L”) and an ICOSL IgV domain as a costimulatory domain (designated “C”) were generated and assessed substantially as described in Example 8. Specifically, the constructs tested in this experiment include: (1) a stacked construct with variant IgV Fc fusion protein (vIgD C-L) containing an NKp30 composed of Ig of consensus NKp30 variant (SEQ ID NO:493) with the IgV domain of ICOSL variant N52H/N57Y/Q100P (ECD set forth in SEQ ID NO: 113 and IgV set forth in SEQ ID NO:201); (2) a stacked construct with the NKp30 wildtype domain Ig domain (SEQ ID NO:214) and V domain of wildtype ICOSL (WT C-L) (SEQ ID NO:196); (3) a construct with wild-type ICOSL IgV domain (WT C domain; SEQ ID NO:196), and (3) a construct with the wildtype NKp30 domain (WT L domain; SEQ ID NO: 214).

[0529] CD32+ K562 cells were engineered to stably express B7-H6 on the cell surface. The cells were then treated with mitomycin and plated with pan T-cells in the presence of absence of 10nM anti-CD3 and stacked variants or control domains at 100, 33, 11, or 3.7 nM. Cells were cultured 3 days before harvesting culture supernatants and measuring human IFN-gamma levels using an ELISA.

[0530] As shown in FIG. 8, both variant and wildtype costimulatory-localizing domain stacks were able to localize to the engineered K562 cells and deliver a co-stimulatory signal to the pan-T-cells to induce IFN-gamma secretion. The stacked construct with variant IgV Fc fusion protein (vIgD C-L) showed increased functional activity results at all concentrations tested, while the individual domain components had no effect when not combined with each other.

## EXAMPLE 19

### Assessment of Delayed Type Hypersensitivity *in vivo*

[0531] Variant ICOSL ECD-Fc fusion molecules were assessed for anti-inflammatory activity *in vivo* in the mouse delayed-type hypersensitivity (DTH) model. Delayed-type hypersensitivity immune reactions were elicited in ovalbumin (OVA)-sensitized mice and response after challenge was assessed. The variant ICOSL ECD-Fc fusion molecules tested contained a variant ECD with the following amino acid substitutions: N52H/N57Y/Q100P (ECD

set forth in SEQ ID NO: 113), N52H/Q100R (ECD set forth in SEQ ID NO: 285), or N52H/N57Y/Q100R/F172S (ECD set forth in SEQ ID NO: 291). The variants were fused to either an Fc backbone containing mutations C220S/L234A/L235E/G237A by EU numbering (designated Fc#1) set forth in SEQ ID NO:477) or an Fc backbone containing mutations C220S/E233P/L234V/L235A/G236del/S267K by EU numbering (designated Fc#2) set forth in SEQ ID NO:478) either with or without a G4S (GGGGS) linker. Table 26 sets forth the tested constructs:

| <b>Table 26: ICOSL ECD-Fc Fusion Constructs</b> |                          |            |                   |
|-------------------------------------------------|--------------------------|------------|-------------------|
|                                                 | ICOSL ECD<br>(SEQ ID NO) | G4S Linker | Fc<br>(SEQ ID NO) |
| N52H/N57Y/Q100P (G4S)-Fc #1                     | 113                      | +          | 477               |
| N52H/Q100R (G4S)-Fc #1                          | 285                      | +          | 477               |
| N52H/N57Y/Q100R/F172S (G4S)-Fc                  | 291                      | +          | 478               |
| N52H/N57Y/Q100R/F172S (G4S)-Fc                  | 291                      | +          | 477               |
| N52H/N57Y/Q100R/F172S-Fc                        | 291                      | -          | 477               |

**[0532]** For sensitization, 8-week old female BALB/c mice were injected subcutaneously with 100µg of OVA emulsified in Sigma Adjuvant (100µL; catatlog number S6322-1VL) at the base of the tail on day 0. On days 1 and 4, the mice were administered variant ICOSL ECD-Fc fusion proteins, 75 µg of CTLA-4 Fc (abatacept), or PBS as negative control by intraperitoneal injection. On day 7 at two to three hours prior to OVA challenge, the mice were further administered PBS as a control, 75µg of CTLA-4 Fc (abatacept from Orencia), or the indicated variant ICOSL polypeptide by intraperitoneal injection. Abatacept and variant ICOSL-Fc fusion molecules were dosed at molar equivalents.

**[0533]** For OVA challenge, an intradermal injection of 10µg OVA in the left ear pinnae in a 10 µL volume of PBS was delivered under gas isoflurane anesthesia 2 - 3 hours following therapeutic treatment. Baseline ear thickness was measured prior to OVA challenge.

**[0534]** On day 8, ear thickness was measured under isoflurane anesthesia using Mitutoyo calipers and the change in ear thickness before and after OVA challenge was determined. As shown in FIG. 9, mice treated with the indicated variant ICOSL ECD-Fc fusion molecules showed significantly less OVA-induced ear swelling as compared to PBS control ( $< 0.0001$  by 1-way ANOVA). There were no significant differences between ear thickness for mice treated with abatacept compared to any of the indicated variant ICOSL ECD-Fc fusion molecules tested or between the variant ICOSL treatments. These results demonstrate that variant ICOSL molecules can reduce immune responses *in vivo*.

## **EXAMPLE 20**

### **Generation and Assessment of Binding and Activity of Variant ICOSL IgSF Domain-Containing Molecules**

**[0535]** Additional variant ICOSL IgSF (e.g. ECD) domain-containing molecules were generated, as described below. In each of the Tables below, the Table indicates amino acid substitutions in the ECD of the variant ICOSL as designated by amino acid position number corresponding to amino acid positions in the respective reference unmodified ICOSL extracellular domain (ECD) sequence set forth in SEQ ID NO:32. In some cases, the removal of the AAA linker sequence of the variant ICOSL ECD-Fc is indicated by “ $\Delta$ AAA”. Column 2 sets forth the SEQ ID NO identifier for each variant ECD domain contained in the variant ECD-Fc fusion molecule.

#### **A. Generation of Additional Variants**

##### **1. Solubility Variants**

**[0536]** From a collection of mutants containing the following mutations including E16V, N30D, K42E, N52H, N52Y, N52S, N57Y, E90A, Q100R, Q100P, L102R, V110D, H115R, F120S, V122A, F138L, I143V, I143T, H152C, K156M, F172S, N194D, C198R, L203P, R221I, I224V, the mutations H115R, F172S and C198R were identified as mutations that may potentially enhance protein solubility or enhance protein expression (‘solubility mutations’). These three mutations (H115R, F172S and C198R) were randomly introduced by site directed mutagenesis into the same set of clones to generate a collection of derivatives that contain one or more of these solubility mutations. Because site directed mutagenesis reaction was carried out

with pooled mutagenic oligos reacted with pooled parental clones in a single reaction, some of the clones also contain some mutations from other parental clones. The generated variants contained between 3 to 10 different amino acid mutations in various combinations, as summarized in Table 27A.

| <b>TABLE 27A: Exemplary variant ICOSL polypeptides</b>   |                      |
|----------------------------------------------------------|----------------------|
| <b>Mutation(s)</b>                                       | <b>ECD SEQ ID NO</b> |
| Wild-type                                                | 32                   |
| N52H/N57Y/Q100R/H115R/C198R                              | 435                  |
| N52H/N57Y/Q100R/F172S/C198R                              | 436                  |
| N52H/N57Y/Q100R/H115R/F172S/C198R                        | 437                  |
| N52H/N57Y/Q100R/H115R/I143V/F172S/C198R                  | 438                  |
| N52H/N57Y/Q100R/L102R/H115R/F172S/C198R                  | 439                  |
| N52H/V122A/F172S/C198R                                   | 440                  |
| N52H/N57Y/Q100R/H115R/F172S/N194D                        | 441                  |
| N52H/N57Y/H115R/F172S/C198R                              | 442                  |
| N52H/N57Y/Q100R/H115R/C198R                              | 443                  |
| N52H/N57Y/H115R                                          | 444                  |
| N52H/N57Y/Q100R/H115R                                    | 445                  |
| N52H/N57Y/Q100R/H115R/F172S/I224V                        | 446                  |
| N52H/N57Y/Q100R/H115R/F172S                              | 447                  |
| N52H/N57Y/Q100R/F172S                                    | 448                  |
| N52H/Q100R/H115R/I143T/F172S                             | 449                  |
| N52H/N57Y/Q100P/H115R/F172S                              | 450                  |
| N52Y/N57Y/Q100P/F172S                                    | 451                  |
| E16V/N52H/N57Y/Q100R/V110D/H115R/C198R                   | 452                  |
| E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R | 453                  |
| N52S/E90A/H115R                                          | 454                  |
| N30D/K42E N52S/H115R                                     | 455                  |
| N30D/K42E/N52S/H115R/C198R/R221I                         | 456                  |
| N30D/K42E/N52S/H115R/C198R                               | 457                  |
| N30D/K42E/N52S/H115R/F172S/N194D                         | 458                  |
| N52S/H115R/F120S/I143V/C198R                             | 459                  |
| N52S/H115R/F172S/C198R                                   | 460                  |
| N52H/N57Y/Q100P/C198R                                    | 461                  |
| N52H/N57Y/Q100P H115R/F172S/C198R                        | 462                  |
| N52H/N57Y/Q100P/F172S/C198R                              | 463                  |
| N52H/N57Y/Q100P/H115R                                    | 464                  |
| N52H/N57Y/Q100P/H115R/C198R                              | 465                  |
| N52H/Q100R/C198R                                         | 466                  |
| N52H/Q100R/H115R/F172S                                   | 467                  |
| N52H/Q100R/H115X/F172S/C198R                             | 468                  |
| N52H/Q100R/H115R/F172S/C198R                             | 469                  |
| N52H/N57Y/Q100R /F172S/C198R                             | 470                  |

## 2. Back Variants

**[0537]** Particular exemplary mutations including N52H, N52Y, N57Y, Q100R, Q100P, F138L, C198R, L203P identified in select variants described in Example 6 were further combined in the ECD backbone of wild-type ICOSL with reference to positions set forth in SEQ ID NO:32 to generate additional combination variants. The generated variants contained between 1 to 3 different amino acid mutations in various combinations as set forth in Table 27B.

| <b>TABLE 27B: Exemplary variant ICOSL polypeptides</b> |                      |
|--------------------------------------------------------|----------------------|
| <b>Mutation(s)</b>                                     | <b>ECD SEQ ID NO</b> |
| Wild-type                                              | 32                   |
| Q100R                                                  | 427                  |
| F138L/L203P                                            | 428                  |
| N52Y/F138L/L203P                                       | 429                  |
| N57Y/Q100R/C198R                                       | 430                  |
| N57Y/F138L/L203P                                       | 431                  |
| N52H                                                   | 110                  |
| N57Y                                                   | 121                  |
| N57Y/Q100P                                             | 122                  |
| Q100R/F138L                                            | 432                  |
| L203P                                                  | 433                  |

## 3. Glycosylation Variants

**[0538]** Exemplary glycosylation mutations selected from N52H, N52Q, N84Q, N119Q, N155H, N155Q, N168Q, N207Q were combined in various permutations in the ECD backbone of wild-type ICOSL with reference to positions set forth in SEQ ID NO:32 to generate additional combination variants. The generated variants contained between 1 to 5 different amino acid mutations in various combinations as set forth in Table 27C. Mutations designated with an “X” indicate either an N or Q at the indicated position.

| <b>TABLE 27C (glyc): Exemplary variant ICOSL polypeptides</b> |                      |
|---------------------------------------------------------------|----------------------|
| <b>Mutation(s)</b>                                            | <b>ECD SEQ ID NO</b> |
| Wild-type                                                     | 32                   |
| N84Q                                                          | 387                  |
| N119Q                                                         | 388                  |
| N168Q                                                         | 389                  |
| N207Q                                                         | 390                  |
| N52Q/N207X                                                    | 391                  |
| N168X/N207X                                                   | 392                  |
| N52Q/N168Q                                                    | 393                  |
| N84Q/N207Q                                                    | 394                  |
| N155Q/N207Q                                                   | 395                  |
| N119Q/N168Q                                                   | 396                  |
| N119Q/N207Q                                                   | 397                  |
| N119Q/N155X                                                   | 398                  |
| N52Q/N84Q                                                     | 399                  |
| N52Q/N119Q                                                    | 400                  |
| N84Q/N119Q                                                    | 401                  |
| N52Q/N84Q/N168Q                                               | 402                  |
| N52Q/N84Q/N207Q                                               | 403                  |
| N84Q/N155Q/N168Q                                              | 404                  |
| N84Q/N168Q/N207Q                                              | 405                  |
| N84Q/N155H/N207Q                                              | 406                  |
| N155Q/N168Q/N207Q                                             | 407                  |
| N119Q N155Q/N168Q                                             | 408                  |
|                                                               | 409                  |
| N119Q/N168Q/N207Q                                             |                      |
| N84Q/N119Q/N207Q                                              | 410                  |
| N119Q/N155H/N207Q                                             | 411                  |
| N84Q/N119Q/N155Q                                              | 412                  |
| N52Q/N119Q/N155Q                                              | 413                  |
| N52H/N84Q/N119Q                                               | 414                  |
| N52H/N84Q/N168X/N207X                                         | 415                  |
| N52Q/N84Q/N155X/N168X                                         | 416                  |
| N52Q/N84Q/N119Q/N168Q                                         | 417                  |
| N84Q/N119Q/N155Q/N168Q                                        | 418                  |
| N84Q/N155Q/N168Q/N207Q                                        | 419                  |
| N84Q/N119Q/N155Q/N207Q                                        | 420                  |
| N52Q/N84Q/N119Q/N207Q                                         | 421                  |
| N52Q/N84Q/N119Q/N155Q                                         | 422                  |
| N52Q/N84Q/N119Q/N155Q/N207Q                                   | 423                  |
| N84Q/N119Q/N155Q/N168Q/N207Q                                  | 424                  |

## B. Binding to Cell-Expressed Counter Structures

[0539] The additional variants were formatted as Fc-fusion proteins as described in Example 4. The variant Fc-fusion molecules were assessed in binding studies to assess specificity and affinity of ICOSL domain variant immunomodulatory proteins for cognate binding partners. Expi293 cells transfected with cognate binding partners, human CD28, ICOS and CTLA4, were used in binding studies as described in Example 6. MFI was determined for each transfectant and compared to the corresponding unmodified (parental) ECD-Fc.

[0540] Results for the binding for exemplary variant ICOSL ECD-Fc fusion molecules are shown in Tables 28A-C. As shown in Table 28A-C, ICOSL IgSF (e.g. ECD) domain variants generated with the various combinations of specific mutations exhibited increased binding for at least one, and in some cases more than one, cognate counter structure ligand.

## C. Bioactivity Characterization with Anti-CD3 Coimmobilization Assay

[0541] The costimulatory bioactivity of generated variant Fc-fusion molecules was also assessed in anti-CD3 coimmobilization assays as described in Example 6. Table 28A-C depicts the ratio of IFN-gamma produced by each variant ECD-Fc compared to the corresponding unmodified (wildtype) ICOSL ECD-Fc in the assay. Mutations designated with an “X” indicate a Q or the wildtype residue corresponding to the indicated position of SEQ ID NO: 32 at the indicated position. As shown, variant Fc-fusion molecules generated exhibited improved activities to increase immunological activity.

| TABLE 28A: Molecule sequences, binding data, and costimulatory bioactivity data of variant ICOSLECD-Fc molecules containing select mutations |                 |                           |                           |                             |                                  |
|----------------------------------------------------------------------------------------------------------------------------------------------|-----------------|---------------------------|---------------------------|-----------------------------|----------------------------------|
| ICOSL Mutations                                                                                                                              | SEQ ID NO (ECD) | Binding                   |                           |                             | Coimmobilization with anti-CD3   |
|                                                                                                                                              |                 | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | IFN-gamma pg/mL (parental ratio) |
| N52H, N57Y, Q100R, F172S, C198R                                                                                                              | 436             | 118145 (1.33)             | 59651 (29.60)             | 178790 (41.12)              | 5059 (37.90)                     |
| N52H, N57Y, Q100R, H115R, F172S, C198R                                                                                                       | 437             | 125341 (1.41)             | 51604 (25.60)             | 211000 (48.53)              | 8218 (61.57)                     |
| N52Y, N57Y, Q100P, F172S                                                                                                                     | 451             | 121280                    | 63663                     | 174224                      | 8123 (60.86)                     |

**TABLE 28A: Molecule sequences, binding data, and costimulatory bioactivity data of variant ICOSLECD-Fc molecules containing select mutations**

| ICOSL Mutations                                                   | SEQ ID NO (ECD) | Binding                   |                           |                             | Coimmobilization with anti-CD3   |
|-------------------------------------------------------------------|-----------------|---------------------------|---------------------------|-----------------------------|----------------------------------|
|                                                                   |                 | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | IFN-gamma pg/mL (parental ratio) |
|                                                                   |                 | (1.37)                    | (31.59)                   | (40.07)                     |                                  |
| E16V, N52H, N57Y, Q100R, V110D, H115R, Y152C, K156M, F172S, C198R | 453             | 107819 (1.22)             | 68883 (34.18)             | 170080 (39.12)              | 8936 (66.95)                     |
| N52S, H115R, F120S, I143V, C198R                                  | 459             | 116235 (1.31)             | 25582 (12.69)             | 22483 (5.17)                | 125 (0.93)                       |
| N52H, N57Y, Q100P, C198R                                          | 461             | 107164 (1.21)             | 56103 (27.84)             | 172319 (39.63)              | 1258 (9.43)                      |
| N52H, N57Y, Q100P, H115R, F172S, C198R                            | 462             | 120864 (1.36)             | 54586 (27.08)             | 176637 (40.63)              | 5507 (41.26)                     |
| N52H, N57Y, Q100P, F172S, C198R                                   | 463             | 117954 (1.33)             | 59376 (29.46)             | 151265 (34.79)              | 3884 (29.10)                     |
| N52H, N57Y, Q100P, H115R                                          | 464             | 126221 (1.42)             | 53321 (26.46)             | 178812 (41.13)              | 4154 (31.13)                     |
| N52H, N57Y, Q100P, H115R, C198R                                   | 465             | 137004 (1.55)             | 55454 (27.51)             | 148417 (34.14)              | 5069 (37.98)                     |
| N52H, Q100R, C198R                                                | 466             | 111428 (1.26)             | 58608 (29.08)             | 116111 (26.71)              | 3729 (27.94)                     |
| N52H, Q100R, H115R, F172S                                         | 467             | 105532 (1.19)             | 58287 (28.92)             | 106295 (24.45)              | 5294 (39.67)                     |
| N52H, Q100R, H115X, F172S, C198R                                  | 468             | 106555 (1.20)             | 73397 (36.42)             | 171815 (39.52)              | 6961 (52.16)                     |
| N52H, Q100R, H115R, F172S, C198R                                  | 469             | 114223 (1.29)             | 66686 (33.09)             | 157154 (36.15)              | 7592 (56.88)                     |
| N52H, N57Y, Q100R, F172S, C198R                                   | 470             | 99350 (1.12)              | 61292 (30.41)             | 182288 (41.93)              | 9167 (68.68)                     |
| N52H, N57Y, Q100R, H115R, F172S, C198R                            | 437             | 114057 (1.29)             | 52011 (25.81)             | 146471 (33.69)              | 6545 (49.04)                     |
| N52H, N57Y, Q100R, H115R, F172S                                   | 447             | 136143 (1.54)             | 66516 (33.00)             | 177376 (40.80)              | 8527 (63.89)                     |
| N52H, N57Y, Q100R, H115R, F172S, C198R                            | 437             | 132970 (1.50)             | 59633 (29.59)             | 133247 (30.65)              | 5999 (44.95)                     |
| Q100R                                                             | 427             | 62064 (0.70)              | 16740 (8.31)              | 29654 (6.82)                | 35 (0.26)                        |
| Q100R ΔAAA                                                        | 427             | 1594 (0.018)              | 16535 (8.20)              | 33457 (7.69)                | 87 (0.65)                        |
| F138L L203P                                                       | 428             | 53804 (0.61)              | 1510 (0.75)               | 2151 (0.49)                 | 35 (0.26)                        |
| F138L L203P ΔAAA                                                  | 428             | 53044 (0.60)              | 1882 (0.93)               | 1623 (0.37)                 | 35 (0.26)                        |

| <b>TABLE 28A: Molecule sequences, binding data, and costimulatory bioactivity data of variant ICOSLECD-Fc molecules containing select mutations</b> |                 |                           |                           |                             |                                  |
|-----------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|---------------------------|---------------------------|-----------------------------|----------------------------------|
| ICOSL Mutations                                                                                                                                     | SEQ ID NO (ECD) | Binding                   |                           |                             | Coimmobilization with anti-CD3   |
|                                                                                                                                                     |                 | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | IFN-gamma pg/mL (parental ratio) |
| N52Y F138L L203P                                                                                                                                    | 429             | 99761 (1.12)              | 47369 (23.50)             | 67300 (15.48)               | 1489 (11.16)                     |
| N52Y F138L L203P ΔAAA                                                                                                                               | 429             | 59576 (0.67)              | 52865 (26.23)             | 66553 (15.31)               | 997 (7.47)                       |
| N57Y Q100R C198R                                                                                                                                    | 430             | 58706 (0.66)              | 57739 (28.65)             | 99426 (22.87)               | 9962 (74.64)                     |
| N57Y Q100R C198R ΔAAA                                                                                                                               | 430             | 98514 (1.11)              | 57694 (28.63)             | 131458 (30.23)              | 6763 (50.67)                     |
| N57Y F138L L203P                                                                                                                                    | 431             | 109472 (1.23)             | 42276 (20.98)             | 64477 (14.83)               | 4979 (37.30)                     |
| N57Y F138L L203P ΔAAA                                                                                                                               | 431             | 97777 (1.10)              | 44924 (22.29)             | 64742 (14.89)               | 6507 (48.75)                     |
| N52H                                                                                                                                                | 110             | 91598 (1.03)              | 58264 (28.91)             | 103025 (23.69)              | 3393 (25.42)                     |
| N57Y                                                                                                                                                | 121             | 109031 (1.23)             | 43754 (21.71)             | 50683 (11.66)               | 4881 (36.57)                     |
| N57Y, Q100P                                                                                                                                         | 122             | 72480 (0.82)              | 60161 (29.85)             | 109522 (25.19)              | 2797 (20.95)                     |
| Q100R, F138L                                                                                                                                        | 432             | 65974 (0.74)              | 4485 (2.23)               | 8136 (1.87)                 | 685 (5.13)                       |
| L203P                                                                                                                                               | 433             | 61554 (0.69)              | 1533 (0.76)               | 2031 (0.47)                 | 2434 (18.24)                     |
| Wildtype ICOSL ECD                                                                                                                                  | 32              | 88625 (1.00)              | 2015 (1.00)               | 4348 (1.00)                 | 133 (1.00)                       |

| <b>TABLE 28B: Molecule sequences, binding data, and costimulatory bioactivity data of variant ICOSLECD-Fc molecules containing select mutations</b> |                 |                           |                           |                             |                                  |
|-----------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|---------------------------|---------------------------|-----------------------------|----------------------------------|
| ICOSL Mutations                                                                                                                                     | SEQ ID NO (ECD) | Binding                   |                           |                             | Coimmobilization with anti-CD3   |
|                                                                                                                                                     |                 | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | IFN-gamma pg/mL (parental ratio) |
| N52H, N57Y, Q100R, H115R                                                                                                                            | 445             | 165027 (1.97)             | 51666 (9.89)              | 287581 (60.27)              | 5858 (20.36)                     |
| N52H, N57Y, Q100R, F172S                                                                                                                            | 448             | 184449 (2.20)             | 51394 (9.84)              | 182109 (38.16)              | 3449 (11.99)                     |
| N52H, N57Y, Q100R, H115R, F172S, I224V                                                                                                              | 446             | 165120 (1.97)             | 46636 (8.93)              | 274026 (57.43)              | 2053 (7.13)                      |

**TABLE 28B: Molecule sequences, binding data, and costimulatory bioactivity data of variant ICOSLECD-Fc molecules containing select mutations**

| ICOSL Mutations                               | SEQ ID NO (ECD) | Binding                   |                           |                             | Coimmobilization with anti-CD3   |
|-----------------------------------------------|-----------------|---------------------------|---------------------------|-----------------------------|----------------------------------|
|                                               |                 | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | IFN-gamma pg/mL (parental ratio) |
| N52H, N57Y, Q100R, H115R, F172S               | 447             | 164750 (1.97)             | 40046 (7.67)              | 259351 (54.35)              | 3722 (12.93)                     |
| N52H, N57Y, Q100R, H115R, C198R               | 435             | 186017 (2.22)             | 39073 (7.48)              | 200505 (42.02)              | 3909 (13.58)                     |
| N52H, N57Y, Q100R, F172S, C198R               | 436             | 181118 (2.16)             | 38233 (7.32)              | 210709 (44.16)              | 1199 (4.17)                      |
| N52H, N57Y, Q100R, H115R, F172S, C198R        | 437             | 155392 (1.85)             | 28828 (5.52)              | 169736 (35.57)              | 3449 (11.99)                     |
| N52H, N57Y, Q100R, H115R, I143V, F172S, C198R | 438             | 139977 (1.67)             | 31459 (6.02)              | 179089 (37.53)              | 1620 (5.63)                      |
| N52H, N57Y, Q100R, L102R H115R, F172S, C198R  | 439             | 146799 (1.75)             | 29636 (5.68)              | 200000 (41.91)              | 2712 (9.43)                      |
| N52H, N57Y, Q100R, H115R F172S, N194D         | 441             | 150863 (1.80)             | 31304 (5.99)              | 167783 (35.16)              | 15607 (54.24)                    |
| N52H, N57Y, H115R, F172S, C198R               | 442             | 126909 (1.51)             | 35803 (6.86)              | 152858 (32.03)              | 5374 (18.67)                     |
| N52H, N57Y, Q100R, H115R, C198R               | 443             | 131730 (1.57)             | 37595 (7.20)              | 139041 (29.14)              | 9306 (32.34)                     |
| N52H, N57Y, H115R                             | 444             | 162632 (1.94)             | 49847 (9.55)              | 266878 (55.93)              | 2918 (10.14)                     |
| N52H, Q100R, H115R, I143T F172S               | 449             | 132873 (1.59)             | 52058 (9.97)              | 186366 (39.06)              | 3086 (10.72)                     |
| N52H, N57Y, Q100P, H115R, F172S               | 450             | 148160 (1.77)             | 46851 (8.97)              | 246636 (51.69)              | 4987 (17.33)                     |
| E16V, N52H, N57Y, Q100R, V110D, H115R, C198R  | 452             | 154036 (1.84)             | 48674 (9.32)              | 212905 (44.62)              | 5095 (17.71)                     |
| N52S, E90A, H115R                             | 454             | 142963 (1.71)             | 3597 (0.69)               | 3772 (0.79)                 | 2241 (7.79)                      |
| N30D, K42E, N52S, H115R, C198R R221I          | 456             | 124095 (1.48)             | 8066 (1.54)               | 7751 (1.62)                 | 417 (1.45)                       |
| N30D, K42E, N52S, H115R, C198R                | 457             | 161734 (1.93)             | 2791 (0.53)               | 2919 (0.61)                 | 841 (2.92)                       |
| N30D, K42E, N52S, H115R, F172S, N194D         | 458             | 117880 (1.41)             | 4395 (0.84)               | 4941 (1.04)                 | 2904 (10.09)                     |
| N30D, K42E, N52S, H115R,                      | 455             | 114107 (1.36)             | 2935 (0.56)               | 2748 (0.58)                 | 549 (1.91)                       |
| N52S, E90A, H115R,                            | 454             | 120450 (1.44)             | 12768 (2.45)              | 23282 (4.88)                | 2890 (10.04)                     |
| N30D, K42E, N52S, H115R                       | 455             | 115273 (1.38)             | 11964 (2.29)              | 22779 (4.77)                | 2241 (7.79)                      |

**TABLE 28B: Molecule sequences, binding data, and costimulatory bioactivity data of variant ICOSLECD-Fc molecules containing select mutations**

| ICOSL Mutations           | SEQ ID NO (ECD) | Binding                   |                           |                             | Coimmobilization with anti-CD3   |
|---------------------------|-----------------|---------------------------|---------------------------|-----------------------------|----------------------------------|
|                           |                 | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | IFN-gamma pg/mL (parental ratio) |
| N52S, H115R, F172S, C198R | 460             | 95537 (1.14)              | 7614 (1.46)               | 21701 (4.55)                | 1458 (5.07)                      |
| Wildtype                  | 32              | 83813 (1.00)              | 5222 (1.00)               | 4772 (1.00)                 | 288 (1.00)                       |

**Table 28C: Molecule sequences, binding data, and costimulatory bioactivity data of variant ICOSLECD-Fc molecules containing glycosylation mutations**

| ICOSL Mutation(s) | SSEQ ID NO (ECD) | Binding                   |                           |                             | Coimmobilization with anti-CD3   |
|-------------------|------------------|---------------------------|---------------------------|-----------------------------|----------------------------------|
|                   |                  | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | IFN-gamma pg/mL (parental ratio) |
| N84Q              | 387              | 34426 (0.94)              | 1755 (1.16)               | 5757 (1.51)                 | 100 (2.03)                       |
| N119Q             | 388              | 30806 (0.84)              | 4102 (2.70)               | 19836 (5.21)                | 81 (1.66)                        |
| N168Q             | 389              | 27041 (0.74)              | 1410 (0.93)               | 18641 (4.90)                | 67 (1.36)                        |
| N207Q             | 390              | 36516 (1.00)              | 11923 (7.86)              | 25701 (6.76)                | 206 (4.20)                       |
| N52Q, N207X       | 391              | 30216 (0.83)              | 12086 (7.97)              | 27952 (7.35)                | 77 (1.56)                        |
| N168X, N207X      | 392              | 37191 (1.02)              | 5787 (3.81)               | 12280 (3.23)                | 104 (2.12)                       |
| N52Q, N168Q       | 393              | 32576 (0.89)              | 12638 (8.33)              | 27167 (7.14)                | 101 (2.06)                       |
| N84Q, N207Q       | 394              | 37176 (1.02)              | 5292 (3.49)               | 3153 (0.83)                 | 31 (0.63)                        |
| N155Q, N207Q      | 395              | 34884 (0.95)              | 1489 (0.98)               | 987 (0.26)                  | 73 (1.48)                        |
| N119Q, N168Q      | 396              | 29099 (0.80)              | 2534 (1.67)               | 11289 (2.97)                | 51 (1.05)                        |
| N119Q, N207Q      | 397              | 32603 (0.89)              | 1861 (1.23)               | 6795 (1.79)                 | 153 (3.12)                       |
| N119Q N155X       | 398              | 38516 (1.05)              | 15318 (10.10)             | 27498 (7.23)                | 173 (3.52)                       |
| N52Q, N84Q        | 399              | 33988 (0.93)              | 1675 (1.10)               | 3525 (0.93)                 | 39 (0.80)                        |

| <b>Table 28C: Molecule sequences, binding data, and costimulatory bioactivity data of variant ICOSLECD-Fc molecules containing glycosylation mutations</b> |                  |                           |                           |                             |                                  |
|------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|---------------------------|---------------------------|-----------------------------|----------------------------------|
| ICOSL Mutation(s)                                                                                                                                          | SSEQ ID NO (ECD) | Binding                   |                           |                             | Coimmobilization with anti-CD3   |
|                                                                                                                                                            |                  | ICOS MFI (parental ratio) | CD28 MFI (parental ratio) | CTLA-4 MFI (parental ratio) | IFN-gamma pg/mL (parental ratio) |
| N52Q, N119Q                                                                                                                                                | 400              | 35729 (0.98)              | 11040 (7.28)              | 26139 (6.87)                | 51 (1.03)                        |
| N84Q, N119Q                                                                                                                                                | 401              | 34777 (0.95)              | 1493 (0.98)               | 2877 (0.76)                 | 39 (0.80)                        |
| N52Q, N84Q, N168Q                                                                                                                                          | 402              | 27021 (0.74)              | 1584 (1.04)               | 958 (0.25)                  | 38 (0.78)                        |
| N52Q, N84Q, N207Q                                                                                                                                          | 403              | 39942 (1.09)              | 13396 (8.83)              | 26360 (6.93)                | 37 (0.76)                        |
| N84Q, N155Q, N168Q                                                                                                                                         | 404              | 27812 (0.76)              | 357 (0.24)                | 466 (0.12)                  | 30 (0.61)                        |
| N84Q, N168Q, N207Q                                                                                                                                         | 405              | 30659 (0.84)              | 737 (0.49)                | 861 (0.23)                  | 25 (0.52)                        |
| N84Q, N155H, N207Q                                                                                                                                         | 406              | 13557 (0.37)              | 685 (0.45)                | 607 (0.16)                  | 29 (0.59)                        |
| N155Q, N168Q, N207Q                                                                                                                                        | 407              | 13999 (0.38)              | 277 (0.18)                | 317 (0.08)                  | 40 (0.82)                        |
| N119Q, N155Q, N168Q                                                                                                                                        | 408              | 36896 (1.01)              | 4094 (2.70)               | 2179 (0.57)                 | 50 (1.02)                        |
| N119Q, N168Q, N207Q                                                                                                                                        | 409              | 29543 (0.81)              | 921 (0.61)                | 3744 (0.98)                 | 72 (1.47)                        |
| N84Q, N119Q, N207Q                                                                                                                                         | 410              | 21357 (0.58)              | 569 (0.38)                | 640 (0.17)                  | 59 (1.20)                        |
| N119Q, N155H, N207Q                                                                                                                                        | 411              | 37310 (1.02)              | 614 (0.40)                | 931 (0.24)                  | 86 (1.75)                        |
| N84Q, N119Q, N155Q                                                                                                                                         | 412              | 2675 (0.07)               | 262 (0.17)                | 291 (0.08)                  | 34 (0.70)                        |
| N52Q, N119Q, N155Q                                                                                                                                         | 413              | 27853 (0.76)              | 552 (0.36)                | 772 (0.20)                  | 42 (0.87)                        |
| N52H, N84Q, N119Q                                                                                                                                          | 414              | 40700 (1.11)              | 4580 (3.02)               | 4601 (1.21)                 | 39 (0.80)                        |
| N52H, N84Q, N168X, N207X                                                                                                                                   | 415              | 8796 (0.24)               | 587 (0.39)                | 481 (0.13)                  | 32 (0.66)                        |
| N52Q, N84Q, N155X, N168X                                                                                                                                   | 416              | 43521 (1.19)              | 6605 (4.35)               | 4811 (1.26)                 | 32 (0.66)                        |
| N52Q, N84Q, N119Q, N168Q                                                                                                                                   | 417              | 39342 (1.07)              | 4519 (2.98)               | 3300 (0.87)                 | 37 (0.76)                        |
| N52Q, N84Q, N119Q, N207Q                                                                                                                                   | 421              | 7011 (0.19)               | 602 (0.40)                | 433 (0.11)                  | 37 (0.75)                        |
| Wildtype ICOSL ECD                                                                                                                                         | 32               | 36602 (1.00)              | 1517 (1.00)               | 3804 (1.00)                 | 49 (1.00)                        |

## EXAMPLE 21

### Generation and Assessment of Fusion Molecules with HER2-Targetting Antibody

**[0542]** This Example describes the generation and assessment of variant ICOSL ECD-Fc fusion molecules conjugated with a tumor targeting agent to form a conjugate (“vIgD conjugate”).

**[0543]** The V-domain only of the ICOSL vIgD (N52H/N57Y/Q100P; set forth in SEQ ID NO:201) was fused to the amino and carboxyl termini of the light chain (FIG. 10A) and heavy chain (FIG. 10B) of a HER2 targeting antibody with intervening GGGSGGGS linkers. Exemplary configurations of vIgD conjugates are shown in FIG. 10C.

**[0544]** To assess HER2 binding, HER2 DNA or mock Expi293 transfectants were stained with titrated amounts of a HER2 targeting antibody containing the variant ICOSL conjugate (vIgD N52H/N57Y/Q100P conjugate) at a concentration of 100pM to 100nM. Control proteins, including wildtype ICOSL ECD-Fc fusion, wildtype PD-L2 IgV-Fc fusion, and variant ICOSL ECD-Fc fusion molecule with mutations at N52H/N57Y/Q100P, were also tested. Mean Fluorescence Intensity (MFI) or percent positive cells was determined for each transfectant as described in Example 6. All IgSF conjugates generated as shown in FIG 11A-B retained binding to HER2 compared to the endogenous level of HER2 expression observed in Expi293 cells. Similarly, vIgD conjugates also showed binding to cognate binding partners of ICOSL including CD28, CTLA-4, and ICOS.

**[0545]** Protein bioactivity and proliferation of human primary T cell in vitro assays were also characterized as described in Example 6. vIgD conjugates were bound overnight to 96-well plates at 30-0.1nM in the presence of 10nM anti-CD3. The plates were washed and 100,000 CFSE-labeled pan T cells were added to the plates and incubated for 72 hours. IFN gamma levels in supernatant were assayed by ELISA. As shown in FIG. 12, vIgD conjugates with the indicated configurations showed greater IFN gamma secretion and proliferation compared to parental wild-type ICOSL ECD-Fc fusion molecule conjugate.

## EXAMPLE 22

### Nanostring Transcriptional Signature of Primary Human T cells

**[0546]** Tissue culture plates were coated with 10 nM anti-CD3 with 40 nM of an Fc-control protein, wild-type ICOSL-Fc, wild-type CD80-Fc, both of these proteins, or a variant ICOSL Fc-fusion proteins with mutations as indicated. Purified human T cells were then plated on the protein coated plates and incubated at 37°C. Cultures from each treatment group described above were harvested at 24, 48 and 72 hours and total RNA was prepared from each cell sample. The RNA was transferred to Nanostring and a Cancer Immune chip was used to quantitate transcripts of 750 gene in each sample. Transcript values were normalized using Nanostring's proprietary software allowing comparison of transcript levels between treatment groups and over the various time points. As shown in FIG. 18 and FIG. 19, the variant ICOSL ECD-Fc polypeptides tested show altered inflammatory activity compared to wildtype CD80 ECD-Fc, wildtype ICOSL ECD-Fc, or a combination of both.

### **EXAMPLE 23**

#### **Generation and Assessment of Fusion Molecules with HER2-Targetting Antibody**

**[0547]** Proliferation of human T-cells co-cultured with VmAbs and HER2 expressing target cells was also characterized. CFSE-labeled pan T-cells were stimulated for 72 hours with K562-derived artificial target cells displaying cell surface anti-CD3 single chain Fv (OKT3) and HER2 in the presence of VmAbs or control proteins. Proliferation was measured by flow cytometric analysis of CFSE-dilution on CD4<sup>+</sup> or CD8<sup>+</sup> stained T-cells. Vmabs were assayed varying either target cell number or the concentration of the VmAb utilized. In the first assay, K562 target cells were titrated from 2500 to 78 cells/well and added to 100,000 T-cells for an effector:target (E:T) range of 40 to 1280:1. VmAbs, parental IgSF domain, or WT ICOSL were added at 1000pM. In the second assay, K562 target cells were added at 625 cells/well to 100,000 T-cells for an effector:target ratio of 160:1. VmAbs or control proteins were titrated and added at 3000 to 37pM. As shown in FIG. 20A and 20B, both configurations of the assay demonstrate VmAbs containing the vIgD-conjugate provide superior proliferation compared to the parental antibody, parental IgSF domain, or WT ICOSL. Additionally, vIgD-conjugates mediated proliferation at low E:T ratios (1280:1) or at low protein concentrations (37pM).

**[0548]** The present invention is not intended to be limited in scope to the particular disclosed embodiments, which are provided, for example, to illustrate various aspects of the invention.

Various modifications to the compositions and methods described will become apparent from the description and teachings herein. Such variations may be practiced without departing from the true scope and spirit of the disclosure and are intended to fall within the scope of the present disclosure.

**[0549]** The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

## WHAT IS CLAIMED:

1. A variant ICOS Ligand (ICOSL) polypeptide, comprising one or more amino acid substitutions N52H, N57Y or Q100R, in an unmodified ICOSL set forth in SEQ ID NO:32 or a portion of SEQ ID NO:32 comprising an IgV domain, wherein the IgV domain is amino acids 19-129 of SEQ ID NO:5; wherein:  
the variant ICOSL exhibits at least 95% sequence identity to SEQ ID NO:32 or the portion thereof comprising the IgV domain; and  
the variant ICOSL polypeptide specifically binds to the ectodomain of human CD28 with increased affinity compared to the binding of the unmodified ICOSL to the ectodomain of human CD28.
2. The variant ICOSL polypeptide of claim 1, wherein the variant ICOSL comprises N52Y/N57Y/F138L/L203P, N52H/N57Y/Q100P, N52H/C198R/T225A, N52H/K92R, N52H/S99G, N52Y/N57Y/Y152C, N52Y/N57Y/H129P/C198R, N52H/L161P/C198R, N52S/T113E, N52H/I143T, N52S/L80P, N52H/N57Y/Q100R, N52H/Q100R, A20V/N52H/N57Y/Q100R/S109G, N52H/N57Y/R61S/Q100R/V110D/L173S, N52H/N57Y/Q100R/V122A, N52H/N57Y/Q100R/F172S, E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/C198R, Q37R/N52H/N57Y/Q100R/V110N/S142F/C198R/D217V/R221G, N52H/N57Y/Q100R/V110D/C198R, N52H/N57Y/Q100R/V110D/V116A/L161M/F172S/S192G/C198R, F27S/N52H/N57Y/V110N, N52S/H94E/L96I/S109N/L166Q, V11E/N30D/N52H/N57Y/H94E/L96I/L98F/N194D/V210A/I218T, N52H/N57Y/H94E/L96I/F120I/S126T/W153R/I218N, N52H/N57Y/Q100R/V110D/A117T/T190S/C198R, N52H/N57Y/Q100R/V110D/F172S/C198R, S25G/F27C/N52H/N57Y/Q100R/V110D/E135K/L173S/C198R, N52H/N57Y/V110A/C198R/R221I, M10I/S13G/N52H/N57Y/D77G/V110A/H129P/I143V/F172S/V193M/C198R, N52H/N57Y/R61C/Y62F/Q100R/V110N/F120S/C198R, N52H/N57Y/Q100R/V110D/N144D/F172S/C198R, N52S/H94E/L98F/Q100R,

N52H/N57Y/Q100R/V110D/C198R/S212G, N52H/N57Y/Q100R/C198R, N52S/N194D, N52H/N57Y/Q100R/L102R/V110D/H115R/C198R, N52H/F78L/Q100R/C198R, N52H/N57Y/R75Q/Q100P/V110D, N52H/N57Y/L74Q/V110D/S192G, N52H/S121G/C198R, T43A/N52H/N57Y/L74Q/D89G/V110D/F172S, N52H/N57Y/Q100R/V110D/S132F/M175T, N52H/N57Y/Q100R/V107I/V110D/I154F/C198R/R221G, N52Y/F138L/L203P, N57Y/Q100R/C198R, Q100R/F138L, N52H/N57Y/Q100R/H115R/C198R, N52H/N57Y/Q100R/H115R/F172S/C198R, N52H/N57Y/Q100R/H115R/I143V/F172S/C198R, N52H/N57Y/Q100R/L102R/H115R/F172S/C198R, N52H/N57Y/Q100R/H115R/F172S/N194D, N52H/N57Y/H115R/F172S/C198R, N52H/N57Y/H115R, N52H/N57Y/Q100R/H115R, N52H/N57Y/Q100R/H115R/F172S/I224V, N52H/N57Y/Q100R/H115R/F172S, N52H/N57Y/Q100R/F172S, N52H/Q100R/H115R/I143T/F172S, N52H/N57Y/Q100P/H115R/F172S, N52Y/N57Y/Q100P/F172S, E16V/N52H/N57Y/Q100R/V110D/H115R/C198R, E16V/N52H/N57Y/Q100R/V110D/H115R/Y152C/K156M/F172S/C198R, N52H/N57Y/Q100P/C198R, , N52H/N57Y/Q100P/F172S/C198R, N52H/N57Y/Q100P/H115R, N52H/N57Y/Q100P/H115R/C198R, N52H/Q100R/C198R, N52H/Q100R/H115R/F172S, N52H/Q100R/H115R/F172S/C198R, or N52H/N57Y/Q100R/F172S/C198R.

3. The variant ICOSL polypeptide of claim 1 or claim 2, comprising the sequence of amino acids set forth in any of SEQ ID NOS: 110, 112, 113, 115, 117-119, 121, 122, 126-128, 135, 283, 285, 287, 289-291, 300-302, 304, 308, 310, 315-318, 321, 322, 364, 365, 367, 372, 375, 376, 381, 427, 430-432, 435, 439, 441-453, and 461-470 or a specific binding fragment thereof; or

the sequence of amino acids set forth in any of SEQ ID NOS: 198, 201-203, 205, 206, 212, 326-334, 337, 386 and 434 or a specific binding fragment thereof.

4. The variant ICOSL polypeptide of claim 1 or claim 2, wherein the variant ICOSL polypeptide specifically binds to the ectodomain of human ICOS and the ectodomain of human CD28 each with increased affinity compared to the binding of the unmodified ICOSL to the ectodomain of human ICOS and human CD28.

5. The variant ICOSL polypeptide of any of claims 1-4, wherein the increased affinity to the ectodomain of human CD28 is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold compared to the unmodified ICOSL.
6. The variant ICOSL polypeptide of any of claims 1-5 that is a soluble protein.
7. The variant ICOSL polypeptide of any of claims 1-6 that is linked to a multimerization domain.
8. The variant ICOSL polypeptide of any of claims 1-5, wherein the variant ICOSL polypeptide further comprises a transmembrane immunomodulatory protein comprising a transmembrane domain linked to the ECD or the specific binding fragment thereof of the variant ICOSL polypeptide, and further comprises a cytoplasmic signaling domain linked to the transmembrane domain.
9. A variant ICOSL-Fc fusion protein comprising a variant ICOSL polypeptide of any of claims 1-6 and an Fc domain.
10. The variant ICOSL-Fc fusion protein of claim 9, wherein the Fc domain is a variant Fc domain with reduced effector function .
11. A variant ICOSL-Fc fusion protein, comprising a variant ICOSL polypeptide linked via a linker to an Fc domain, wherein:
  - (i) the variant ICOSL polypeptide comprises amino acid substitution N52H, N57Y and Q100R in the amino acid sequence set forth in SEQ ID NO:32 or a portion thereof comprising an IgV domain, wherein the IgV domain is amino acids 19-129 of SEO ID NO:5; and
  - (ii) the Fc domain is a variant human IgG1 Fc with reduced effector function.
12. A conjugate, comprising a variant ICOSL polypeptide of any of claims 1-8 or a variant ICOSL-Fc fusion protein of any of claims claim 9-11 linked to a moiety.

13. The conjugate of claim 12, wherein the moiety is a targeting moiety that specifically binds to a molecule on the surface of a cell, optionally wherein the cell is an immune cell or a tumor cell.

14. The conjugate of claim 12 or claim 13, wherein the moiety is an antibody or antigen-binding fragment.

15. The conjugate of claim 14 that is a fusion protein.

16. A nucleic acid molecule(s), encoding a variant ICOSL polypeptide of any of claims 1-8 or a variant ICOSL-Fc fusion protein of any of claims 9-11.

17. A vector, comprising the nucleic acid molecule(s) of claim 16.

18. A cell, comprising the nucleic acid of claim 16 or the vector of claim 17.

19. A method of producing a variant ICOSL polypeptide or a variant ICOSL-Fc fusion protein, comprising introducing the nucleic acid molecule of claim 16 or vector of claim 17 into a host cell under conditions to express the protein in the cell.

20. The method of claim 19, further comprising isolating or purifying the variant ICOSL polypeptide or the variant ICOSL-Fc fusion protein from the cell.

21. An *in vitro* method of engineering a cell expressing a variant ICOSL variant polypeptide, comprising introducing a nucleic acid molecule encoding the variant ICOSL polypeptide of any of claims 1-8 or a variant ICOSL-Fc fusion protein of any of claims 9-11 into a host cell under conditions in which the polypeptide is expressed in the cell.

22. An engineered cell, comprising the variant ICOSL polypeptide of any of claims 1-8, the a variant ICOSL-Fc fusion protein of any of claims 9-11, the nucleic acid molecule of claim 16 or the vector of claim 17.

23. The engineered cell of claim 22, wherein the cell is an immune cell.
24. The engineered cell of claim 22 or claim 23, further comprising a chimeric antigen receptor (CAR) or an engineered T-cell receptor.
25. An infectious agent, comprising a nucleic acid molecule encoding a variant ICOSL polypeptide of any of claims 1-8 or a variant ICOSL-Fc fusion protein of any of claims 9-11.
26. The infectious agent of claim 25, wherein the infectious agent is a bacteria or a virus.
27. A pharmaceutical composition, comprising the variant ICOSL polypeptide of any of claims 1-8, a variant ICOSL-Fc fusion protein of any of claims 9-11, a conjugate of any of claims 12-15, an engineered cell of any of claims 22-24 or an infectious agent of claim 25 or claim 26, and a pharmaceutically acceptable excipient.
28. An article of manufacture comprising the pharmaceutical composition of claim 27 in a vial.
29. A kit comprising the pharmaceutical composition of claim 27, the article of manufacture of claim 28, and instructions for use.
30. Use of the pharmaceutical composition of claim 27 for the preparation of a medicament for the treatment of an autoimmune or inflammatory disease in a mammalian subject.
31. A pharmaceutical composition of claim 27 for use in modulating an immune response in a mammalian subject, wherein an autoimmune or inflammatory disease is to be treated by modulating the immune response.

32. The use of claim 30 or the pharmaceutical composition of claim 31, wherein the autoimmune or inflammatory disease is selected from inflammatory bowel disease, transplant, Crohn's disease, ulcerative colitis, multiple sclerosis, asthma, rheumatoid arthritis, psoriasis, graft versus host disease (GVHD), or systemic erythematosus (SLE).