CD40L-SPECIFIC TN3-DERIVED SCAFFOLDS AND METHODS OF USE THEREOF

REFERENCE TO RELATED APPLICATIONS

[0001] This application contains subject matter related, in part, to U.S. Provisional Application No. 61/546,028, filed on October 11, 2011, U.S. Provisional Application No. 61/323,708, filed on April 13, 2010; International Application No. PCT/US2011/032184, filed on April 12, 2011; International Application No. PCT/US2011/032188, filed on April 12, 2011; and PCT Application No. PCT/US2008/012398, filed on October 10, 2008 and published as International Publication No. WO 2009/058379 A2. Each of the above-referenced applications are incorporated herein by reference in their entireties.

REFERENCE TO SEQUENCE LISTING

[0002] This application incorporates by reference a Sequence Listing submitted with the application via EFS-Web as a test filed entitled ” CD40L-101WO1_SL.txt” created on October 3, 2012 and having a size of 232 kilobytes.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] The present invention relates in general to the field of antibody mimetics, specifically to scaffolds derived from the third fibronectin type III domain of human Tenascin C useful, for example, for the generation of products having novel binding characteristics. In particular, the invention relates to CD40L-specific Tn3 scaffolds, methods of making such scaffolds, and methods of use for diagnosis and treatment of systemic lupus erythematosus and other autoimmune and/or inflammatory disorders.

Background Art

[0004] This invention relates to CD40L-specific protein scaffolds that bind to CD40L, useful, for example, for the treatment of autoimmune and/or inflammatory disorders.

[0005] Biomolecules capable of specific binding to a desired target epitope are of great importance as therapeutics, research, and medical diagnostic tools. A well known example of this class of molecules is the antibody. Antibodies can be selected that bind

specifically and with affinity to almost any structural epitope. However, classical antibodies are structurally complex heterotetrameric molecules with are difficult to express in simple eukaryotic systems. As a result, most antibodies are produced using complex and expensive mammalian cell expression systems.

[0006] Proteins having relatively defined three-dimensional structures, commonly referred to as protein scaffolds, may be used as reagents for the design of engineered products. One particular area in which such scaffolds are useful is the field of antibody mimetic design. Antibody mimetics, *i.e.*, small, non-antibody protein therapeutics, capitalize on the advantages of antibodies and antibody fragments, such as high affinity binding of targets and low immunogenicity and toxicity, while avoiding some of the shortfalls, such as the tendency for antibody fragments to aggregate and be less stable than full-length IgGs.

[0007] These drawbacks can be addressed by using antibody fragments created by the removal of parts of the antibody native fold. However, this often causes aggregation when amino acid residues which would normally be buried in a hydrophobic environment such as an interface between variable and constant domain become exposed to the solvent. One example of a scaffold-based antibody mimetic is based on the structure of a Fibronectin type III domain (FnIII), a domain found widely across phyla and protein classes, such as in mammalian blood and structural proteins. The design and use of FnIII scaffolds derived from the third FnIII domain of human tenascin C is described in PCT applications PCT/US2011/032184 and PCT/US2011/032188.

[0008] CD40L is a member of the TNF family of molecules which is primarily expressed on activated T cells (including Th0, Th1, and Th2 subtypes, and forms homotrimers similar to other members of this family. Further, CD40L has also been found expressed on Mast cells, and activated basophils and eosinophils. CD40L binds to the CD40 receptor (CD40R) on antigen-presenting cells (APC), which leads to many effects depending on the target cell type. In general, CD40L plays the role of a costimulatory molecule and induces activation in APC in association with T cell receptor stimulation by MHC molecules on the APC.

[0009] Signaling through the CD40 receptor by CD40L initiates a cascade of events that result in the activation of the CD40 receptor-bearing cells and optimal CD4+ T cell priming. More specifically, the cognate interaction between CD40L and the CD40 receptor promotes the differentiation of B cells into antibody secreting cells and memory B cells (Burkly, In Adv. Exp. Med. Bio., Vol. 489., D. M. Monroe, U. Hedner, M. R. Hoffman, C. Negrier, G. F. Savidge, and G. C. I. White, eds. Klower Academic/Plenum Publishers, 2001,

p. 135). Additionally, the interaction between CD40L and the CD40 receptor promotes cell-mediated immunity through the activation of macrophages and dendritic cells and the generation of natural killer cells and cytotoxic T lymphocytes (see Burkly, supra).

[0010] The interaction between CD40L and the CD40 receptor has been shown to be important in several experimentally induced autoimmune diseases, such as collagen-induced arthritis, experimental allergic encephalomyelitis, oophoritis, colitis, drug-induced lupus nephritis. Specifically, it has been shown that disease induction in all of these models can be blocked with CD40L antagonists at the time of antigen administration. The blockade of disease using anti-CD40L antagonists has also been seen in animal models of spontaneous autoimmune disease, including insulin- dependent diabetes and lupus nephritis, as well as in graft-vs-host disease, transplant, pulmonary fibrosis, and atherosclerosis disease models.

[0011] Disruption of the CD40L/CD40R pathway via CD40L blockade has been shown to be beneficial in many autoimmune mediated diseases (for example, but not limited to systemic lupus erythermatosis (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), inflammatory bowel disease (IBD) and allograft rejection. For example, treatment with anti-CD40L antibodies prevented or improved nephritis in a collagen-induced arthritis mouse model (Mohan *et al.* J. Immuno. 154:1470). Additionally, anti-CD40L antibodies preserved renal function in SNF1 mice with established nephritis. (Kalled *et al.* J. Immuno. 160:2158). CD40L levels correlate closely with clinical disease severity (*i.e.*, reduction of inflammation), and damage in target tissue in both non-humans and humans.

[0012] SLE is a progressive and sometimes fatal autoimmune disease. The diverse presentations of lupus range from rash and arthritis through anemia and thrombocytopenia to even psychosis. There is clear evidence showing that many arms of the immune system are involved in the inflammatory process leading to kidney, skin, brain disease and thrombosis. One characteristic feature of SLE is the loss of B cell tolerance and autoantibodies are prominent in patients with this disease. In lupus kidney disease, anti-double-stranded DNA autoantibodies can form antibody nucleosome complexes and settle in the renal glomerular basement membrane. These immune complexes in turn activate complement, which can lead to glomerulonephritis.

[0013] Expression of CD40R as well as CD40L has been found elevated in patients with SLE. The increased costimulatory signal likely contributes to the pathological inflammatory response found in the SLE. SLE T cells have spontaneously increased activation associated with a reduced threshold of activation to self-antigens. Further, these cells are hyporesponsive to further antigenic stimulation, are resistant to apoptosis, have

increased survival after activation and have many altered intracellular signaling pathways. Following CD40R activation on APCs by T cell CD40L, both APC and T cells become activated, produce cytokines and in SLE contribute to the production of pathogenic autoantibodies and tissue injury (lupus nephritis). Blockade of the CD40R/CD40L pathway is effective, alone or in combination, in blocking disease in lupus-prone mice. In patients with SLE, a humanized anti-CD40L antibody reduced anti-dsDNA and B cells, proteinuria, and improved SLE disease severity.

[0014] However, targeting CD40L with traditional antibodies has raised significant safety concerns. For example, a study with anti-CD40L antibody 5c8 (BIOGEN®) in patients suffering with chronic refractory idiopathic thrombocytopenic purpura (ITP) was placed on hold because of reported thromboebolic complications (Davidson *et al*. *Arth Rheu*, 43:S271). Further, additional trials with alternative antibodies directed against CD40L gave rise to other thrombotic related complications (Davis *et al*. *Arth Rheu*, 43:S281; Schuler, *Transplantation*, 77:717). Given the complications with antibody-directed antagonism of CD40L, there is an unmet need to target and antagonize CD40L with a non-antibody alternative. Thus, targeting CD40L with a Tn3-based scaffold is an attractive alternative by avoiding Fab2 and/or Fc-mediated platelet aggregation and the downstream side effects.

[0015] Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[0016] The invention provides a Tn3 scaffold comprising a CD40L-specific monomer subunit, wherein the monomer subunit comprises seven beta strands designated A, B, C, D, E, F, and G, and six loop regions designated AB, BC, CD, DE, EF, and FG, and wherein the Tn3 scaffold specifically binds to CD40L. In some embodiments, the Tn3 scaffold comprises a single CD40L-specific monomer subunit. In other embodiments, the Tn3 scaffold comprises two CD40L-specific monomer subunits connected in tandem. In some specific embodiments, the Tn3 scaffold comprises two CD40L-specific monomer subunits which are directly connected.

[0017] In some embodiments, two CD40L-specific monomer subunits are connected by a linker. In other embodiments, the linker comprises a peptide linker, which can be a flexible peptide linker. In some embodiments, the peptide linker comprises a $(G_mX)_n$ sequence wherein X is Serine (S), Alanine (A), Glycine (G), Leu (L), Isoleucine (I), or Valine

(V); m and n are integer values; m is 1, 2, 3 or 4; and, n is 1, 2, 3, 4, 5, 6, or 7. In some embodiments, the peptide linker comprises SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 142 or SEQ ID NO: 143.

[0018] In some embodiments, the binding of a Tn3 scaffold comprising two CD40L-specific monomer subunits to CD40L is improved over that of a Tn3 scaffold comprising a single CD40L-specific monomer subunit. In other embodiments, the binding of a Tn3 scaffold comprising two CD40L-specific monomer subunits to CD40L improves the action on the target over that of a Tn3 scaffold comprising a single CD40L-specific monomer subunit. In other embodiments, the improvement in the binding of the Tn3 scaffold to CD40L is an improvement in binding affinity, an improvement in binding avidity, or both. In certain embodiments, the binding affinity of a Tn3 scaffold comprising two CD40L-specific monomer subunits to CD40L and the Tn3 scaffold protein stability are improved over those of a Tn3 scaffold comprising a single CD40L-specific monomer subunit. In some embodiments, the binding avidity of a Tn3 scaffold comprising two CD40L-specific monomer subunits for CD40L and the Tn3 scaffold protein stability are improved over those of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

[0019] In some embodiments, at least one CD40L-specific monomer subunit in a Tn3 scaffold is bound to a linker, or to a heterologous moiety. In other embodiments, a linker or a heterologous moiety in a Tn3 scaffold is conjugated to the N-terminus or the C-terminus of a CD40L-specific monomer subunit. In certain embodiments, the linker bound to a CD40L-specific monomer subunit in a Tn3 scaffold comprises a peptide linker, which in some embodiments can be a flexible peptide linker. This peptide linker can comprise in certain embodiments a $(G_mX)_n$ sequence wherein X is Serine (S), Alanine (A), Glycine (G), Leucine (L), Isoleucine (I), or Valine (V); m and n are integers; m is 1, 2, 3 or 4; and, n is 1, 2, 3, 4, 5, 6, or 7. In some embodiments, the peptide linker bound to a CD40L-specific monomer subunit in a Tn3 scaffold comprises SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 142, or SEQ ID NO: 143.

[0020] In some embodiments, the Tn3 scaffold comprises a linker which comprises a functional moiety. In some embodiments, this functional moiety is an immunoglobulin or a fragment thereof. In certain embodiments, this immunoglobulin or fragment thereof comprises an Fc domain. In some embodiments, this Fc domain fails to induce at least one FcγR-mediated effector function. In some embodiments, this at least one FcγR-mediated effector function is ADCC.

[0021] In some embodiments, the Tn3 scaffold comprises at least one CD40L-specific monomer subunit bound to a heterologous moiety. In some embodiments, this heterologous moiety comprises a composition selected from the group consisting of: a protein, a peptide, a protein domain, a linker, a drug, a toxin, a cytotoxic agent, an imaging agent, a radionuclide, a radioactive compound, an organic polymer, an inorganic polymer, a polyethylene glycol (PEG), biotin, an albumin, a human serum albumin (HSA), a HSA FcRn binding portion, an antibody, a domain of an antibody, an antibody fragment, a single chain antibody, a domain antibody, an albumin binding domain, an enzyme, a ligand, a receptor, a binding peptide, a non-FnIII scaffold, an epitope tag, a recombinant polypeptide polymer, a cytokine, and a combination of two or more of said moieties.

[0022] In some embodiments, the Tn3 scaffold comprises at least one CD40L-specific monomer subunit conjugated to PEG. In some embodiments, the Tn3 scaffold comprises at least one CD40L-specific monomer subunit conjugated to an albumin. In certain embodiments, this albumin is human serum albumin (HSA). In other embodiments, this HSA is a variant HSA. In some specific embodiments, the amino acid sequence of the variant HSA is SEQ ID NO: 133. In other embodiments, the variant HSA has at least one improved property compared with a native HSA or a native HSA fragment. In certain embodiments, the improved property is an altered plasma half-life compared with the plasma half-life of a native HSA or a native HSA fragment. In some embodiments, the altered plasma half-life is a longer plasma half-life compared with the plasma half-life of a native HSA or a native HSA fragment. In other embodiments, the altered plasma half-life is a shorter plasma half-life compared with the plasma half-life of a native HSA or a native HSA fragment.

[0023] In some embodiments, the Tn3 scaffold is fused to an HSA variant comprising at least one amino acid substitution in HSA domain III. In other embodiments, the Tn3 scaffold is fused to an HSA variant comprising the sequence of full-length mature HSA (SEQ ID NO: 133 or 138) or a fragment thereof, except for at least one amino acid substitution, numbered relative to the position in full length mature HSA, at a position selected from the group consisting of 407, 415, 463, 500, 506, 508, 509, 511, 512, 515, 516, 521, 523, 524, 526, 535, 550, 557, 573, 574, and 580; wherein the at least one amino acid substitution does not comprise a lysine (K) to glutamic acid (E) at position 573, and wherein the Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to such HSA variant.

[0024] In some embodiments, the Tn3 scaffold is fused to an HSA variant wherein at least one amino acid substitution, numbered relative to the position in full length mature

HSA, is at a position selected from the group consisting of 463, 508, 523, and 524, wherein said Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to said HSA variant. In some embodiments, the HSA variant comprises the sequence of full-length mature HSA (SEQ ID NO: 133 or 138) or a fragment thereof, except for at least one amino acid substitution, numbered relative to the position in full length mature HSA, selected from the group consisting of: (a) substitution of Leucine (L) at position 407 to Asparagine (N) or Tyrosine (Y); (b) substitution of Valine (V) at position 415 to Threonine (T); (c) substitution of Leucine (L) at position 463 to Asparagine (N); (d) substitution of Lysine (K) at position 500 to Arginine (R); (e) substitution of Threonine (T) at position 506 to Tyrosine (Y); (f) substitution of Threonine (T) at position 508 to Arginine (R); (g) substitution of Phenylalanine (F) at position 509 to Methionine (M) or Tryptophan (W); (h) substitution of Alanine (A) at position 511 to Phenylalanine (F); (i) substitution of Aspartic Acid (D) at position 512 to Tyrosine (Y); (j) substitution of Threonine (T) at position 515 to Glutamine (Q); (k) substitution of Leucine (L) at position 516 to Threonine (T) or Tryptophan (W); (l) substitution of Arginine (R) at position 521 to Tryptophan (W); (m) substitution of Isoleucine (I) at position 523 to Aspartic Acid (D), Glutamic Acid (E), Glycine (G), Lysine (K), or Arginine (R); (n) substitution of Lysine (K) at position 524 to Leucine (L); (o) substitution of Glutamine (Q) at position 526 to Methionine (M); (p) substitution of Histidine (H) at position 535 to Proline (P); (q) substitution of Aspartic Acid (D) at position 550 to Glutamic Acid (E); (r) substitution of Lysine (K) at position 557 to Glycine (G); (s) substitution of Lysine (K) at position 573 to Phenylalanine (F), Histidine (H), Proline (P), Tryptophan (W), or Tyrosine (Y); (t) substitution of Lysine (K) at position 574 to Asparagine (N); (u) substitution of Glutamine (Q) at position 580 to Lysine (K); and, (v) a combination of two or more of said substitutions, wherein said Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to said HSA variant.

[0025] In some embodiments, the Tn3 scaffold is fused to an HSA variant comprising the sequence of full-length mature HSA (SEQ ID NO: 133 or 138) or a fragment thereof, except for at least one amino acid substitution, numbered relative to the position in full length mature HSA, selected from the group consisting of: (a) substitution of Leucine (L) at position 463 to Asparagine (N); (b) substitution of Threonine (T) at position 508 to Arginine (R); (c) substitution of Isoleucine (I) at position 523 to Aspartic Acid (D), Glutamic Acid (E), Glycine (G), Lysine (K), or Arginine (R); (d) substitution of Lysine (K) at position 524 to Leucine (L); and, (e) a combination of two or more of said substitutions, wherein said

Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to said HSA variant.

[0026] In some embodiments, the Tn3 scaffold comprises at least two identical CD40L-specific monomer subunits. In other embodiments, the Tn3 scaffold comprises at least two different CD40L-specific monomer subunits. In some embodiments, the Tn3 scaffold is a CD40 receptor agonist. In other embodiments, the Tn3 scaffold is a CD40 receptor antagonist.

[0027] In some embodiments, the Tn3 scaffold comprises at least two CD40L-specific monomer subunits which specifically bind to the same CD40L epitope. In other embodiments, the Tn3 scaffold comprises at least two CD40L-specific monomer subunits which specifically bind to different CD40L epitopes. In some embodiments, these different CD40L epitopes are non-overlapping epitopes. In other embodiments, these different CD40L epitopes are overlapping epitopes.

[0028] In some embodiments, the Tn3 scaffold binds to at least two CD40L molecules. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit which binds to at least two CD40L molecules.

[0029] In some embodiments, the beta strands of at least one CD40L-specific monomer subunit of the Tn3 scaffold have at least 90% sequence identity to the beta strands of SEQ ID NO: 3. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising a A beta strand comprising SEQ ID NO: 11, or comprising a A beta strand comprising SEQ ID NO: 11 except for at least one mutation. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising a B beta strand comprising SEQ ID NO: 12, or comprising a B beta strand comprising SEQ ID NO: 12 except for at least one mutation. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising a C beta strand comprising SEQ ID NO: 13 or 14, or comprising a C beta strand comprising SEQ ID NO: 13 or 14 except for at least one mutation, and wherein the cysteine in SEQ ID NO: 13 or 14 is not substituted. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising a D beta strand comprising SEQ ID NO: 15, or comprising a D beta strand comprising SEQ ID NO: 15 except for at least one mutation.

[0030] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising an E beta strand comprising SEQ ID NO: 16, or comprising an E beta strand comprising SEQ ID NO: 16 except for at least one mutation. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising an

F beta strand comprising SEQ ID NO: 17, or comprising an F beta strand comprising SEQ ID NO: 17 except for at least one mutation, and wherein the cysteine in SEQ ID NO: 17 is not substituted. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising a G beta strand comprising SEQ ID NO: 18, or comprising a G beta strand comprising SEQ ID NO: 18 except for at least one mutation.

[0031] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising the amino acid sequence:

IEV$(X_{AB})_n$ALITW$(X_{BC})_n$CELX$_1$YGI$(X_{CD})_n$TTIDL$(X_{DE})_n$YSI$(X_{EF})_n$YEVSLIC$(X_{FG})_n$KETFTT

wherein: X_{AB}, X_{BC}, X_{CD}, X_{DE}, X_{EF}, and X_{FG} represent the amino acid residues present in the sequences of the AB, BC, CD, DE, EF, and FG loops, respectively; X_1 represents amino acid residue A or T; and, the length of the loop *n* is an integer between 2 and 26.

[0032] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the AB loop comprises SEQ ID NO: 4 or SEQ ID NO: 136, the sequence of the CD loop comprises SEQ ID NO: 6, and the sequence of the EF loop comprises SEQ ID NO: 8 or SEQ ID NO: 137. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the BC loop comprises a sequence selected from the group consisting of SEQ ID NOs: 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, and 168. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the DE loop comprises a sequence selected from the group consisting of SEQ ID NOs: 94, 95, 96, 97, 98, and 169. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the FG loop comprises a sequence selected from the group consisting of SEQ ID NOs: 9, 99, 139, and 170.

[0033] In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the BC loop comprises a sequence selected from the group consisting of SEQ ID NOs: 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, and 174. In certain embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the DE loop comprises a sequence selected from the group consisting of SEQ ID NOs: 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, and 175. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the FG loop comprises a sequence selected from the groups consisting of SEQ ID NOs: 129, 130, and

177. In certain embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the AB loop comprises a sequence selected from the group consisting of SEQ ID NOs: 4 and 136.

[0034] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit, wherein the sequence of the BC loop comprises SEQ ID NO: 83, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 83, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 99. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 84, the sequence of the DE loop comprises SEQ ID NO: 95, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139.

[0035] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 85, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 86, the sequence of the DE loop comprises SEQ ID NO: 96, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 87, the sequence of the DE loop comprises SEQ ID NO: 97, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139.

[0036] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 88, the sequence of the DE loop comprises SEQ ID NO: 95, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 89, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 90, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139.

[0037] In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 91, the sequence of the DE loop comprises SEQ ID NO: 95, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 92, the sequence of the DE loop comprises SEQ ID NO: 98, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 93, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139.

[0038] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 100, the sequence of the DE loop comprises SEQ ID NO: 118, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 101, the sequence of the DE loop comprises SEQ ID NO: 119, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 102, the sequence of the DE loop comprises SEQ ID NO: 120, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 103, the sequence of the DE loop comprises SEQ ID NO: 121, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the AB loop comprises SEQ ID NO: 136.

[0039] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 104, the sequence of the DE loop comprises SEQ ID NO: 122, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 105, the sequence of the DE loop comprises SEQ ID NO: 121, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 106, the sequence of the DE loop comprises SEQ ID NO: 123, and the sequence of the FG loop

comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the AB loop comprises SEQ ID NO: 136.

[0040] In certain embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 107, the sequence of the DE loop comprises SEQ ID NO: 123, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 108, the sequence of the DE loop comprises SEQ ID NO: 118, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 109, the sequence of the DE loop comprises SEQ ID NO: 123, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 110, the sequence of the DE loop comprises SEQ ID NO: 121, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the AB loop comprises SEQ ID NO: 136.

[0041] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 111, the sequence of the DE loop comprises SEQ ID NO: 123, and the sequence of the FG loop comprises SEQ ID NO: 130. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 108, the sequence of the DE loop comprises SEQ ID NO: 121, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 112, the sequence of the DE loop comprises SEQ ID NO: 124, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 113, the sequence of the DE loop comprises SEQ ID NO: 125, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the AB loop comprises SEQ ID NO: 136.

[0042] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 114, the sequence of the DE loop comprises SEQ ID NO: 118, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the Tn3 scaffold comprises a CD40L-

specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 115, the sequence of the DE loop comprises SEQ ID NO: 126, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 116, the sequence of the DE loop comprises SEQ ID NO: 127, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the BC loop comprises SEQ ID NO: 117, the sequence of the DE loop comprises SEQ ID NO: 128, and the sequence of the FG loop comprises SEQ ID NO: 129. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein the sequence of the AB loop comprises SEQ ID NO: 136.

[0043] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising a sequence selected from the group consisting of SEQ ID NO: 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42 and 146. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprises the amino acid sequence:

IEVKDVTDTTALITWX_1D$X_2X_3X_4X_5X_6X_7X_8$CELTYGIKDVPGDRTTIDLWX_9HX_{10}AX_{11}YSIGNLKPDTEYEVSLICRX_{12}GDMSSNPAKETFTT (SEQ ID NO: 167)

wherein: (a) X_1 represents amino acid residue serine (S) or leucine (L); (b) X_2 represents amino acid residue aspartic acid (D) or glutamic acid (E); (c) X_3 represents amino acid residue histidine (H), isoleucine (I), valine (V), phenylalanine (F) or tryptophan (W); (d) X_4 represents amino acid residue alanine (A), glycine (G), glutamic acid (E) or aspartic acid (D); (e) X_5 represents amino acid residue glutamic acid (E), leucine (L), glutamine (Q), serine (S), aspartic acid (D) or asparagine (N); (f) X_6 represents amino acid residue phenylalanine (F) or tyrosine (Y); (g) X_7 represents amino acid residue isoleucine (I), valine (V), histidine (H), glutamic acid (E) or aspartic acid (D); (h) X_8 represents amino acid residue glycine (G), tryptophan (W) or valine (V); (i) X_9 represents amino acid residue tryptophan (W), phenylalanine (F) or tyrosine (Y); (j) X_{10} represents amino acid residue serine (S), glutamine (Q), methionine (M) or histidine (H); (k) X_{11} represents amino acid residue tryptophan (W) or histidine (H); and, (l) X_{12} represents amino acid residue arginine (R) or serine (S).

[0044] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising a sequence selected from the group consisting of SEQ ID NO: 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, and 82.

[0045] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit comprising the amino acid sequence:

IEVX$_1$DVTDTTALITWX$_2$X$_3$RSX$_4$X$_5$X$_6$X$_7$X$_8$X$_9$X$_{10}$CELX$_{11}$YGIKDVPGDRTTIDLX$_{12}$X$_{13}$X$_{14}$X$_{15}$YVHYSIGNLKPDTX$_{16}$YEVSLICLTTDGTYX$_{17}$NPAKETFTT (SEQ ID NO: 171)

wherein:

(a) X$_1$ represents amino acid residue lysine (K) or glutamic acid (E);

(b) X$_2$ represents amino acid residue threonine (T) or isoleucine (I);

(c) X$_3$ represents amino acid residue asparagine (N) or alanine (A);

(d) X$_4$ represents amino acid residue serine (S), leucine (L), alanine (A), phenylalanine (F) or tyrosine (Y);

(e) X$_5$ represents amino acid residue tyrosine (Y), alanine (A), glycine (G), valine (V), isoleucine (I) or serine (S);

(f) X$_6$ represents amino acid residue tyrosine (Y), serine (S), alanine (A) or histidine (H);

(g) X$_7$ represents amino acid residue asparagine (N), aspartic acid (D), histidine (H) or tyrosine (Y);

(h) X$_8$ represents amino acid residue leucine (L), phenylalanine (F), histidine (H) or tyrosine (Y);

(i) X$_9$ represents amino acid residue histidine (H), proline (P), serine (S), leucine (L) or aspartic acid (D);

(j) X$_{10}$ represents amino acid residue glycine (G), phenylalanine (F), histidine (H) or tyrosine (Y);

(k) X$_{11}$ represents amino acid residue alanine (A) or threonine (T);

(l) X$_{12}$ represents amino acid residue serine (S), asparagine (N), glutamic acid (E), asparagine (R) or aspartic acid (D);

(m) X$_{13}$ represents amino acid residue serine (S), glutamine (Q), threonine (T), asparagine (N) or alanine (A);

(n) X$_{14}$ represents amino acid residue proline (P), valine (V), isoleucine (I) or alanine (A) or no amino acid;

(o) X$_{15}$ represents amino acid residue isoleucine (I) or no amino acid;

(p) X$_{16}$ represents amino acid residue glutamic acid (E) or lysine (K); and,

(q) X$_{17}$ represents amino acid residue serine (S) or asparagine (N).

[0046] In some embodiments, the Tn3 scaffold comprises a sequence selected from the group consisting of SEQ ID NOs: 134, 135, 205, 206, 207 and 208. In other embodiments, the Tn3 scaffold consists of a sequence selected from the group consisting of SEQ ID NO: 134, 135, 205, 206, 207 and 208.

[0047] In some embodiments, the Tn3 scaffold comprises a sequence selected from the group consisting of SEQ ID NOs: 201, 202, 203, and 204. In some embodiments, the Tn3 scaffold consists of a sequence selected from the group consisting of SEQ ID NOs: 201, 202, 203, and 204. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein said CD40L-specificity is towards human CD40L. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit wherein said CD40L-specificity is towards membrane bound CD40L (SEQ ID NO: 1), soluble CD40L (SEQ ID NO: 2), or a fragment thereof. In some specific embodiments, the Tn3 scaffold binds CD40L and prevents binding of CD40L to CD40.

[0048] The invention also provides a method of altering an activity in a CD40L expressing cell comprising contacting the cell with a Tn3 scaffold, wherein the Tn3 scaffold binds CD40L and prevents binding of CD40L to CD40. In some embodiments, the Tn3 scaffold binds to CD40L with an affinity (K_d) of about 1 μM or less, or about 500 nM or less, or about 100 nM or less, or about 50 nM or less, or about 25 nM or less, or about 10 nM or less, or about 5 nM or less, or about 2 nM or less.

[0049] In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit which specifically binds to a CD40L epitope comprising amino acids located at positions 142 to 155, 200 to 230, or 247 to 251 of SEQ ID NO: 2. In some embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit that interacts with CD40L amino acids E142, Y146, M148, N151, L155, R200, R203, and E230. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit that interacts with CD40L amino acids R203, I204, V247, H249, and T251. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit that interacts with CD40L amino acids E142, Y146, M148, N151, L155, which are located in a first CD40L molecule, and with CD40L amino acids R200, R203, and E230, which are located in a second CD40L molecule. In other embodiments, the Tn3 scaffold comprises a CD40L-specific monomer subunit that interacts with CD40L amino acids R203 and I204, which are located in a first CD40L molecule, and with CD40L amino acids V247, H249, and T251, which are located in a second CD40L molecule.

[0050] The invention also provides polypeptides comprising one or more CD40L-specific Tn3 monomer, including but not limited to the serum albumin fusions described herein.

[0051] The invention also provides an isolated nucleic acid molecule encoding a CD40L-specific Tn3 scaffold, an expression vector comprising the nucleic acid molecule encoding a CD40L-specific Tn3 scaffold, and a host cell comprising such vector. The invention also provides a method of producing a Tn3 scaffold comprising culturing the host cell under conditions in which the CD40L-specific Tn3 scaffold encoded by the nucleic acid molecule is expressed.

[0052] The invention also provides a pharmaceutical composition comprising a CD40L-specific Tn3 scaffold and a pharmaceutically acceptable excipient. The invention also provides a method of preventing, treating, ameliorating, or managing autoimmune disease in a patient in need thereof comprising administering an effective amount of a pharmaceutical composition comprising a CD40L-specific Tn3 scaffold.

[0053] The invention also provides a method of reducing the frequency or quantity of corticosteroid administered to a patient with an autoimmune disease comprising administering to the patient a therapeutically effective amount of a pharmaceutical composition comprising a CD40L-specific Tn3 scaffold.

[0054] The autoimmune disease treated by the administration of a CD40L-specific Tn3 scaffold can be alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diseases of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, Sjogren's syndrome, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation, sepsis, rheumatoid arthritis, peritonitis, Crohn’s disease, reperfusion injury, septicemia, endotoxic shock, cystic fibrosis, endocarditis, psoriasis, arthritis (*e.g.*, psoriatic arthritis), anaphylactic shock, organ ischemia, reperfusion injury, spinal cord injury and allograft rejection. autoimmune thrombocytopenia, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatrical pemphigoid, CREST syndrome, cold agglutinin disease, Crohn's disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, glomerulonephritis, Graves' disease, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis,

idiopathic thrombocytopenia purpura (ITP), IgA neuropathy, juvenile arthritis, lichen planus, lupus erythematosus, Meniere's disease, mixed connective tissue disease, multiple sclerosis, type 1 or immune-mediated diabetes mellitus, myasthenia gravis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychrondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynauld's phenomenon, Reiter's syndrome, Rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, lupus erythematosus, takayasu arteritis, temporal arteristis/giant cell arteritis, ulcerative colitis, uveitis, vasculitides such as dermatitis herpetiformis vasculitis, vitiligo, and Wegener's granulomatosis.

[0055] In some specific embodiments, the autoimmune disease treated by the administration of a CD40L-specific Tn3 scaffold is Systemic Lupus Erythematosus (SLE).

[0056] Methods of treatment with CD40L-specific Tn3 scaffolds can further comprise an additional therapy, such as immunotherapy, biological therapy, chemotherapy, radiation therapy, or small molecule drug therapy.

[0057] The invention also provides a protein crystal comprising a Tn3 scaffold consisting of SEQ ID NO: 20 in a complex with soluble CD40L (SEQ ID NO: 2) wherein the crystal has a crystal lattice in a $P2_12_12_1$ orthorhombic space group and unit cell dimensions, +/- 0.1%, of a=85.69 Å , b=90.64 Å , c=95.56 Å. In some embodiments, the asymmetric unit of the crystal comprises a trimer of CD40L and three molecules of Tn3 scaffold. In other embodiments, the crystals diffract X-rays for a determination of structure coordinates to a resolution of a value equal to or less that 3.2 Å.

[0058] The invention also provides a protein crystal comprising a Tn3 scaffold consisting of SEQ ID NO: 68 in a complex with soluble CD40L (SEQ ID NO: 2) wherein the crystal has a crystal lattice in a $P2_13$ cubic space group and unit cell dimensions, +/- 0.1%, of a=b=c=97.62 Å. In some embodiments, the asymmetric unit of the crystal comprises one CD40L molecule and one Tn3 scaffold molecule. In other embodiments, the crystal diffracts X-rays for a determination of structure coordinates to a resolution of a value equal to or less than 2.7 Å.

[0059] The invention also provides a protein crystal comprising a Tn3 scaffold consisting of SEQ ID NO: 28 or 146 in a complex with soluble CD40L (SEQ ID NO: 2) wherein the crystal has a crystal lattice in a P321 space group and unit cell dimensions, +/- 0.1%, of a=95.53 Å , b=93.53 Å , c=66.69 Å. In some embodiments the asymmetric unit of the crystal comprises one CD40L molecule and one Tn3 scaffold molecule. In other

embodiments the crystal diffracts X-rays for a determination of structure coordinates to a resolution of a value equal to or less than 2.8 Å

[0060] The invention also provides a protein crystal comprising two different Tn3 scaffolds consisting of SEQ ID NO: 68 and SEQ ID NO: 28 or 146 in a complex with soluble CD40L (SEQ ID NO: 2) wherein the crystal has a crystal lattice in a $P2_1$ cubic space group and unit cell dimensions, +/- 0.1%, of a=80.32 Å, b=143.48 Å, c=111.27 Å, β=98.22 Å. In some embodiments the asymmetric unit of the crystal comprises two CD40L trimers and six of each Tn3 scaffold molecule. In other embodiments the crystal diffracts X-rays for a determination of structure coordinates to a resolution of a value equal to or less than1.9Å

[0061] In some embodiments, the protein crystal is produced by using sitting-drop vapor diffusion. The invention also provides a method of making a protein crystal, comprising: (a) mixing a volume of a solution comprising a Tn3 scaffold comprising a CD40L-specific monomer subunit in a complex with CD40L with a volume of a reservoir solution comprising a precipitant; and (b) incubating the mixture obtained in step (a) in a closed container, under conditions suitable for crystallization until the protein crystal forms. In some embodiments, the method to produce the protein crystal comprises using sitting-drop vapor diffusion.

[0062] In some embodiments, the method to make a protein crystal is used to produce crystals comprising the CD40L-specific Tn3 monomer subunits of SEQ ID NO: 20, SEQ ID NO: 28, SEQ ID NO: 68 or SEQ ID NO: 146.

[0063] The invention also provides a machine-readable data storage medium comprising a data storage material encoded with machine-readable instructions for: (a) transforming data into a graphical three-dimensional representation for the structure of a portion of a protein crystal of a Tn3 scaffold comprising a CD40L-specific monomer subunit complexed with CD40L; and, (b) causing the display of said graphical three-dimensional representation. In some embodiments, such Tn3 scaffold comprises SEQ ID NO: 20, SEQ ID NO: 28, SEQ ID NO: 68 or SEQ ID NO: 146. In other embodiments, such protein crystal is:

[0064] (a) a protein crystal comprising a Tn3 scaffold consisting of SEQ ID NO: 20 in a complex with soluble CD40L (SEQ ID NO: 2) wherein the crystal has a crystal lattice in a $P2_12_12_1$ orthorhombic space group and unit cell dimensions, +/- 0.1%, of a=85.69 Å , b=90.64 Å , c=95.56 Å;

[0065] (b) a protein crystal comprising a Tn3 scaffold consisting of SEQ ID NO: 68 in a complex with soluble CD40L (SEQ ID NO: 2) wherein the crystal has a crystal lattice in a $P2_13$ cubic space group and unit cell dimensions, +/- 0.1%, of a=b=c=97.62 Å; or

[0066] (c) a protein crystal comprising a Tn3 scaffold consisting of SEQ ID NO: 20 and a Tn3 scaffold consisting of SEQ ID NO: 68, wherein both Tn3 scaffold are in a complex with soluble CD40L (SEQ ID NO: 2)

[0067] (d) a protein crystal comprising a Tn3 scaffold consisting of SEQ ID NO: 28 or 146 in a complex with soluble CD40L (SEQ ID NO: 2) wherein the crystal has a crystal lattice in a P321 space group and unit cell dimensions, +/- 0.1%, of a=95.53 Å , b=93.53 Å , c=66.69 Å

[0068] (e) a protein crystal comprising two different Tn3 scaffolds consisting of SEQ ID NO: 68 and SEQ ID NO: 28 or 146 in a complex with soluble CD40L (SEQ ID NO: 2) wherein the crystal has a crystal lattice in a $P2_1$ cubic space group and unit cell dimensions, +/- 0.1%, of a=80.32 Å, b=143.48 Å, c=111.27 Å, β=98.22 Å.

BRIEF DESCRIPTION OF THE DRAWINGS

[0069] For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

[0070] FIG. 1A shows the inhibition of murine CD40L (MuCD40L)-induced CD86 expression measured using a D10G4.1/PBMC (Peripheral Blood mononuclear Cell) assay. The M13 mouse CD40L-specific Tn3 scaffold, its M31 affinity optimized variant (approximately 20x affinity improvement), the anti-CD40L MR1 monoclonal antibody, and a negative control were assayed. IC_{50} values are also shown.

[0071] FIG. 1B shows CD40L inhibition in a murine NfkB assay. The assay uses NIHT3T cells expressing murine CD40R and containing an NfkB-Luciferase reporter construct. Addition of CD40L results in signaling (measured by luciferase activity) that is inhibited by both the MR1 anti-CD40L antibody and by the M31 CD40L-specific Tn3 scaffold.

[0072] FIG. 2A shows the design of CD40L-specific tandem bivalent Tn3 scaffolds and Serum Albumin (SA) fusion constructs.

[0073] FIG. 2B shows the SDS-PAGE analysis of a purified monovalent M13 construct (CD40L-specific Tn3 construct), or tandem bivalent scaffolds with linkers containing 1, 3, 5 or 7 Gly_4Ser units (denoted as GS) joining two M13 Tn3 monomer subunits. The monovalent M13 construct was run in lane 2, the dimeric construct with 1 GS unit (C1) was run in lanes 3 and 7, the dimeric constrict with 3 GS units (C2) was run in lanes

4 and 8, the dimeric construct with 5 GS units (C3) was run in lanes 5 and 9, and the dimeric construct with 7 GS units (C4) was run in lanes 6 and 10. Samples were run either non-reduced conditions (lanes 2-6) or reduced conditions (lanes 7-10).

[0074] FIG. 2C shows the competitive inhibition of murine CD40L binding to murine CD40 receptor immobilized on a biosensor chip by murine CD40L-specific monovalent (M13) or bivalent tandem scaffolds (M13-xGS-M13, wherein x is 1, 3, 5 or 7, corresponding to bivalent scaffolds with linkers containing 1, 3, 5 or 7 Gly_4Ser units). The half maximal inhibitory concentration (IC_{50}) for the various constructs is also indicated.

[0075] FIG. 2D shows the inhibitory effect of murine CD40L-specific Tn3 monovalent (M13) and bivalent tandem scaffolds, on murine CD40L-induced CD86 expression on B cells. IC_{50} values are provided for all Tn3 constructs and for the MR1 anti-murine CD40L antibody.

[0076] FIG. 3A shows high expression levels of murine CD40L-specific tandem bivalent Tn3 scaffold fused to mouse serum albumin (MSA) in HEK 293 cells. These constructs have 1 (G_4S) repeat in the linker between the Tn3 scaffold units and 3 (G_4S) repeats in the linker between the Tn3 scaffold and MSA. In addition, the construct contains a N49Q mutation into each of the M13 and M31 scaffolds to remove a potential N-linked glycosylation site. 10 μl culture supernatant taken 3 or 6 days after transfection were run on an SDS-PAGE gel along with known quantities of the purified protein. Expression level was estimated to 200 mg/l 6 days post transfection. Purification was carried out by IMAC through a C-terminal His-tag.

[0077] FIG. 3B shows inhibition of murine CD40L (MuCD40L)-induced CD86 expression measured using a D10G4.1/PBMC cell assay. A CD40L-specific tandem bivalent Tn3 scaffold (M13-1GS-M13), the same construct fused to mouse serum albumin (MSA) (M13-1GS-M13-MSA), and the MR1 anti-murine CD40L monoclonal antibody were assayed. IC_{50} values are provided for all constructs.

[0078] FIG. 3C shows inhibition of murine CD40L (MuCD40L)-induced CD86 expression measured using a D10G4.1/PBMC cell assay. A CD40L-specific tandem bivalent Tn3 scaffold (M13-1GS-M13) fused to mouse serum albumin (MSA) (M13-1GS-M13-MSA), an affinity matured variant of the M13 scaffold conjugated to MSA (M31Mono-MSA), a tandem bivalent scaffold comprising the M31 affinity optimized variant conjugated to MSA (M31-1GS-M31-MSA), a negative control tandem bivalent scaffold that does not

bind murine CD40L (D1-1GS-D1-MSA), and the MR1 monoclonal antibody were assayed. IC_{50} values are provided.

[0079] FIG. 4A shows the pharmacokinetics of several murine CD40L specific constructs in mouse as determined by ELISA. The plasma half-life ($t_{1/2}$) values for each construct are indicated.

[0080] FIG. 4B shows the pharmacokinetics of the human CD40L specific 342-HSA and a 342-HSA variant comprising the substitution of Leu at position 463 with Asn (L463N) and the substitution of Lys at position 524 with Leu (K524L) in Cynomolgus monkey as determined by ELISA.

[0081] FIG. 5A shows B cell maturation in the germinal centers (GC) from a sheep red blood cell (SRBC) immunization assay. The MR1 monoclonal anti-murine CD40L antibody was assayed.

[0082] FIG. 5B shows B cell maturation in the germinal centers (GC) from a sheep red blood cell (SRBC) immunization assay. M31-derived monovalent and bivalent constructs fused to MSA were assayed. The D1-D1 bivalent construct conjugated to MSA was used a negative control.

[0083] FIG. 5C shows B cell maturation in periphery (nonGC) from a sheep red blood cell (SRBC) immunization assay. M31-derived monovalent and bivalent constructs fused to MSA were assayed. The D1-D1 bivalent construct conjugated to MSA was used a negative control.

[0084] FIG. 5D shows the percentage (% CD4) and number (# CD4) of CD4 positive cells from a sheep red blood cell (SRBC) immunization assay. M31-derived monovalent and bivalent constructs fused to MSA, and the MR1 anti-CD40L monoclonal antibodies were assayed. The D1-D1 bivalent construct conjugated to MSA was used a negative control.

[0085] FIG. 5E shows the percentage (% CD44hi) and number (#CD44hi) of CD44hi positive cells from a sheep red blood cell (SRBC) immunization assay. M31-derived monovalent and bivalent constructs fused to MSA and the MR1 anti-CD40L monoclonal antibodies were assayed. The D1-D1 bivalent construct conjugated to MSA was used a negative control

[0086] FIG. 5F shows the amount of anti-SRBC IgG from a sheep red blood cell (SRBC) immunization assay. M31-derived monovalent and bivalent constructs fused to MSA assayed. The D1-D1 bivalent construct conjugated to MSA was used a negative control.

[0087] FIG. 5G shows the anti-KLH IgM titers from a KLH-specific T cell dependent antibody response (TDAR) model. 342-derived monovalent and bivalent constructs fused to HSA were assayed.

[0088] FIG. 5H shows the anti-KLH IgG titers from a KLH-specific T cell dependent antibody response (TDAR) model. 342-derived monovalent and bivalent constructs fused to HSA were assayed.

[0089] FIG. 6A shows the inhibitory effect of human CD40L-specific monovalent Tn3 monomer scaffolds 309 and 311 on human CD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells.

[0090] FIG. 6B shows the inhibitory effect of human CD40L-specific monovalent Tn3 monomer scaffolds 309 and 311 on human CD40L-stimulated B-cell proliferation.

[0091] FIG. 6C the inhibitory effect of human CD40L-specific monovalent Tn3 monomer scaffolds 309 and 311 on plasma cell number in T/B cell co-cultures. Tn3 scaffold 309 was also shown to bind activated primary T cells by FACS (data not shown). A D1 scaffold ("Neg Tn3") was used as control. Two monoclonal antibodies against CD40L, designated aCD40L(RE) and aCD40L(Bio) (Biogen's 5c8 anti-human CD40L monoclonal antibody) were also used as controls.

[0092] FIG. 7A shows that human CD40L-specific monovalent Tn3 scaffolds 309 and 311 have similar biophysical characteristics. Both scaffolds are monodispersed as measured by SEC.

[0093] FIG. 7B shows that human CD40L-specific monovalent Tn3 scaffolds 309 and 311 have similar biophysical characteristics. Both scaffolds have similar thermostability as the parent Tn3 scaffold (designated Tn3(wild type) in the graph) as measured by differential scanning calorimetry (DSC).

[0094] FIG. 8A shows inhibition of human CD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells. Monovalent (311) and bivalent (311_3GS and 311_7GS) human CD40L-specific Tn3 scaffolds were assayed. IC_{50} values for each construct are shown.

[0095] FIG. 8B shows inhibition of human CD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells. Monovalent (309) and bivalent (309_3GS and 309_7GS) human CD40L-specific Tn3 scaffolds were assayed, as well as Biogen's 5c8 anti-human CD40L monoclonal antibody. IC_{50} values for each construct and the antibody are shown.

[0096] FIG. 9A shows the design of a representative human CD40L-specific tandem bivalent Tn3 scaffold fused to human serum albumin (HSA). "GGGGG" (SEQ ID NO: 148) and "GGGGA" (SEQ ID NO: 149) are alternative linkers to the "GGGGS" linkers (SEQ ID NO: 147).

[0097] FIG. 9B shows a test purification from 293F cells over an IEX column. The shoulder fraction (<10% of the major peak) contains O-glycosylated protein linked to serine residues present in the linkers.

[0098] FIG. 9C shows inhibition in the human CD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells. A bivalent (309) human CD40L-specific Tn3 scaffold, the same scaffold fused to HSA, and Biogen's 5c8 anti-human CD40L monoclonal antibody were assayed.

[0099] FIG. 9D shows inhibition in the human CD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells. Three bivalent (309) human CD40L-specific Tn3 scaffolds were tested. Three (G_4S) repeats were present in the linker between the human CD40L-specific subunits (309 in this example) while the linker between the 309 subunits and the HSA was varied from 1 to 3 (G_4S) repeats. Biogen's 5c8 anti-human CD40L monoclonal antibody was also assayed.

[0100] FIG. 10A shows the effect of mutating loop sequences of 309 (left panel) and 311 (right panel) on CD40L binding. Binding indicates signal strength in the binding assay. WT is the variant with the original lead sequence (parent Tn3 sequence), whereas BC, DE and FG denotes variants in which the BC, DE, or FG loop sequence has been changed to the parent Tn3 sequence as present in human Tenascin C.

[0101] FIG. 10B shows inhibition profiles of a panel of affinity optimized scaffolds as measured by the human CD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells. Human CD40L-specific Tn3 clone 309 monomers were affinity optimized. Affinity optimized monomers are designated as clone 340 to clone 349. The clone 309wtFG construct had the entire FG loop replaced with the FG loop of the parent Tn3 scaffold. The 5c8 anti-CD40L monoclonal antibody was also assayed.

[0102] FIG. 10C shows inhibition profiles as measured by the human CD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells. The profile of human CD40L-specific Tn3 311 monomer, its K4E variant, and a negative control are shown.

[0103] FIG. 10D shows inhibition profiles as measured by the human CD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1

cells. The profile of human CD40L-specific Tn3 311K4E monomer, the affinity optimized 311K4E_12 monomer, and the 5c8 anti-CD40L monoclonal antibody are shown. IC_{50}'s for the two constructs and the antibody are also presented.

[0104] FIG. 11A and FIG. 11B show a multiple sequence alignment of the parental CD40L-specific Tn3 scaffold 309, the 309FGwt variant, and the affinity optimized variants 340 to 349. Amino acid residues 1 to 42 are shown in FIG. 11A, and amino acid residues 43 to 83 are shown in FIG. 11B. The variant loops are shaded. The consensus amino acid sequence is presented below the multiple sequence alignment. The aligned sequences correspond to the amino acid sequences of Tn3 scaffold clones 309 (SEQ ID NO: 20), 309FGwt (SEQ ID NO: 22), 340 (SEQ ID NO: 24), 341 (SEQ ID NO: 26), 342 (SEQ ID NO: 28), 343 (SEQ ID NO: 30), 344 (SEQ ID NO: 32), 345 (SEQ ID NO: 34), 346 (SEQ ID NO: 36), 347 (SEQ ID NO: 38), 348 (SEQ ID NO: 40), and 349 (SEQ ID NO: 42).

[0105] FIG. 12A and FIG. 12B show as multiple sequence alignment of the parental CD40L-specific Tn3 scaffold 311, the 311K4E variant, and the affinity optimized variants 311K4E_1 to 311K4E_21. Amino acid residues 1 to 44 are shown in FIG. 12A, and amino acid residues 45 to 87 are shown in FIG. 12B. The variant loops are shaded. Amino acid variations outside the shadowed loops are boxed. A consensus sequence is presented below the multiple sequence alignment. The aligned sequences correspond to the amino acid sequences of Tn3 scaffold clones 311 (SEQ ID NO: 44), 311K4E (SEQ ID NO: 46), 311K4E_1 (SEQ ID NO: 48), 311K4E_2 (SEQ ID NO: 50), 311K4E_2 (SEQ ID NO: 52), 311K4E_3 (SEQ ID NO: 54), 311K4E_4 (SEQ ID NO: 56), 311K4E_5 (SEQ ID NO: 58), 311K4E_7 (SEQ ID NO: 60), 311K4E_8 (SEQ ID NO: 62), 311K4E_9 (SEQ ID NO: 64), 311K4E_10 (SEQ ID NO: 66), 311K4E_11 (SEQ ID NO: 68), 311K4E_12 (SEQ ID NO: 70), 311K4E_13 (SEQ ID NO: 72), 311K4E_14 (SEQ ID NO: 74), 311K4E_15 (SEQ ID NO: 76), 311K4E_16 (SEQ ID NO: 78), 311K4E_19 (SEQ ID NO: 80), 311K4E_20 (SEQ ID NO: 82), and 311K4E_21 (SEQ ID NO: 84).

[0106] FIG. 13 shows a human NfkB inhibition assay that uses HEK293 cells expressing human CD40 receptor and containing an NfkB-Luciferase reporter construct. Addition of human CD40L results in signaling (measured by luciferase activity) that can be inhibited by CD40L binding molecules. The CD40L-specific Tn3 scaffolds 340 and 342, as well as the 5c8 anti-CD40L monoclonal antibody were assayed.

[0107] FIG. 14 shows binding of human CD40L-specific Tn3 scaffolds to 24h anti-CD3/28 activated human CD4+ T Cells. A monovalent 342 scaffold fused to HSA

(designated 342-HSA) and a bivalent 342 scaffold fused to HSA (designated 342-342-HSA) were assayed.

[0108] FIG. 15 shows inhibition of primary human T/B cell proliferation at day 3. A monovalent 340 scaffold fused to HSA (340-HSA), a monovalent 342 scaffold fused to HSA (342-HSA), and a bivalent 342 scaffold fused to HSA (342-342-HSA) were assayed. IC_{50} values for each construct are shown.

[0109] FIG. 16A shows an aggregation assay on washed platelets. The graph shows a representative ADP induced aggregation positive control for a donor (Top three traces respectively) ADP: 0.5μM, 1μM, and 2μM) along with the immune complex (IC) of 5c8 monoclonal antibody (600nM) and soluble human CD40L (200nM).

[0110] FIG. 16B shows an aggregation assay on washed platelets. The graph shows lack of aggregation when preformed immuno complexes of 309-309 bivalent scaffolds (not fused to HSA) and soluble human CD40L were used. The concentration of human CD40L (soluble form) was kept constant at 600 nM and the concentration of the scaffold constructs was varied from 200 nM to 800 nM.

[0111] FIG. 16C an aggregation assay on washed platelets. The graph shows lack of aggregation when preformed immuno complexes of 342 monovalent scaffolds fused to HSA and soluble human CD40L were used. The concentration of human CD40L (soluble form) was kept constant at 600 nM and the concentration of the scaffold constructs was varied from 100 nM to 400 nM. The graph also shows rapid aggregation induced by the immune complex of Biogen 5c8 monoclonal antibody and soluble human CD40L.

[0112] FIG. 17A shows a ribbon representation of the crystal structure of soluble CD40L in a complex with the CD40L-specific Tn3 309 monomer scaffold. CD40L forms a trimer (polypeptides A, B and C). Each 309 scaffold (polypeptides D, E and F, circled) makes contact with two CD40L polypeptides. The specific contacts between each 309 scaffold and the first and second CD40L polypeptides are listed. This is a "top-down" view of the structure.

[0113] FIG. 17B shows a ribbon representation of the crystal structure of soluble CD40L in a complex with the CD40L-specific Tn3 311K4E_12 monomer scaffold. CD40L forms a trimer (polypeptides A, B and C). Each 311K4E_12 monomer scaffold (polypeptides D, E and F, circled) makes contact with two CD40L polypeptides. The specific contacts between each 311K4E_12 monomer scaffold and the first and second CD40L polypeptides are listed. This is a "top-down" view of the structure.

[0114] FIG. 17C shows a ribbon representation illustrating that the 311K4E_12 and 309 scaffolds (circled) bind to different epitopes located in different parts of the CD40L trimer complex. Both scaffolds bind in the same groove that would interact with the CD40 receptor. This is a "side" view of the structure.

[0115] FIG. 17D shows a ribbon representation of the crystal structure of soluble CD40L in a complex with the CD40L-specific Tn3 342 monomer scaffold. Only one CD40L and one 342 monomer scaffold are shown. The specific contacts between the 342 monomer scaffold and the first CD40L polypeptides are listed.

[0116] FIG. 17E shows a ribbon representation illustrating that the 342 and 311K4E_12 scaffolds can bind simultaneously to different epitopes located in different parts of the CD40L trimer complex. Both scaffolds bind in the same groove that would interact with the CD40 receptor. This is a "side" view of the structure.

[0117] FIG. 18A shows the location of the contacts between amino acids in the CD40L-specific Tn3 311K4E_12 monomer scaffold (SEQ ID NO: 68) and a trimer formed by soluble CD40L (SEQ ID NO: 2) molecules as shown in FIG. 17A. Each scaffold makes contact with 2 CD40L molecules. The CD40L sequence (SEQ ID NO: 2) is shown. Dotted underline = cytoplasmic domain; Solid underline = signal anchor type II membrane protein; Double underline = portion co-crystalized with Tn3 scaffold; Dark shading = residues on 1st CD40L that contact the Tn3; Light shading = residues on 2nd CD40L that contact the Tn3.

[0118] FIG. 18B shows the location of the contacts between amino acids in the CD40L-specific Tn3 309 monomer scaffold and CD40L trimer as shown in FIG. 17B. Each scaffold makes contact with 2 CD40L molecules. The CD40L sequence (SEQ ID NO: 2) is shown. Dotted underline = cytoplasmic domain; Solid underline = signal anchor type II membrane protein; Double underline = portion co-crystalized with Tn3 scaffold; Dark shading = reisudes on 1st CD40L that contact the Tn3; Light shading = residues on 2nd CD40L that contact the Tn3; double boxed residue is contact with FG loop of 309 scaffold, which likely is not conserved in clones having a wildtype FG loop.

[0119] FIG. 19. Panel A shows an exemplary chromatogram of the elution of Tn3 scaffold (309 or 311K4E_12), CD40L and the complex between them off the size exclusion Superdex 200 10/300 GL column. Panel B shows crystals of the 309-CD40L complex. The crystal shown grew to dimensions up to 0.15 × 0.15 × 0.1 mm. Panel C shows crystals of the 311K4E_12-CD40L complex.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0120] Before describing the present invention in detail, it is to be understood that this invention is not limited to specific compositions or process steps, as such can vary. It must be noted that, as used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the context clearly dictates otherwise. The terms "a" (or "an"), as well as the terms "one or more," and "at least one" can be used interchangeably herein.

[0121] Furthermore, “and/or” where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A," (alone) and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following embodiments: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0122] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this invention.

[0123] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects or embodiments of the invention, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0124] It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0125] Amino acids are referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, are referred to by their commonly accepted single-letter codes.

[0126] The term "epitope" as used herein refers to a protein determinant capable of binding to a scaffold of the invention. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

[0127] The terms "fibronectin type III (FnIII) domain," "FnIII domain" and "FnIII scaffold" refer to polypeptides homologous to the human fibronectin type III domain having at least 7 beta strands which are distributed between two beta sheets, which themselves pack against each other to form the core of the protein, and further containing solvent exposed loops which connect the beta strands to each other. There are at least three such loops at each edge of the beta sheet sandwich, where the edge is the boundary of the protein perpendicular to the direction of the beta strands. In certain embodiments, an FnIII domain comprises 7 beta strands designated A, B, C, D, E, F, and G linked to six loop regions designated AB, BC, CD, DE, EF, and FG, wherein a loop region connects each beta strand.

[0128] The term "Tn3 scaffold" used herein, refers to molecules comprising at least one FnIII scaffold wherein the A beta strand comprises SEQ ID NO: 11, the B beta strand comprises SEQ ID NO: 12, the C beta strand SEQ ID NO: 13 or 14, the D beta strand comprises SEQ ID NO: 15, the E beta strand comprises SEQ ID NO: 16, the F beta strand comprises SEQ ID NO: 17, and the beta strand G comprises SEQ ID NO: 18, wherein at least one loop is a non-naturally occurring variant of the loops in the “parent Tn3 scaffold.” In certain embodiments, one or more of the beta strands of a Tn3 module comprise at least one amino acid substitution except that the cysteine residues in the C beta strand (e.g., the cysteine in SEQ ID NOs: 13 or 14) and F beta strands (SEQ ID NO: 17) are not substituted.

[0129] The term “parent Tn3” as used herein refers to an FnIII scaffold comprising SEQ ID NO: 3, i.e., a thermally stabilized cysteine-engineered FnIII scaffold derived from the 3rd FnIII domain of human tenascin C.

[0130] The terms "multimer" or "multimeric scaffold" refer to a molecule that comprises at least two FnIII scaffolds in association. The scaffolds forming a multimeric scaffold can be linked through a linker that permits each scaffold to function independently.

[0131] The terms "monomer," "monomer subunit" or "monomer scaffold" refer to a molecule that comprises only one FnIII scaffold.

[0132] The term "CD40L-specific monomer subunit" as used herein refers to a Tn3 monomer derived from a "parent Tn3" wherein the Tn3 monomer specifically binds to CD40L or a fragment thereof, e.g., a soluble form of CD40L.

[0133] The term "DNA" refers to a sequence of two or more covalently bonded, naturally occurring or modified deoxyribonucleotides.

[0134] The term "fusion protein" refers to a protein that includes (i) one or more scaffolds of the invention joined to (ii) a second, different protein (*i.e.*, a "heterologous" protein).

TABLE 1: Sequences and SEQ ID NOs of components of "parent Tn3"

Name/Brief Description	Sequence	SEQ ID NO
Tn3	IEVKDVTDTTALITWFKPLAEIDGCELT YGIKDVPGDRTTIDLTEDENQYSIGNLK PDTEYEVSLICRRGDMSSNPAKETFTT (cys residues of disulfide bond are underlined)	3
3rd FnIII of tenascin C, AB loop (Tn3)	KDVTDTT	4
3rd FnIII of tenascin C, BC loop (Tn3)	FKPLAEIDG	5
3rd FnIII of tenascin C, CD loop (Tn3)	KDVPGDR	6
3rd FnIII of tenascin C, DE loop (Tn3)	TEDENQ	7
3rd FnIII of tenascin C, EF loop (Tn3)	GNLKPDTE	8
3rd FnIII of tenascin C, FG loop (Tn3)	RRGDMSSNPA	9
3rd FnIII of tenascin C, beta strand A (Tn3)	RLDAPSQIEV	10
3rd FnIII of tenascin C, beta strand A (Tn3) N-terminal truncation	IEV	11
3rd FnIII of tenascin C, beta strand B (Tn3)	ALITW	12
3rd FnIII of tenascin C, beta strand C (Tn3 variant)	CELAYGI	13
3rd FnIII of tenascin C, beta strand C (Tn3)	CELTYGI	14
3rd FnIII of tenascin C, beta strand D (Tn3)	TTIDL	15
3rd FnIII of tenascin C, beta strand E (Tn3)	YSI	16
3rd FnIII of tenascin C, beta strand F (Tn3)	YEVSLIC	17
3rd FnIII of tenascin C, beta strand G (Tn3)	KETFTT	18

[0135] The term "heterologous moiety" is used herein to indicate the addition of a composition to a Tn3 scaffold of the invention wherein the composition is not normally part of an FnIII domain. Exemplary heterologous moieties include proteins, peptides, protein domains, linkers, drugs, toxins, imaging agents, radioactive compounds, organic and inorganic polymers, and any other compositions which might provide an activity that is not inherent in the FnIII domain itself, including, but are not limited to, polyethylene glycol (PEG), a cytotoxic agent, a radionuclide, imaging agent, biotin, a dimerization domain (e.g.

leucine zipper domain), human serum albumin (HSA) or an FcRn binding portion thereof, a domain or fragment of an antibody (e.g., antibody variable domain, a CH1 domain, a Ckappa domain, a Clambda domain, a CH2, or a CH3 domain), a single chain antibody, a domain antibody, an albumin binding domain, an IgG molecule, an enzyme, a ligand, a receptor, a binding peptide, a non-FnIII scaffold, an epitope tag, a recombinant polypeptide polymer, a cytokine, and the like.

[0136] The term "linker" as used herein refers to any molecular assembly that joins or connects two or more scaffolds. The linker can be a molecule whose function is to act as a "spacer" between modules in a scaffold, or it can also be a molecule with additional function (i.e., a "functional moiety"). A molecule included in the definition of "heterologous moiety" can also function as a linker.

[0137] The terms "linked" and "fused" are used interchangeably. These terms refer to the joining together of two or more scaffolds, heterologous moieties, or linkers by whatever means including chemical conjugation or recombinant means.

[0138] The terms "domain" or "protein domain" refer to a region of a protein that can fold into a stable three-dimensional structure, often independently of the rest of the protein, and which can be endowed with a particular function. This structure maintains a specific function associated with the domain's function within the original protein, e.g., enzymatic activity, creation of a recognition motif for another molecule, or to provide necessary structural components for a protein to exist in a particular environment of proteins. Both within a protein family and within related protein superfamilies, protein domains can be evolutionarily conserved regions. When describing the component of a multimeric scaffold, the terms "domain," "monomeric scaffold," "monomer subunit," and "module" can be used interchangeably. By “native FnIII domain” is meant any non-recombinant FnIII domain that is encoded by a living organism.

[0139] A "protein sequence" or "amino acid sequence" means a linear representation of the amino acid constituents in a polypeptide in an amino-terminal to carboxyl-terminal direction in which residues that neighbor each other in the representation are contiguous in the primary structure of the polypeptide.

[0140] The term "nucleic acid" refers to any two or more covalently bonded nucleotides or nucleotide analogs or derivatives. As used herein, this term includes, without limitation, DNA, RNA, and PNA. “Nucleic acid” and “polynucleotide” are used interchangeably herein.

[0141] The term "polynucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, e.g., messenger RNA (mRNA) or plasmid DNA (pDNA). The term "isolated" nucleic acid or polynucleotide refers to a nucleic acid molecule, DNA or RNA that has been removed from its native environment. For example, a recombinant polynucleotide encoding, e.g., a scaffold of the invention contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of polynucleotides of the present invention. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid can be or can include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

[0142] The term "pharmaceutically acceptable" refers to a compound or protein that can be administered to an animal (for example, a mammal) without significant adverse medical consequences.

[0143] The term "physiologically acceptable carrier" refers to a carrier which does not have a significant detrimental impact on the treated host and which retains the therapeutic properties of the compound with which it is administered. One exemplary physiologically acceptable carrier is physiological saline. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and are described, for example, in Remington's Pharmaceutical Sciences, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, Pa..

[0144] By a "polypeptide" is meant any sequence of two or more amino acids linearly linked by amide bonds (peptide bonds) regardless of length, post-translation modification, or function. "Polypeptide," "peptide," and "protein" are used interchangeably herein. Thus, peptides, dipeptides, tripeptides, or oligopeptides are included within the definition of "polypeptide," and the term "polypeptide" can be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide can be derived from a natural biological source or produced by

recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. A polypeptide can be generated in any manner, including by chemical synthesis.

[0145] Also included as polypeptides of the present invention are fragments, derivatives, analogs, or variants of the foregoing polypeptides, and any combination thereof. Variants can occur naturally or be non-naturally occurring. Non-naturally occurring variants can be produced using art-known mutagenesis techniques. Variant polypeptides can comprise conservative or non-conservative amino acid substitutions, deletions, or additions. Also included as "derivatives" are those peptides that contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids.

[0146] By "randomized" or "mutated" is meant including one or more amino acid alterations, including deletion, substitution or addition, relative to a template sequence. By "randomizing" or "mutating" is meant the process of introducing, into a sequence, such an amino acid alteration. Randomization or mutation can be accomplished through intentional, blind, or spontaneous sequence variation, generally of a nucleic acid coding sequence, and can occur by any technique, for example, PCR, error-prone PCR, or chemical DNA synthesis. The terms “randomizing”, “randomized”, “mutating”, “mutated” and the like are used interchangeably herein.

[0147] By a “cognate” or "cognate, non-mutated protein" is meant a protein that is identical in sequence to a variant protein, except for the amino acid mutations introduced into the variant protein, wherein the variant protein is randomized or mutated.

[0148] By "RNA" is meant a sequence of two or more covalently bonded, naturally occurring or modified ribonucleotides. One example of a modified RNA included within this term is phosphorothioate RNA.

[0149] The terms "scaffold of the invention" or "scaffolds of the invention" as used herein, refers to multimeric Tn3 scaffolds as well as monomeric Tn3 scaffolds. The term “target” refers to a compound recognized by a specific scaffold of the invention. The terms “target” and “antigen” are used interchangeably herein. The term "specificity" as used herein, e.g., in the terms "specifically binds" or "specific binding," refers to the relative affinity by which a Tn3 scaffold of the invention binds to one or more antigens via one or more antigen binding domains, and that binding entails some complementarity between one or more antigen binding domains and one or more antigens. According to this definition, a Tn3 scaffold of the invention is said to "specifically bind" to an epitope when it binds to that epitope more readily than it would bind to a random, unrelated epitope.

[0150] An “affinity matured” scaffold is a scaffold with one or more alterations, generally in a loop, which result in an improvement in the affinity of the Tn3 scaffold for an epitope compared to a parent Tn3 scaffold which does not possess those alteration(s).

[0151] The term "affinity" as used herein refers to a measure of the strength of the binding of a certain Tn3 scaffold of the invention to an individual epitope.

[0152] The term "avidity" as used herein refers to the overall stability of the complex between a population of Tn3 scaffolds of the invention and a certain epitope, i.e., the functionally combined strength of the binding of a plurality of Tn3 scaffolds with the antigen. Avidity is related to both the affinity of individual antigen-binding domains with specific epitopes, and also the valency of the scaffold of the invention.

[0153] The term "action on the target" refers to the binding of a Tn3 scaffold of the invention to one or more targets and to the biological effects resulting from such binding. In this respect, multiple antigen binding units in a Tn3 scaffold can interact with a variety of targets and/or epitopes and, for example, bring two targets physically closer, trigger metabolic cascades through the interaction with distinct targets, etc. With reference to CD40L, "action on the target" refers to the effect achieved, for example, by the enhancement, stimulation or activation, of one or more biological activities of CD40L.

[0154] The term "valency" as used herein refers to the number of potential antigen-binding modules, e.g., the number of FnIII modules in a scaffold of the invention. When a Tn3 scaffold of the invention comprises more than one antigen-binding module, each binding module can specifically bind, e.g., the same epitope or a different epitope, in the same target or different targets.

[0155] The term "disulfide bond" as used herein includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group.

[0156] The term “immunoglobulin” and "antibody" comprises various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon. It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA IgG, or IgE, respectively. Modified versions of each of these classes are readily discernible to the skilled artisan. As used herein, the term "antibody" includes but not limited to an intact antibody, a modified antibody, an antibody VL or VL domain, a CHl domain, a Ckappa domain, a Clambda domain, an Fc domain (see below), a CH2, or a CH3 domain.

[0157] As used herein, the term "Fc domain" domain refers to a portion of an antibody constant region. Traditionally, the term Fc domain refers to a protease (e.g., papain) cleavage product encompassing the paired CH2, CH3 and hinge regions of an antibody. In the context of this disclosure, the term Fc domain or Fc refers to any polypeptide (or nucleic acid encoding such a polypeptide), regardless of the means of production, that includes all or a portion of the CH2, CH3 and hinge regions of an immunoglobulin polypeptide.

[0158] As used herein, the term "modified antibody" includes synthetic forms of antibodies which are altered such that they are not naturally occurring, e.g., antibodies that comprise at least two heavy chain portions but not two complete heavy chains (as, e.g., domain deleted antibodies or minibodies); multispecific forms of antibodies (e.g., bispecific, trispecific, etc.) altered to bind to two or more antigens or to different epitopes of a single antigen). In addition, the term "modified antibody" includes multivalent forms of antibodies (e.g., trivalent, tetravalent, etc., antibodies that to three or more copies of the same antigen). (See, e.g., Antibody Engineering, Kontermann & Dubel, eds., 2010, Springer Protocols, Springer).

[0159] The term “in vivo half-life” is used in its normal meaning, i.e., the time at which 50% of the biological activity of a polypeptide is still present in the body/target organ, or the time at which the activity of the polypeptide is 50% of its initial value. As an alternative to determining functional in vivo half-life, "serum half-life" may be determined, i.e., the time at which 50% of the polypeptide molecules circulate in the plasma or bloodstream prior to being cleared. Determination of serum-half-life is often more simple than determining functional in vivo half-life and the magnitude of serum-half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternative terms to serum half-life include "plasma half-life," circulating half-life, circulatory half-life, serum clearance, plasma clearance, and clearance half-life. The functionality to be retained is normally selected from procoagulant, proteolytic, co-factor binding, receptor binding activity, or other type of biological activity associated with the particular protein.

[0160] The term "increased" with respect to the functional in vivo half-life or plasma half-life is used to indicate that the relevant half-life of the polypeptide is statistically significantly increased relative to that of a reference molecule (for example an unmodified polypeptide), as determined under comparable conditions.

[0161] The term "decreased" with respect to the functional in vivo half-life or plasma half-life is used to indicate that the relevant half-life of the polypeptide is statistically

significantly decreased relative to that of a reference molecule (for example an unmodified polypeptide), as determined under comparable conditions.

[0162] The term "expression" as used herein refers to a process by which a gene produces a biochemical, for example, a scaffold of the invention or a fragment thereof. The process includes any manifestation of the functional presence of the gene within the cell including, without limitation, gene knockdown as well as both transient expression and stable expression. It includes without limitation transcription of the gene into one or more mRNAs, and the translation of such mRNAs into one or more polypeptides. If the final desired product is a biochemical, expression includes the creation of that biochemical and any precursors.

[0163] An “expression product” can be either a nucleic acid, e.g., a messenger RNA produced by transcription of a gene, or a polypeptide. Expression products described herein further include nucleic acids with post transcriptional modifications, e.g., polyadenylation, or polypeptides with post translational modifications, e.g., methylation, glycosylation, the addition of lipids, association with other protein subunits, proteolytic cleavage, and the like.

[0164] The term "vector" or "expression vector" is used herein to mean vectors used in accordance with the present invention as a vehicle for introducing into and expressing a desired expression product in a host cell. As known to those skilled in the art, such vectors can easily be selected from the group consisting of plasmids, phages, viruses and retroviruses. In general, vectors compatible with the instant invention will comprise a selection marker, appropriate restriction sites to facilitate cloning of the desired nucleic acid and the ability to enter and/or replicate in eukaryotic or prokaryotic cells.

[0165] The term "host cells" refers to cells that harbor vectors constructed using recombinant DNA techniques and encoding at least one expression product. In descriptions of processes for the isolation of an expression product from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of the expression product unless it is clearly specified otherwise, i.e., recovery of the expression product from the "cells" means either recovery from spun down whole cells, or recovery from the cell culture containing both the medium and the suspended cells.

[0166] The terms "treat" or "treatment" as used herein refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder in a subject, such as the progression of an inflammatory disease or condition. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease,

stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable.

[0167] The term "treatment" also means prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

[0168] The terms "subject," "individual," "animal," "patient," or "mammal" refer to any individual, patient or animal, in particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sports, or pet animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, cows, and so on.

[0169] The term “CD40L” as used herein refers without limitations to CD40L expressed on the surface of T-cells, recombinantly expressed CD40L, CD40L expressed and purified form E.coli or other suitable recombinant protein expression systems, aglycosylated CD40L, and soluble fragments of CD40L. As used herein, "CD40L" also refers to MegaCD40L. MegaCD40L™ is a high activity construct in which two trimeric CD40 ligands are artificially linked via the collagen domain of ACRP30/adiponectin. This construct very effectively simulates the natural membrane-assisted aggregation of CD40L in vivo. It provides a simple and equally potent alternative to [CD40L+enhancer] combinations (Alexis biochemicals). The term "CD40L" refers to monomeric forms of CD40L as well as oligomeric forms, e.g., trimeric CD40L.

[0170] The term “CD40L” refers both to the full length CD40L and to soluble fragments, e.g., extracellular domain forms of CD40L resulting from proteolysis. Amino acid sequences of membrane-bound and soluble forms of human CD40L (Swissprot: P29965) are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

[0171] The terms “CD40L antagonist” or “antagonist” are used in the broadest sense, and includes any molecule that partially or fully inhibits, decreases or inactivates one or more biological activities of CD40L, and biologically active variants thereof, in vitro, in situ, or in vivo. For instance, a CD40L antagonist may function to partially or fully inhibit, decrease or inactivate one or more biological activities of one or more CD40L molecules, or one or more CD40L molecules bound to CD40 or other targets, in vivo, in vitro or in situ, as a result of its binding to CD40L.

[0172] The term "CD40L agonist" or "agonist" is used in the broadest sense, and includes any molecule that partially or fully enhances, stimulates or activates one or more biological activities of CD40L, and biologically active variants thereof, in vitro, in situ, or in vivo. For instance, a CD40L agonist may function to partially or fully enhance, stimulate or activate one or more biological activities of one or more CD40L molecules, or one or more CD40L molecules bound to CD40R or other targets, in vivo, in vitro or in situ, as a result of its binding to CD40L.

[0173] The term "crystal" as used herein, refers to one form of solid state of matter in which atoms are arranged in a pattern that repeats periodically in three-dimensions, typically forming a lattice.

[0174] The term "space group symmetry," as used herein, refers to the whole symmetry of the crystal that combines the translational symmetry of a crystalline lattice with the point group symmetry. A "space group" is designated by a capital letter identifying the lattice group (P, A, F, etc.) followed by the point group symbol in which the rotation and reflection elements are extended to include screw axes and glide planes. Note that the point group symmetry for a given space group can be determined by removing the cell centering symbol of the space group and replacing all screw axes by similar rotation axes and replacing all glide planes with mirror planes. The point group symmetry for a space group describes the true symmetry of its reciprocal lattice.

[0175] The term "unit cell," as used herein, means the atoms in a crystal that are arranged in a regular repeated pattern, in which the smallest repeating unit is called the unit cell. The entire structure can be reconstructed from knowledge of the unit cell, which is characterized by three lengths (a, b, and c) and three angles (α, β, and γ). The quantities a and b are the lengths of the sides of the base of the cell and γ is the angle between these two sides. The quantity c is the height of the unit cell. The angles α and β describe the angles between the base and the vertical sides of the unit cell.

[0176] The term "machine-readable data storage medium," as used herein, means a data storage material encoded with machine-readable data, wherein a machine is programmed with instructions for using such data and is capable of displaying data in the desired format, for example, a graphical three-dimensional representation of molecules or molecular complexes.

[0177] The term "X-ray diffraction pattern" means the pattern obtained from X-ray scattering of the periodic assembly of molecules or atoms in a crystal. X-ray crystallography

is a technique that exploits the fact that X-rays are diffracted by crystals. X-rays have the proper wavelength (in the Angstrom range, approximately 10^{-8} cm) to be scattered by the electron cloud of an atom of comparable size. Based on the diffraction pattern obtained from X-ray scattering of the periodic assembly of molecules or atoms in the crystal, the electron density can be reconstructed. Additional phase information can be extracted either from the diffraction data or from supplementing diffraction experiments to complete the reconstruction (the phase problem in crystallography). A model is the progressively built into the experimental electron density, refined against the data to produce an accurate molecular structure. X-ray structure coordinates define a unique configuration of points in space. Those of skill in the art understand that a set of structure coordinates for a protein or a protein-ligand complex, or a portion thereof, define a relative set of points that, in turn, define a configuration in three dimensions. A similar or identical configuration can be defined by an entirely different set of coordinates, provided the distances and angles between coordinates remain essentially the same. In addition, a configuration of points can be defined by increasing or decreasing the distances between coordinates by a scalar factor, while keeping the angles essentially the same.

[0178] The term "crystal structure," as used herein, refers to the three-dimensional or lattice spacing arrangement of repeating atomic or molecular units in a crystalline material. The crystal structure of a crystalline material can be determined by X-ray crystallographic methods, see, for example, "Principles of Protein X-Ray Crystallography" by Jan Drenth, Springer Advanced Texts in Chemistry, Springer Verlag, 2nd ed., February 199, ISBN: 0387985875, and "Introduction to Macromolecular Crystallography" by Alexander McPherson, Wiley-Liss, Oct. 18, 2002, ISBN: 0471251224.

[0179] The term "effector function" refers to those biological activities of an antibody or antibody fragment attributable to the Fc region (a native Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; downregulation of cell surface receptors (e.g., B cell receptors); and B cell activation.

[0180] The term "antibody-dependent cell-mediate cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cells with cytotoxins.

[0181] The term “Fc receptor” or “FcR” describes a receptor that binds to the Fc region of an antibody. The FcR can be a native sequence human FcR. The FcR can bind to an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. The term also includes the neonatal receptor FcRn.

[0182] The term “consensus sequence” refers to a protein sequence showing the most common amino acids at a particular position after multiple sequences are aligned. A consensus sequence is a way of representing the results of a multiple sequence alignment, where related sequences are compared to each other. The consensus sequence shows which residues are most abundant in the alignment at each position, and the degree of variability at each position.

Introduction

[0183] CD40L (also known as CD154, CD40 ligand, gp39 or TBAM) is a 33 kDa, Type II membrane glycoprotein (Swiss-ProtAcc-No P29965). Additionally, shorter 18 kDa CD40L soluble forms exist, (also known as sCD40L or soluble CD40L). These soluble forms of CD40L are generated by proteolytic processing of the membrane bound protein, but the cellular activity of the soluble species is weak in the absence of higher order oligomerization (e.g., trimerization).

[0184] The present invention provides a family of recombinant, non-naturally occurring protein scaffolds (Tn3 scaffolds) capable of binding to CD40L. In particular, the proteins described herein can be used to display defined loops which are analogous to the complementarity-determining regions ("CDRs") of an antibody variable region. These loops can be subjected to randomization or restricted evolution to generate diversity capable of binding to a multitude of target compounds. The Tn3 scaffolds can be used as monomers or can be assembled into multimer scaffolds capable of binding to CD40L.

[0185] In specific embodiments, the invention provides CD40L-specific binders which are useful for preventing ameliorating, detecting, diagnosing, or monitoring diseases, such as but not limited to autoimmune disease. In other specific embodiments, CD40L-specific Tn3 scaffolds of the invention are useful for the treatment of autoimmune diseases and conditions. In some embodiments, autoimmune diseases may include, but are not limited to systemic lupus erythematosis (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), inflammatory bowel disease (IBD) and allograft rejection.

[0186] The Tn3 scaffolds of the invention comprise CD40L-specific monomer subunits derived from the third FnIII domain of human tenascin C, in which at least one non-naturally occurring intramolecular disulfide bond has been engineered. The monomer subunits that make up the Tn3 scaffolds of the invention correctly fold independently of each other, retain their binding specificity and affinity, and each of the monomeric scaffolds retains its functional properties. When monomer subunits are assembled in high valency multimeric Tn3 scaffolds the monomer subunits correctly fold independently of each other, retain their binding specificity and affinity, and each one of the monomers retains its functional properties.

[0187] Tn3 scaffolds of the invention comprising more than one monomer subunit can bind to multiple epitopes, e.g., (i) bind to multiple epitopes in a single target, (ii) bind to a single epitope in multiple targets, (iii) bind to multiple epitopes located on different subunits of one target, or (iv) bind to multiple epitopes on multiple targets, thus increasing avidity.

[0188] In addition, due to the possibility of varying the distance between multiple monomers via linkers, multimeric Tn3 scaffolds are capable of binding to multiple target molecules on a surface (either on the same cell/surface or in different cells/surfaces). As a result of their ability to bind simultaneously to more than one target, a Tn3 multimeric scaffold of the invention can be used to modulate multiple pathways, cross-link receptors on a cell surface, bind cell surface receptors on separate cells, and/or bind target molecules or cells to a substrate.

[0189] In addition, the present invention provides affinity matured scaffolds wherein the affinity of a scaffold for a specific target is modulated via mutation. Also, the invention provides methods to produce the scaffolds of the invention as well as methods to engineer scaffolds with desirable physicochemical, pharmacological, or immunological properties. Furthermore, the present invention provides uses for such scaffolds and methods for therapeutic, prophylactic, and diagnostic use.

The FnIII Structural Motif

[0190] The Tn3 scaffolds of the present invention are based on the structure of a type III fibronectin module (FnIII), a domain found widely across all three domains of life and viruses, and in multitude of protein classes. In specific embodiments, the scaffolds of the invention are derived from the third FnIII domain of human tenascin C (see International Application No. International Application No. PCT/US2008/012398, published as WO

2009/058379; PCT/US2011/032184, published as WO 2011/130324; and International Application No. PCT/US2011/032188, published as WO2011130328).

[0191] In one specific embodiment, the Tn3 scaffolds of the invention comprise a CD40L-specific monomer subunit derived from a parent Tn3 scaffold. The overall tridimensional fold of the monomer is closely related to that of the smallest functional antibody fragment, the variable region of the heavy chain (VH), which in the single domain antibodies of camels and camelids (e.g., llamas) comprises the entire antigen recognition unit.

[0192] The Tn3 monomer subunits of the invention and the native FnIII domain from tenascin C are characterized by the same tridimensional structure, namely a beta-sandwich structure with three beta strands (A, B, and E) on one side and four beta strands (C,D, F, and G) on the other side, connected by six loop regions. These loop regions are designated according to the beta-strands connected to the N- and C- terminus of each loop. Accordingly, the AB loop is located between beta strands A and B, the BC loop is located between strands B and C, the CD loop is located between beta strands C and D, the DE loop is located between beta strands D and E, the EF loop is located between beta strands E and F, and the FG loop is located between beta strands F and G. FnIII domains possess solvent exposed loop s tolerant of randomization, which facilitates the generation of diverse pools of protein scaffolds capable of binding specific targets with high affinity.

[0193] In one aspect of the invention, Tn3 monomer subunits are subjected to directed evolution designed to randomize one or more of the loops which are analogous to the complementarity-determining regions (CDRs) of an antibody variable region. Such a directed evolution approach results in the production of antibody-like molecules with high affinities for targets of interest, e.g., CD40L.

[0194] In addition, in some embodiments the Tn3 scaffolds described herein can be used to display defined exposed loops (for example, loops previously randomized and selected on the basis of target binding) in order to direct the evolution of molecules that bind to such introduced loops. This type of selection can be carried out to identify recognition molecules for any individual CDR-like loop or, alternatively, for the recognition of two or all three CDR-like loops combined into a nonlinear epitope binding moiety. A set of three loops (designated BC, DE, and FG), which can confer specific target binding, run between the B and C strands; the D and E strands, and the F and G beta strands, respectively. The BC, DE, and FG loops of the third FnIII domain of human tenascin C are 9, 6, and 10 amino acid residues long, respectively. The length of these loops falls within the narrow range of the cognate antigen-recognition loops found in antibody heavy chains, that is, 7-10, 4-8, and 4-28

amino acids in length, respectively. Similarly, a second set of loops, the AB, CD, and EF loops (7, 7, and 8, amino acids in length respectively) run between the A and B beta strands; the C and D beta strands; and the E and F beta strands, respectively.

[0195] Once randomized and selected for high affinity binding to a target, the loops in the Tn3 monomer scaffold may make contacts with targets equivalent to the contacts of the cognate CDR loops in antibodies. Accordingly, in some embodiments the AB, CD, and EF loops are randomized and selected for high affinity binding to one or more targets, e.g., CD40L. In some embodiments, this randomization and selection process may be performed in parallel with the randomization of the BC, DE, and FG loops, whereas in other embodiments this randomization and selection process is performed in series.

CD40L-Specific Monomeric Subunits

[0196] The invention provides CD40L-specific recombinant, non-naturally occurring Tn3 scaffolds comprising, a plurality of beta strand domains linked to a plurality of loop regions, wherein one or more of said loop regions vary by deletion, substitution or addition of at least one amino acid from the cognate loops in wild type Tn3 (SEQ ID NO: 3) (see TABLE 1).

[0197] To generate improved CD40L-specific Tn3 monomer subunits with novel binding characteristics, parent Tn3 is subjected to amino acid additions, deletions or substitutions. It will be understood that, when comparing the sequence of a CD40L-specific Tn3 monomer subunit to the sequence of parent Tn3, the same definition of the beta strands and loops is utilized. In some embodiments, the CD40L-specific Tn3 monomer subunits of the invention comprise the amino acid sequence:

$IEV(X_{AB})_nALITW(X_{BC})_nCELX_1YGI(X_{CD})_nTTIDL(X_{DE})_nYSI(X_{EF})_nYEVSLIC(X_{FG})_n$ KETFTT

wherein:

(a) X_{AB}, X_{BC}, X_{CD}, X_{DE}, X_{EF}, and X_{FG} represent the amino acid residues present in the sequences of the AB, BC, CD, DE, EF, and FG loops, respectively;

(b) X_1 represents amino acid residue alanine (A) or threonine (T); and,

(c) length of the loop *n* is an integer between 2 and 26.

TABLE 2: Loop Sequences of Tn3 Clones Used in These Studies

Clone	AB Loop SEQ ID NO	BC Loop SEQ ID NO	CD Loop SEQ ID NO	DE Loop SEQ ID NO	EF Loop SEQ ID NO	FG Loop SEQ ID NO*
PARENT Tn3						
Tn3	4	5	6	7	8	9
309 FAMILY						
309FGwt	4	83	6	94	8	9
309	4	83	6	94	8	99
340	4	84	6	95	8	9
341	4	85	6	94	8	9
342	4	86	6	96	8	9
343	4	87	6	97	8	9
344	4	88	6	95	8	9
345	4	89	6	94	8	9
346	4	90	6	94	8	9
347	4	91	6	95	8	9
348	4	92	6	98	8	9
349	4	93	6	94	8	9
309FGwt consensus	4	168	6	169	8	170
311 FAMILY*						
311	4	100	6	118	8	129
311K4E	136	100	6	118	137	129
311K4E_1	136	101	6	119	8	129
311K4E_2	136	102	6	120	8	129
311K4E_3†	136	103	6	121	8	129
311K4E_4†	136	104	6	122	8	129
311K4E_5†	136	105	6	121	8	129
311K4E_7	136	106	6	123	8	129
311K4E_8†	136	107	6	123	8	129
311K4E_9	136	108	6	118	8	129
311K4E_10†	136	109	6	123	8	129
311K4E_11	136	110	6	121	8	129
311K4E_12†	136	111	6	123	8	130
311K4E_13	136	108	6	121	8	129
311K4E_14	136	112	6	124	8	129
311K4E_15	136	113	6	125	8	129
311K4E_16	136	114	6	118	8	129
311K4E_19	136	115	6	126	8	129
311K4E_20	136	116	6	127	8	129
311K4E_21	136	117	6	128	8	129
311 consensus	173	174	6	175	176	177

† Clones comprising a C beta strand having the sequence CELAYGI (SEQ ID NO: 14), all other clones comprise a C beta strand having the sequence CELTYGI (SEQ ID NO: 13).

* In some variants in the 309 family, e.g., 342, the FG loop can be replaced with SEQ ID NO: 139.

** In some variants in the 311 family, the BC loop can be engineered to replace the tyrosine at position 21. It is specifically contemplated that the replacement amino acid residues can have a small side chain.

[0198] In some embodiments, the CD40L-specific Tn3 monomer subunits of the invention consist of the amino acid sequence:

$IEV(X_{AB})_nALITW(X_{BC})_nCELX_1YGI(X_{CD})_nTTIDL(X_{DE})_nYSI(X_{EF})_nYEVSLIC(X_{FG})_n$ KETFTT

wherein:

(a) X_{AB}, X_{BC}, X_{CD}, X_{DE}, X_{EF}, and X_{FG} represent the amino acid residues present in the sequences of the AB, BC, CD, DE, EF, and FG loops, respectively;

(b) X_1 represents amino acid residue alanine (A) or threonine (T); and,

(c) length of the loop *n* is an integer between 2 and 26.

[0199] In one embodiment, the beta strands of the CD40L-specific Tn3 monomer scaffold have at least 90% sequence identity to the beta strands of the parent Tn3 scaffold (SEQ ID NO: 3). To calculate such percentage of sequence identify, amino acid sequences are aligned applying methods known in the art. The percentage of sequence identity is defined as the ratio between (a) the number of amino acids located in beta strands which are identical in the sequence alignment and (b) the total number of amino acids located in beta strands.

[0200] In one embodiment, the sequence of the AB loop comprises SEQ ID NO: 4 or SEQ ID NO: 136. In another embodiment, the sequence of the CD loop comprises SEQ ID NO: 6. In another embodiment, the sequence of the EF loop comprises SEQ ID NO: 8 or SEQ ID NO: 137. In one embodiment, the sequence of the AB loop consists of SEQ ID NO: 4 or SEQ ID NO: 136. In another embodiment, the sequence of the CD loop consists of SEQ ID NO: 6. In another embodiment, the sequence of the EF loop consists of SEQ ID NO: 8 or SEQ ID NO: 137.

[0201] In one embodiment, the sequence of the BC loop comprises a sequence selected from the group consisting of SEQ ID NOs: 83, 84, 85, 86, 87, 88, 89, 90, 91, 92 and 93. In another embodiment, the sequence of the BC loop consists of a sequence selected from the group consisting of SEQ ID NOs: 83, 84, 85, 86, 87, 88, 89, 90, 91, 92 and 93.

[0202] In one embodiment, the sequence of the DE loop comprises a sequence selected from the group consisting of SEQ ID NOs: 94, 95, 96, 97 and 98. In another embodiment, the sequence of the DE loop consists of a sequence selected from the group consisting of SEQ ID NOs: 94, 95, 96, 97 and 98.

[0203] In one embodiment, the sequence of the FG loop comprises a sequence selected from the group consisting of SEQ ID NOs: 9, 99, and 139. In another embodiment,

the sequence of the FG loop consists of a sequence selected from the group consisting of SEQ ID NOs: 9, 99, and 139.

[0204] In one embodiment, the sequence of the BC loop comprises a sequence selected from the group consisting of SEQ ID NOs: 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116 and 117. In another embodiment, the sequence of the BC loop consists of a sequence selected from the group consisting of SEQ ID NOs: 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116 and 117.

[0205] In some embodiments, the sequence of the DE loop comprises a sequence selected from the group consisting of SEQ ID NOs: 118, 119, 120, 121, 122, 123, 124, 125, 126, 127 and 128. In other embodiments, the sequence of the DE loop consists of a sequence selected from the group consisting of SEQ ID NOs: 118, 119, 120, 121, 122, 123, 124, 125, 126, 127 and 128.

[0206] In some embodiments, the sequence of the FG loop comprises a sequence selected from the groups consisting of SEQ ID NOs: 129 and 130. In other embodiments, the sequence of the FG loop consists of a sequence selected from the groups consisting of SEQ ID NOs: 129 and 130.

[0207] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 83, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In some embodiments, the sequence of the BC loop consists of SEQ ID NO: 83, the sequence of the DE loop consists of SEQ ID NO: 94, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0208] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 83, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 99. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 83, the sequence of the DE loop consists of SEQ ID NO: 94, and the sequence of the FG loop consists of SEQ ID NO: 99.

[0209] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 84, the sequence of the DE loop comprises SEQ ID NO: 95, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 84, the sequence of the DE loop consists of SEQ ID NO: 95, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0210] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 85, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists

of SEQ ID NO: 85, the sequence of the DE loop consists of SEQ ID NO: 94, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0211] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 86, the sequence of the DE loop comprises SEQ ID NO: 96, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 86, the sequence of the DE loop consists of SEQ ID NO: 96, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0212] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 87, the sequence of the DE loop comprises SEQ ID NO: 97, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 87, the sequence of the DE loop consists of SEQ ID NO: 97, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0213] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 88, the sequence of the DE loop comprises SEQ ID NO: 95, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 88, the sequence of the DE loop consists of SEQ ID NO: 95, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0214] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 89, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 89, the sequence of the DE loop consists of SEQ ID NO: 94, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0215] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 90, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 90, the sequence of the DE loop consists of SEQ ID NO: 94, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0216] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 91, the sequence of the DE loop comprises SEQ ID NO: 95, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 91, the sequence of the DE loop consists of SEQ ID NO: 95, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0217] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 92, the sequence of the DE loop comprises SEQ ID NO: 98, and the sequence of the FG loop

comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 92, the sequence of the DE loop consists of SEQ ID NO: 98, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0218] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 93, the sequence of the DE loop comprises SEQ ID NO: 94, and the sequence of the FG loop comprises SEQ ID NO: 9 or 139. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 93, the sequence of the DE loop consists of SEQ ID NO: 94, and the sequence of the FG loop consists of SEQ ID NO: 9 or 139.

[0219] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 168, the sequence of the DE loop comprises SEQ ID NO: 169, and the sequence of the FG loop comprises SEQ ID NO: 170. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 168, the sequence of the DE loop consists of SEQ ID NO: 169, and the sequence of the FG loop consists of SEQ ID NO: 170.

[0220] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 100, the sequence of the DE loop comprises SEQ ID NO: 118, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 100, the sequence of the DE loop consists of SEQ ID NO: 118, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0221] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 101, the sequence of the DE loop comprises SEQ ID NO: 119, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 101, the sequence of the DE loop consists of SEQ ID NO: 119, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0222] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 102, the sequence of the DE loop comprises SEQ ID NO: 120, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 102, the sequence of the DE loop consists of SEQ ID NO: 120, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0223] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 103, the sequence of the DE loop comprises SEQ ID NO: 121, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence

of the BC loop consists of SEQ ID NO: 103, the sequence of the DE loop consists of SEQ ID NO: 121, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0224] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 104, the sequence of the DE loop comprises SEQ ID NO: 122, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 104, the sequence of the DE loop consists of SEQ ID NO: 122, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0225] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 105, the sequence of the DE loop comprises SEQ ID NO: 121, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 105, the sequence of the DE loop consists of SEQ ID NO: 121, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0226] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 106, the sequence of the DE loop comprises SEQ ID NO: 123, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 106, the sequence of the DE loop consists of SEQ ID NO: 123, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0227] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 107, the sequence of the DE loop comprises SEQ ID NO: 123, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 107, the sequence of the DE loop consists of SEQ ID NO: 123, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0228] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 108, the sequence of the DE loop comprises SEQ ID NO: 118, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 108, the sequence of the DE loop consists of SEQ ID NO: 118, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0229] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 109, the sequence of the DE loop

comprises SEQ ID NO: 123, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 109, the sequence of the DE loop consists of SEQ ID NO: 123, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0230] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 110, the sequence of the DE loop comprises SEQ ID NO: 121, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 110, the sequence of the DE loop consists of s SEQ ID NO: 121, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0231] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 111, the sequence of the DE loop comprises SEQ ID NO: 123, and the sequence of the FG loop comprises SEQ ID NO: 130. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 111, the sequence of the DE loop consists of SEQ ID NO: 123, and the sequence of the FG loop consists of SEQ ID NO: 130.

[0232] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 108, the sequence of the DE loop comprises SEQ ID NO: 121, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 108, the sequence of the DE loop consists of SEQ ID NO: 121, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0233] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 112, the sequence of the DE loop comprises SEQ ID NO: 124, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 112, the sequence of the DE loop consists of SEQ ID NO: 124, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0234] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 113, the sequence of the DE loop comprises SEQ ID NO: 125, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 113, the sequence of the DE loop consists of SEQ ID NO: 125, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0235] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 114, the sequence of the DE loop comprises SEQ ID NO: 118, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 114, the sequence of the DE loop consists of SEQ ID NO: 118, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0236] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 115, the sequence of the DE loop comprises SEQ ID NO: 126, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 115, the sequence of the DE loop consists of SEQ ID NO: 126, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0237] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 116, the sequence of the DE loop comprises SEQ ID NO: 127, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 116, the sequence of the DE loop consists of SEQ ID NO: 127, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0238] In some embodiments, the sequence of the AB loop comprises SEQ ID NO: 136, the sequence of the BC loop comprises SEQ ID NO: 117, the sequence of the DE loop comprises SEQ ID NO: 128, and the sequence of the FG loop comprises SEQ ID NO: 129. In other embodiments, the sequence of the AB loop consists of SEQ ID NO: 136, the sequence of the BC loop consists of SEQ ID NO: 117, the sequence of the DE loop consists of SEQ ID NO: 128, and the sequence of the FG loop consists of SEQ ID NO: 129.

[0239] In some embodiments, the sequence of the BC loop comprises SEQ ID NO: 174, the sequence of the DE loop comprises SEQ ID NO: 175, and the sequence of the FG loop comprises SEQ ID NO: 177. In other embodiments, the sequence of the BC loop consists of SEQ ID NO: 174, the sequence of the DE loop consists of SEQ ID NO: 175, and the sequence of the FG loop consists of SEQ ID NO: 177.

[0240] In some embodiments, the CD40L-specific monomer subunit comprises a sequence selected from the group consisting of SEQ ID NO: 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42 and 146. In other embodiments, the CD40L-specific monomer subunit consists of a sequence selected from the group consisting of SEQ ID NO: 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42 and 146.

[0241] In some embodiments, the CD40L-specific monomer subunit comprises SEQ ID NO: 28 or 146. In other embodiments, the CD40L-specific monomer subunit consists of SEQ ID NO: 28 or 146.

[0242] In some embodiments, the CD40L-specific Tn3 monomer subunits of the invention comprise the amino acid sequence:

IEVKDVTDTTALITWX$_1$DX$_2$X$_3$X$_4$X$_5$X$_6$X$_7$X$_8$CELTYGIKDVPGDRTTIDLWX$_9$HX$_{10}$AX$_{11}$YSIGNLKPDTEYEVSLICRX$_{12}$GDMSSNPAKETFTT (SEQ ID NO: 167)

wherein:

(a) X$_1$ represents amino acid residue serine (S) or leucine (L);

(b) X$_2$ represents amino acid residue aspartic acid (D) or glutamic acid (E);

(c) X$_3$ represents amino acid residue histidine (H), isoleucine (I), valine (V), phenylalanine (F) or tryptophan (W);

(d) X$_4$ represents amino acid residue alanine (A), glycine (G), glutamic acid (E) or aspartic acid (D);

(e) X$_5$ represents amino acid residue glutamic acid (E), leucine (L), glutamine (Q), serine (S), aspartic acid (D) or asparagine (N);

(f) X$_6$ represents amino acid residue phenylalanine (F) or tyrosine (Y);

(g) X$_7$ represents amino acid residue isoleucine (I), valine (V), histidine (H), glutamic acid (E) or aspartic acid (D);

(h) X$_8$ represents amino acid residue glycine (G), tryptophan (W) or valine (V);

(i) X$_9$ represents amino acid residue tryptophan (W), phenylalanine (F) or tyrosine (Y);

(j) X$_{10}$ represents amino acid residue serine (S), glutamine (Q), methionine (M) or histidine (H);

(k) X$_{11}$ represents amino acid residue tryptophan (W) or histidine (H); and,

(l) X$_{12}$ represents amino acid residue arginine (R) or serine (S).

[0243] In some embodiments, the CD40L-specific Tn3 monomer subunits of the invention consist of the amino acid sequence:

IEVKDVTDTTALITWX$_1$DX$_2$X$_3$X$_4$X$_5$X$_6$X$_7$X$_8$CELTYGIKDVPGDRTTIDLWX$_9$HX$_{10}$AX$_{11}$YSIGNLKPDTEYEVSLICRX$_{12}$GDMSSNPAKETFTT (SEQ ID NO: 167)

wherein:

(a) X$_1$ represents amino acid residue serine (S) or leucine (L);

(b) X$_2$ represents amino acid residue aspartic acid (D) or glutamic acid (E);

(c) X_3 represents amino acid residue histidine (H), isoleucine (I), valine (V), phenylalanine (F) or tryptophan (W);

(d) X_4 represents amino acid residue alanine (A), glycine (G), glutamic acid (E) or aspartic acid (D);

(e) X_5 represents amino acid residue glutamic acid (E), leucine (L), glutamine (Q), serine (S), aspartic acid (D) or asparagine (N);

(f) X_6 represents amino acid residue phenylalanine (F) or tyrosine (Y);

(g) X_7 represents amino acid residue isoleucine (I), valine (V), histidine (H), glutamic acid (E) or aspartic acid (D);

(h) X_8 represents amino acid residue glycine (G), tryptophan (W) or valine (V);

(i) X_9 represents amino acid residue tryptophan (W), phenylalanine (F) or tyrosine (Y);

(j) X_{10} represents amino acid residue serine (S), glutamine (Q), methionine (M) or histidine (H);

(k) X_{11} represents amino acid residue tryptophan (W) or histidine (H); and,

(l) X_{12} represents amino acid residue arginine (R) or serine (S).

[0244] In some embodiments, the CD40L-specific monomer subunit comprises a sequence selected from the group consisting of SEQ ID NO: 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, and 82. In some embodiments, the CD40L-specific monomer subunit consists of a sequence selected from the group consisting of SEQ ID NO: 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, and 82.

[0245] In some embodiments, the CD40L-specific Tn3 monomer subunits of the invention comprise the amino acid sequence:

IEVX$_1$DVTDTTALITWX$_2$X$_3$RSX$_4$X$_5$X$_6$X$_7$X$_8$X$_9$X$_{10}$CELX$_{11}$YGIKDVPGDRTTIDLX$_{12}$X$_{13}$X$_{14}$X$_{15}$YVHYSIGNLKPDTX$_{16}$YEVSLICLTTDGTYX$_{17}$NPAKETFTT (SEQ ID NO: 171)

wherein:

(a) X_1 represents amino acid residue lysine (K) or glutamic acid (E);

(b) X_2 represents amino acid residue threonine (T) or isoleucine (I);

(c) X_3 represents amino acid residue asparagine (N) or alanine (A);

(d) X_4 represents amino acid residue serine (S), leucine (L), alanine (A), phenylalanine (F) or tyrosine (Y);

(e) X_5 represents amino acid residue tyrosine (Y), alanine (A), glycine (G), valine (V), isoleucine (I) or serine (S);

(f) X_6 represents amino acid residue tyrosine (Y), serine (S), alanine (A) or histidine (H);

(g) X_7 represents amino acid residue asparagine (N), aspartic acid (D), histidine (H) or tyrosine (Y);

(h) X_8 represents amino acid residue leucine (L), phenylalanine (F), histidine (H) or tyrosine (Y);

(i) X_9 represents amino acid residue histidine (H), proline (P), serine (S), leucine (L) or aspartic acid (D);

(j) X_{10} represents amino acid residue glycine (G), phenylalanine (F), histidine (H) or tyrosine (Y);

(k) X_{11} represents amino acid residue alanine (A) or threonine (T);

(l) X_{12} represents amino acid residue serine (S), asparagine (N), glutamic acid (E), asparagine (R) or aspartic acid (D);

(m) X_{13} represents amino acid residue serine (S), glutamine (Q), threonine (T), asparagine (N) or alanine (A);

(n) X_{14} represents amino acid residue proline (P), valine (V), isoleucine (I) or alanine (A) or no amino acid;

(o) X_{15} represents amino acid residue isoleucine (I) or no amino acid;

(p) X_{16} represents amino acid residue glutamic acid (E) or lysine (K); and,

(q) X_{17} represents amino acid residue serine (S) or asparagine (N).

[0246] In some embodiments, the CD40L-specific Tn3 monomer subunits of the invention consist of the amino acid sequence:

IEVX$_1$DVTDTTALITWX$_2$X$_3$RSX$_4$X$_5$X$_6$X$_7$X$_8$X$_9$X$_{10}$CELX$_{11}$YGIKDVPGDRTTIDLX$_{12}$X$_{13}$X$_{14}$X$_{15}$YVHYSIGNLKPDTX$_{16}$YEVSLICLTTDGTYX$_{17}$NPAKETFTT (SEQ ID NO: 171)

wherein:

(a) X_1 represents amino acid residue lysine (K) or glutamic acid (E);

(b) X_2 represents amino acid residue threonine (T) or isoleucine (I);

(c) X_3 represents amino acid residue asparagine (N) or alanine (A);

(d) X_4 represents amino acid residue serine (S), leucine (L), alanine (A), phenylalanine (F) or tyrosine (Y);

(e) X_5 represents amino acid residue tyrosine (Y), alanine (A), glycine (G), valine (V), isoleucine (I) or serine (S);

(f) X_6 represents amino acid residue tyrosine (Y), serine (S), alanine (A) or histidine (H);

(g) X_7 represents amino acid residue asparagine (N), aspartic acid (D), histidine (H) or tyrosine (Y);

(h) X_8 represents amino acid residue leucine (L), phenylalanine (F), histidine (H) or tyrosine (Y);

(i) X_9 represents amino acid residue histidine (H), proline (P), serine (S), leucine (L) or aspartic acid (D);

(j) X_{10} represents amino acid residue glycine (G), phenylalanine (F), histidine (H) or tyrosine (Y);

(k) X_{11} represents amino acid residue alanine (A) or threonine (T);

(l) X_{12} represents amino acid residue serine (S), asparagine (N), glutamic acid (E), asparagine (R) or aspartic acid (D);

(m) X_{13} represents amino acid residue serine (S), glutamine (Q), threonine (T), asparagine (N) or alanine (A);

(n) X_{14} represents amino acid residue proline (P), valine (V), isoleucine (I) or alanine (A) or no amino acid;

(o) X_{15} represents amino acid residue isoleucine (I) or no amino acid;

(p) X_{16} represents amino acid residue glutamic acid (E) or lysine (K); and,

(q) X_{17} represents amino acid residue serine (S) or asparagine (N).

[0247] In some embodiments, a CD40L-specific monomer scaffold comprise a Tn3 module wherein one or more of the beta strands comprise at least one amino acid substitution except that the cysteine residues in the C and F beta strands (SEQ ID NOs: 13 or 14; and SEQ ID NO: 17, respectively) may not be substituted.

[0248] The loops connecting the various beta strands of a CD40L-specific monomer subunit can be randomized for length and/or sequence diversity. In one embodiment, a CD40L-specific monomer subunit has at least one loop that is randomized for length and/or sequence diversity. In one embodiment, at least one, at least two, at least three, at least four, at least five or at least six loops of a CD40L-specific monomer subunit are randomized for length and/or sequence diversity. In one embodiment, at least one loop of a CD40L-specific monomer subunit is kept constant while at least one additional loop is randomized for length and/or sequence diversity. In another embodiment, at least one, at least two, or all three of loops AB, CD, and EF are kept constant while at least one, at least two, or all three of loops BC, DE, and FG are randomized for length or sequence diversity. In another embodiment, at

least one, at least two, or at least all three of loops AB, CD, and EF are randomized while at least one, at least two, or all three of loops BC, DE, and FG are randomized for length and/or sequence diversity. In still another embodiment, at least one, at least two, at least three of loops, at least 4, at least five, or all six of loops AB, CD, EF, BC, DE, and FG are randomized for length or sequence diversity.

[0249] In some embodiments, one or more residues within a loop are held constant while other residues are randomized for length and/or sequence diversity. In some embodiments, one or more residues within a loop are held to a predetermined and limited number of different amino acids while other residues are randomized for length and/or sequence diversity. Accordingly, a CD40L-specific monomer subunit of the invention can comprise one or more loops having a degenerate consensus sequence and/or one or more invariant amino acid residues.

[0250] In one embodiment, the CD40L-specific monomer subunit of the invention comprises an AB loop which is randomized. In another embodiment, the CD40L-specific monomer subunit of the invention comprises a BC loop which is randomized. In one embodiment, the CD40L-specific monomer subunit of the invention comprises a CD loop which is randomized. In one embodiment, the CD40L-specific monomer subunit of the invention comprises a DE loop which is randomized. In one embodiment, the CD40L-specific monomer subunit of the invention comprises an EF loop which is randomized.

[0251] In certain embodiments, the CD40L-specific monomer subunit of the invention comprises a FG loop which is held to be at least one amino acid residue shorter than the cognate FG loop of the third FnIII domain of human tenascin C and is further randomized at one or more positions.

[0252] In specific embodiments, at least one of loops BC, DE, and FG is randomized, wherein the A beta strand comprises SEQ ID NO:10 or 11, the B beta strand comprises SEQ ID NO:12, the C beta strand comprises SEQ ID NO:13 or 14, the D beta strand comprises SEQ ID NO:15, the E beta strand comprises SEQ ID NO:16, the F beta strand comprises SEQ ID NO:17, and the G beta strand comprises SEQ ID NO:18, the AB loop comprises SEQ ID NO:4 or 136, the CD loop comprises SEQ ID NO:6 and the EF loop comprises SEQ ID NO:8 or 137.

[0253] In other specific embodiments, at least one of loops AB, CD, and EF are randomized, wherein the A beta strand comprises SEQ ID NO:10 or 11, the B beta strand comprises SEQ ID NO:12, the C beta strand comprises SEQ ID NO:13 or 14, the D beta strand comprises SEQ ID NO:15, the E beta strand comprises SEQ ID NO:16, the F beta

strand comprises SEQ ID NO:17, and the G beta strand comprises SEQ ID NO:18, the BC loop comprises SEQ ID NO:5, the DE loop comprises SEQ ID NO:7 and the FG loop comprises SEQ ID NO:9 or 139.

Enhanced Scaffold Stability

[0254] The stability of Tn3 scaffolds of the invention may be increased by many different approaches. In some embodiments, Tn3 scaffolds of the invention can be stabilized by elongating the N- and/or C-terminal regions. The N- and/or C-terminal regions can be elongated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 amino acids. In other embodiments, the Tn3 scaffolds of the invention can be stabilized by introducing an alteration that increases serum half-life, as described herein. In yet another embodiment, the Tn3 scaffolds of the invention comprise an addition, deletion or substitution of at least one amino acid residue to stabilize the hydrophobic core of the scaffold.

[0255] Tn3 scaffolds of the invention can be effectively stabilized by engineering non-natural disulfide bonds as disclosed in International Patent Application No. PCT/US2011/032184. In some embodiments, scaffolds of the invention comprise non-naturally occurring disulfide bonds, as described in PCT Publication No: WO 2009/058379. A bioinformatics approach may be utilized to identify candidate positions suitable for engineering disulfide bonds.

[0256] In one embodiment, a Tn3 monomer subunit of the invention comprises at least one, at least two, at least three, at least four, or at least five non-naturally occurring intramolecular disulfide bonds. In one embodiment, a Tn3 monomer subunit of the invention comprises at least one non-naturally occurring intramolecular disulfide bond, wherein said at least one non-naturally occurring disulfide bond stabilizes the monomer. In yet another embodiment, Tn3 scaffolds of the invention comprise at least one non-naturally occurring disulfide bond, wherein the bond is located between two distinct monomer or multimer Tn3 scaffolds, *i.e.*, the disulfide bond is an intermolecular disulfide bond. For example, a disulfide bond can link distinct scaffolds (for example, two CD40L-specific monomer scaffolds), a Tn3 scaffold and a linker, a Tn3 scaffold and an Fc domain, or a Tn3 scaffold and an antibody or fragment thereof.

[0257] In some embodiments, Tn3 scaffolds of the invention comprise at least one non-naturally occurring intermolecular disulfide bond that links a Tn3 monomer subunit and an isolated heterologous moiety, a Tn3 monomer subunit and a heterologous moiety fused or

conjugated to the same Tn3 scaffold, or a Tn3 monomer subunit and a heterologous moiety fused or conjugated to a different Tn3 scaffold.

[0258] In some embodiments, Tn3 scaffolds of the invention comprise a disulfide bond that forms a Tn3 multimeric scaffold of at least 2, at least 3, at least 4 or more monomer subunits.

[0259] In another embodiment, Tn3 scaffolds of the invention may comprise an elongation of the N and/or C terminal regions. In one embodiment, the Tn3 scaffold of the invention comprises an alteration to increase serum half-life, as described herein. In yet another embodiment, the scaffolds of the invention comprise an addition, deletion or substitution of at least one amino acid residue to stabilize the hydrophobic core of the scaffold.

Stability Measurements

[0260] The stability of the Tn3 monomer subunits of the invention, isolated or as part of a multimeric Tn3 scaffold, can be readily measured by techniques well known in the art, such as thermal (T_m) and chaotropic denaturation (such as treatment with urea, or guanidine salts), protease treatment (such as treatment with thermolysin) or another art accepted methodology to determine protein stability. A comprehensive review of techniques used to measure protein stability can be found, for example in "Current Protocols in Molecular Biology" and "Current Protocols in Protein Science" by John Wiley and Sons. 2007.

Multimeric Tn3 Scaffolds

[0261] One aspect of the present invention provides multimeric Tn3 scaffolds comprising at least two Tn3 monomer subunits of the invention joined in tandem, and wherein at least one of the monomers is a CD40L-specific monomer subunit. Such multimeric Tn3 scaffolds can be assembled in multiple formats. In a specific aspect, the invention provides multimeric Tn3 scaffolds, wherein at least two CD40L-specific monomer subunits are connected in tandem via a peptide linker. In some embodiments, the multimeric Tn3 scaffold exhibits an increase in the valency and/or avidity of target binding, or other action of the target(s). In some embodiments, the increase in valency and/or avidity of target binding is accomplished when multiple monomer subunits bind to the same target. In some embodiments, the increase in valency improves a specific action on the target, such as increasing the dimerization of a target protein.

[0262] In a specific embodiment, a multimeric Tn3 scaffold of the invention comprises at least two CD40L-specific monomer subunits connected in tandem, wherein each CD40L-specific monomer subunit binds at least one target, and wherein each CD40L-specific monomer subunit comprises a plurality of beta strands linked to a plurality of loop regions, wherein at least one loop is a non-naturally occurring variant of the cognate loop in the parent Tn3 scaffold (SEQ ID NO: 3).

[0263] In one embodiment, multimeric Tn3 scaffolds are generated through covalent binding between CD40L-specific monomer subunits, for example, by directly linking the CD40L-specific monomer subunits, or by the inclusion of a linker, *e.g.*, a peptide linker. In particular examples, covalently bonded Tn3 scaffolds are generated by constructing fusion genes that encode the CD40L-specific monomer subunits or, alternatively, by engineering codons for cysteine residues into CD40L-specific monomer subunits and allowing disulfide bond formation to occur between the expression products.

[0264] In one embodiment, multimeric Tn3 scaffolds of the invention comprise at least two CD40L-specific monomer subunits that are connected directly to each other without any additional intervening amino acids. In another embodiment, multimeric Tn3 scaffolds of the invention comprise at least two CD40L-specific monomer subunits that are connected in tandem via a linker, *e.g.*, a peptide linker.

[0265] In a specific embodiment, multimeric Tn3 scaffolds of the invention comprise at least two CD40L-specific monomer subunits that are connected in tandem via a peptide linker, wherein the peptide linker comprises 1 to about 1000, or 1 to about 500, or 1 to about 250, or 1 to about 100, or 1 to about 50, or 1 to about 25, amino acids. In a specific embodiment, the multimeric Tn3 scaffold comprises at least two CD40L-specific monomer subunits that are connected in tandem via a peptide linker, wherein the peptide linker comprises 1 to about 20, or 1 to about 15, or 1 to about 10, or 1 to about 5, amino acids.

[0266] In a specific embodiment, the multimeric Tn3 scaffold comprises at least two CD40L-specific monomer subunits that are connected in tandem via a linker, *e.g.*, a peptide linker, wherein the linker is a functional moiety**.** The functional moiety will be selected based on the desired function and/or characteristics of the multimeric Tn3 scaffold. For example, a functional moiety useful for purification (*e.g.*, a histidine tag) may be used as a linker. Functional moieties useful as linkers include, but are not limited to, polyethylene glycol (PEG), a cytotoxic agent, a radionuclide, imaging agent, biotin, a dimerization domain, human serum albumin (HSA) or an FcRn binding portion thereof, a domain or fragment of an antibody, a single chain antibody, a domain antibody, an albumin binding domain, an IgG

molecule, an enzyme, a ligand, a receptor, a binding peptide, a non-Tn3 scaffold, an epitope tag, a recombinant polypeptide polymer, a cytokine, and the like. Specific peptide linkers and functional moieties which may be used as linkers are disclosed *infra*.

[0267] In specific embodiments, the functional moiety is an immunoglobulin or a fragment thereof. In some embodiments, the immunoglobulin or fragment thereof comprises an Fc domain. In some embodiments, the Fc domain fails to induce at least one FcγR-mediated effector function, such as ADCC (Antibody-dependent cell-mediated cytotoxicity). It is known in the art that the Fc domain maybe altered to reduce or eliminate at least one FcγR-mediated effector function, see, for example, U.S. Pat. Nos. 5,624,821 and 6,737,056.

[0268] In some embodiments, the multimeric Tn3 scaffold comprises at least two CD40L-specific monomer subunits that are connected via one or more linkers, wherein the linkers interposed between each CD40L-specific monomer subunit can be the same linkers or different linkers. In some embodiments, a linker can comprise multiple linkers, which can be the same linker or different linkers. In some embodiments, when a plurality of linkers are concatenated, some or all the linkers can be functional moieties.

Scaffold Binding Stoichiometry

[0269] In some embodiments, a monomeric or multimeric Tn3 scaffold can comprise a CD40L-specific monomer subunit specific for different epitopes, which can be different epitopes on a single CD40L molecule or on different CD40L target molecules. In some embodiments, a multimeric Tn3 scaffold can comprise CD40L-specific monomer subunits wherein each subunit targets one or more different epitopes on one or more CD40L molecules.

[0270] In other embodiments, a monomeric or multimeric Tn3 scaffold can bind two or more different epitopes on the same CD40L molecule. In some embodiments, the different epitopes are non-overlapping epitopes. In other embodiments, the different epitopes are overlapping epitopes.

[0271] In yet another specific embodiment, a monomeric or multimeric Tn3 scaffold can bind one or more epitopes on a CD40L molecule and additionally bind one or more epitopes on a second CD40L molecule. In some embodiments, the different target molecules are part of an oligomeric complex, *e.g.*, a trimeric CD40L complex.

[0272] In still another specific embodiment, a monomeric or multimeric Tn3 scaffold can bind to a single epitope on a CD40L trimer. In yet another embodiment, a

monomeric or multimeric Tn3 scaffold can bind to the same epitope on at least two CD40L trimers.

[0273] In certain embodiments, a monomeric or multimeric Tn3 scaffold can bind the same epitope on two or more copies of a CD40L molecule on an adjacent cell surface. In certain embodiments, a monomeric or multimeric Tn3 scaffold can bind the same epitope on two or more copies of a CD40L molecule in solution. In some embodiments, a monomeric or multimeric Tn3 scaffold can bind to the same epitope or different epitopes on CD40L with the same or different binding affinities and/or avidities.

[0274] In another embodiment, a monomeric or multimeric Tn3 scaffolds can bind to epitopes on one or more copies of CD40L and achieve or enhance (*e.g.*, synergistically) a desired action on the target, *e.g.*, prevent binding to a receptor or prevent oligomerization.

[0275] In addition, when a monomeric or multimeric Tn3 scaffold of the invention comprises a plurality of CD40L-specific monomer subunits, *e.g.*, different monomers wherein each monomer targets different epitopes on CD40L, such monomer subunits can be arranged according to a certain pattern or special orientation to achieve or enhance a certain biological effect. Such combinations of monomeric subunits can be assembled and subsequently evaluated using methods known in the art.

Fusions

[0276] The invention provides Tn3 scaffolds wherein at least one CD40L-specific monomer subunit can be fused to a heterologous moiety. In this context the heterologous moiety is not used to link the scaffolds as a spacer but may provide additional functionality to the Tn3 scaffold. In some embodiments, a heterologous moiety can also function as a linker. The present invention encompasses the use of Tn3 scaffolds conjugated or fused to one or more heterologous moieties, including but not limited to, peptides, polypeptides, proteins, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules. Accordingly, the invention provides polypeptides comprising one or more CD40L-specific Tn3 monomer, including but not limited to the fusion proteins described herein.

[0277] The present invention encompasses the use of Tn3 scaffolds recombinantly fused or chemically conjugated to a heterologous protein or polypeptide or fragment thereof. Conjugation includes both covalent and non-covalent conjugation. In some embodiments, a Tn3 scaffold can be fused or chemically conjugated to a polypeptide of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100,

at least 200, at least 300, at least 500, or at least 1000 amino acids) to generate fusion proteins.

[0278] The fusion or conjugation of a Tn3 scaffold to one or more heterologous moieties can be direct, *i.e.*, without a linker interposed between a Tn3 scaffold and a heterologous moiety, or via one or more linker sequences described herein. In some embodiments, scaffolds can be used to target heterologous polypeptides to particular cell types, either *in vitro* or *in vivo*, by fusing or conjugating the Tn3 scaffolds to antibodies specific for particular cell surface receptors in the target cells.

[0279] Tn3 scaffolds fused or conjugated to heterologous polypeptides can also be used in *in vitro* immunoassays and purification methods using methods known in the art. See, *e.g.*, International Publication No. WO 93/21232; European Patent No. EP 439,095; Naramura *et al.*, Immunol. Lett. 39:91-99, 1994; U.S. Pat. No. 5,474,981; Gillies *et al.*, PNAS 89:1428-1432, 1992; and Fell *et al.*, J. Immunol. 146:2446-2452, 1991

[0280] In some embodiments, the Tn3 scaffolds can be integrated with the human immune response by fusing or conjugating a scaffold with an immunoglobulin or domain thereof including, but not limited to, the constant region of an IgG (Fc), *e.g.*, through the N or C-terminus. Similarly, a fusion between a Tn3 scaffold and a complement protein, such as CIq, can be used to target cells.

[0281] Various publications describe methods for obtaining physiologically active molecules whose half-lives are modified by introducing an FcRn-binding polypeptide into the molecules (see, *e.g.*, WO 97/43316; U.S. Pat. No. 5,869,046; U.S. Pat. No. 5,747,035; WO 96/32478; and WO 91/14438), by fusing the molecules with antibodies whose FcRn-binding affinities are preserved but affinities for other Fc receptors have been greatly reduced (see, *e.g.*, WO 99/43713), or by fusing the molecules with FcRn binding domains of antibodies (see, *e.g.*, WO 00/09560; U.S. Pat. No. 4,703,039). Specific techniques and methods of increasing half-life of physiologically active molecules can also be found in U.S. Patent No. 7,083,784. Specifically, it is contemplated that the Tn3 scaffolds can be fused to an Fc region from an IgG, wherein the Fc region comprises amino acid residue mutations M252Y/S254T/T256E or H433K/N434F/Y436H, wherein amino acid positions are designated according to the Kabat numbering schema. It is specifically contemplated the fusion of a Tn3 scaffold to an Fc domain variant not capable of inducing ADCC.

[0282] In some embodiments, the half-life of the Tn3 scaffold can be increased by genetically fusing the Tn3 scaffold with an intrinsically unstructured recombinant

polypeptide (*e.g.*, an XTEN™ polypeptide) or by conjugation with polyethylene glycol (PEG).

[0283] In some embodiments, the Tn3 scaffold can be fused with molecules that increase or extend *in vivo* or serum half-life. In some embodiments, the scaffold can be fused or conjugated with albumin, such as human serum albumin (HSA), a neonatal Fc receptor (FcRn) binding fragment thereof, PEG, polysaccharides, antibodies, complement, hemoglobin, a binding peptide, lipoproteins and other factors to increase its half-life in the bloodstream and/or its tissue penetration. Any of these fusions may be generated by standard techniques, for example, by expression of the fusion protein from a recombinant fusion gene constructed using publicly available gene sequences.

[0284] In some embodiments, a property of the Tn3 scaffold can be improved by conjugation or fusion to an HSA variant, *i.e.*, a molecule derived from full length HSA (SEQ ID NO: 139) comprising at least an amino acid substitution, a deletion, or a sequence truncation.

[0285] In some embodiments, the property improved by conjugation with an HSA variant is plasma half-life. The improvement in plasma half-life of the Tn3 scaffold can be an alteration in that property such as an increase or decrease in plasma half-life, or changes in other pharmacokinetic parameters. In some embodiments, the HSA variant is a mutant derived from full length HSA (SEQ ID NO: 138). In a specific embodiment, the HSA variant comprises a substitution of cysteine at position 34 to serine (SEQ ID NO: 133). HSA variants that can be used to modify the plasma half-life of a Tn3 scaffold are described, *e.g.*, in International Publications WO 2011/103076 and WO 2011/051489. In some embodiments, the plasma half-life of a Tn3 scaffold of the invention is increased by fusing it with an HSA variant comprising at least one amino acid substitution in domain III of HSA.

[0286] In some embodiments, the Tn3 scaffold of the invention comprises an HSA variant comprising the sequence of full-length mature HSA (SEQ ID NO: 138) or a fragment thereof, except for at least one amino acid substitution, numbered relative to the position in full length mature HSA, at a position selected from the group consisting of 407, 415, 463, 500, 506, 508, 509, 511, 512, 515, 516, 521, 523, 524, 526, 535, 550, 557, 573, 574, and 580; wherein the at least one amino acid substitution does not comprise a lysine (K) to glutamic acid (E) at position 573, and wherein the Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to the HSA variant.

[0287] In some other embodiments, at least one amino acid substitution, numbered relative to the position in full length mature HSA, is at a position selected from the group consisting of 463, 508, 523, and 524, wherein the Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to the HSA variant.

[0288] In other embodiments, a Tn3 scaffold of the invention comprises an HSA variant comprising the sequence of full-length mature HSA (SEQ ID NO: 133 or 138) or a fragment thereof, except for at least one amino acid substitution, numbered relative to the position in full length mature HSA, selected from the group consisting of:

(a) substitution of Leucine (L) at position 407 to Asparagine (N) or Tyrosine (Y);

(b) substitution of Valine (V) at position 415 to Threonine (T);

(c) substitution of Leucine (L) at position 463 to Asparagine (N);

(d) substitution of Lysine (K) at position 500 to Arginine (R);

(e) substitution of Threonine (T) at position 506 to Tyrosine (Y);

(f) substitution of Threonine (T) at position 508 to Arginine (R);

(g) substitution of Phenylalanine (F) at position 509 to Methionine (M) or Tryptophan (W);

(h) substitution of Alanine (A) at position 511 to Phenylalanine (F);

(i) substitution of Aspartic Acid (D) at position 512 to Tyrosine (Y);

(j) substitution of Threonine (T) at position 515 to Glutamine (Q);

(k) substitution of Leucine (L) at position 516 to Threonine (T) or Tryptophan (W);

(l) substitution of Arginine (R) at position 521 to Tryptophan (W);

(m) substitution of Isoleucine (I) at position 523 to Aspartic Acid (D), Glutamic Acid (E), Glycine (G), Lysine (K), or Arginine (R);

(n) substitution of Lysine (K) at position 524 to Leucine (L);

(o) substitution of Glutamine (Q) at position 526 to Methionine (M);

(p) substitution of Histidine (H) at position 535 to Proline (P);

(q) substitution of Aspartic Acid (D) at position 550 to Glutamic Acid (E);

(r) substitution of Lysine (K) at position 557 to Glycine (G);

(s) substitution of Lysine (K) at position 573 to Phenylalanine (F), Histidine (H), Proline (P), Tryptophan (W), or Tyrosine (Y);

(t) substitution of Lysine (K) at position 574 to Asparagine (N);

(u) substitution of Glutamine (Q) at position 580 to Lysine (K); and,

(v) a combination of two or more of said substitutions,

wherein said Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to said HSA variant.

[0289] In some embodiments, the Tn3 scaffold comprises a HSA variant which comprises the sequence of full-length mature HSA (SEQ ID NO: 133 or 138) or a fragment thereof, except for at least one amino acid substitution, numbered relative to the position in full length mature HSA, selected from the group consisting of:

(a) substitution of Leucine (L) at position 463 to Asparagine (N);

(b) substitution of Threonine (T) at position 508 to Arginine (R);

(c) substitution of Isoleucine (I) at position 523 to Aspartic Acid (D), Glutamic Acid (E), Glycine (G), Lysine (K), or Arginine (R);

(d) substitution of Lysine (K) at position 524 to Leucine (L); and,

(e) a combination of two or more of said substitutions,

wherein said Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to said HSA variant.

[0290] Moreover, the Tn3 scaffolds of the invention can be fused to marker sequences, such as a peptide to facilitate purification. In some embodiments, the marker amino acid sequence is a poly-histidine peptide (His-tag), *e.g.*, a octa-histidine-tag (His-8-tag) or hexa-histidine-tag (His-6-tag) such as the tag provided in a pQE expression vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, Calif, 91311), among other vectors, many of which are commercially available. As described in Gentz *et al.*, Proc. Natl. Acad. Sci. USA 86:821-824, 1989, for instance, poly-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, a hemagglutinin ("HA") tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (see, *e.g.*, Wilson *et al.*, Cell 37:767, 1984), a FLAG tag, a Strep-tag, a myc-tag, a V5 tag, a GFP-tag, an AU1-tag, an AU5-tag, an ECS-tag, a GST-tag, or an OLLAS tag.

[0291] Additional fusion proteins comprising Tn3 scaffolds of the invention may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling").

[0292] DNA shuffling may be employed to alter the action of Tn3 scaffolds on the target (*e.g.*, generate scaffolds with higher affinities and lower dissociation rates). Tn3 scaffolds may be altered by random mutagenesis by error-prone PCR, random nucleotide insertion, or other methods prior to recombination. One or more portions of a polynucleotide encoding a scaffold, which bind to a specific target may be recombined with one or more

components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

Antibody and Fc Domain Fusions

[0293] In some embodiments, the Tn3 scaffold of the invention comprises a CD40L-specific monomer subunit fused to a domain or fragment of an antibody (*e.g.*, an IgG), including, but not limited to, an Fc domain.

[0294] In some embodiments, only one CD40L-specific monomer subunit is conjugated or fused to a domain or fragment of an antibody. For instance, a single a CD40L-specific monomer subunit can be fused to the N-terminus of a polypeptide of a domain or fragment of an antibody (*e.g.*, a heavy chain or a light chain of an antibody). In other embodiments, Tn3 scaffolds are created by fusing or conjugating one or more CD40L-specific monomer subunits to the N-terminus and/or the C-terminus a polypeptide of a domain or fragment of an antibody (*e.g.*, a heavy chain and/or a light chain of an antibody, or an Fc domain).

[0295] In some embodiments, some or all the a CD40L-specific monomer subunits fused to a domain or fragment of an antibody are identical. In some other embodiments, some or all the a CD40L-specific monomer subunit fused to a domain or fragment of an antibody are different.

[0296] In a specific embodiment, the Tn3 scaffold of the invention comprises one CD40L-specific monomer subunit fused to an Fc domain. In other embodiments, the Tn3 scaffold of the invention comprises at least two CD40L-specific monomer subunits fused to an Fc domain. In one specific embodiment, two of the CD40L-specific monomer subunits fused to an Fc domain are identical. In one specific embodiment, two of the CD40L-specific monomer subunits fused to an Fc domain are different. In one specific embodiment, two CD40L-specific monomer subunits fused to an Fc domain are connected to each other in tandem, and one of the CD40L-specific monomer subunits is fused to the Fc domain.

[0297] In some embodiments, different Tn3 scaffolds of the invention can be dimerized by the use of Fc domain mutations which favor the formation of heterodimers. It is known in the art that variants of the Fc region (*e.g.*, amino acid substitutions and/or additions and/or deletions) enhance or diminish effector function of the antibody and can alter the pharmacokinetic properties (*e.g.* half-life) of the antibody. Thus, in certain embodiments, the Tn3 scaffolds of the invention comprise Fc domain(s) that comprise an altered Fc region in which one or more alterations have been made in the Fc region in order

to change functional and/or pharmacokinetic properties of the Tn3 scaffold. In certain embodiments, the Tn3 scaffolds of the invention comprise Fc domain(s) that comprise an altered Fc region in which one or more alterations have been made in the Fc region in order reduce or eliminate at least one Fc□R-mediated effector function.

[0298] It is also known that the glycosylation of the Fc region can be modified to increase or decrease effector function and/or anti-inflammatory activity. Accordingly, in one embodiment a Tn3 scaffold of the invention comprise an Fc region with altered glycosylation of amino acid residues in order to change cytotoxic and/or anti-inflammatory properties of the Tn3 scaffolds.

Tn3 Scaffold Topologies

[0299] The Tn3 scaffolds of the invention can be fused to the C-terminus of the Fc domains, antibody light chains, and antibody heavy chains in any suitable spatial arrangement. See, *e.g.*, International Publication PCT/US2011/032184 for a detailed description of contemplated scaffold topologies.

Generation of Scaffolds of the Invention

[0300] The Tn3 scaffolds described herein may be used in any technique for evolving new or improved target binding proteins. In one particular example, the target is immobilized on a solid support, such as a column resin or microtiter plate well, and the target contacted with a library of candidate scaffold-based binding proteins. Such a library may consist of clones constructed from a Tn3 scaffold, through randomization of the sequence and/or the length of the CDR-like loops.

[0301] In this regard, bacteriophage (phage) display is one well known technique which allows one to screen large oligopeptide libraries to identify member(s) of those libraries which are capable of specifically binding to a target. Phage display is a technique by which variant polypeptides are displayed as fusion proteins to the coat protein on the surface of bacteriophage particles (Scott, J. K. and Smith, G. P. (1990) Science 249: 386). A bioinformatics approach may be employed to determine the loop length and diversity preferences of naturally occurring FnIII domains. Using this analysis, the preferences for loop length and sequence diversity may be employed to develop a "restricted randomization" approach. In this restricted randomization, the relative loop length and sequence preferences are incorporated into the development of a library strategy. Integrating the loop length and sequence diversity analysis into library development results in a restricted randomization (*i.e.*

certain positions within the randomized loop are limited in which amino acid could reside in that position).

[0302] The invention also provides recombinant libraries comprising diverse populations of non-naturally occurring Tn3 scaffolds. In one embodiment, the libraries comprise non-naturally occurring Tn3 scaffolds comprising, a plurality of beta strand domains linked to a plurality of loop regions, wherein one or more of said loops vary by deletion, substitution or addition by at least one amino acid. In a specific embodiment, the libraries comprise Tn3 scaffolds derived from the wild type Tn3 scaffold.

[0303] As detailed above, the loops connecting the various beta strands of the scaffolds may be randomized for length and/or sequence diversity. In one embodiment, the libraries of the invention comprise Tn3 scaffolds having at least one loop that is randomized for length and/or sequence diversity. In one embodiment, at least one, at least two, at least three, at least four, at least five or at least six loops of the Tn3 scaffolds are randomized for length and/or sequence diversity. In one embodiment, at least one loop is kept constant while at least one additional loop is randomized for length and/or sequence diversity. In another embodiment, at least one, at least two, or all three of loops AB, CD, and EF are kept constant while at least one, at least two, or all three of loops BC, DE, and FG are randomized for length or sequence diversity. In another embodiment, at least one, at least two, or at least all three of loops AB, CD, and EF are randomized while at least one, at least two, or all three of loops BC, DE, and FG are randomized for length and/or sequence diversity.

[0304] In a specific embodiment, the libraries of the invention comprise FnIII scaffolds, wherein the A beta strand comprises SEQ ID NO: 10 or 11, the B beta strand comprises SEQ ID NO: 12, the C beta strand comprises SEQ ID NO: 13 or 14, the D beta strand comprises SEQ ID NO: 15, the E beta strand comprises SEQ ID NO: 16, the F beta strand comprises SEQ ID NO: 17, and the G beta strand comprises SEQ ID NO: 18.

[0305] In a specific embodiment, the libraries of the invention comprise FnIII scaffolds, wherein the A beta strand consists of SEQ ID NO: 10 or 11, the B beta strand consists of SEQ ID NO: 12, the C beta strand consists of SEQ ID NO: 13 or 14, the D beta strand consists of SEQ ID NO: 15, the E beta strand consists of SEQ ID NO: 16, the F beta strand consists of SEQ ID NO: 17, and the G beta strand consists of SEQ ID NO: 18.

[0306] In a specific embodiment, the libraries of the invention comprise FnIII scaffolds, wherein the A beta strand consists essentially of SEQ ID NO: 10 or 11, the B beta strand consists essentially of SEQ ID NO: 12, the C beta strand consists essentially of SEQ ID NO: 13 or 14, the D beta strand consists essentially of SEQ ID NO: 15, the E beta strand

consists essentially of SEQ ID NO: 16, the F beta strand consists essentially of SEQ ID NO: 17, and the G beta strand consists essentially of SEQ ID NO: 18.

[0307] As detailed above, one or more residues within a loop may be held constant while other residues are randomized for length and/or sequence diversity. Optionally or alternatively, one or more residues within a loop may be held to a predetermined and limited number of different amino acids while other residues are randomized for length and/or sequence diversity. Accordingly, libraries of the invention comprise Tn3 scaffolds that may comprise one or more loops having a degenerate consensus sequence and/or one or more invariant amino acid residues. In another embodiment, the libraries of the invention comprise Tn3 scaffolds having BC loops which are randomized. In another embodiment, the libraries of the invention comprise Tn3 scaffolds having BC loops which are randomized. In still another embodiment, the libraries of the invention comprise Tn3 scaffolds having BC loops which are randomized.

[0308] In one embodiment the libraries of the invention comprise Tn3 scaffolds having DE loops which are randomized. In one embodiment, the libraries of the invention comprise Tn3 scaffolds having FG loops which are randomized. In another embodiment, the libraries of the invention comprise FnIII scaffolds having FG loops which are randomized.

[0309] In a specific embodiment, the libraries of the invention comprise scaffolds, wherein the scaffolds comprise the amino acid sequence:

$IEV(X_{AB})_nALITW(X_{BC})_nCELX_1YGI(X_{CD})_nTTIDL(X_{DE})_nYSI(X_{EF})_nYEVSLIC(X_{FG})_n$ KETFTT

wherein:

(a) X_{AB}, X_{BC}, X_{CD}, X_{DE}, X_{EF}, and X_{FG} represent the amino acid residues present in the sequences of the AB, BC, CD, DE, EF, and FG loops, respectively;

(b) X_1 represents amino acid residue A or T; and,

(c) length of the loop *n* is an integer between 2 and 26.

[0310] In some embodiments, the libraries of the invention comprise CD40L-specific Tn3 monomer subunits of the invention comprising the amino acid sequence:

$IEVKDVTDTTALITWX_1DX_2X_3X_4X_5X_6X_7X_8CELTYGIKDVPGDRTTIDLWX_9HX_{10}AX_{11}YSIGNLKPDTEYEVSLICRX_{12}GDMSSNPAKETFTT$ (SEQ ID NO: 167)

wherein:

(a) X_1 represents amino acid residue serine (S) or leucine (L);

(b) X_2 represents amino acid residue aspartic acid (D) or glutamic acid (E);

(c) X_3 represents amino acid residue histidine (H), isoleucine (I), valine (V), phenylalanine (F) or tryptophan (W);

(d) X_4 represents amino acid residue alanine (A), glycine (G), glutamic acid (E) or aspartic acid (D);

(e) X_5 represents amino acid residue glutamic acid (E), leucine (L), glutamine (Q), serine (S), aspartic acid (D) or asparagine (N);

(f) X_6 represents amino acid residue phenylalanine (F) or tyrosine (Y);

(g) X_7 represents amino acid residue isoleucine (I), valine (V), histidine (H), glutamic acid (E) or aspartic acid (D);

(h) X_8 represents amino acid residue glycine (G), tryptophan (W) or valine (V);

(i) X_9 represents amino acid residue tryptophan (W), phenylalanine (F) or tyrosine (Y);

(j) X_{10} represents amino acid residue serine (S), glutamine (Q), methionine (M) or histidine (H);

(k) X_{11} represents amino acid residue tryptophan (W) or histidine (H); and,

(l) X_{12} represents amino acid residue arginine (R) or serine (S).

[0311] In some embodiments, the libraries of the invention comprise CD40L-specific Tn3 monomer subunits of the invention comprising the amino acid sequence:

IEVX$_1$DVTDTTALITWX$_2$X$_3$RSX$_4$X$_5$X$_6$X$_7$X$_8$X$_9$X$_{10}$CELX$_{11}$YGIKDVPGDRTTIDLX$_{12}$X$_{13}$X$_{14}$X$_{15}$YVHYSIGNLKPDTX$_{16}$YEVSLICLTTDGTYX$_{17}$NPAKETFTT (SEQ ID NO: 171)

wherein:

(a) X_1 represents amino acid residue lysine (K) or glutamic acid (E);

(b) X_2 represents amino acid residue threonine (T) or isoleucine (I);

(c) X_3 represents amino acid residue asparagine (N) or alanine (A);

(d) X_4 represents amino acid residue serine (S), leucine (L), alanine (A), phenylalanine (F) or tyrosine (Y);

(e) X_5 represents amino acid residue tyrosine (Y), alanine (A), glycine (G), valine (V), isoleucine (I) or serine (S);

(f) X_6 represents amino acid residue tyrosine (Y), serine (S), alanine (A) or histidine (H);

(g) X_7 represents amino acid residue asparagine (N), aspartic acid (D), histidine (H) or tyrosine (Y);

(h) X_8 represents amino acid residue leucine (L), phenylalanine (F), histidine (H) or tyrosine (Y);

(i) X_9 represents amino acid residue histidine (H), proline (P), serine (S), leucine (L) or aspartic acid (D);

(j) X_{10} represents amino acid residue glycine (G), phenylalanine (F), histidine (H) or tyrosine (Y);

(k) X_{11} represents amino acid residue alanine (A) or threonine (T);

(l) X_{12} represents amino acid residue serine (S), asparagine (N), glutamic acid (E), asparagine (R) or aspartic acid (D);

(m) X_{13} represents amino acid residue serine (S), glutamine (Q), threonine (T), asparagine (N) or alanine (A);

(n) X_{14} represents amino acid residue proline (P), valine (V), isoleucine (I) or alanine (A) or no amino acid;

(o) X_{15} represents amino acid residue isoleucine (I) or no amino acid;

(p) X_{16} represents amino acid residue glutamic acid (E) or lysine (K); and,

(q) X_{17} represents amino acid residue serine (S) or asparagine (N).

[0312] The invention further provides methods for identifying a recombinant Tn3 scaffold that binds a target, *e.g.*, CD40L, and has increased stability or improved action on the target, *e.g.*, CD40L, as compared to a parent Tn3 scaffold by screening the libraries of the invention.

[0313] In certain embodiments, the method for identifying a recombinant Tn3 scaffold having increased protein stability as compared to a parent Tn3 scaffold, and which specifically binds a target, comprises:

[0314] contacting the target ligand with a library of the invention under conditions suitable for forming a scaffold:target ligand complex;

[0315] obtaining from the complex, the scaffold that binds the target ligand;

[0316] determining if the stability of the scaffold obtained in step (b) is greater than that of the wild type Tn3 scaffold.

[0317] The same method can be used to identify a recombinant Tn3 scaffold with improved binding affinity, avidity, etc. to the target. In one embodiment, in step (a) the scaffold library of the invention is incubated with immobilized target. In one embodiment, in step (b) the scaffold:target ligand complex is washed to remove non-specific binders, and the tightest binders are eluted under very stringent conditions and subjected to PCR to recover the sequence information. It is specifically contemplated that the binders and/or sequence

information obtained in step (b) can be used to create a new library using the methods disclosed herein or known to one of skill in the art, which may be used to repeat the selection process, with or without further mutagenesis of the sequence. In some embodiments, a number of rounds of selection may be performed until binders of sufficient affinity for the antigen are obtained.

[0318] A further embodiment of the invention is a collection of isolated nucleic acid molecules encoding a library comprising the scaffolds of the invention and as described above.

[0319] The scaffolds of the invention may be subjected to affinity maturation. In this art-accepted process, a specific binding protein is subject to a scheme that selects for increased affinity for a specific target (see Wu *et al.*, Proc. Natl. Acad. Sci. USA. 95(11):6037-42). The resultant scaffolds of the invention may exhibit binding characteristics at least as high as compared to the scaffolds prior to affinity maturation.

[0320] The invention also provides methods of identifying the amino acid sequence of a protein scaffold capable of binding to target so as to form a scaffold:target complex. In one embodiment, the method comprises: (a) contacting a library of the invention with an immobilized or separable target; (b) separating the scaffold:target complexes from the free scaffolds; (c) causing the replication of the separated scaffolds of (b) so as to result in a new polypeptide display library distinguished from that in (a) by having a lowered diversity and by being enriched in displayed scaffolds capable of binding the target; d) optionally repeating steps (a), and (b) with the new library of (c); and e) determining the nucleic acid sequence of the region encoding the displayed scaffold of a species from (d) and hence deducing the peptide sequence capable of binding to the target.

[0321] In another embodiment, the Tn3 scaffolds of the invention may be further randomized after identification from a library screen. In one embodiment, methods of the invention comprise further randomizing at least one, at least two, at least three, at least four, at least five or at least six loops of a scaffold identified from a library using a method described herein. In another embodiment, the further randomized scaffold is subjected to a subsequent method of identifying a scaffold capable of binding a target. This method comprises (a) contacting said further randomized scaffold with an immobilized or separable target, (b) separating the further randomized scaffold:target complexes from the free scaffolds, (c) causing the replication of the separated scaffolds of (b), optionally repeating steps (a)-(c), and (d) determining the nucleic acid sequence of the region encoding said

further randomized scaffold and hence, deducing the peptide sequence capable of binding to the target.

[0322] In a further embodiment, the further randomized scaffolds comprise at least one, at least two, at least three, at least four, at least five, or at least six randomized loops which were previously randomized in the first library. In an alternate further embodiment, the further randomized scaffolds comprise at least one, at least two, at least three, at least four, at least five, or at least six randomized loops which were not previously randomized in the first library.

[0323] The invention also provides a method for obtaining at least two Tn3 scaffolds that bind to at least one or more targets. This method allows for the screening of agents that act cooperatively to elicit a particular response. It may be advantageous to use such a screen when an agonistic activity requiring the cooperation of more than one scaffold is required. This method allows for the screening of cooperative agents without the reformatting of the library to form multimeric complexes. In one embodiment, the method of the invention comprises contacting a target ligand with a library of the invention under conditions that allow a scaffold:target ligand complex to form, engaging said scaffolds with a crosslinking agent (defined as an agent that brings together, in close proximity, at least two identical or distinct scaffolds) wherein the crosslinking of the scaffolds elicits a detectable response and obtaining from the complex, said scaffolds that bind the target. In a further embodiment, the crosslinking agent is a scaffold specific antibody, or fragment thereof, an epitope tag specific antibody of a fragment thereof, a dimerization domain, such as Fc region, a coiled coil motif (for example, but not limited to, a leucine zipper), a chemical crosslinker, or another dimerization domain known in the art.

[0324] The invention also provides methods of detecting a compound utilizing the Tn3 scaffolds of the invention. Based on the binding specificities of the Tn3 scaffolds obtained by library screening, it is possible to use such Tn3 scaffolds in assays to detect the specific target in a sample, such as for diagnostic methods. In one embodiment, the method of detecting a compound comprises contacting said compound in a sample with a Tn3 scaffold of the invention, under conditions that allow a compound: scaffold complex to form and detecting said scaffold, thereby detecting said compound in a sample. In further embodiments, the scaffold is labeled (*i.e.*, radiolabel, fluorescent, enzyme-linked or colorimetric label) to facilitate the detection of the compound.

[0325] The invention also provides methods of capturing a compound utilizing the Tn3 scaffolds of the invention. Based on the binding specificities of the Tn3 scaffolds

obtained by library screening, it is possible to use such Tn3 scaffolds in assays to capture the specific target in a sample, such as for purification methods. In one embodiment, the method of capturing a compound in a sample comprises contacting said compound in a sample with a scaffold of the invention under conditions that allow the formation of a compound:scaffold complex and removing said complex from the sample, thereby capturing said compound in said sample. In further embodiments, the Tn3 scaffold is immobilized to facilitate the removing of the compound:scaffold complex.

[0326] In some embodiments, Tn3 scaffolds isolated from libraries of the invention comprise at least one, at least two, at least four, at least five, at least six, or more randomized loops. In some embodiments, isolated Tn3 scaffold loop sequences may be swapped from a donor scaffold to any loop in a FnIII receiver scaffold included, but not limited to, a Tn3 receiver scaffold (for example, an FG loop sequence from a donor scaffold may be transferred to any loop in a receiver FnIII scaffold). In specific embodiments, isolated loop sequences may be transferred to the cognate loop in the receiving scaffold (for example, an FG loop sequence from a donor scaffold may be transferred to an FnIII receiver scaffold in the FG loop position). In some embodiments, isolated loop sequences may be "mix and matched" randomly with various receiver scaffolds.

[0327] In other embodiments, isolated Tn3 scaffolds sequences may be identified by the loop sequence. For example, a library is used to pan against a particular target and a collection of specific binders are isolated. The randomized loop sequences may be characterized as specific sequences independently of the Tn3 scaffold background (*i.e.*, the scaffold that binds target X wherein said scaffold comprises an FG loop sequence of SEQ ID NO:X). In alternative embodiments, where a scaffold exhibits two loop sequences that bind target X, the loop sequences may be characterized as binding target X in the absence of the scaffold sequence. In other words, it is contemplated that scaffolds isolated from a library that bind a particular target may be expressed as the variable loop sequences that bind that target independent of the scaffold backbone. This process would be analogous to the concept of CDRs in variable regions of antibodies.

Affinity Maturation

[0328] The development of Tn3 scaffolds of the invention may involve one or more *in vitro* or *in vivo* affinity maturation steps. In some embodiments, Tn3 monomer subunits can undergo a single step of affinity maturation. In other embodiments, Tn3 monomer subunits can under two or more steps of affinity maturation. Any affinity maturation

approach can be employed that results, in general, in amino acid changes in a parent Tn3 scaffold, or specifically amino acid changes in a parent Tn3 scaffold's loops that improve the binding of the affinity matured Tn3 scaffold to the desired antigen.

[0329] These amino acid changes can, for example, be achieved via random mutagenesis, "walk though" mutagenesis, and "look through" mutagenesis. Such mutagenesis can be achieved by using, for example, error-prone PCR, “mutator” strains of yeast or bacteria, incorporation of random or defined nucleic acid changes during *ab initio* synthesis of all or part of a FnIII-based binding molecule. Methods for performing affinity maturation and/or mutagenesis are described, for example, in U.S. Pat. Nos. 7,195,880; 6,951,725; 7,078,197; 7,022,479; 5,922,545; 5,830,721; 5,605,793, 5,830,650; 6,194,550; 6,699,658; 7,063,943; 5,866,344 and PCT Publication WO06023144.

[0330] Such affinity maturation methods may further require that the stringency of the antigen-binding screening assay is increased to select for Tn3 scaffolds with improved affinity for an antigen. Art recognized methods for increasing the stringency of a protein-protein interaction assay can be used here. In one embodiment, one or more of the assay conditions are varied (for example, the salt concentration of the assay buffer) to reduce the affinity of the Tn3 scaffold for the desired antigen. In another embodiment, the length of time permitted for the Tn3 scaffold to bind to the desired antigen is reduced.

[0331] In another embodiment, a competitive binding step can be added to the protein-protein interaction assay. For example, the Tn3 scaffold can be first allowed to bind to a desired immobilized antigen. A specific concentration of non-immobilized antigen is then added which serves to compete for binding with the immobilized antigen such that the Tn3 scaffolds with the lowest affinity for antigen are eluted from the immobilized antigen resulting in selection of Tn3 scaffolds with improved antigen binding affinity. The stringency of the assay conditions can be further increased by increasing the concentration of non-immobilized antigen is added to the assay.

[0332] Screening methods may also require multiple rounds of selection to enrich for one or more Tn3 scaffolds with improved antigen binding. In one embodiment, at each round of selection further amino acid mutations are introduce into the Tn3 scaffold. In another embodiment, at each round of selection the stringency of binding to the desired antigen is increased to select for Tn3 scaffolds with increased affinity for antigen.

[0333] In some embodiments, affinity maturation is performed by saturation mutagenesis of portions of the BC, DE, and FG loops of Tn3. In some embodiments,

saturation mutagenesis is performed using Kunkel mutagenesis. In other embodiments, saturation mutagenesis is performed by using PCR.

[0334] In some embodiments, at least one, at least two, at least three, at least four, at least five, or more than five rounds of affinity maturation are applied. In some embodiments, saturation mutagenesis is applied to only one loop, whereas in some other embodiments, only one loop or a portion of a loop is mutated during one round of affinity maturation. In some embodiments, more than one loop or portions of one or more than loop are mutated during the same round of affinity maturation.

[0335] In other embodiments, the BC, DE, and FG loops mutated simultaneously during the same round of affinity maturation.

[0336] In the case of the monomers to assemble into multimeric Tn3 scaffolds binding to different epitopes of the same target, each binding specificity can be screened independently.

[0337] In some embodiments, the loops are randomized using a phage display library. In some embodiments, the binding of a Tn3 scaffold to a desired target can be determined using methods recognized in the art. Also, the amino acid sequences of the Tn3 scaffolds identified in the screens can be determined using art recognized methods.

[0338] In some embodiments, the monomeric affinity matured scaffolds of the invention exhibit an increased in affinity for CD40L of at least 5-fold, at least 10-fold, at least 20-fold, at least 40-fold, at least 6o-fold, at least 80-fold, or at least 100-fold or more compared to the same Tn3 scaffold prior to affinity maturation, as measured by Surface Plasmon Resonance or by other assays known in the art. In some embodiments, the monomeric affinity matured scaffolds of the invention have a dissociation constant (K_d) of less than 5 μM, less than 1 μM, less than 500 μM, less than 250 μM, less than 100 μM, or less than 50 μM, as measured by Surface Plasmon Resonance or by other assays known in the art.

[0339] These affinity maturation methods can be applied to develop Tn3 scaffolds with desirable improved binding properties such as increased affinity or other desirable characteristics, such as favorable pharmacokinetic properties, high potency, low immunogenicity, increased or decreased cross-reactivity, etc.

Generation of Tandem Repeats

[0340] Linking of tandem constructs, a dimer formed by linking two CD40L-specific monomer subunits, may be generated by ligation of oligonucleotides at restriction

sites using restriction enzymes known in the art, including but not limited to type II and type IIS restriction enzymes.

[0341] The multimeric Tn3 scaffolds of the invention may comprise a linker at the C-terminus and/or the N-terminus and/or between domains as described herein. Further, scaffolds of the invention comprising at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8 or polypeptide scaffolds may be fused or conjugated to a dimerization domain, including but not limited to an antibody moiety selected from:

(i) a Fab fragment, having VL, CL, VH and CH1 domains;

(ii) a Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain;

(iii) a Fd fragment having VH and CH1 domains;

(iv) a Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain;

(v) a Fv fragment having the VL and VH domains of a single arm of an antibody;

(vi) a dAb fragment which consists of a VH domain;

(vii) isolated CDR regions;

(viii) F(ab')2 fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region;

(ix) single chain antibody molecules (e.g., single chain Fv; scFv);

(x) a "diabody" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain;

(xi) a "linear antibody" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions;

(xii) a full length antibody; and

(xiii) an Fc region comprising CH2-CH3, which may further comprise all or a portion of a hinge region and/or a CH1 region..

Tn3 Scaffold Production

[0342] Recombinant expression of a Tn3 scaffold of the invention requires construction of an expression vector containing a polynucleotide that encodes the Tn3 scaffold. Once a polynucleotide encoding a Tn3 scaffold has been obtained, the vector for the production of the Tn3 scaffold may be produced by recombinant DNA technology using

techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing a Tn3 scaffold encoding nucleotide sequence are described herein. Methods that are well known to those skilled in the art can be used to construct expression vectors containing scaffold polypeptide coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding a Tn3 scaffold of the invention, operably linked to a promoter.

[0343] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce a Tn3 scaffold of the invention. Thus, the invention includes host cells containing a polynucleotide encoding a scaffold of the invention, operably linked to a heterologous promoter. Suitable host cells include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli* and *B. subtilis*).

[0344] A variety of host-expression vector systems may be utilized to express the Tn3 scaffolds of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express a scaffold of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing scaffold coding sequences or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, NSO, and 3T3 cells).

[0345] Methods useful for the production of the Tn3 scaffolds of the invention are disclosed, for example, in International Patent Application Publication No WO 2009/058379. Once a scaffold of the invention has been produced by recombinant expression, it may be purified by any method known in the art for purification of a protein.

[0346] In some embodiments, scaffolds of the invention can be produced in an aglycosylated form by replacing amino acid residues that can be glycosylated during recombinant expression. In one specific embodiment, serine amino acids in a glycine-serine linker (*e.g.*, SEQ ID NO: 131 or SEQ ID NO: 132) can be replaced by other amino acids residues such as alanine, glycine, leucine, isoleucine or valine (see, *e.g.*, SEQ ID NOs: 140, 141, 142 and 143) in order to prevent glycosylation during recombinant expression. In some specific embodiments, an N-glycosylation site is removed from a Tn3 scaffolds of the

invention. In other embodiments, a scaffold of the invention can be deglycosylated after recombinant expression. Methods of *in vitro* deglycosylation after recombinant expression using, *e.g.*, enzymatic cocktails are known in the art (for example, the PFGase F, Enodo F Multi, Orela O-linked Glycan Release, Enzymatic CarboRelease, and Enzymatic DeGlycoMx deglycosylation kits marketed by QA-bio, Palm Desert, CA).

[0347] Production of the Tn3 scaffolds of the invention in the research laboratory can be scaled up to produce scaffolds in analytical scale reactors or production scale reactors, as described in U.S. Patent Publication No. US 2010-0298541 A1.

Scalable production of secreted Tn3 scaffolds

[0348] The Tn3 scaffolds of the invention can be produced intracellularly or as a secreted form. In some embodiments, the secreted scaffolds are properly folded and fully functional. Tn3 scaffolds of the invention can be produced by a scalable process. In some embodiments, scaffolds can be produced by a scalable process of the invention in the research laboratory that can be scaled up to produce the scaffolds of the invention in analytical scale bioreactors (for example, but not limited to 5L, 10L, 15L, 30L, or 50L bioreactors). In other embodiments, the Tn3 scaffolds can be produced by a scalable process of the invention in the research laboratory that can be scaled up to produce the Tn3 scaffolds of the invention in production scale bioreactors (for example, but not limited to 75L, 100L, 150L, 300L, or 500L). In some embodiments, the scalable process of the invention results in little or no reduction in production efficiency as compared to the production process performed in the research laboratory.

Linkers

[0349] The monomer subunits in a multimeric Tn3 scaffold can be connected by protein and/or nonprotein linkers, wherein each linker is fused to at least two monomer subunits. A suitable linker can consist of a protein linker, a nonprotein linker, and combinations thereof. Combinations of linkers can be homomeric or heteromeric. In some embodiments, a multimeric Tn3 scaffold of the invention comprises a plurality of monomer subunits wherein are all the linkers are identical. In other embodiments, a multimeric Tn3 scaffold comprises a plurality of monomer subunits wherein at least one of the linkers is functionally or structurally different from the rest of the linkers. In some embodiments, linkers can themselves contribute to the activity of a multimeric Tn3 scaffold by participating directly or indirectly in the binding to a target.

[0350] In some embodiments, the protein linker is a polypeptide. The linker polypeptide should have a length, which is adequate to link two or more monomer subunits in such a way that they assume the correct conformation relative to one another so that they retain the desired activity.

[0351] In one embodiment, the polypeptide linker comprises 1 to about 1000 amino acids residues, 1 to about 50 amino acid residues, 1-25 amino acid residues, 1-20 amino acid residues, 1-15 amino acid residues, 1-10 amino acid residues, 1- 5 amino acid residues, 1-3 amino acid residues. The invention further provides nucleic acids, such as DNA, RNA, or combinations of both, encoding the polypeptide linker sequence. The amino acid residues selected for inclusion in the polypeptide linker should exhibit properties that do not interfere significantly with the activity or function of the multimeric Tn3 scaffold of the invention. Thus, a polypeptide linker should on the whole not exhibit a charge which would be inconsistent with the activity or function of the Tn3 multimeric scaffold of the invention, or interfere with internal folding, or form bonds or other interactions with amino acid residues in one or more of the monomer subunits which would seriously impede the binding of the multimeric Tn3 scaffold of the invention to CD40L.

[0352] The use of naturally occurring as well as artificial peptide linkers to connect polypeptides into novel linked fusion polypeptides is well known in the literature. Accordingly, the linkers fusing two or more monomer subunits are natural linkers, artificial linkers, or combinations thereof. In some embodiments, the amino acid sequences of all peptide linkers present in a Tn3 multimeric scaffold of the invention are identical. In other embodiments, the amino acid sequences of at least two of the peptide linkers present in a multimeric Tn3 scaffold of the invention are different.

[0353] In some embodiments, a polypeptide linker possesses conformational flexibility. In some embodiments, a polypeptide linker sequence comprises a $(G\text{-}G\text{-}G\text{-}G\text{-}X)_m$ amino acid sequence where X is Alanine (A), Serine (S), Glycine (G), Isoleucine (I), Leucine (L) or Valine (V) and m is a positive integer (see, *e.g.*, SEQ ID NO: 209). In a specific embodiment, a polypeptide linker sequence comprises a $(G\text{-}G\text{-}G\text{-}G\text{-}S)_m$ amino acid sequence where m is a positive integer (see, *e.g.*, SEQ ID NO: 147) . In another specific embodiment, a polypeptide linker sequence comprises a $(G\text{-}G\text{-}G\text{-}G\text{-}G)_m$ amino acid sequence where m is a positive integer (see, *e.g.*, SEQ ID NO: 148). In still another specific embodiment, a polypeptide linker sequence comprises a $(G\text{-}G\text{-}G\text{-}G\text{-}A)_m$ amino acid sequence where m is a positive integer (see, *e.g.*, SEQ ID NO: 149). In some embodiments, a polypeptide linker is an inherently unstructured natural or artificial polypeptide (see, *e.g.*, Schellenberger *et al.*,

Nature Biotechnol. 27:1186-1190, 2009; see also, Sickmeier *et al.*, Nucleic Acids Res. 35:D786-93, 2007).

[0354] The peptide linker can be modified in such a way that an amino acid residue comprising an attachment group for a non-polypeptide moiety is introduced. Examples of such amino acid residues may be a cysteine residue (to which the non-polypeptide moiety is then subsequently attached) or the amino acid sequence may include an *in vivo* N-glycosylation site (thereby attaching a sugar moiety (*in vivo*) to the peptide linker).

[0355] In some embodiments, the amino acid sequences of all peptide linkers present in the polypeptide multimer are identical. Alternatively, the amino acid sequences of all peptide linkers present in the polypeptide multimer may be different.

Labeling or Conjugation of Tn3 Scaffolds

[0356] The Tn3 scaffolds of the invention can be used in non-conjugated form or conjugated to at least one of a variety of heterologous moieties to facilitate target detection or for imaging or therapy. The Tn3 scaffolds of the can be labeled or conjugated either before or after purification, when purification is performed.

[0357] Many heterologous moieties lack suitable functional groups to which Tn3 scaffolds of the invention can be linked. Thus, in some embodiments, the effector molecule is attached to the scaffold through a linker, wherein the linker contains reactive groups for conjugation. In some embodiments, the heterologous moiety conjugated to a Tn3 scaffold of the invention can function as a linker. In other embodiments, the moiety is conjugated to the Tn3 scaffold via a linker that can be cleavable or non-cleavable. In one embodiment, the cleavable linking molecule is a redox cleavable linking molecule, such that the linking molecule is cleavable in environments with a lower redox potential, such as the cytoplasm and other regions with higher concentrations of molecules with free sulfhydryl groups. Examples of linking molecules that may be cleaved due to a change in redox potential include those containing disulfides.

[0358] In some embodiments, Tn3 scaffolds of the invention are engineered to provide reactive groups for conjugation. In such scaffolds, the N-terminus and/or C-terminus can also serve to provide reactive groups for conjugation. In other embodiments, the N-terminus can be conjugated to one moiety (such as, but not limited to PEG) while the C-terminus is conjugated to another moiety (such as, but not limited to biotin), or vice versa.

[0359] The term "polyethylene glycol" or "PEG" means a polyethylene glycol compound or a derivative thereof, with or without coupling agents, coupling or activating

moieties (*e.g.*, with thiol, triflate, tresylate, aziridine, oxirane, N-hydroxysuccinimide or a maleimide moiety). The term "PEG" is intended to indicate polyethylene glycol of a molecular weight between 500 and 150,000 Da, including analogues thereof, wherein for instance the terminal OH-group has been replaced by a methoxy group (referred to as mPEG).

[0360] The Tn3 scaffolds of the invention can be derivatized with polyethylene glycol (PEG). PEG is a linear, water-soluble polymer of ethylene oxide repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights which typically range from about 500 daltons to about 40,000 daltons. In a specific embodiment, the PEGs employed have molecular weights ranging from 5,000 daltons to about 20,000 daltons. PEGs coupled to the scaffolds of the invention can be either branched or unbranched. See, for example, Monfardini, C. *et al.* 1995 Bioconjugate Chem 6:62-69. PEGs are commercially available from Nektar Inc., Sigma Chemical Co. and other companies. Such PEGs include, but are not limited to, monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S- NHS), monomethoxypolyethylene glycol-amine (MePEG-NH2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM).

[0361] Briefly, the hydrophilic polymer which is employed, for example, PEG, is capped at one end by an unreactive group such as a methoxy or ethoxy group. Thereafter, the polymer is activated at the other end by reaction with a suitable activating agent, such as cyanuric halides (for example, cyanuric chloride, bromide or fluoride), carbonyldiimidazole, an anhydride reagent (for example, a dihalo succinic anhydride, such as dibromosuccinic anhydride), acyl azide, p-diazoniumbenzyl ether, 3-(p-diazoniumphenoxy)-2-hydroxypropylether) and the like. The activated polymer is then reacted with a polypeptide as described herein to produce a polypeptide derivatized with a polymer. Alternatively, a functional group in the Tn3 scaffolds of the invention can be activated for reaction with the polymer, or the two groups can be joined in a concerted coupling reaction using known coupling methods. It will be readily appreciated that the polypeptides of the invention can be derivatized with PEG using a myriad of other reaction schemes known to and used by those of skill in the art. A PEG can be coupled to a scaffold of the invention at one or more functional groups at either end of the Tn3 scaffold or within the Tn3 scaffold. In certain embodiments, the PEG is coupled at either the N-terminus or the C-terminus.

[0362] In other embodiments, Tn3 scaffolds of the invention, analogs or derivatives thereof may be conjugated to a diagnostic or detectable agent. Such Tn3 scaffolds can be useful for monitoring or prognosing the development or progression of a disease as part of a clinical testing procedure, such as determining the efficacy of a particular therapy.

[0363] The present invention further encompasses uses of Tn3 scaffolds conjugated to a therapeutic moiety. A Tn3 scaffold may be conjugated to a therapeutic moiety such as a cytotoxin, *e.g.*, a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, *e.g.*, alpha- emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells.

Assaying Tn3 Scaffolds

[0364] The binding affinity and other binding properties of a Tn3 scaffold to an antigen may be determined by a variety of *in vitro* assay methods known in the art including for example, equilibrium methods (*e.g.*, enzyme-linked immunoabsorbent assay (ELISA) or kinetics (*e.g.*, BIACORE® analysis), and other methods such as indirect binding assays, competitive binding assays, gel electrophoresis and chromatography (*e.g.*, gel filtration). These and other methods may utilize a label on one or more of the components being examined and/or employ a variety of detection methods including but not limited to chromogenic, fluorescent, luminescent, or isotopic labels. A detailed description of binding affinities and kinetics can be found in Paul, W.E., ed., Fundamental Immunology, 4th Ed., Lippincott-Raven, Philadelphia (1999).

[0365] In some embodiments, Tn3 scaffolds of the invention specifically bind a target with specific kinetics. In some embodiments, Tn3 scaffolds of the invention may have a dissociation constant or K_d (k_{off}/k_{on}) of less than 1×10^{-2}M, 1×10^{-3}M, 1×10^{-4}M, 1×10^{-5}M, 1×10^{-6}M, 1×10^{-7}M, 1×10^{-8}M, 1×10^{-9}M, 1×10^{-10}M, 1×10^{-11}M, 1×10^{-12}M, 1×10^{-13}M, 1×10^{-14}M or less than 1×10^{-15}M. In specific embodiments, Tn3 scaffolds of the invention have a K_d of 500 μM, 100 μM, 500 nM, 100 nM, 1 nM, 500 pM, 100 pM or less as determined by a BIAcore assay® or by other assays known in the art.

[0366] In an alternative embodiment, the affinity of the Tn3 scaffolds of the invention is described in terms of the association constant (K_a), which is calculated as the ratio k_{on}/k_{off}, of at least $1\times10^{2}M^{-1}$, $1\times10^{3}M^{-1}$, $1\times10^{4}M^{-1}$, $1\times10^{5}M^{-1}$, $1\times10^{6}M^{-1}$, $1\times10^{7}M^{-1}$, $1\times10^{8}M^{-1}$, $1\times10^{9}M^{-1}$, $1\times10^{10}M^{-1}$ $1\times10^{11}M^{-1}$ $1\times10^{12}M^{-1}$, $1\times10^{13}M^{-1}$, $1\times10^{14}M^{-1}$, $1\times10^{15}M^{-1}$, or at least 5 X $l0^{15}$ M^{-1}.

[0367] In certain embodiments the rate at which the Tn3 scaffolds of the invention dissociate from a target epitope may be more relevant than the value of the K_d or the K_a. In some embodiments, the Tn3 scaffolds of the invention have a k_{off} of less than 10^{-3} s^{-1}, less than $5x10^{-3}$ s^{-1}, less than 10^{-4} s^{-1}, less than $5x10^{-4}$ s^{-1}, less than 10^{-5} s^{-1}, less than $5x10^{-5}$ s^{-1}, less than 10^{-6} s^{-1}, less than $5x10^{-6}$ s^{-1}, less than 10^{-7} s^{-1}, less than $5x10^{-7}$ s^{-1}, less than 10^{-8} s^{-1}, less than $5x10^{-8}$ s^{-1}, less than 10^{-9} s^{-1}, less than $5x10^{-9}$ s^{-1}, or less than 10^{-10} s^{-1}.

[0368] In certain other embodiments, the rate at which the Tn3 scaffolds of the invention associate with a target epitope may be more relevant than the value of the K_d or the K_a. In this instance, the Tn3 scaffolds of the invention bind to a target with a k_{on} rate of at least 10^5 $M^{-1}s^{-1}$, at least $5x10^5$ $M^{-1}s^{-1}$, at least 10^6 $M^{-1}s^{-1}$, at least 5 x 10^6 $M^{-1}s^{-1}$, at least 10^7 $M^{-1}s^{-1}$, at least 5 x 10^7 $M^{-1}s^{-1}$, or at least 10^8 $M^{-1}s^{-1}$, or at least 10^9 $M^{-1}s^{-1}$.

[0369] Tn3 scaffolds of the invention may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

CD40L-specific Tn3 Scaffolds

[0370] The invention provides Tn3 scaffolds that specifically bind to CD40L. In specific embodiments, scaffolds of the invention specifically bind to human CD40L. In other specific embodiments, Tn3 scaffolds of the invention bind to CD40L homologs from mouse, chicken, Rhesus, cynomolgus, rat, or rabbit. In some embodiments, Tn3 scaffolds of the invention bind to an exposed epitope of CD40L. Such embodiments include CD40L endogenously expressed on cells and/or cells transfected to ectopically express the receptor.

[0371] In some embodiments, Tn3 scaffolds of the invention recognize epitopes displayed on a monomeric CD40L. In other embodiments, Tn3 scaffolds of the invention recognize epitopes displayed on a trimeric form of CD40L. In other embodiments, Tn3 scaffolds of the invention recognize epitopes displayed on a membrane bound CD40L. In other embodiments, Tn3 scaffolds of the invention recognize epitopes displayed on soluble CD40L.

[0372] In yet other embodiments, Tn3 scaffolds of the invention bind monomeric CD40L and prevent or interfere with oligomerization of CD40L molecules. In yet other embodiments, scaffolds of the invention reduce or inhibit interaction of CD40L with CD40. In other embodiments, Tn3 scaffolds of the invention agonize cellular signaling mediated by

CD40L. In yet other embodiments, Tn3 scaffolds of the invention antagonize cellular signaling mediated by CD40L.

[0373] The invention also provides methods of modulating CD40L activity using the Tn3 scaffolds described herein. In some embodiments, methods of the invention comprise contacting a CD40L with CD40L-specific scaffolds and blocking the interaction between CD40 and CD40L. In other embodiments, methods of the invention comprise contacting a cell expressing CD40L with a CD40L-specific Tn3 scaffold and preventing proteolytic cleavage of CD40L from the cell surface. In other embodiments, methods of the invention comprise contacting a CD40L monomer with a CD40L-specific Tn3 scaffold and preventing CD40L oligomerization. In other embodiments, dimerization or oligomerization of CD40L may be achieved through the use of multimeric Tn3 scaffolds.

[0374] In some embodiments, methods of the invention comprise the administration of a CD40L specific scaffold that reduces a CD40-mediated immune response (see, *e.g.*, Elqueta *et al.* 229: 152-172, 2009), or a downstream signaling pathway initiated by CD40 binding to CD40L, as measured by routine assays known in the art.

[0375] Without wishing to be bound by any particular theory, CD40L scaffolds of the present invention could function by preventing binding of CD40L to CD40, by binding and sequestering soluble CD40L, by altering the interaction of CD40L with CD40 but not preventing binding, by preventing or enhancing metalloprotease-mediated enzymatic cleavage of CD40L from the cell surface to yield soluble CD40L, by preventing or enhancing cell surface CD40L endocytosis, etc.

Specific CD40L Binding Sequences

[0376] In some embodiments, the Tn3 scaffold of the invention comprise CD40L-specific monomer subunits comprising at least one, at least two, at least three, at least four, at least five, or at least six loop sequences that bind to CD40L.

[0377] In some embodiments, CD40L-specific monomer subunits comprise at least one, at least two, at least three, at least four, at least five, or at least six loop sequences of CD40L-binding monomer clones selected from: 309 (parental 309 family clone isolated from naiive Tn3 library; SEQ ID NO: 20), 309FGwt (parental 309 clone with humanized FG loop; SEQ ID NO: 22), 340 (affinity matured 309 clone; SEQ ID NO: 24), 341 (affinity matured 309 clone; SEQ ID NO: 26), 342 (affinity matured 309 clone; SEQ ID NO: 28 or SEQ ID NO: 146), 343 (affinity matured 309 clone; SEQ ID NO: 30), 344 (affinity matured 309 clone; SEQ ID NO: 32), 345 (affinity matured 309 clone; SEQ ID NO: 34), 346 (affinity

matured 309 clone; SEQ ID NO: 36), 347 (affinity matured 309 clone; SEQ ID NO: 38), 348 (affinity matured 309 clone; SEQ ID NO: 40), 349 (affinity matured 309 clone; SEQ ID NO: 42), 311 (parental 311 family clone isolated from naiive Tn3 library; SEQ ID NO: 44), 311K4E (variant 311 family clone from first round of affinity maturation; SEQ ID NO: 46); 311K4E_1 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 48), 311K4E_2 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 50), 311K4E_3 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 52), 311K4E_4 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 54), 311K4E_5 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 56), 311K4E_7 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 58), 311K4E_8 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 60), 311K4E_9 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 62), 311K4E_10 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 64), 311K4E_11 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 66), 311K4E_12 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 68), 311K4E_13 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 70), 311K4E_14 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 72), 311K4E_15 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 74), 311K4E_16 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 76), 311K4E_19 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 78), 311K4E_20 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 80), and 311K4E_21 (variant 311 family clone from second round of affinity maturation; SEQ ID NO: 82).

[0378] In some embodiments, CD40L-specific monomer subunits comprise at least one loop sequence selected from the loop sequences listed in TABLE 2. In other embodiments, CD40L-specific monomer subunits comprise at least one BC loop sequence selected from the BC loop sequences listed in TABLE 2. In other embodiments, CD40L-specific monomer subunits comprise at least one DE loop sequence selected from the DE loop sequences listed in TABLE 2. In other embodiments, CD40L-specific monomer subunits comprise at least one FG loop sequence selected from the FG loop sequences listed in TABLE 2.

[0379] In some embodiments, CD40L-specific monomer subunits comprise a BC loop sequence selected from the BC loop sequences listed in TABLE 2; and a DE loop

sequence selected from the DE loop sequences listed in TABLE 2. In other embodiments, CD40L-specific monomer subunits comprise a BC loop sequence selected from the BC loop sequences listed in TABLE 2; and an FG loop sequence selected from the FG loop sequences listed in TABLE 2. In other embodiments, CD40L-specific monomer subunits comprise a DE loop sequence selected from the DE loop sequences listed in TABLE 2; and an FG loop sequence selected from the FG loop sequences listed in TABLE 2. In some embodiments, a CD40L-specific monomer subunits comprises loop sequences corresponding to loop sequences from one, two or three different Tn3 clones.

[0380] In certain embodiments, where the CD40L-specific monomer scaffold sequence contains a linker and/or a Histidine tag (*e.g.*, a His-8 tag) at the C-terminus of the sequence, or additional N-terminal amino acids, these C-terminal linker and/or Histidine tag and additional N-terminal amino acids can be removed, the corresponding amino acid sequence thus containing a deletion of the C-terminal linker and His tag sequences and the N-terminal additional amino acid or amino acids.

[0381] In some embodiments, the CD40L-specific Tn3 scaffold comprises a single monomer subunit, *e.g.*, the 342 clone sequence (affinity matured 309 clone; SEQ ID NO: 28 and/or SEQ ID NO: 146). In other embodiments, the CD40L-specific scaffold comprises more than one monomer subunits, *e.g.*, two 342 clone monomer subunits (SEQ ID NO: 28 and/or SEQ ID NO: 146) in tandem (see, *e.g.*, SEQ ID NO: 135). In specific embodiments, Tn3 scaffolds of the invention are conjugated to a variant HSA (see, *e.g.*, SEQ ID NO: 134 and SEQ ID NO: 135). In further embodiments, the HSA can be conjugated at either the N-terminus or the C-terminus of the multimeric Tn3 scaffold.

[0382] In a specific embodiment, the CD40L-specific Tn3 scaffold comprises a single 311K4E_12 monomer subunit, a GS linker, and a C34S HSA variant (see, *e.g.*, SEQ ID NO: 201). In another specific embodiment, the CD40L-specific Tn3 scaffold comprises a single 311K4E_12 monomer subunit with a beta strand C CELTYG variant, an all glycine linker, and a C34S HSA variant (see, *e.g.*, SEQ ID NO: 202). In another specific embodiment, the CD40L-specific Tn3 scaffold comprises two 311K4E_12 subunits in tandem, and two GS linkers, wherein one GS linker connects the subunits to each other and a second GS linker connects one subunit to a C34S HSA variant (see, *e.g.*, SEQ ID NO: 203). In yet another specific embodiment, the CD40L-specific Tn3 scaffold comprises two 311K4E_12 subunits in tandem, and two all glycine linkers, wherein one all glycine linker connects the subunits to each other and a second all glycine linker connects one subunit to a C34S HSA variant (see, *e.g.*, SEQ ID NO: 204).

[0383] In one specific embodiment, the CD40L-specific Tn3 scaffold comprises two 309 subunits connected in tandem via a GS linker (see, *e.g.*, SEQ ID NO: 205). In another specific embodiment, the CD40L-specific Tn3 scaffold comprises a single 309 subunit connected to a C34S HSA variant (see, e.g., SEQ ID NO: 206). In another specific embodiment, the CD40L-specific Tn3 scaffold comprises two 309 subunits in tandem, and two GS linkers, wherein one GS linker connects the subunits to each other and a second GS linker connects one subunit to a C34S HSA variant (see, *e.g.*, SEQ ID NO: 207).

[0384] In a specific embodiment, the CD40L-specific Tn3 scaffold comprises a single 342 monomer subunit, a GS linker, and a C34S HSA variant (see, *e.g.*, SEQ ID NO: 134). In another specific embodiment, the CD40L-specific Tn3 scaffold comprises a single 342 monomer subunit, an all glycine linker, and a C34S HSA variant (see, *e.g.*, SEQ ID NO: 144). In another specific embodiment, the CD40L-specific Tn3 scaffold comprises two 342 subunits in tandem, and two GS linkers, wherein one GS linker connects the subunits to each other and a second GS linker connects one subunit to a C34S HSA variant (see, *e.g.*, SEQ ID NO: 135). In yet another specific embodiment, the CD40L-specific Tn3 scaffold comprises two 342 subunits in tandem, and two all glycine linkers, wherein one all glycine linker connects the subunits to each other and a second all glycine linker connects one subunit to a C34S HSA variant (see, *e.g.*, SEQ ID NO: 145). In yet another specific embodiment, the CD40L-specific Tn3 scaffold comprises two 342 subunits connected in tandem by a GS linker (see, *e.g.*, SEQ ID NO: 208).

[0385] In a specific embodiment, the CD40L-specific Tn3 scaffold comprises In another specific embodiment, the CD40L-specific Tn3 scaffold comprises a 311 subunit, or a subunit derived from 311 (e.g., 311K4E_12) and a 309 subunit, or a subunit derived from 309 (e.g., 342) in tandem and two GS linkers, wherein one GS linker connects the subunits to each other and a second GS linker connects one subunit to a C34S HSA variant (see, *e.g.*, SEQ ID NO: 135). In yet another specific embodiment, the CD40L-specific Tn3 scaffold comprises a 311 subunit, or a subunit derived from 311 (e.g., 311K4E_12) and a 309 subunit, or a subunit derived from 309 (e.g., 342) in tandem, and two all glycine linkers, wherein one all glycine linker connects the subunits to each other and a second all glycine linker connects one subunit to a C34S HSA variant (see, *e.g.*, SEQ ID NO: 145).

[0386] Examples of CD40L-specific tandem bivalent Tn3 scaffolds and Serum Albumin (SA) fusions are shown in FIG. 2A (also see FIG. 9A). Although specific linkers are provided in FIG. 2A, other linkers are contemplated as provided herein. Although wild type mature SA may be used, *e.g.*, murine serum albumin (MSA) or human serum albumin

(HSA), it is contemplated that one or more Cysteine (C) amino acid residues in the mature SA may be substituted, for example with Serine (S), Alanine (A), Glycine (G), etc.

[0387] Representative constructs are shown below. The sequence of the SA is underlined. Linkers are boxed. It will be understood that numerous variations are within the scope of the invention. For example, the linkers may be altered (several non-limited examples are provided herein), the first one or two N-terminal amino acid residues (SQ) may be absent and/or substituted with alternative amino acid residues, a tag (e.g., 6xHis tag) may be incorporated, alternative CD40L-specific scaffolds (e.g., those based on the 10th Fn3 domain of fibronectin) may be utilized in a similar construct, etc.

342 Monovalent HSA construct 1 (SEQ ID NO: 134)

[342 monomer]-$(G_4S)_2$ linker-HSA_{C34S}

SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKALVLI
AFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYG
EMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARR
HPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQK
FGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYIC
ENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDV
FLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQ
NLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKR
MPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAE
TFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKE
TCFAEEGKKLVAASQAALGL

342 Monovalent HSA construct 2 (SEQ ID NO: 144)

[342 monomer]-G_{10} linker-HSA_{C34S}:

SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGGGGGGGDAHKSEVAHRFKDLGEENFKALVLI
AFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYG
EMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARR
HPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQK
FGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYIC
ENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDV
FLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQ
NLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKR
MPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAE
TFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKE
TCFAEEGKKLVAASQAALGL

342 Bivalent HSA Construct 1 (SEQ ID NO: 135)

[342 monomer]-$(G_4S)_3$ linker-[342 monomer]-$(G_4S)_2$ linker-HSA_{C34S}:

SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGSGGGGSGGGGSRLDAPSQIEVKDVTDTTALI
TWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDTEYEVSLICRSGDMS
SNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHV
KLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNE
CFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKR
YKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLS

QRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCE
KPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGE
YKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQL
CVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKE
RQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQ
AALGL

342 Bivalent HSA Construct 2 (SEQ ID NO: 145)

[342 monomer]-G_{15} linker-[342 monomer]-G_{10} linker-HSA_{C34S}:

SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGGGGGGGGGGGGRLDAPSQIEVKDVTDTTALI
TWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDTEYEVSLICRSGDMS
SNPAKETFTTGGGGGGGGGGDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHV
KLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNE
CFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKR
YKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLS
QRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCE
KPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGE
YKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQL
CVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKE
RQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQ
AALGL

311K4E_12 Monovalent HSA Construct 1 (SEQ ID NO: 201)

[311K4E_12 monomer]-$(G_4S)_2$ linker-HSA_{C34S}:

SQIEVEDVTDTTALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLK
PDTEYEVSLICLTTDGTYNNPAKETFTT**GGGGSGGGGS**DAHKSEVAHRFKDLGEENFKAL
VLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRE
TYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEI
ARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCAS
LQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAK
YICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEA
KDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVE
EPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPE
AKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEF
NAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKAD
DKETCFAEEGKKLVAASQAALGL

311K4E_12 Monovalent HSA Construct 2 (SEQ ID NO: 202)

[311K4E_12 monomer]-G_{10} linker-HSA_{C34S}:

SQIEVEDVTDTTALITWTNRSSYSNLHGCELTYGIKDVPGDRTTIDLNQPYVHYSIGNLK
PDTEYEVSLICLTTDGTYNNPAKETFTT**GGGGGGGGGG**DAHKSEVAHRFKDLGEENFKAL
VLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRE
TYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEI
ARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCAS
LQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAK
YICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEA
KDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVE
EPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPE
AKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEF

NAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKAD
DKETCFAEEGKKLVAASQAALGL

311K4E_12 Bivalent HSA Construct 1 (SEQ ID NO: 203)

[311K4E_12 monomer]-G_4S_3 linker-[311K4E_12 monomer]-$(G_4S)_2$ linker-HSA_{C34S}:

SQIEVEDVTDTTALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLK
PDTEYEVSLICLTTDGTYNNPAKETFTTGGGGSGGGGSGGGGSRLDAPSQIEVEDVTDTT
ALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKPDTEYEVSLICL
TTDGTYNNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQS
PFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQ
EPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPEL
LFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAW
AVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSK
LKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEY
ARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCEL
FEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLS
VVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADIC
TLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKK
LVAASQAALGL

311K4E_12 Bivalent HSA Construct 2 (SEQ ID NO: 204)

[311K4E_12 monomer]-G_{15} linker-[311K4E_12 monomer]-G_{10} linker-HSA_{C34S}:

SQIEVEDVTDTTALITWTNRSSYSNLHGCELTYGIKDVPGDRTTIDLNQPYVHYSIGNLK
PDTEYEVSLICLTTDGTYNNPAKETFTT**GGGGGGGGGGGGGGG**RLDAPSQIEVEDVTDTT
ALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKPDTEYEVSLICL
TTDGTYNNPAKETFTT**GGGGGGGGGG**DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQS
PFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQ
EPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPEL
LFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAW
AVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSK
LKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEY
ARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCEL
FEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLS
VVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADIC
TLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKK
LVAASQAALGL

Pharmaceutical Compositions

[0388] In another aspect, the present invention provides a composition, for example, but not limited to, a pharmaceutical composition, containing one or a combination of Tn3 scaffolds of the present invention, formulated together with a pharmaceutically acceptable carrier. Such compositions may include one or a combination of, for example, but not limited to two or more different Tn3 scaffolds of the invention. For example, a pharmaceutical composition of the invention may comprise a combination of Tn3 scaffolds that bind to different epitopes on the target antigen or that have complementary activities. In a specific

embodiment, a pharmaceutical composition comprises a single monomer Tn3 scaffold of the invention. In a specific embodiment, a pharmaceutical composition comprises a multimeric Tn3 scaffold of the invention. In still another specific embodiment, a pharmaceutical composition comprises dimer Tn3 scaffold of the invention.

[0389] Pharmaceutical compositions of the invention also can be administered in combination therapy, such as, combined with other agents. For example, the combination therapy can include a Tn3 scaffold of the present invention combined with at least one other therapy wherein the therapy may be immunotherapy, chemotherapy, radiation treatment, or drug therapy. The pharmaceutical compounds of the invention may include one or more pharmaceutically acceptable salts.

Methods of Using Scaffolds

[0390] The Tn3 scaffolds of the present invention have *in vitro* and *in vivo* diagnostic and therapeutic utilities. For example, these molecules can be administered to cells in culture, *e.g.*, *in vitro* or *ex vivo*, or in a subject, *e.g.*, *in vivo*, to treat, prevent or diagnose a variety of disorders.

[0391] The invention also provides methods of using the Tn3 scaffolds of the invention. The present invention also encompasses the use of the Tn3 scaffolds of the invention for the prevention, diagnosis, management, treatment or amelioration of one or more symptoms associated with diseases, disorders of diseases or disorders, including but not limited to cancer, inflammatory and autoimmune diseases, infectious diseases either alone or in combination with other therapies. The invention also encompasses the use of the Tn3 scaffolds of the invention conjugated or fused to a moiety (*e.g.*, therapeutic agent or drug) for prevention, management, treatment or amelioration of one or more symptoms associated with diseases, disorders or infections, including but not limited to cancer, inflammatory and autoimmune diseases, infectious diseases either alone or in combination with other therapies.

[0392] The invention also provides methods of targeting epitopes not easily accomplished with traditional antibodies. For example, in one embodiment, the Tn3 scaffolds the invention may be used to first target an adjacent antigen and while binding, another binding domain may engage the cryptic antigen.

[0393] The invention also provides methods of using the Tn3 scaffolds to bring together distinct cell types. In one embodiment, the proteins of the invention may bind a target cell with one binding domain and recruit another cell via another binding domain. In another embodiment, the first cell may be a cancer cell and the second cell is an immune

effector cell such as an NK cell. In another embodiment, the Tn3 scaffolds of the invention may be used to strengthen the interaction between two distinct cells, such as an antigen presenting cell and a T cell to possibly boost the immune response.

[0394] The invention also provides methods of using the Tn3 scaffolds to deplete a cell population. In one embodiment, methods of the invention are useful in the depletion of the following cell types: eosinophil, basophil, neutrophil, T cell, B cell, mast cell, monocytes and tumor cells.

[0395] The invention also provides methods of using Tn3 scaffolds as diagnostic reagents. Such diagnostic reagents are could be used to test for the presence or absence of CD40L, the presence of CD40 receptor, the binding efficiency of CD40L to CD40 receptor, free CD40L in a patient, free CD40L in a sample, or bound CD40L to CD40 receptor in a sample.

[0396] The Tn3 scaffolds of the invention and compositions comprising the same are useful for many purposes, for example, as therapeutics against a wide range of chronic and acute diseases and disorders including, but not limited to, autoimmune and/or inflammatory diseases. The compositions and methods of the invention described herein are useful for the prevention or treatment of autoimmune disorders and/or inflammatory disorders.

[0397] Examples of autoimmune and/or inflammatory disorders include, but are not limited to, alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diseases of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, Sjogren's syndrome, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation, sepsis, rheumatoid arthritis, peritonitis, Crohn’s disease, reperfusion injury, septicemia, endotoxic shock, cystic fibrosis, endocarditis, psoriasis, arthritis (*e.g.*, psoriatic arthritis), anaphylactic shock, organ ischemia, reperfusion injury, spinal cord injury and allograft rejection. autoimmune thrombocytopenia, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatrical pemphigoid, CREST syndrome, cold agglutinin disease, Crohn's disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, glomerulonephritis, Graves' disease, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic

thrombocytopenia purpura (ITP), IgA neuropathy, juvenile arthritis, lichen planus, lupus erythematosus, Meniere's disease, mixed connective tissue disease, multiple sclerosis, type 1 or immune-mediated diabetes mellitus, myasthenia gravis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychrondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynauld's phenomenon, Reiter's syndrome, Rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, lupus erythematosus, takayasu arteritis, temporal arteristis/giant cell arteritis, ulcerative colitis, uveitis, vasculitides such as dermatitis herpetiformis vasculitis, vitiligo, and Wegener's granulomatosis.

[0398] Examples of inflammatory disorders include, but are not limited to, asthma, encephilitis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), allergic disorders, septic shock, pulmonary fibrosis, undifferentitated spondyloarthropathy, undifferentiated arthropathy, arthritis, inflammatory osteolysis, and chronic inflammation resulting from chronic viral or bacterial infections. The compositions and methods of the invention can be used with one or more conventional therapies that are used to prevent, manage or treat the above diseases.

[0399] The invention provides methods for preventing, managing, treating or ameliorating cancer, autoimmune, inflammatory or infectious diseases or one or more symptoms or one or more symptoms thereof, said methods comprising administering to a subject in need thereof one or more Tn3 scaffolds of the invention in combination with one or more of therapeutic agents that are not cancer therapeutics (a.k.a., non-cancer therapies).

[0400] Examples of such agents include, but are not limited to, anti-emetic agents, anti-fungal agents, anti-bacterial agents, such as antibiotics, anti-inflammatory agents, and anti-viral agents. Non-limiting examples of anti-emetic agents include metopimazin and metochlopramide. Non-limiting examples of antifungal agents include azole drugs, imidazole, triazoles, polyene, amphotericin and ryrimidine. Non-limiting examples of anti-bacterial agents include dactinomycin, bleomycin, erythromycin, penicillin, mithramycin, cephalosporin, imipenem, axtreonam, vancomycin, cycloserine, bacitracin, chloramphenicol, clindamycin, tetracycline, streptomycin, tobramycin, gentamicin, amikacin, kanamycin, neomycin, spectinomycin, trimethoprim, norfloxacin, refampin, polymyxin, amphotericin B, nystatin, ketocanazole, isoniazid, metronidazole and pentamidine. Non-limiting examples of antiviral agents include nucleoside analogs (*e.g.*, zidovudine, acyclivir, gangcyclivir, vidarbine, idoxuridine, trifluridine and ribavirin), foscaret, amantadine, rimantadine,

saquinavir, indinavir, ritonavir, interferon ("IFN")-α,β or γ and AZT. Non-limiting examples of anti-inflammatory agents include non-steroidal anti-inflammatory drugs ("NSAIDs"), steroidal anti-inflammatory drugs, beta-agonists, anti-cholingenic agents and methylxanthines.

[0401] In one embodiment, the invention comprises compositions capable of treating chronic inflammation. In one embodiment, the compositions are useful in the targeting of immune cells for destruction or deactivation. In one embodiment, the compositions are useful in targeting activated T cells, dormant T cells, B cells, neutrophils, eosiniphils, basophils, mast cells, or dendritic cells. In another embodiment, the invention comprises compositions capable of decreasing immune cell function. In another embodiment, the compositions are capable of ablating immune cell function.

[0402] In another embodiment, the invention comprises compositions useful for treatment of diseases of the gastrointestinal tract. The scaffolds of the invention exhibit a high level of stability under low pH conditions. The stability at low pH suggests that the composition will be suitable for oral administration for a variety of gastrointestinal disorders, such as irritable bowel syndrome, gastroesophageal reflux, intestinal pseudo-obstructions, dumping syndrome, intractable nausea, peptic ulcer, appendicitis, ischemic colitis, ulcerative colitis, gastritis, *Helicobacter pylori* disease, Crohn's disease, Whipple's disease, celiac sprue, diverticulitis, diverticulosis, dysphagia, hiatus hernia, infections esophageal disorders, hiccups, rumination and others.

[0403] The invention further provides combinatorial compositions and methods of using such compositions in the prevention, treatment, reduction, or amelioration of disease or symptoms thereof. The Tn3 scaffolds of the invention may be combined with conventional therapies suitable for the prevention, treatment, reduction or amelioration of disease or symptoms thereof. Exemplary conventional therapies can be found in the Physician's Desk Reference (56th ed., 2002 and 57th ed., 2003). In some embodiments, Tn3 scaffolds of the invention may be combined with chemotherapy, radiation therapy, surgery, immunotherapy with a biologic (antibody or peptide), small molecules, or another therapy known in the art. In some embodiments, the combinatorial therapy is administered together. In other embodiments, the combinatorial therapy is administered separately.

[0404] The invention also provides methods of diagnosing diseases. The Tn3 scaffolds of the invention which bind a specific target associated with a disease may be implemented in a method used to diagnose said disease. In one embodiment, the Tn3

scaffolds of the invention are used in a method to diagnose a disease in a subject, said method comprising obtaining a sample from the subject, contacting the target with the Tn3 scaffold in said sample under conditions that allow the target:scaffold interaction to form, identifying the target:scaffold complex and thereby detecting the target in the sample. In other embodiments, the disease to be diagnosed is described herein.

[0405] The invention also provides methods of imaging specific targets. In one embodiment, Tn3 scaffolds of the invention conjugated to imaging agents such as green-fluorescent proteins, other fluorescent tags (Cy3, Cy5, Rhodamine and others), biotin, or radionuclides may be used in methods to image the presence, location, or progression of a specific target. In some embodiments, the method of imaging a target comprising a Tn3 scaffold of the invention is performed in vitro. In other embodiments, the method of imaging a target comprising a Tn3 scaffold of the invention is performed in vivo. In other embodiments, the method of imaging a target comprising a Tn3 scaffold of the invention is performed by MRI, PET scanning, X-ray, fluorescence detection or by other detection methods known in the art.

[0406] The invention also provides methods of monitoring disease progression, relapse, treatment, or amelioration using the scaffolds of the invention. In one embodiment, methods of monitoring disease progression, relapse, treatment, or amelioration is accomplished by the methods of imaging, diagnosing, or contacting a compound/target with a Tn3 scaffold of the invention as presented herein.

Pharmaceutical Dosing and Administration

[0407] To prepare pharmaceutical or sterile compositions including a Tn3 scaffold of the invention, a scaffold is mixed with a pharmaceutically acceptable carrier or excipient. For administration compositions are preferably pyrogen-free which are substantially free of endotoxins and/or related pyrogenic substances. Selecting an administration regimen for a therapeutic depends on several factors, including the serum or tissue turnover rate of the entity, the level of symptoms, the immunogenicity of the entity, and the accessibility of the target cells in the biological matrix. In certain embodiments, an administration regimen maximizes the amount of therapeutic delivered to the patient consistent with an acceptable level of side effects.

[0408] Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular

patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

[0409] A composition of the present invention may also be administered via one or more routes of administration using one or more of a variety of methods known in the art. In certain embodiments, the Tn3 scaffolds of the invention can be formulated to ensure proper distribution *in vivo*.

Equivalents

[0410] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

EXAMPLES

[0411] The invention is now described with reference to the following examples. These examples are illustrative only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teachings provided herein.

Example 1

Construction of a 3 loop library on the Parent Tn3 Scaffold

[0412] A library was constructed based upon the parent Tn3 scaffold, described in International Patent Application Publ. No. WO 2009/058379, wherein it is designated "Tn3

SS4." The library contained randomized regions of the BC, DE and FG loops. This design incorporated characterized sequence and loop length diversity into the Tn3 library, consistent with patterns of diversity described for natural FnIII domains, three different lengths for the BC and FG loops, and used a "NHT" mixed codon scheme for introducing diversity into the library (H = A, T, C). .This scheme generated 12 codons that coded for 12/20 amino acids (see TABLE 3), that is, each codon coded for a unique amino acid. Moreover, there were no stop or Cysteine (Cys) codons.

TABLE 3

A	AAT = Asn	ATT = Ile	ACT = Thr
G	GAT = Asp	GTT = Val	GCT = Ala
C	CAT = His	CTT = Leu	CCT = Pro
T	TAT = Tyr	TTT = Phe	TCT = Ser
	A	T	C

[0413] The library diversity was generated using the degenerate oligonucleotides shown in TABLE 4.

TABLE 4

Oligo	Loop	Sequence	SEQ ID NO
BC9 NHT	BC	ACCGCGCTGATTACCTGGNHTNHTSCGNHTGSTNHTNHTNHTGGC TGTGAACTGACCTATGGCATTAAA	178
BC11 NHT	BC	ACCGCGCTGATTACCTGGNHTNHTBSTNHTNHTNHTNHTNHTNHT NHTGGCTGTGAACTGACCTATGGCATTAAA	179
BC12 NHT	BC	ACCGCGCTGATTACCTGGNHTVMACCGNHTNHTNHTRRCRGCNHT VTTNHTGGCTGTGAACTGACCTATGGCATTAAA	180
DE NHT	DE	CGATCGCACCACCATAGATCTGNHTNHTNHTNHTNHTNHTTATAG CATTGGTAACCTGAAACCG	181
FG9 NHT	FG	GAATATGAAGTGAGCCTGATTTGCNHTAMSNHTNHTGGTNHTNHT NHTKCGAAAGAAACCTTTACCACCGGTG	182
FG10 NHT	FG	GAATATGAAGTGAGCCTGATTTGCNHTAMSNHTNHTNHTNHTRGC NHTCCGGCGAAAGAAACCTTTACCACCGGTG	183
FG11 NHT	FG	GAATATGAAGTGAGCCTGATTTGCNHTAMSNHTNHTGGTNHTNHT AGCAACCCGGCGAAAGAAACCTTTACCACCGGTG	184

Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G; V = A/C/G; M = A/C; K = G/T

[0414] The library was assembled using the oligonucleotides shown in TABLE 5.

TABLE 5

Oligo	Sequence	SEQ ID NO
BCX-DE bridge v2	CAGATCTATGGTGGTGCGATCGCCCGGCACATCTTTAATGCCAT AGGTCAGTTCACA	185
DE-FGX bridge v2	GCAAATCAGGCTCACTTCATATTCGGTATCCGGTTTCAGGTTAC CAATGCTAT	186
KpnI amp rev v2	CGGGTCGGTTGGGGTACCGCCACCGGTGGTAAAGGTTTCTTT	187
KpnI reverse v2	CGGGTCGGTTGGGGTA	188
BC library amp v2	GGCCCAGCCGGCCATGGCCGCCATTGAAGTGAAAGATGTGACCG ATACCACCGCGCTGATTACCTGG	189

[0415] A mix of the degenerate oligonucleotides (equimolar ratios of the oligonucleotides corresponding to the BC and FG loops, respectively), BCX-DE bridge v2, DE-FGX bridge v2, and KpnI amp rev v2, was assembled in a 20 cycle PCR reaction without an excess of external primers. This product was diluted and amplified in a regular PCR reaction using the primers BC library amp v2 and KpnI reverse v2. The resulting PCR product generated a complete Tn3 gene which was then digested with *NcoI* and *KpnI* and ligated into the phage display vector (described in WO 2009/058379). The DNA was transformed into *E. coli* by electroporation. The final diversity of the library was estimated to be about 7.9×10^{10} members.

[0416] After electroporation, the library was incubated for 1 hour at 37°C with shaking. M13K07 helper phage was added and after one hour the cells were diluted to a larger volume and grown at 37°C with shaking overnight. The next day phage were removed and concentrated from the supernatant by precipitation with PEG 8000.

Example 2

Panning libraries for Human CD40L-specific Tn3 scaffolds

[0417] Phage displayed Tn3 libraries containing $>10^{10}$ unique sequences were panned against CD40L. The diversity in these libraries was derived from sequence and length variability in the BC, DE and FG loops which are analogous to the three CDR loops within an

antibody variable domain. Selection of lead Tn3 proteins was performed by panning of libraries on recombinant biotinylated human CD40L and a CD40L overexpressing CHO cell line. Alternate rounds of panning against these two reagents were used to ensure leads would recognize the recombinant extracellular domain as well as native, membrane-anchored CD40L.

[0418] Recombinant human CD40L (Human MegaCD40L; Axxora) was biotinylated with EZ-Link sulfo-NHS-biotin (Pierce, Rockford, IL) using a 5–fold molar excess of the biotinylation reagent. After incubation for 1 hour at room temperature, the sample was dialyzed in PBS overnight to remove unconjugated biotin. 10 µg biotinylated CD40L was immobilized on M280 streptavidin beads (Dynal, Carlsbad, CA), followed by blocking in PBS containing 10 mg/ml BSA for 2 hours. Input consisted of libraries developed as described in Example 1 or additionally, libraries developed using standard construction techniques, such as described in WO 2009/058379.

[0419] Phages were blocked in PBS containing 10 mg/ml BSA for 2 hours. The blocked input was added to blocked M280 streptavidin control beads (without target) and incubated on rocker for 2 hours at room temperature to deplete the library of binders to the beads. The depleted library was then added to the CD40L coated beads and incubated for 2 hours at room temperature on a rocking platform. After three washes with PBST (PBS + 0.1% Tween) to remove unbound phage, the beads were added to exponentially growing *E. coli* XL-1 Blue cells, which were subsequently co-infected with M13KO7 helper phage in 60 ml 2xYT medium containing 50µg/ml carbenicillin. After growing overnight at 37^{o}C with shaking, phage were harvested by PEG precipitation from the overnight culture media.

[0420] The second round of panning (Round 2) was performed on a CD40L overexpressing CHO cell line. The phage library was blocked in 3% BSA/PBS rocking at room temperature for 1 hour. Cells were detached with Accutase (Invitrogen), washed 2x with 5 ml PBS, and 10^7 cells were blocked in 1 ml 3%BSA/PBS rocking at room temperature for 30 minutes. The blocked cells were spun down at 500xg, 5 minutes, gently resuspended in the blocked phage library solution, and incubated 1 hour at room temperature. Unbound phage was removed by gently washing the cells 3 times in 1 ml 3% BSA/PBS and once in PBS, pelleting cells by centrifuging 500xg for 5 minutes in a microcentrifuge using a fresh Eppendorf tube for each wash. The cell pellet was added directly to exponentially growing *E. coli* XL-1 Blue, which were then processed as described for Round 1.

[0421] Panning Round 3 was performed as described for Round 1, except bound phages were eluted by addition of 100 mM HCl followed by neutralization with 1M Tris-

HCl, pH 8. Eluted, neutralized phage was used to infect *E. coli* XL1 Blue cells as described for Round 1.

[0422] Panning Round 4 was again carried out on cells as described for Round 2, except 5 washes in 3% BSA/PBS were conducted. Round 5 was done using 5 μg of biotinylated MegaCD40L, but otherwise as described for Round 3.

[0423] After 5 rounds of panning, screening of resulting Tn3 variants as soluble protein was performed. Amplified and PEG precipitated phage stocks were used in a PCR to amplify a pool of fragments encompassing the encoded Tn3 sequences. This fragment pool was digested with *NcoI* + *KpnI* and cloned into the corresponding *NcoI* - *KpnI* sites of plasmid pSec-oppA(L25M)-Tn3 (see, for example, WO 2009/058379). Auto-inducing MagicMedia (Invitrogen) containing carbenicillin (100 μg/ml) in 96 well deep-well plates were inoculated with *E. coli* BL21 DE3 cells transformed with the pSec-oppA(L25M)-Tn3 derived constructs. Cultures were grown for 18 hours shaking at 37ºC, and cells were separated from the media by centrifugation. The media containing secreted, soluble Tn3 variants was used directly in a screening assay for CD40L binding.

[0424] Ten sets of 96 clones were screened to identify Tn3 proteins that specifically bound to recombinant CD40L. Briefly, the screening assay utilized capture of soluble His-tagged Tn3 variants secreted into media through binding to an anti-His antibody immobilized in wells of microplates. After capture, media and excess protein were washed away and the interaction between captured Tn3 variants and CD40L was monitored by utilizing biotinylated Human MegaCD40L and measuring the remaining target (after washing the plate) by SA-HRP and conventional ELISA reagents.

[0425] In the capture step, the immobilized anti-His antibody was saturated with Tn3, and the molar amount of captured Tn3 in each well became virtually identical irrespectively of the expression levels of individual clones. This normalization of Tn3 levels resulted in assay levels proportional with the efficiency of target interaction and unaffected by potential differences in protein expression levels.

[0426] Positives from this assay were sequenced to identify 34 unique Tn3 sequences that bound recombinant CD40L. From the panel of unique CD40L-binding Tn3 sequences, a subset of 24 clones that had a robust assay signal and good expression levels judging by SDS-PAGE of the culture supernatants underwent re-expression and small scale purification.

[0427] Briefly, Superbroth media containing carbenicillin (100 μg/mL) with 1% glucose) was inoculated with *E. coli* BL21 DE3 cells transformed with the pSec-oppA(L25M)-Tn3 derived constructs. Cultures were grown at 37ºC to an optical density (O.D.) of 0.5-0.8 then induced with 0.2 mM IPTG. After shaking at 37ºC for 5 hours, cells were separated from the media by centrifugation. Purification of Tn3 scaffolds from the media was effected by batch purification using Ni-NTA Superflow (Qiagen), washing in 2x PBS with 20 mM imidazole, and elution with 2x PBS with 250 mM imidazole. Samples were dialyzed in PBS, and concentrations determined by UV absorbance at 280 nm according to Gill and von Hippel (Anal. Biochem. 182: 319, 1989).

[0428] Based on assay ranking and SEC behavior, expression of 8 leads was scaled up and purified to low endotoxin levels (< 1 EU/mg) for testing in a functional cell assay.

[0429] Two Tn3 clones (designated 309 and 311) showed similar activity in biochemical and cell-based assays (FIGS. 6A, 6B, 6C), and were 3-5 fold more potent than the nearest rival clone.

[0430] Human CD40L-specific monovalent Tn3 scaffolds 309 and 311 inhibited total B cell number, plasma cell number and Ig class switching (FIGS. 6A, 6B and 6C). FIG. 6 A shows the inhibitory effect of 309 and 311 on HuCD40L-induced CD86 expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells; FIG. 6B shows inhibition of HuCD40L stimulated B-cell proliferation by 309 and 311; and FIG. 6C C shows inhibition of plasma cell number in T/B cell co-cultures. 309 was also shown to bind activated primary T cells by FACS (data not shown). PBMCs were stimulated by recombinant human MegaCD40L (Axxora) or human CD40L expressing Jurkat cells (D1.1, ATCC), and the percentage of CD19+/CD86+ cells was measured by FACS after 24 hours.

[0431] The 309 and 311 lead clones were monodispersed and did not display any tendency to aggregate or form higher order oligomers in solution as determined by size exclusion chromatography (SEC) analysis of purified samples (FIG. 7A).

[0432] The thermal stabilities of the 309 and 311 lead clones were determined by differential scanning calorimetry (DSC) using protein samples at 1 mg/mL in PBS pH 7.2 and compared to the thermal stability of the parental Tn3 protein (FIG. 7B). The 309 and 3011 lead clones had T_m's of 70 ± 1°C which was only slightly below the T_m of parent Tn3 (72°C).

[0433] As no murine cross-reactive clones were identified a similar panning process as described above was carried out to identify the murine specific Tn3 designated M13. M13 also showed activity in a PBMC cell based assay (see FIG. 1A)

Example 3

CD40L-specific Tn3 Scaffold Lead Optimization

[0434] Affinity optimization was used to increase the potency of the selected Tn3 leads. In general, one or more rounds of mutagenesis within the Tn3 loops contacting the target were conducted, with selection of improved variants from combinatorial phage display libraries.

3.1 Loop swapping

[0435] In order to determine which of the 3 loops in the two leads were involved in interaction with CD40L, constructs were generated in which each single loop sequence was changed to the parent Tn3 loop sequence as found in human tenascin C. Activities of the mutated variants were compared to the original variants in a binding assay conducted as described for the screening assay described above (FIG. 10A). For both leads mutating the BC and DE loops resulted in a complete loss of binding to CD40L, whereas changing the FG loop to the parent Tn3 sequence had either no effect (for 309) or limited effect (311) on binding. Thus, the BC and DE loop appeared to contain the sequences mainly responsible for contacting CD40L, and were thus primarily selected for affinity optimization.

3.2 CD40L-specific Tn3 Scaffold 309 Lead Optimization

[0436] As the loop swapping experiment indicated the 309 FG-loop sequence could be substituted with the parent Tn3 FG-loop sequence without substantial loss of binding potency, it was decided to use this construct (termed 309FGwt) as a backbone for affinity maturation. This would eliminate non-essential mutations deviating from the parent tenascin C sequence in order to reduce possible immunogenicity risk. It should be noted that the parent Tn3 FG-loop sequence contained an RGD motif which was later eliminated by a mutation in the final lead molecules. Three BC loop libraries and one DE-loop library were generated.

[0437] For the three BC loop libraries three rounds of PCR were done using the degenerated oligos BC9 PCR, BC 9-loop NNK and 309 BC-loop NNKdope (TABLE 6) together with the reverse primer Kpn1 amp rev v2 (TABLE 5) using a 309FGwt derived template in which the BC-loop codons had been replaced with stop codons. Subsequently PCR amplification of those fragments with the primers BC library amp v2 (TABLE 5) and KpnI reverse v2 gave the full length Tn3 library fragment.

[0438] For the DE-loop library a PCR amplification with DE PCR and KpnI amp reverse v2 on a 309FGwt derived template (in which the DE-loop codons had been replaced with stop codons) gave a fragment containing the randomized DE loop and wild type Tn3 FG-loop which was combined with a fragment encoding the Tn3 region upstream of the DE loop generated by PCR with BC library amp v2 and BCX-DE bridge v2 on a 309 template. The two fragments were joined in an overlap PCR with the external primers BC library amp v2 and KpnI reverse v2.

TABLE 6: DNA oligonucleotides used for 309FGwt LO library generation

Oligo	Sequence	SEQ ID NO
BC9 PCR	5'-ACCGCGCTGATTACCTGGTCT1213111GGCTGTGAACTGACCTATGGCATTAAAGATG	190
BC 9-loop NNK	5'-ACCGCGCTGATTACCTGGNNKNNKSMGNNKGSTNNKNNKNNKGGCTGTGAACTGACCTATGGCATTAAA-3'	191
309 BC-loop NNKdope	5'-ACCGCGCTGATTACCTGG76K45K45K77K44K65K78T45K44KTGTGAACTGACCTATGGCATTAAA-3'	192
DE PCR	5'- GATGTGCCGGGCGATCGCACCACCATAGATCTG111111TATAGCATTGGTAACCTGAAACCGG-3'	193
Upstr BCloop Rev	CCAGGTAATCAGCGCGGTGGTAT	194
BC shuffle rev	CAGATCTATGGTGGTGCGATCGC	195
DE shuffle FWD	TGTGAACTGACCTATGGCATTAAAGATGT	196

1= Codons for all 19aa(-cys) 2= Codons for Ala/Pro 3= Codons for Ala/Gly;

4= 70%G10%A10%C10%T 5= 10%G,70%A,10%C,10%T

6=10%G,10%A,70%C, 10%T 7= 10%G,10%A,10%C,70%T

8= 70%A15%C15%T K = 50%G/50%T

[0439] The NcoI-KpnI fragments were cloned into the phage display vector, and phage library generated as described in Example 1.

[0440] The four libraries were panned separately on Biotinylated Human MegaCD40L as described for the first round in Example 2, using 4 μg CD40L in Round 1 and 1 μg in Round 2. After amplification of phage output after Round 2, single-stranded DNA was isolated using a Qiagen Spin M13 kit (Qiagen, Valencia, CA), and the pools of BC-loop containing fragments from the BC loop libraries were amplified using BC lib amp v2 and BC shuffle rev, whereas the pool of DE-loop containing fragments was amplified from the DE-loop library using primers DE-shuffle FWD and KpnI reverse v2. The PCR fragments were gel-purified and assembled through their overlapping sequence using the external primers BC lib amp v2 and KpnI reverse v2. The resulting PCR fragment was used to generate a library in the phage vector as previously described. This library was panned for a total of 5 rounds on biotinylated Human MegaCD40L as described in Example 2, except the libraries were initially contacted with a target at a concentration of 50 nM, 20 nM, 20 nM, and 10 nM (in a total volume of 50 μl) for Rounds 1 through 4 for 2 hours prior to incubation with blocked M280 streptaviding magnetic beads for 10 minutes followed by washing..

[0441] Outputs were pool cloned into the *NcoI* - *KpnI* sites of plasmid pSec-oppA(L25M)-Tn3 pSec, and sixteen 96 well plates were screened for CD40L binding using soluble protein in the screening assay described above. The 270 highest scoring clones were cherry-picked, re-assayed and sequenced. Ten clones were chosen for further characterization based on binding assay and sequence analysis. This included assessment of potency in the PBMC assay, K_d determination for binding to CD40L in a biosensor assay, thermodynamic stability determined by differential scanning calorimetry, and tendency to aggregate or form higher order oligomers in solution by size exclusion chromatography analysis. Results are summarized in TABLE 7. Sequences of the 309 and 309FGwt clones aligned with the ten optimized clones (designated 340, 341, 342, 343, 344, 345, 346, 347, 348 and 349) are shown in FIGS. 11A and 11B.

[0442] Affinity matured variants showed 1-3 logs higher potency than the 309 clone, retained high stability as measured by DSC, and most were monodispersed as measured by SEC.

TABLE 7

Variant	PBMC IC50 (nM)	Kd (nM)	SEC Profile	Tm, DSC (°C)
309	226	191	OK	72
309FGwt	760	nd	OK	71
340	0.7	2.2	OK	77
341	0.7	nd	OK(?)	71
342	0.7	1.4	OK	73
343	0.6	2.0	OK	69 (?)
344	1.3	nd	OK	(65+78.5)
345	37.3	39	OK	72
346	9.0	14.9	OK	71
347	11.0	10.7	OK	70
348	1.0	1.8	?	nd
349	38.2	21	OK(?)	nd

[0443] PBMC assays were performed by stimulating PBMCs with human CD40L-expressing Jurkat cells (D1.1, ATCC), and the percentage of CD19+/CD86+ cells was measured by FACS after 24 hours. This assay was used to test and rank the panel of leading Tn3 scaffolds to emerge from prioritization based on biochemical criteria. Results of the PBMC assays are shown in FIG. 10B, and summarized in TABLE 7.

[0444] Affinity measurements were performed on the ProteOn XPR36 protein interaction array system (Bio-Rad, Hercules, CA) with GLC sensor chip at 25ºC. ProteOn phosphate buffered saline with 0.005% Tween 20, pH 7.4 (PBS/Tween) was used as running buffer. Human MegaCD40L was immobilized on the chip at a surface density of approximately 2300 RU. Two-fold dilutions of the Tn3 variants (340, 342, 343, 345, 346, 347, 348, and 349) were prepared in PBS/Tween/0.5mg/ml BSA, pH 7.4 (from 150 to 4.7 nM). Samples of each concentration were injected into the six analyte channels at a flow rate of 30 µl/min. for 300 seconds. The K_d was determined by using the equilibrium analysis setting within the ProteOn software. Results are shown in TABLE 7.

[0445] The ten TCD40L-specific Tn3 variants were analyzed for stability by DSC. Briefly, DSC measurements were conducted on a VP-Capillary DSC (MicroCal). Proteins were exchanged into PBS (pH 7.2) through extensive dialysis, and adjusted to a concentration of 0.25-0.5 mg/ml for DSC analysis. Samples were scanned from 20-95°C at a scan rate of 90°C/hour, with no repeat scan. The results are shown in TABLE 7.

[0446] Up to a 300-fold improvement in IC_{50} over 309, and over 1000-fold improvement over the 309FGwt backbone used for lead optimization library generation was obtained for the best clones. Seven clones had K_d's in the single digit nM range.

3.3 CD40L-specific Tn3 Scaffold 311 Lead Optimization

Prior to conducting the loop usage experiment mentioned previously (see FIG. 10A)

[0447] an initial attempt at a lead optimization library focused on introducing diversity in the FG-loop of 311, generating and screening the resulting phage display library. Screening was conducted after 4 rounds of panning on Biotinylated human MegaCD40L as previously described, and it was found that a majority of the positive hits contained a fortuitous mutation of residue K4 to E in the N-terminal constant region of Tn3, upstream of the BC-loop. No obvious consensus among the FG-loop sequences of positive hits was detected. Introducing the single K4E mutation into 311 resulted in an approximately 100-fold increase in potency (from approximately 4 μM to 36 nM) in the PBMC assay (see FIG. 10C).

[0448] As the loop swap experiment indicated that the BC and DE loop sequences were required for binding to CD40L, these loops were then targeted in the 311K4E backbone for further affinity maturation. Two separate libraries were generated. One library targeted the BC-loop with a strategy where each residue had a 50% chance of being wild type 311 sequence, and approximately 50% chance of being one of the other 11 NHT encoded residues. The other library completely randomized the 6-residue DE-loop.

[0449] For the BC-loop library, the oligonucleotides BC11-311Gly and BC11-311NHT (TABLE 8) were used in PCR reactions with the reverse primer KpnI amp rev v2 (TABLE 5) on a 311 derived template in which the BC-loop codons had been replaced with stop codons to generate fragments including BC, DE and FG loops. Finally, amplification of a 1:1 mixture of these fragments with the primers BC library amp K4E and KpnI amp rev v2 gave the full length Tn3 library fragment.

[0450] The DE-loop library was generated as the 309FGwt DE-loop library described above, except a 311 derived template was used in the PCR reactions and the BC library amp K4E primer was used in the final PCR amplification.

TABLE 8. Oligos employed for 311K4E lead optimization library generation.

Oligo	Sequence	SEQ ID
BC11-311Gly	5’- ACCGCGCTGATTACCTGG26T25TV1T46T46T45T45T25T37T35TGGCTGTGAACTGACCTATGGCATTAAA-3’	197
BC11-311NHT	5’-ACCGCGCTGATTACCTGG26T25TV1T46T46T45T45T25T37T35TNHTTGTGAACTGACCTATGGCATTAAA-3’	198

BC library amp K4E	5’-GGCCCAGCCGGCCATGGCCGCCATTGAAGTGGAAG ATGTGACCGATACCACCGCGCTGATTACCTGG-3’	199
BC11-311Gly	5’- ACCGCGCTGATTACCTGG26T25TV1T46T46T45T45T25 T37T35TGGCTGTGAACTGACCTATGGCATTAAA-3’	197
BC11-311NHT	5’-ACCGCGCTGATTACCTGG26T25TV1T46T46T45T45T25 T37T35TNHTTGTGAACTGACCTATGGCATTAAA-3’	198
BC library amp K4E	5’-GGCCCAGCCGGCCATGGCCGCCATTGAAGTGGAAG ATGTGACCGATACCACCGCGCTGATTACCTGG-3’	199

1=70%G, 10%A, 10%C, 10%T 2=10%G, 70%A, 10%C, 10%T

3=10%G, 10%A, 70%C, 10%T 4=10%G, 10%A, 10%C, 70%T

5=70%A, 15%C, 15%T 6=15%A, 70%C, 15%T

7=15%A, 15%C, 70%T V=33%A, 33%C, 33%G

H=33%A, 33%C, 33%T

[0451] The full length library fragments were digested with NcoI and KpnI, cloned into the phage display vector, and phage libraries generated as described in Example 1.

[0452] The two libraries were panned separately on Biotinylated human MegaCD40L as described in Example 1, using 10 □g of protein in Round 1, 5 □g in Round 2, and 5 □g in Round 3. After amplification of phage output after Round 3, single stranded DNA was isolated using a Qiagen kit (Qiagen, Valencia, CA), and the two libraries were shuffled as described for the 309FGwt libraries. The shuffled library was panned for a total of 5 rounds on biotinylated human MegaCD40L as described above for the 309FGwt shuffled library using 100 nM , 20 nM , 4 nM, 1 nM and 1nM of target for Rounds 1 through 5.

[0453] Outputs were pool cloned into the soluble secretion vector as previously described, and five 96-well plates were screened for CD40L binding using the soluble protein screening assay described previously. Positive hits were identified relative to the signal obtained with the 311K4E backbone variant. The 18 highest scoring unique clones, designated 311K4E_1, 311K4E_2, 311K4E_3, 311K4E_4311K4E_5, 311K4E_7, 311K4E_8, 311K4E_9, 311K4E_10, 311K4E_11, 311K4E_12, 311K4E_13, 311K4E_14, 311K4E_15, 311K4E_16, 311K4E_19, 311K4E_20 and 311K4E_21 (sequences shown in FIG. 12A and FIG. 12B) were assayed as crude unpurified proteins for off-rate ranking. Off-rate estimates of unpurified Tn3 scaffolds were performed on the ProteOn XPR36 protein interaction array system (Bio-Rad, Hercules, CA) in a biosensor assay with CD40L immobilized on a chip. Mega human CD40L was immobilized on a GLC chip (BioRad) and all variants diluted to an

estimated concentration of 80 nM, injected at a flow rate of 30 μl/min for 300 seconds with the dissociation time set to 1200 seconds. PBS, 0.005% Tween20, 3mM EDTA, pH7.4 was used as running buffer. Off-rates were ranked by visual inspection of the sensorgrams. A subset of four variants which displayed the slowest off-rates,, 311K4E_3, 311K4E_11, 311K4E_12 and 311K4E_15, was purified, and K_d values were determined to be between 1.1 and 6.4 nM (TABLE 9).

TABLE 9. K_d of 311K4E and 4 affinity purified variants binding to human CD40L.

311 Variant	K_d (nM)
311K4E	18
311K4E_12	1.1
311K4E_11	6.3
311K4E_15	1.6
311K4E_3	6.4

[0454] As indicated in FIG. 10D, the decreased K_d (from 18 nM to 1 nM) of 311K4E_12 corresponded to a 12-fold increase in potency in the PBMC assay relative to the 311K4E backbone.

[0455] In conclusion, the lead optimization campaigns of initial hits 309 and 311 from the naïve libraries lead to single-digit nM binders of CD40L.

[0456] A similar optimization campaign was performed on the murine-specific M13 molecule (data not shown). The resulting optimized murine CD40L-specific molecule (designated M31) showed an approximately 20-fold increase in potency in the PBMC assay over the parent molecule (FIG. 1A).

Example 4

Expression and purification of untagged CD40L-specific Tn3-HSA fusions

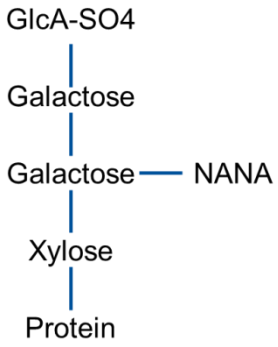
[0457] Tn3 constructs fused to HSA as outlined in FIG. 2A and 9A were expressed in mammalian 293F cell line by transient transfection. Tn3-HSA fusion expression constructs were generated based on an in-house generated mammalian expression vector. To increase product homogeneity, a mutant form of HSA (designated HSA C34S) was employed in

which the unpaired, partially exposed cysteine 34 had been mutated to serine (Zhao *et al.*, 2009, Eur. J. Pharm. Biopharm. 72: 405-11).

[0458] The fusion protein could be purified in a one-step purification by ion exchange chromatography (IEX). An example of elution of 309-309-HSA from a Q-HP column (GE HealthCare) by a salt gradient is shown in FIG. 9B. In addition to the main peak, minor later eluting peaks were seen (constituting less than 10% of the total peak area). Mass spectrometry analysis indicated these minor side peaks were enriched for O-glycosylated 309-309-HSA species. Fractions containing the main peak were pooled and used for subsequent activity assays.

[0459] For larger scale purifications, the IEX step mentioned above was preceded by capturing the Tn3-HSA fusions from the culture supernatant by affinity chromatography using HSA affinity matrixes, *e.g.*, HiTrap Blue HP (GE HealthCare). After washing, HSA fusion protein could be eluted with an Octenoic Acid containing buffer. Eluate was loaded onto the Q-HP column after 3-fold dilution in phosphate buffer.

[0460] Analysis of the minor peak(s) revealed the presence of O-linked carbohydrate moieties. The O-glycan were proposed to be a heterogeneous mix of carbohydrates derived from a previously reported O-xylosylated core structure (Wakabayashi et al., 1999, J. Biol. Chem. 274:5436-5442) shown below:



[0461] The predominate site of attachment was determined to be in the GGGGS linker between the Tn3 domains. The glycan was also found to be present to a lesser extent in the GGGGS linker found between the Tn3 and HSA. The levels of O-glycan were therefore higher in the bivalent constructs as compared to the monovalent constructs and were higher in material produced in HEK cells as compared to CHO cells. The levels also varied between the different Tn3 constructs. Thus, the level of O-glycan may be reduced through careful host cell section, for example use of CHO cells or other cells found to produce material with lower levels of O-glycan. In addition, material containing the O-glycan can be removed via purification methods to yield a more

homogenous product lacking the O-glycan. Alternatively, the linker may be modified to remove the primary site(s) of O-glycan attachment, for example by mutating the Ser residue to a Gly. The linkers in several constructs were reengineered to have one or more GGGGG linker No O-glycans of any type were detected in material having the GGGGG linker(s) and no difference in activity was seen (data not show).

Example 5

Extension of Serum Half-Life of CD40L-specific Tn3 Scaffolds

[0462] Fusion to serum albumin was explored as a strategy to extend the serum half-life of CD40L-specific Tn3 scaffolds. In order to determine the pharmacokinetic (PK) properties of murine CD40L specific Tn3-MSA fusions a mouse PK assay was conducted. MSA fusions were chosen for studies of surrogate molecules over the corresponding HSA fusions since mouse FcRn binds HSA considerably weaker than it binds MSA, resulting in decreased recycling from endosomes and consequently increased turnover (Andersen *et al.* J. Biol. Chem. 285, 4826-4836, 2010).

[0463] HEK 293 cells were used for expression of mouse CD40L-specific tandem bivalent Tn3 scaffold fused to MSA. High levels of expression were observed (FIG. 3A). These constructs had a (G_4S) linker between the Tn3 scaffold units and 3 (G_4S) repeats in the linker between the scaffold and MSA. In addition, a N49Q mutation was introduced into each of the M13 and M31 scaffolds to remove a potential N-linked glycosylation site. This mutation did not affect the potency of these scaffolds (data not shown). Expression level was estimated to 200 mg/L 6 days post transfection. Purification was carried out by IMAC through a C-terminal His-tag. The yield of purified protein was estimated to be 125 mg/L culture supernatant.

[0464] When MSA was fused to bivalent M13 scaffolds, an 8-fold decrease in potency compared to the M13 dimeric scaffold without MSA was observed (FIG. 3B). A bivalent scaffold comprising affinity matured M31 fused to MSA was 140 times more potent than the corresponding bivalent M13 scaffold fused to MSA, approximately 900 times more potent than the monovalent M31 MSA fusion, and had a potency comparable to the MR1 anti murine CD40L monoclonal antibody (FIG. 3C).

[0465] To determine the PK properties of CD40L-specific Tn3-MSA fusions a mouse PK analysis was carried out. Protein constructs were administered intravenously at 10

mg/kg in 5-7 week old female CD-1 mice. Each mouse was bled 150 μl at various time points and serum concentration of Tn3-HSA fusion determined by an ELISA assay. Briefly, Nunc MaxiSorp plates were coated with anti-FLAG M2 antibody (Agilent), blocked in 4% milk in PBS+0.1%Tween (PBST) and incubated with murine MegaCD40L (Axxora). The MegaCD40L was immobilized through its FLAG tag. Serum samples and protein standards were diluted in 4% milk PBST and added after washing plate in PBST. After incubation the plate was washed in PBST and rabbit anti-TN3 polyclonal antibody (Covance) was used to detect the Tn3-HSA fusion constructs using Goat anti-rabbit HRP-conjugated antibody (Jackson ImmunoResearch) in a standard ELISA protocol. Concentrations in serum samples were determined based on linear regression of standard curves generated by assays of dilution of the same Tn3-MSA fusion construct. Concentrations were determined as an average for 3 different mice.

[0466] As seen in FIG. 4A, M31-MSA and M13-M13-MSA had half-lives of 38 and 31 hours, respectively, whereas the M31-M31-HSA construct had a half-life of 12 hours. In comparison, the M13-M13 tandem construct by itself (not fused to MSA) displayed a half-life of 30 minutes (not shown).

[0467] In contrast to the observations in mouse scaffolds, when HSA was fused to human CD40L specific scaffolds there was no significant decrease in potency. FIG. 9C shows that there was no significant decrease in potency by fusing a human CD40L-specific bivalent Tn3 scaffold comprising two 309 monomer to HSA as measured in PMBC assays.

[0468] The PK properties of the human CD40L-specific 342-HSA monomer construct were compared to those of a 342-HSA variant comprising two substitutions (L463N and K524L) to enhance serum half-life in Cynomolgus monkey following a single intravenous injection. Protein constructs were administered via slow bolus injection at 10 mg/kg to male Cynomolgus monkeys weighing 2-5 kg. 1 mL of blood/animal/time point was collected from a peripheral vessel at predose, 5 minutes and 30 minutes post dose; 2, 12, 24, and 48 hours post dose; and on Days 4, 8, 11, 15, 22, 29, 36, 43 and 57. Serum concentration of Tn3-HSA fusion determined by an ELISA assay. Briefly, Nunc MaxiSorp plates were coated with anti-FLAG M2 antibody (Agilent), blocked in 4% milk in PBS+0.1%Tween (PBST) and incubated with human MegaCD40L (Axxora). The MegaCD40L was immobilized through its FLAG tag. Serum samples and protein standards were diluted in 4% milk PBST and added after washing plate in PBST. After incubation the plate was washed in PBST and rabbit anti-TN3 polyclonal antibody (Covance) was used to detect the Tn3-HSA

fusion constructs using Goat anti-rabbit HRP-conjugated antibody (Jackson ImmunoResearch) in a standard ELISA protocol. Concentrations in serum samples were determined based on linear regression of standard curves generated by assays of dilution of the same Tn3-HSA fusion construct. Concentrations are plotted in FIG. 4B. The half-life of the 342-HSA construct was about 7 days, while the 342-HSA L463N/K524L variant construct showed an increased half-life of 13-17 days during the initial linear phase (FIG. 4B). After 30 days, the serum concentrations of the 342-HSA L463N/K524L variant construct dropped off more rapidly as compared to the wild type HSA construct. These observations may indicate some immunogenicity of this construct in monkeys.

Example 6

Characterization of CD40L-specific Tn3 Scaffolds

6.1 Experimental methods

[0469] 6.1.1 PBMC stimulation assay: Blood was obtained from healthy donors according to MedImmune safety guidelines. PBMCs were isolated via CPT tubes (spin 1500g for 20 minutes) and 1 x 10^6 PBMCs (per condition) were stimulated by recombinant human MegaCD40L (Axxora) or human CD40L expressing Jurkat cells (D1.1, ATCC). The percentage of CD19+/CD86+ cells was measured by FACS after 24 hours stimulation. This assay was used to test and rank the panel of leading Tn3 scaffolds to emerge from prioritization based on biochemical criteria. The assay can also be performed with a murine CD40L-expressing cell line (D10.G4) or murine MegaCD40L (Axxora ALX522120) in place of the human cell or recombinant protein stimulation as murine ligand cross reacts with human receptor.

[0470] 6.1.2 MurineCD40R/NFκB assay: NFκB reporter NIH3T3 cells (Panomics NFκB reporter system and in-house mCD40R transfection) were stimulated with murine MegaCD40L (Axxora, cat. ALX522120) recombinant protein or CD40L over-expressing D10.G4 cells (ATCC) for 24 hours with or without Tn3 scaffolds. Bright-Glow (Promega E2610) was added according to manufacturer's directions. The readout was luminescence (700) via the NFκB reporter activation performed on an EnVision system (Perkin Elmer).

[0471] 6.1.3 Human CD40R/NFκB assay: Reporter HEK293 cells (Panomics and in-house) were stimulated with MegaCD40L (Axxora ALX522110) recombinant protein or CD40L overexpressing D1.1 Jurkat subclone cells (ATCC) for 24 hours with or without Tn3

scaffolds. Bright-Glow (Promega E2610) was added according to manufacturers directions. The readout was luminescence (700) via the NFκB reporter activation performed on an EnVision system (Perkin Elmer).

[0472] 6.1.4 Dual cell assay: Primary T/B cells were isolated from various donors. Anti-CD3 stimulated, mitomycin C treated human CD4+ T cells ($1x10^5$) were cultured with purified human B cells ($5x10^4$). Readouts were as follows: Day 2: Activation markers (FACS), Day 5: B cell proliferation (ATP metabolite, Cell-Titer Glo, Invitrogen), Day 7: plasma cell differentiation (FACS) Day 7: Ig production (ELISAs, R&D Systems).

[0473] 6.1.5 Platelet aggregation assays: Adenosine diphosphate (ADP) was from Chrono-Log (Havertown, PA, USA). All other products were at least reagent grade. Blood samples were collected from healthy volunteers in 12.9 mM sodium citrate and centrifuged at 150xg for 15 minutes to obtain PRP (platelet rich plasma). After separation of PRP, tubes were centrifuged again at 1,200xg for 15 minutes to obtain PPP (platelet poor plasma). Platelets were washed using the method described by Mustard *et al.* (Br. J. Haematol. 22:193-204, 1972), and re-suspended in Tyrode's solution containing $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, 0.1% dextrose, 0.35% bovine serum albumin, 0.05 U/mL apyrase, pH 7.35. Platelet aggregation was studied using a light transmission aggregometer (Chrono-Log 700-4DR, Chrono-Log Corporation, Havertown, PA, USA) and recorded for 10 min after stimulation of platelets with the indicated platelet agonists as described. Tn3 scaffolds were pre-incubated with the soluble CD40L (sCD40L) to form immunocomplexes prior to addition.

[0474] 6.1.6 Immunization assays: Sheep Red Blood Cells (SRBC) were purchased from Colorado Serum (Denver, CO) and diluted 10-fold in HBSS medium immediately before use. Mice were immunized with 0.2 ml of diluted SRBC on day 0. Primary Germinal center (GC) response in challenged mice was assayed 14 days after immunization via FACS (GC B cells, non-GC B-cells, and all T cell subsets). Tn3 scaffolds and controls were administered on days 9-13 in 24 hour increments as indicated.

[0475] 6.1.7 KLH-specific T cell dependent antibody response (TDAR) assays: Cynomolgus Monkeys (Macaca fascicularis) of Chinese origin and weighing 3.1 - 4.6 kg, (Covance Research Products, Alice TX) were dosed intravenously (saphenous or cephalic vein) once weekly with the indicated dose (0.5, 5, 40 mg/kg) of inhibitor (342-monomer-Tn3-HSA and 342-342 bivalent-Tn3-HSA or control/vehicle. KLH (Lot No. MD158678A, Supplier Pierce Biotechnologies, Rockford, Illinois) was reconstituted with the appropriate amount of sterile water for injection (Supplier Midwest Veterinary Supply, Norristown,

Pennsylvania) under sterile conditions. Vials were swirled to mix and then pooled together into a sterile vial. 1 mL of KLH solution (10 mg/mL) was administered subcutaneously on each animal's back, to the left of the midline on two occasions (Day 1 and Day 29), within 1 hour of the end of the test or control article administration. Blood samples for further analysis were obtained from all animals at the following time points: pretest, Days 4, 6, 8, 11, 15, 22, 25, 32, 34, 36, 39, 43, 46, 50 and 57. Samples collected on Days 8, 15 and 22 were taken prior to dosing. Evaluation of KLH-specific IgM and IgG antibody titers were done at days 8, 11 and 15. The titers of KLH-specific IgM and IgG antibodies at day 15 are shown in Figures 5G and 5H, respectively. The cutpoint titration method utilized an ELISA format to detect anti-KLH antibodies in monkey serum. The samples were incubated with KLH, which was immobilized on an ELISA plate. After incubation, the plates were washed, and the bound antibodies were detected with Goat anti-Monkey IgG-HRP or IgM-HRP and then visualized with tetramethylbenzidine (TMB).

[0476] For all experiments utilizing animals currently acceptable practices of good animal husbandry were followed e.g., Guide for the Care and Use of Laboratory Animals; National Academy Press, 2011. Huntingdon Life Sciences, East Millstone, New Jersey is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Animals were monitored by the technical staff for any conditions requiring possible veterinary care and treated as necessary.

6.2 CD40L specific Tn3 Scaffolds Functionally Neutralize CD40L: CD40 Interactions.

[0477] CD40L-expressing T cells engage CD40-expressing B cells resulting in the activation of NFκB signaling pathway (Zangani, 2009). Thus, an NFκB-luciferase reporter cell line was used to determine if the anti-CD40L-Tn3 molecules could inhibit signaling downstream of CD40 engagement. HEK293 cells expressing human CD40L and the reporter were stimulated with either human or murine MegaCD40L at EC_{90} (Effective Concentration that results in 90% inhibition; *i.e.*, 1.5 μg/ml for human MegaCD40L, and 3 μg/ml for murine MegaCD40L).

[0478] The human-specific 342 molecule inhibited human CD40L-induced NFκB activity with an IC_{50} of 1.5nM (FIG. 13). The murine-specific M31 molecule, neutralized murine CD40L-induced NFκB activity with an IC_{50} = 1.6 nM (FIG. 1B). The positive control anti-CD40L monoclonal antibodies 5c8 (anti human CD40L) and MR1 (anti murine CD40L) both performed about 10-fold better than the monomeric Tn3 scaffolds with IC_{50}'s

of 0.200 nM +/- SD (lowest threshold of the assay). This could be in part due to the bivalent nature of the monoclonal antibodies contributing to the avidity of the interaction with their respective CD40L's.

6.3 Dimeric CD40L specific Tn3 Scaffolds exhibit improved binding.

[0479] Experimental data indicates that the binding of a CD40L-specific Tn3 bivalent scaffold was improved over that of a CD40L-specific Tn3 monomer scaffold. The binding of the CD40L-specific Tn3 bivalent scaffold to CD40L improved the action on the target, in some cases by approximately 3 logs over that of a CD40L specific Tn3 monomer scaffold in vitro, as shown in FIG. 2C and FIG. 2D (murine), and FIG. 8A and FIG. 8B (human).

[0480] FIG. 2C shows the competitive inhibition of murine CD40L binding to murine CD40 receptor immobilized on a biosensor chip by murine CD40L-specific monovalent (M13) or bivalent tandem scaffolds. M13 monomers were linked with varying length peptide linkers comprising one (1GS), three (3GS), five (5GS) or seven (7GS) "GGGGS" repeats. The IC_{50} of the M13-1GS-M13 scaffold was 29 nM, whereas the IC_{50} of the monomer M13 scaffold was 71 nM. The IC_{50} of divalent M13 scaffolds with longer linkers were dramatically lower (5-6 nM).

[0481] FIG. 2D shows the inhibitory effect of murine CD40L-specific monovalent (M13) or bivalent tandem scaffolds on murine CD40L-induced CD86 expression on B cells. The bivalent scaffolds were approximately 3 logs more potent than the monovalent scaffolds.

[0482] FIGS. 8A and 8B show that human CD40L-specific Tn3 scaffolds 309 and 311 displayed enhanced potency in a bivalent tandem format. The bivalent 311 scaffolds (FIG. 8A) and the bivalent 309 scaffolds (FIG. 8B) showed approximately a 7-fold and a 500-1000-fold improvement, respectively, in inhibition of human CD40L-induced expression on CD19 positive human PBMCs stimulated with Jurkat D1.1 cells. The bivalent 309 scaffolds were comparable in potency to Biogen's 5c8 anti human CD40L monoclonal antibody.

[0483] Solubility, stability and ease of purification was not disrupted with the addition of varying length peptide linkers comprising one (1GS), three (3GS), five (5GS) or seven (7GS) "GGGGS" repeats (see FIG. 2B).

6.4 CD40L Specific Tn3 Scaffolds Binding and Function.

[0484] In addition to the biochemical binding described above, it was important to verify that these novel Tn3 scaffolds were able to bind endogenous CD40L expressed on primary T cells following activation. T cells were isolated from multiple donors and activated as described. After 24 hours, CD40L expression was upregulated as determined by staining with 5c8 (human-specific) monoclonal antibody and MR1 (murine-specific) monoclonal antibody (data not shown). The CD40L specific Tn3 scaffold molecules were able to detect comparable levels of CD40L expression as the monoclonal antibodies confirming that these molecules can bind native protein.

[0485] One of the functional consequences of the CD40L:CD40 interaction is the up regulation of co-stimulatory molecules on B cells (Yellin e*t al.*, J. Exp. Med. 6:1857-1864, 1995). The CD40L-directed Tn3 molecules were tested for their ability to prevent this. Cell lines endogenously expressing human or murine CD40L (D1.1 Jurkat subclone or D10.G4 respectively) were used to stimulate peripheral blood mononuclear cells (PBMC). Once stimulated, the activation of B cells was assessed by measuring the percentage of CD86 up regulation by CD19+ B cells via flow cytometry. In this assay, the positive control monoclonal antibodies were able to inhibit the CD19+ percentage of cells with CD86 expression with IC_{50}s of 0.170 nM (5c8) and 0.230 nM (MR1). The human-specific optimized Tn3, 342 was able to antagonize CD86 up regulation with IC_{50} values= 0.700nM (n = 5 donors) (see FIG. 10B and TABLE 7).

[0486] The murine-specific optimized Tn3 M31 had and IC_{50} of 1.5nM. These similar results were observed when Mega-CD40L recombinant protein was used to stimulate PBMCs. The exprimetal data demonstrated that both molecules, whether murine or human specific, cannot only inhibit the main signaling pathway within a cell (NFκB), but also one of its most important functional roles: T-B cell interactions. This inhibitory action can counteract CD40L's contribution in many auto immune diseases and conditions.

6.5 Anti-CD40L Tn3's inhibit B cell proliferation and plasma cell differentiation following T/B co-culture.

[0487] Interactions of CD40L on T cells with CD40-expressing B cells are a fundamental aspect of T cell help which facilitates the development of adaptive immune responses (Banchereau, 1994; Oxenius, 1996, van Kooten & Banchereau, 1997). To model this, the anti-hCD40L Tn3-HSA fusions of 340, 342 and 342-342 dimer were evaluated in

primary cell co-cultures of T cells and B cells where anti-CD3 stimulated, mitomycin C treated human CD4+ T cells were cultured with purified human B cells. The ability of the B cells to proliferate at day four to differentiate into plasma cells (PC) by day seven and switch their antibody class of production were measured (PC and antibody data not shown) (FIG. 15) (Ettinger, 2007). The CD40L-specific Tn3 scaffold 342-342-HSA was able to reduce T cell induced proliferation by at least 50% as compared to the cell proliferation of B-cells in the absence of scaffolds, or in the presence of a non-specific control scaffold. Proliferation is a pre-cursor, signal one, to plasma cell differentiation, upon CD40L:CD40 ligation. Inhibition of plasma cell differentiation and antibody class switching (data not shown) were also observed.

6.6 *In vivo* disruption of the CD40:CD40L axis.

[0488] The central role of CD40L:CD40R interactions in T-dependent immune responses have been well characterized (Noelle, 1992; Renshaw, 1994, Wykes, 2003). The murine CD40L-specific Tn3 scaffold M31 (M31-MSA and M31-M31-MSA) was used to evaluate the effects of these novel molecules in a T-dependent immunization model by immunizing mice (intravenously) at day zero with Sheep Red Blood Cells (SRBC).

[0489] On days 9-13, mice were injected intraperitoneally daily with the indicated dose of inhibitor and at day 14 splenic and lymph node GC B cells were quantitated. Daily dosing was required given the short $T_{1/2}$ of this molecule in vivo, 31 hours (FIG. 4). It is well established that CD40L controls humoral responses such as the generation of germinal centers in anatomical sites such as the spleen and lymph nodes from previous findings (Jacob, 1991). Here, the disruption of the CD40L:CD40 axis contribution to that formation was observed in a dose dependent manner with M31-MSA versus naïve or our non-specific control, D1-MSA, as shown by the percentage of GC B cells.

[0490] Even at 10 mg/kg M31-MSA was able to abolish the percent of GC B cells (FIG. 5B) as well as the MR1 monoclonal antibody (FIG. 5A). Other sub-populations of cells appeared normal including specific T-cell populations assuring that the results observed were not due to T cell depletion (FIG. 5C, FIG. 5D, FIG. 5E). In addition, the results from the anti-SRBC Ig ELISA data mirrored those of the germinal center B cell data (FIG. 5F). Taken together, these data indicated that the murine-specific Tn3 scaffold M31-MSA can abrogate reactions driven via CD40 signaling.

[0491] Similarly, the human CD40L-specific Tn3 scaffold 342 (342-HSA and 342-342-HSA) was used to evaluate the effects of these novel molecules in KLH-specific T cell

dependent antibody response (TDAR) model in Cynomolgus Monkeys. Here, the disruption of the CD40L:CD40 axis results in suppression of antibody generation to the KLH antigen in a dose dependent manner. As shown in Figures 5G and 5H the 342-bivalent construct suppressed the levels of IgM and IgG antibodies at 0.5 mg/kg (mpk) and nearly complete suppression was seen at 5 mg/kg. The 342-monomer construct also suppressed the levels of IgM and IgG but at higher concentrations with nearly complete suppression seen at 40 mg/kg. These data indicated that the human-specific Tn3 scaffold constructs 342-HSA and 342-342-HSA can both abrogate reactions driven via CD40 signaling.

6.7 Human CD40L-specific Tn3 scaffolds do not induce platelet aggregation.

[0492] Human clinical trials with anti-CD40L monoclonal antibodies were halted when thromboembolisms occurred in several patients (Davidson *et al*. *Arth Rheu*, 43:S271). Subsequent pre-clinical analyses suggested this to be an on-target class effect of anti-CD40L monoclonal antibodies. Thus it was important to test the human CD40L-specific Tn3 scaffolds in platelet aggregation assays.

[0493] When a ratio of three molecules of physiological CD40L to one molecule of the anti-CD40L monoclonal antibody was used, pro-aggregator effects were observed in citrated Platelet Rich Plasma (PRP), washed platelets, and whole blood (FIG. 16A). These effects were mediated by monoclonal antibody Fc domain dependent interactions subsequent to CD40L binding (data not shown). In the absence of the Fc domain fusion, no aggregation was observed. No aggregation was observed in multiple donors with any of the human CD40L specific Tn3 scaffolds either as dimers or as HSA fusion proteins (FIG. 16B and FIG. 16C).

[0494] Deleterious side-effects as observed in the clinical trials were observed by creating a soluble CD40L/anti-CD40L monoclonal antibody immunocomplex in the presence of platelets (FIG. 16A and 5C8 trace on FIG. 16C). Another example of this can be seen in the histology of the transgenic human FcγRIIa murine study (Francis *et al.*, 2010). Upon administration of soluble CD40L/monoclonal antibody immune complexes, an abundance of thrombi was seen within the lung tissue within minutes after administration. However, when duplicated with anti-CD40L Tn3 scaffolds, normal histology was present in the lung in accordance with the control samples (data not shown).

Example 7

Fibronectin type III domains engineered to bind CD40L: cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of two complexes.

[0495] Recombinant human soluble CD40L was co-crystallized with two CD40L-specific Tn3 monomer scaffolds, 309 and 311K4E-12, both isolated as CD40L binders from phage display libraries. The crystals diffracted to 3.1 and 2.9 Å respectively. In addition, recombinant human soluble CD40L was co-crystalized with the optimized Tn3 monomer 342 alone and with both the 342 monomer and the 311K4E_12 monomer. The crystals for these structures diffracted to 2.8 and 1.9 Å respectively. The corresponding crystal structures help to understand the interaction between Tn3 scaffolds and CD40L and can be used to design higher affinity CD40L binders and tandem constructs binding multiple epitopes.

7.1 Expression and purification of Tn3 molecules and human soluble CD40L

[0496] To produce tagless Tn3 molecules for crystallization, the proteins were expressed in *E. coli* using an in-house IPTG-inducible vector designed to secrete recombinantly expressed proteins into the periplasmic space. This vector has a Ptac promoter, OppA signal peptide mutant L25/M (MTNITKRSLVAAGVLAALMAGNVAMA) (SEQ ID NO: 210), a C-terminal 8xHis-tag in addition to a thrombin cleavage site. The Tn3 sequences were subcloned between signal peptide and thrombin cleavage site.

[0497] Expressed secreted His-tagged proteins were purified using Ni-NTA resin according to the manufacturer's instructions (Qiagen, Valencia, California, USA,) and then cleaved by thrombin followed by Ni-NTA affinity purification again to remove the uncut intact protein and the cut His-tagged fragment. This purification step was followed by ion-exchange step using HiTrap Q columns (GE Healthcare, Piscataway, New Jersey, USA) performed on Äkta Purifier (GE Healthcare, Piscataway, New Jersey, USA). The purified tagless Tn3 proteins show greater than 95% purity and homogeneity based on SDS-PAGE and SEC results.

[0498] Human soluble CD40L (113-261, UNIPROT: P29965) gene was synthesized by GeneArt with an N-terminal 6xHis-tag and was cloned into an in house mammalian expression vector under the control of the cytomegalovirus major immediate early (hCMVie) promoter (Boshart *et al.*, Cell 41: 521–530,1985). The CD40L gene was cloned in frame with a CD33 signal peptide. The EBNA and Ori P genes in the vector were used to increase

protein expression. The CD40L gene also incorporated a SV40 poly-A sequence to allow proper processing of its mRNA 3'-end. The construct was transiently transfected into 293F suspension cells (human embryonic kidney cells [HEK] grown in 293 Freestyle Medium and using 293 Fectin, Invitrogen, Carlsbad, California, USA). Cells were grown using standard protocol and media was harvested after 4 and 8 days. The soluble CD40L protein was then purified using Ni-NTA resin followed by an ion-exchange step using Hi-Trap SP FF column (GE Healthcare, Piscataway, New Jersey, USA) and dialysis against 50mM Tris pH 7.5, 50mM NaCl.

[0499] To prepare complexes the Tn3 molecule, either 309 or 311K4E_12 or 342, was mixed with CD40L in a 1.1:1 ratio, concentrated using Vivaspin concentrators (30,000 Da cut-off; GE Healthcare, Piscataway, New Jersey, USA) to approximately 10 mg/ml and subjected to size-exclusion chromatography (SEC) using Superdex 200 10/300 GL column (GE Healthcare, Piscataway, New Jersey, USA) pre-equilibrated with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.02% NaN3 (FIG. 19, panel A). After the separation step the complex was concentrated to 18 mg/ml and subjected to crystallization. The 342-311K4E_12-CD40L complex was prepared essentially as described above using a 1.1:1.1:1 ratio of the three components.

7.2 Crystallization screening and optimization

[0500] Sitting drop crystallization experiments were set up in 96-well Intelli-plates (Art Robbins Instruments, Sunnyvale, California, USA) using a Phoenix crystallization robot (Art Robbins Instruments, Sunnyvale, California, USA) by mixing 300 nL volumes of well solution and protein complex solution in the drop compartment and letting it equilibrate against 50 μL of well solution. Commercial crystallization screens from Hampton Research (Aliso Viejo, California, USA), Emerald BioSystems (Bainbridge Island, Washington, USA) and Molecular Dimensions (Apopka, Florida, USA) were used.

[0501] Crystallization of the 309-CD40L, 342-CD40L and 342-311K4E_12-CD40L complexes each required an optimization step which included additional screening using Additive Screen HT (Hampton Research, Aliso Viejo, California, USA). In the optimization step the well solution of the 96-well plate was filled with 80% of successful solution from the initial screening and 20 % of respective additive. The drop was made of 300 nL of protein solution and 300 nL of the new well solution after thorough mixing of the latter. The diffraction quality crystals were harvested directly from 96-well plate. For cryo-preservation

the crystal was transferred into three consecutive solutions of mother liquor with increasing glycerol concentrations.

[0502] The diffraction quality 311-CD40L crystals grew at the initial screen out of solution that did not require addition of cryo agent.

7.3 X-Ray diffraction and data collection

[0503] X-ray diffraction patterns for the 309-CD40L complex were collected from single crystal at the Beamline 5.0.3 of the Advanced Light Source in Lawrence Berkeley National Laboratory (University of California, Berkeley) equipped with ADSC Q315R CCD X-Ray detector (Area Detector Systems Corporation, Poway, California, USA). 360 consecutive images with oscillation range of 0.5º were collected at crystal-to-detector distance of 300 mm and an exposure time of 0.8 seconds.

[0504] X-ray diffraction patterns for the 311K4E_12-CD40L, 342-CD40L and 342-311K4E_12-CD40L complexes were collected from single crystals at the Beamline 31-ID-D of the Advanced Photon Source in Argonne National Laboratory (University of Chicago, Chicago, IL) equipped with a Rayonix 225 HE detector (Rayonix LLC, Evanston, Illinois, USA). 180 consecutive images with oscillation range of 1º were collected at crystal-to-detector distance of 300 mm and an exposure time of 0.8 seconds.

[0505] Reduction and scaling for all data sets were performed using HKL2000 suite (Otwinowski & Minor, Methods in Enzymology, 276:307-326. 1997).

7.4 Results and discussion

[0506] The most reproducible crystallization condition of the 309-CD40L complex appeared to be B5 (0.2 M $NaNO_3$, 20% PEG3350) in Peg/Ion Screen (Hampton Research). Further optimization with Additive Screen yielded diffraction quality crystal from the A1 condition (0.1M BaCl2·2H2O). The crystal shown in FIG. 19, panel B was harvested from a 96-well plate, and cooled in liquid Nitrogen after transfer to the mother liquor solution supplemented with 20% Glycerol.

[0507] Space group symmetry: The crystal belonged to orthorhombic space group P212121 with cell parameters a=85.69 Å, b=90.64 Å, c=95.56 Å and diffracted to 3.1 Å. The asymmetric unit is expected to contain a trimer of CD40L and three 309 molecules with VM value about 2.3 Å3/Da.

[0508] For the 311K4E_12-CD40L crystallization the Cryo I & II screen (Emerald BioStructures) yielded number of conditions which required neither optimization nor cryo

preservation. A single crystal (FIG. 19, panel C) from condition F7 (40% PEG 600, 0.1M CH3COONa, 0.2M MgCl2) was used for data collection.

[0509] Space group symmetry: The crystal belonged to cubic space group P213 with cell parameter 97.62 Å and diffracted to 2.6 Å. The asymmetric unit contains one CD40L and one 311K4E_12 molecule with VM value about 2.9 Å3/Da.

[0510] 342-CD40L space group symmetry: The crystal belonged to space group P321 with cell parameters a=93.53Å, b=93.53Å, c=66.69Å, resolution 2.8 Å. The asymmetric unit contains one CD40L monomer and one 342 monomer.

[0511] 342-311K4E_12-CD40L space group symmetry: The crystal belonged to space group P21 with cell parameters a=80.32Å, b=143.48Å, c=111.27Å, β=98.22º, resolution 1.9Å. The asymmetric unit contains two CD40 trimers, six 342 monomers, and six 311K4E-12 monomers.

[0512] Data statistics for all the structures are shown in TABLE 10.

TABLE 10. X-Ray data collection statistics.

	309-CD40L	311K4E-12
Wavelength, Å	0.9793	0.9793
Resolution, Å	50.0-3.05 (3.16-3.05) [a]	50.0-2.94
Space group	$P2_12_12_1$	$P2_13$
Cell parameters, Å	a=85.69, b=90.64, c=95.56	a=97.62
Total reflections	94,024	128,140
Unique reflections	14,555	6720
Average redundancy	6.5 (6.4) [a]	19.2 (19.7)
Completeness, %	100.0 (100.0) [a]	99.4 (100.0)
R_{sym}	0.097 (0.443) [a]	0.114 (0.785)
Mean I/σ (I)	17.2 (4.6) [a]	20.1 (2.4)

[a] Values in parentheses correspond to the highest resolution shell

	342-CD40L	342-311K4E_12-CD40L
Wavelength, Å	0.9793	0.9793
Resolution, Å	50.0-2.8 (2.83-2.82) [a]	144.5-1.9 (1.96-1.95) [a]
Space group	P321	$P2_1$
Cell parameters, Å	a=93.53, b=93.53, c=66.69	a=80.32, b=143.48, c=111.27, β=98.22º
Total reflections	66,038 (549) [a]	733,814 (1806) [a]
Unique reflections	8,406 (88) [a]	179,232 (1806) [a]
Average redundancy	7.9 (6.2) [a]	4.1 (4.2) [a]
Completeness, %	99.9 (100.0) [a]	99.7 (99.6) [a]
R_{sym}	0.19 (0.79) [a]	0.06 (0.57) [a]
Mean I/σ (I)	8.1 (1.4) [a]	14.5 (3.0) [a]

[a] Values in parentheses correspond to the highest resolution shell

[0513] CD40L formed a trimer (polypeptides A, B, and C in FIG. 17A). Each 309 Tn3 scaffold (polypeptides D, E, and F in FIG. 17A) made contact with two CD40L polypeptides. The crystal structure revealed that there are six specific contacts between each 309 scaffold and the first and second CD40L polypeptides. Aspartic acid 17 in the BC makes contact with threonine 251 in the first CD40L. Glutamic acid 18 in the BC loop makes contact with arginine 203 in the first CD40L and with isoleucine 204 in the second CD40L. Serine 47 in the DE loop makes contact with histidine 249 in the first CD40L. Tryptophan 49 in the DE loop makes contact with valine 247 in the first CD40L. Aspartic acid 70 in the FG loop makes contact with serine 185 in the second CD40L (see FIG. 17A). CD40L amino acid residues contacting the 311 scaffold are also shown in FIG. 18A.

[0514] An in the case of 309, each 311K4E_12 monomer scaffold (polypeptides A, B, and C in FIG. 17B) makes contact with two CD40L polypeptides. The crystal structure revealed that there are 19 specific contacts between each 311K4E_12 scaffold and the first and second CD40L polypeptides. Asparagine 17 in the BC loop makes contacts with tyrosine 146 and glutamic acid 142 in the first CD40L. Arginine 18 in the BC loop makes contact with glutamic acid 142, tyrosine 146, and methionine 148 in the first CD40L. Serine 19 in the BC loop makes contact with glutamic acid 142 and leucine 155 in the first CD40L. Serine 22 in the BC loop makes contact with asparagine 151 in the first CD40L. Histidine 15 in the BC loop makes contact with tyrosine 146 in the first CD40L. Histidine 51 in the DE loop makes contact with tyrosine 146 in the first CD40L and with glutamic acid 230 in the second CD40L. Valine 50 in the DE loop makes contact with glutamic acid 230 in the second CD40L. The N-terminal region of the 311K4E_12 monomer scaffold is connected to the second CD40L. Arginine 200 in the second CD40L makes contact with threonine 7, aspartic acid 8, and threonine 10 in the N-terminal region of 311K4E_12. Arginine 203 in the second CD40L makes contact with glutamic acid 4 and aspartic acid 5. CD40L amino acid residues contacting the 309 scaffold are also shown in FIG. 18B.

[0515] The crystal structures of CD40L in complexes with 309 and 311K4E_12 showed that the 311K4E_12 and 309 monomer scaffolds bind to different epitopes located in different parts of the CD40L trimer complex (FIG. 17C). The structures showed that both scaffolds bind in the same groove that would interact with the CD40 receptor.

[0516] The crystal structure of a 342 with CD40L is provided in Figure 17D and shows that while 342 binds on the same part of CD40L specific changes in the contact

residues are seen as compared to the parental 309 clone. Specifically, in 342 aspartate 18 in the BC loop makes contact with threonine 251 of CD40L and histidine 47 of the DE loop makes contact with histidine 249 of CD40L, histidine 48 of the DE loop makes contact with histidine 249, serine 245 and serine 248 of CD40L, and histidine 50 of the DE loop makes contact with valine 247 of CD40L.

[0517] The crystal structure of a 342 and 311K4E_12 with CD40L demonstrates that both scaffolds can bind simultaneously to their respective epitopes which are located in different parts of the CD40L trimer complex (FIG 17E). The contacts for each of the separate scaffolds (as described above) are maintained.

[0518] The examples shown above illustrate various aspects of the invention and practice of the methods of the invention. These examples are not intended to provide an exhaustive description of the many different embodiments of the invention. Thus, although the invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, those of ordinary skill in the art will realize readily that many changes and modifications can be made without departing from the spirit or scope of the appended claims.

SEQUENCES

SEQ ID NO: 1
CD40L sp|P29965|CD40L_HUMAN – Membrane form
Cytoplasmic domain = 1-20
Signal anchor type II membrane protein region = 21-46
Soluble form = 113-261
MIETYNQTSPRSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRLDKIEDERNLH
EDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFEMQKGDQNP
QIAAHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIYAQVTFCSN
REASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVN
VTDPSQVSHGTGFTSFGLLKL

SEQ ID NO: 2
CD40L– Soluble form, corresponds also to the co-crystallized construct
MQKGDQNPQIAAHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIY
AQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQ
PGASVFVNVTDPSQVSHGTGFTSFGLLKL

SEQ ID No: 3
Tn3 (with unmodified loops)
IEVKDVTDTTALITWFKPLAEIDGCELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDTE

YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 4
3rd FnIII of tenascin C, AB loop (Tn3)
KDVTDTT

SEQ ID NO: 5
3rd FnIII of tenascin C, BC loop (Tn3)
FKPLAEIDG

SEQ ID NO: 6
3rd FnIII of tenascin C, CD loop (Tn3)
KDVPGDR

SEQ ID NO: 7
3rd FnIII of tenascin C, DE loop (Tn3)
TEDENQ

SEQ ID NO: 8
3rd FnIII of tenascin C, EF loop (Tn3)
GNLKPDTE

SEQ ID NO: 9
3rd FnIII of tenascin C, FG loop (Tn3); also in 309FGwt, 340,341, 342, 343, 344, 345, 346, 347, 348, and 349 clones
RRGDMSSNPA

SEQ ID NO: 10
3rd FnIII of tenascin C, beta strand A (Tn3)
RLDAPSQIEV

SEQ ID NO: 11
3rd FnIII of tenascin C, beta strand A (Tn3) N-terminal truncation
IEV

SEQ ID NO: 12
3rd FnIII of tenascin C, beta strand B (Tn3)
ALITW

SEQ ID NO: 13
3rd FnIII of tenascin C, beta strand C (Tn3 variant)
CELAYGI

SEQ ID NO: 14
3rd FnIII of tenascin C, beta strand C (Tn3)
CELTYGI

SEQ ID NO: 15
3rd FnIII of tenascin C, beta strand D (Tn3)
TTIDL

SEQ ID NO: 16
3rd FnIII of tenascin C, beta strand E (Tn3)
YSI

SEQ ID NO: 17
3rd FnIII of tenascin C, beta strand F (Tn3)
YEVSLIC

SEQ ID NO: 18
3rd FnIII of tenascin C, beta strand G (Tn3)

KETFTT

SEQ ID NO: 19
Clone 309 - Parental clone isolated from naiive Tn3 library
AIEVKDVTDTTALITWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDT
EYEVSLICYTDQEAGNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 20
Clone 309 - Parental clone isolated from naiive Tn3 library (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDTE
YEVSLICYTDQEAGNPAKETFTT

SEQ ID NO: 21
Clone 309FGwt - Parental clone with "humanized" FG loop
AIEVKDVTDTTALITWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 22
Clone 309FGwt - Parental clone with "humanized" FG loop (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 23
Clone 340 - Affinity Mature variant
AIEVKDVTDTTALITWSDDFDNYEWCELTYGIKDVPGDRTTIDLWYHMAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 24
Clone 340 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDDFDNYEWCELTYGIKDVPGDRTTIDLWYHMAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 25
Clone 341 - Affinity Mature variant
AIEVKDVTDTTALITWSDDFADYVWCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 26
Clone 341 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDDFADYVWCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 27
Clone 342 - Affinity Mature variant (w/WT FG loop)
AIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 28
Clone 342 - Affinity Mature variant (w/WT FG loop; w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 29
Clone 343 - Affinity Mature variant
AIEVKDVTDTTALITWLDDWGSYHVCELTYGIKDVPGDRTTIDLWYHQAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 30
Clone 343 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWLDDWGSYHVCELTYGIKDVPGDRTTIDLWYHQAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 31
Clone 344 - Affinity Mature variant
AIEVKDVTDTTALITWSDEVGDYVVCELTYGIKDVPGDRTTIDLWYHMAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 32
Clone 344 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDEVGDYVVCELTYGIKDVPGDRTTIDLWYHMAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 33
Clone 345 - Affinity Mature variant
AIEVKDVTDTTALITWSDDFAEYVGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 34
Clone 345 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDDFAEYVGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 35
Clone 346 - Affinity Mature variant
AIEVKDVTDTTALITWSDDFEEYVVCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 36
Clone 346 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDDFEEYVVCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 37
Clone 347 - Affinity Mature variant
AIEVKDVTDTTALITWSDEVGQYVGCELTYGIKDVPGDRTTIDLWYHMAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 38
Clone 347 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDEVGQYVGCELTYGIKDVPGDRTTIDLWYHMAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 39
Clone 348 - Affinity Mature variant
AIEVKDVTDTTALITWSDDIGLYVWCELTYGIKDVPGDRTTIDLWFHQAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 40
Clone 348 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDDIGLYVWCELTYGIKDVPGDRTTIDLWFHQAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 41
Clone 349 - Affinity Mature variant
AIEVKDVTDTTALITWSDEHAEFIGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDT
EYEVSLICRRGDMSSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 42
Clone 349 - Affinity Mature variant (w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWSDEHAEFIGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDTE
YEVSLICRRGDMSSNPAKETFTT

SEQ ID NO: 43
Clone 311 - Parental clone isolated from naiive Tn3 library
AIEVKDVTDTTALITWTNRSSYYNLHGCELTYGIKDVPGDRTTIDLSSPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 44
Clone 311 - Parental clone isolated from naiive Tn3 library(w/o N-term A, and C-term linker and His8 tag)
IEVKDVTDTTALITWTNRSSYYNLHGCELTYGIKDVPGDRTTIDLSSPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 45
Clone 311K4E - Variant from first round of affinity maturation
AIEVEDVTDTTALITWTNRSSYYNLHGCELTYGIKDVPGDRTTIDLSSPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 46
Clone 311K4E - Variant from first round of affinity maturation(w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYYNLHGCELTYGIKDVPGDRTTIDLSSPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 47
Clone 311K4E_1 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWINRSYYADLHGCELTYGIKDVPGDRTTIDLDQIYVHYSIGNLKP
DTKYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 48
Clone 311K4E_1 - Clone Variant from second round of affinity maturation(w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWINRSYYADLHGCELTYGIKDVPGDRTTIDLDQIYVHYSIGNLKPD
TKYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 49
Clone 311K4E_2 – Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYSHLDGCELTYGIKDVPGDRTTIDLSAAIYVHYSIGNLK
PDTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 50
Clone 311K4E_2 – Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYSHLDGCELTYGIKDVPGDRTTIDLSAAIYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 51
Clone 311K4E_3 – Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWINRSSYHNFPHCELAYGIKDVPGDRTTIDLNSPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 52
Clone 311K4E_3 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWINRSSYHNFPHCELAYGIKDVPGDRTTIDLNSPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 53
Clone 311K4E_4 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYSNHLGCELAYGIKDVPGDRTTIDLNNIYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 54
Clone 311K4E_4 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYSNHLGCELAYGIKDVPGDRTTIDLNNIYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 55
Clone 311K4E_5 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYSNFHGCELAYGIKDVPGDRTTIDLNSPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 56
Clone 311K4E_5 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYSNFHGCELAYGIKDVPGDRTTIDLNSPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 57
Clone 311K4E_7 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSFYSNLHGCELTYGIKDVPGDRTTIDLNQPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 58
Clone 311K4E_7 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSFYSNLHGCELTYGIKDVPGDRTTIDLNQPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 59
Clone 311K4E_8 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYAYLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 60
Clone 311K4E_8 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYAYLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 61
Clone 311K4E_9 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWINRSSYANLHGCELTYGIKDVPGDRTTIDLSSPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 62
Clone 311K4E_9 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWINRSSYANLHGCELTYGIKDVPGDRTTIDLSSPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 63
Clone 311K4E_10 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYANYHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 64
Clone 311K4E_10 - Clone Variant from second round of affinity maturation
(w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYANYHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 65
Clone 311K4E_11 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYANLPGCELTYGIKDVPGDRTTIDLNSPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 66
Clone 311K4E_11 - Clone Variant from second round of affinity maturation
(w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYANLPGCELTYGIKDVPGDRTTIDLNSPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 67
Clone 311K4E_12 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKP
DTEYEVSLICLTTDGTYNNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 68
Clone 311K4E_12 - Clone Variant from second round of affinity maturation
(w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKPD
TEYEVSLICLTTDGTYNNPAKETFTT

SEQ ID NO: 69
Clone 311K4E_13 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWINRSSYANLHGCELTYGIKDVPGDRTTIDLNSPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 70
Clone 311K4E_13 - Clone Variant from second round of affinity maturation
(w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWINRSSYANLHGCELTYGIKDVPGDRTTIDLNSPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 71
Clone 311K4E_14 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTARSAYSHHHYCELTYGIKDVPGDRTTIDLRQPYVHYSIGNLKP
DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 72
Clone 311K4E_14 - Clone Variant from second round of affinity maturation
(w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTARSAYSHHHYCELTYGIKDVPGDRTTIDLRQPYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 73
Clone 311K4E_15 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYANYHHCELTYGIKDVPGDRTTIDLELYVHYSIGNLKPD
TEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 74

Clone 311K4E_15 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYANYHHCELTYGIKDVPGDRTTIDLELYVHYSIGNLKPDT EYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 75
Clone 311K4E_16 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYSDLPGCELTYGIKDVPGDRTTIDLSSPYVHYSIGNLKP DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 76
Clone 311K4E_16 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYSDLPGCELTYGIKDVPGDRTTIDLSSPYVHYSIGNLKPD TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 77
Clone 311K4E_19 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTHRSAYSNHSFCELTYGIKDVPGDRTTIDLNTPYVHYSIGNLKP DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 78
Clone 311K4E_19 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTHRSAYSNHSFCELTYGIKDVPGDRTTIDLNTPYVHYSIGNLKPD TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 79
Clone 311K4E_20 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSLYANFHGCELTYGIKDVPGDRTTIDLEQVYVHYSIGNLKP DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 80
Clone 311K4E_20 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSLYANFHGCELTYGIKDVPGDRTTIDLEQVYVHYSIGNLKPD TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 81
Clone 311K4E_21 - Clone Variant from second round of affinity maturation
AIEVEDVTDTTALITWTNRSSYSNLPGCELTYGIKDVPGDRTTIDLNQVYVHYSIGNLKP DTEYEVSLICLTTDGTYSNPAKETFTTGGGTLGHHHHHHHH

SEQ ID NO: 82
Clone 311K4E_21 - Clone Variant from second round of affinity maturation (w/o N-term A, and C-term linker and His8 tag)
IEVEDVTDTTALITWTNRSSYSNLPGCELTYGIKDVPGDRTTIDLNQVYVHYSIGNLKPD TEYEVSLICLTTDGTYSNPAKETFTT

SEQ ID NO: 83
Clone 309 and 309FGwt - BC Loop
SDEFGHYDG

SEQ ID NO: 84
Clone 340 - BC Loop
SDDFDNYEW

SEQ ID NO: 85
Clone 341 - BC Loop
SDDFADYVW

SEQ ID NO: 86
Clone 342 - BC Loop
SDDFGEYVW

SEQ ID NO: 87
Clone 343 - BC Loop
LDDWGSYHV

SEQ ID NO: 88
Clone 344 - BC Loop
SDEVGDYVV

SEQ ID NO: 89
Clone 345 - BC Loop
SDDFAEYVG

SEQ ID NO: 90
Clone 346 - BC Loop
SDDFEEYVV

SEQ ID NO: 91
Clone 347 – BC Loop
SDEVGQYVG

SEQ ID NO: 92
Clone 348 – BC Loop
SDDIGLYVW

SEQ ID NO: 93
Clone 349 - BC Loop
SDEHAEFIG

SEQ ID NO: 94
Clone 309, 309FGwt, 341, 345, 346, 349 – DE Loop
WWHSAW

SEQ ID NO: 95
Clone 340, 344, 347 – DE Loop
WYHMAW

SEQ ID NO: 96
Clone 342 - DE Loop
WYHHAH

SEQ ID NO: 97
Clone 343 – DE Loop
WYHQAW

SEQ ID NO: 98
Clone 348 – DE Loop
WFHQAW

SEQ ID NO: 99
Clone 309 – FG Loop
YTDQEAGNPA

SEQ ID NO: 100
Clone 311, 311K4E - BC Loop
TNRSSYYNLHG

SEQ ID NO: 101

Clone 311K4E_1 - BC Loop
INRSYYADLHG

SEQ ID NO: 102
Clone 311K4E_2 - BC Loop
TNRSSYSHLDG

SEQ ID NO: 103
Clone 311K4E_3 - BC Loop
INRSSYHNFPH

SEQ ID NO: 104
Clone 311K4E_4 - BC Loop
TNRSSYSNHLG

SEQ ID NO: 105
Clone 311K4E_5 - BC Loop
TNRSSYSNFHG

SEQ ID NO: 106
Clone 311K4E_7 - BC Loop
TNRSFYSNLHG

SEQ ID NO: 107
Clone 311K4E_8 - BC Loop
TNRSSYAYLHG

SEQ ID NO: 108
Clone 311K4E_9, 311K4E_13 - BC Loop
INRSSYANLHG

SEQ ID NO: 109
Clone 311K4E_10 - BC Loop
TNRSSYANYHG

SEQ ID NO: 110
Clone 311K4E_11 - BC Loop
TNRSSYANLPG

SEQ ID NO: 111
Clone 311K4E_12 - BC Loop
TNRSSYSNLHG

SEQ ID NO: 112
Clone 311K4E_14 - BC Loop
TARSAYSHHHY

SEQ ID NO: 113
Clone 311K4E_15 - BC Loop
TNRSSYANYHH

SEQ ID NO: 114
Clone 311K4E_16 - BC Loop
TNRSSYSDLPG

SEQ ID NO: 115
Clone 311K4E_19 - BC Loop
THRSAYSNHSF

SEQ ID NO: 116
Clone 311K4E_20 - BC Loop

TNRSLYANFHG

SEQ ID NO: 117
Clone 311K4E_21 - BC Loop
TNRSSYSNLPG

SEQ ID NO: 118
Clone 311, 311K4E, 311K4E_9, 311K4E_16 - DE Loop
SSPYVH

SEQ ID NO: 119
Clone 311K4E_1 - DE Loop
DQIYVH

SEQ ID NO: 120
Clone 311K4E_2 - DE Loop
SAAIYVH

SEQ ID NO: 121
Clone 311K4E_3, 311K4E_5, 311K4E_11, 311K4E_13 - DE Loop
NSPYVH

SEQ ID NO: 122
Clone 311K4E_4 - DE Loop
NNIYVH

SEQ ID NO: 123
Clone 311K4E_7, 311K4E_8, 311K4E_10, 311K4E_12 - DE Loop
NQPYVH

SEQ ID NO: 124
Clone 311K4E_14 - DE Loop
RQPYVH

SEQ ID NO: 125
Clone 311K4E_15 - DE Loop
ELYVH

SEQ ID NO: 126
Clone 311K4E_19 - DE Loop
NTPYVH

SEQ ID NO: 127
Clone 311K4E_20 - DE Loop
EQVYVH

SEQ ID NO: 128
Clone 311K4E_21 - DE Loop
NQVYVH

SEQ ID NO: 129
Clone 311, 311K4E, 311K4E_1, 311K4E_2,311K4E_3, 311K4E_4, 311K4E_5,
311K4E_7, 311K4E_8, 311K4E_9, 311K4E_10, 311K4E_11, 311K4E_13,311K4E_14,
311K4E_15, 311K4E_16, 311K4E_19, 311K4E_20, 311K4E_21 - FG Loop
LTTDGTYSNPA

SEQ ID NO: 130
Clone 311K4E_12 - FG Loop
LTTDGTYNNPA

SEQ ID NO: 131

2GS Linker – (Gly4Ser)2
GGGGSGGGGS

SEQ ID NO: 132
3GS Linker – (Gly4Ser)3
GGGGSGGGGSGGGGS

SEQ ID NO: 133
HSA C34S mutant
Cys->Ser mutation location is underlined
DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAE
NCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEV
DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP
KLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTK
VHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPA
DLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKC
CAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVST
PTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTES
LVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKAT
KEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL

SEQ ID NO: 134
342-2GS-HSAC34S - Monovalent Construct
HSAC34S is underlined
SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKALVLI
AFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYG
EMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARR
HPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQK
FGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYIC
ENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDV
FLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQ
NLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKR
MPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAE
TFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKE
TCFAEEGKKLVAASQAALGL

SEQ ID NO: 135
342-3GS-342-2GS-HSAC34S Bivalent Construct
HSA is underlined
SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGSGGGGSGGGGSRLDAPSQIEVKDVTDTTALI
TWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDTEYEVSLICRSGDMS
SNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHV
KLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNE
CFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKR
YKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLS
QRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCE
KPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGE
YKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQL
CVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKE
RQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQ
AALGL

SEQ ID NO: 136
311 clone family AB loop N-term E variant
EDVTDTT

SEQ ID NO: 137
311 clone family EF loop C-term K variant

GNLKPDTK

SEQ ID NO: 138
HSA human full-length
DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQ**C**PFEDHVKLVNEVTEFAKTCVADESAE
NCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEV
DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP
KLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTK
VHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPA
DLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKC
CAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVST
PTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTES
LVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKAT
KEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL

SEQ ID NO: 139
309 FG loop variant (RR->RS mutant); may be present in 342 constructs
RSGDMSSNPA

SEQ ID NO: 140
2GX Linker – (Gly4X)2; X = Ala, Gly, Leu, Ile, Val
GGGGXGGGGX

SEQ ID NO: 141
3GX Linker – (Gly4X)3; X = Ala, Gly, Leu, Ile, Val
GGGGXGGGGXGGGGX

SEQ ID NO: 142
G10 Linker – (Gly4Gly)2
GGGGGGGGGG

SEQ ID NO: 143
G15 Linker – (Gly4Gly)3
GGGGGGGGGGGGGGG

SEQ ID NO: 144
342-G10-HSAC34S - Monovalent construct 2 – all Gly linkers
HSA is underlined
SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGGGGGGGDAHKSEVAHRFKDLGEENFKALVLI
AFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYG
EMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARR
HPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQK
FGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYIC
ENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDV
FLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQ
NLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKR
MPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAE
TFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKE
TCFAEEGKKLVAASQAALGL

SEQ ID NO: 145
342-G15-342-G10-HSAC34S Bivalent construct 2 – all Gly linkers
HSA is underlined
SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGGGGGGGGGGGGRLDAPSQIEVKDVTDTTALI
TWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDTEYEVSLICRSGDMS
SNPAKETFTTGGGGGGGGGGDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHV
KLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNE
CFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKR

YKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLS
QRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCE
KPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGE
YKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQL
CVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKE
RQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQ
AALGL

SEQ ID NO: 146
Clone 342 - Affinity Mature variant (w/FG loop variant RR->RS underlined)
IEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDTE
YEVSLICRSGDMSSNPAKETFTT

SEQ ID NO: 147
Gly-Ser linker module, **$(G_4S)_n$** where n = 1-7; the $(G_4S)_n$ module wherein n=1 is shown
GGGGS

SEQ ID NO: 148
Gly linker module **$(G_5)_n$** where n = 1-7; the $(G_5)_n$ module wherein n=1 is shown
GGGGG

SEQ ID NO: 149
Gly-Ala linker module, **$(G_4A)_n$** where n = 1-7; the $(G_4A)_n$ module wherein n=1 is shown
GGGGA

SEQ ID NO: 150
Poly-Histidine Tag **(H_8) -** An optional component of the Tn3 scaffolds useful for purification maybe combined with additional linker residues.
HHHHHHHH

SEQ ID NO: 151
Linker-Poly-Histidine Tag - An optional component of the Tn3 scaffolds useful for purification
GGGGSHHHHHHHH

SEQ ID NO: 152
Mature MSA wild type
EAHKSEIAHRYNDLGEQHFKGLVLIAFSQYLQKCSYDEHAKLVQEVTDFAKTCVADESAA
NCDKSLHTLFGDKLCAIPNLRENYGELADCCTKQEPERNECFLQHKDDNPSLPPFERPEA
EAMCTSFKENPTTFMGHYLHEVARRHPYFYAPELLYYAEQYNEILTQCCAEADKESCLTP
KLDGVKEKALVSSVRQRMKCSSMQKFGERAFKAWAVARLSQTFPNADFAEITKLATDLTK
VNKECCHGDLLECADDRAELAKYMCENQATISSKLQTCCDKPLLKKAHCLSEVEHDTMPA
DLPAIAADFVEDQEVCKNYAEAKDVFLGTFLYEYSRRHPDYSVSLLLRLAKKYEATLEKC
CAEANPPACYGTVLAEFQPLVEEPKNLVKTNCDLYEKLGEYGFQNAILVRYTQKAPQVST
PTLVEAARNLGRVGTKCCTLPEDQRLPCVEDYLSAILNRVCLLHEKTPVSEHVTKCCSGS
LVERRPCFSALTVDETYVPKEFKAETFTFHSDICTLPEKEKQIKKQTALAELVKHKPKAT
AEQLKTVMDDFAQFLDTCCKAADKDTCFSTEGPNLVTRCKDALA

SEQ ID NO: 153
Mature MSA-C34S/C579S Cys mutant; mutated residues underlined
EAHKSEIAHRYNDLGEQHFKGLVLIAFSQYLQKSSYDEHAKLVQEVTDFAKTCVADESAA
NCDKSLHTLFGDKLCAIPNLRENYGELADCCTKQEPERNECFLQHKDDNPSLPPFERPEA
EAMCTSFKENPTTFMGHYLHEVARRHPYFYAPELLYYAEQYNEILTQCCAEADKESCLTP
KLDGVKEKALVSSVRQRMKCSSMQKFGERAFKAWAVARLSQTFPNADFAEITKLATDLTK
VNKECCHGDLLECADDRAELAKYMCENQATISSKLQTCCDKPLLKKAHCLSEVEHDTMPA
DLPAIAADFVEDQEVCKNYAEAKDVFLGTFLYEYSRRHPDYSVSLLLRLAKKYEATLEKC

CAEANPPACYGTVLAEFQPLVEEPKNLVKTNCDLYEKLGEYGFQNAILVRYTQKAPQVST
PTLVEAARNLGRVGTKCCTLPEDQRLPCVEDYLSAILNRVCLLHEKTPVSEHVTKCCSGS
LVERRPCFSALTVDETYVPKEFKAETFTFHSDICTLPEKEKQIKKQTALAELVKHKPKAT
AEQLKTVMDDFAQFLDTCCKAADKDTCFSTEGPNLVTRSKDALA

SEQ ID NO: 154
Clone M13; BC, DE, FG loops are underlined
IEVKDVTDTTALITWHDAFGYDFGCELTYGIKDVPGDRTTIDLPDHFHNYSIGNLKPDT
EYEVSLICANDHGFDSNPAKETFTT

SEQ ID NO: 155
Clone M13N49Q; N49Q mutation underlined
IEVKDVTDTTALITWHDAFGYDFGCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPDT
EYEVSLICANDHGFDSNPAKETFTT

SEQ ID NO: 156
M13N49Q-1GS-M13N49Q bivalent Construct; N49Q mutation underlined
SQIEVKDVTDTTALITWHDAFGYDFGCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPD
TEYEVSLICANDHGFDSNPAKETFTTTGGGGSRLDAPSQIEVKDVTDTTALITWHDAFGY
DFGCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPDTEYEVSLICANDHGFDSNPAKET
FTT

SEQ ID NO: 157
M13N49Q-3GS-MSA-C34S/C579S monovalent construct; mutations underlined
SQIEVKDVTDTTALITWHDAFGYDFGCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPD
TEYEVSLICANDHGFDSNPAKETFTTGGGGSGGGGSGGGGSEAHKSEIAHRYNDLGEQHF
KGLVLIAFSQYLQKSSYDEHAKLVQEVTDFAKTCVADESAANCDKSLHTLFGDKLCAIPN
LRENYGELADCCTKQEPERNECFLQHKDDNPSLPPFERPEAEAMCTSFKENPTTFMGHYL
HEVARRHPYFYAPELLYYAEQYNEILTQCCAEADKESCLTPKLDGVKEKALVSSVRQRMK
CSSMQKFGERAFKAWAVARLSQTFPNADFAEITKLATDLTKVNKECCHGDLLECADDRAE
LAKYMCENQATISSKLQTCCDKPLLKKAHCLSEVEHDTMPADLPAIAADFVEDQEVCKNY
AEAKDVFLGTFLYEYSRRHPDYSVSLLLRLAKKYEATLEKCCAEANPPACYGTVLAEFQP
LVEEPKNLVKTNCDLYEKLGEYGFQNAILVRYTQKAPQVSTPTLVEAARNLGRVGTKCCT
LPEDQRLPCVEDYLSAILNRVCLLHEKTPVSEHVTKCCSGSLVERRPCFSALTVDETYVP
KEFKAETFTFHSDICTLPEKEKQIKKQTALAELVKHKPKATAEQLKTVMDDFAQFLDTCC
KAADKDTCFSTEGPNLVTRSKDALA

SEQ ID NO: 158
M13N49Q-1GS-M13N49Q-3GS-MSA-C34S/C579S bivalent construct; mutations
underlined
SQIEVKDVTDTTALITWHDAFGYDFGCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPD
TEYEVSLICANDHGFDSNPAKETFTTTGGGGSRLDAPSQIEVKDVTDTTALITWHDAFGY
DFGCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPDTEYEVSLICANDHGFDSNPAKET
FTTGGGGSGGGGSGGGGSEAHKSEIAHRYNDLGEQHFKGLVLIAFSQYLQKSSYDEHAKL
VQEVTDFAKTCVADESAANCDKSLHTLFGDKLCAIPNLRENYGELADCCTKQEPERNECF
LQHKDDNPSLPPFERPEAEAMCTSFKENPTTFMGHYLHEVARRHPYFYAPELLYYAEQYN
EILTQCCAEADKESCLTPKLDGVKEKALVSSVRQRMKCSSMQKFGERAFKAWAVARLSQT
FPNADFAEITKLATDLTKVNKECCHGDLLECADDRAELAKYMCENQATISSKLQTCCDKP
LLKKAHCLSEVEHDTMPADLPAIAADFVEDQEVCKNYAEAKDVFLGTFLYEYSRRHPDYS
VSLLLRLAKKYEATLEKCCAEANPPACYGTVLAEFQPLVEEPKNLVKTNCDLYEKLGEYG
FQNAILVRYTQKAPQVSTPTLVEAARNLGRVGTKCCTLPEDQRLPCVEDYLSAILNRVCL
LHEKTPVSEHVTKCCSGSLVERRPCFSALTVDETYVPKEFKAETFTFHSDICTLPEKEKQ
IKKQTALAELVKHKPKATAEQLKTVMDDFAQFLDTCCKAADKDTCFSTEGPNLVTRSKDA
LA

SEQ ID NO: 159
Clone M31; BC, DE, FG loops are underlined
IEVKDVTDTTALITWHDPSGYDFWCELTYGIKDVPGDRTTIDLPDHFHNYSIGNLKPDTE
YEVSLICANDHGFDSYPAKETFTT

SEQ ID NO: 160

Clone M31N49Q; N49Q mutation underlined
IEVKDVTDTTALITWHDPSGYDFWCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPDTE
YEVSLICANDHGFDSYPAKETFTT

SEQ ID NO: 161
M31N49Q-1GS-M31N49Q bivalent construct; N49Q mutation underlined
SQIEVKDVTDTTALITWHDPSGYDFWCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPD
TEYEVSLICANDHGFDSYPAKETFTTTGGGGSRLDAPSQIEVKDVTDTTALITWHDPSGY
DFWCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPDTEYEVSLICANDHGFDSYPAKET
FTT

SEQ ID NO: 162
M31N49Q-3GS-MSA-C34S/C579S monovalent construct; mutations underlined
SQIEVKDVTDTTALITWHDPSGYDFWCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPD
TEYEVSLICANDHGFDSYPAKETFTTGGGGSGGGGSGGGGSEAHKSEIAHRYNDLGEQHF
KGLVLIAFSQYLQKSSYDEHAKLVQEVTDFAKTCVADESAANCDKSLHTLFGDKLCAIPN
LRENYGELADCCTKQEPERNECFLQHKDDNPSLPPFERPEAEAMCTSFKENPTTFMGHYL
HEVARRHPYFYAPELLYYAEQYNEILTQCCAEADKESCLTPKLDGVKEKALVSSVRQRMK
CSSMQKFGERAFKAWAVARLSQTFPNADFAEITKLATDLTKVNKECCHGDLLECADDRAE
LAKYMCENQATISSKLQTCCDKPLLKKAHCLSEVEHDTMPADLPAIAADFVEDQEVCKNY
AEAKDVFLGTFLYEYSRRHPDYSVSLLLRLAKKYEATLEKCCAEANPPACYGTVLAEFQP
LVEEPKNLVKTNCDLYEKLGEYGFQNAILVRYTQKAPQVSTPTLVEAARNLGRVGTKCCT
LPEDQRLPCVEDYLSAILNRVCLLHEKTPVSEHVTKCCSGSLVERRPCFSALTVDETYVP
KEFKAETFTFHSDICTLPEKEKQIKKQTALAELVKHKPKATAEQLKTVMDDFAQFLDTCC
KAADKDTCFSTEGPNLVTRSKDALA

SEQ ID NO: 163
M31N49Q-1GS-M31N49Q-3GS-MSA-C34S/C579S bivalent construct; mutations
underlined
SQIEVKDVTDTTALITWHDPSGYDFWCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPD
TEYEVSLICANDHGFDSYPAKETFTTTGGGGSRLDAPSQIEVKDVTDTTALITWHDPSGY
DFWCELTYGIKDVPGDRTTIDLPDHFHQYSIGNLKPDTEYEVSLICANDHGFDSYPAKET
FTTGGGGSGGGGSGGGGSEAHKSEIAHRYNDLGEQHFKGLVLIAFSQYLQKSSYDEHAKL
VQEVTDFAKTCVADESAANCDKSLHTLFGDKLCAIPNLRENYGELADCCTKQEPERNECF
LQHKDDNPSLPPFERPEAEAMCTSFKENPTTFMGHYLHEVARRHPYFYAPELLYYAEQYN
EILTQCCAEADKESCLTPKLDGVKEKALVSSVRQRMKCSSMQKFGERAFKAWAVARLSQT
FPNADFAEITKLATDLTKVNKECCHGDLLECADDRAELAKYMCENQATISSKLQTCCDKP
LLKKAHCLSEVEHDTMPADLPAIAADFVEDQEVCKNYAEAKDVFLGTFLYEYSRRHPDYS
VSLLLRLAKKYEATLEKCCAEANPPACYGTVLAEFQPLVEEPKNLVKTNCDLYEKLGEYG
FQNAILVRYTQKAPQVSTPTLVEAARNLGRVGTKCCTLPEDQRLPCVEDYLSAILNRVCL
LHEKTPVSEHVTKCCSGSLVERRPCFSALTVDETYVPKEFKAETFTFHSDICTLPEKEKQ
IKKQTALAELVKHKPKATAEQLKTVMDDFAQFLDTCCKAADKDTCFSTEGPNLVTRSKDA
LA

SEQ ID NO: 164
Clone D1 - Negative control Tn3
IEVKDVTDTTALITWSPGERIWMFTGCELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPD
TEYEVSLICPNYERISNPAKETFTTT

SEQ ID NO: 165
D1-1GS-D1-3G-MSA-C34S/C579S bivalent construct; mutations underlined
SQIEVKDVTDTTALITWSPGERIWMFTGCELTYGIKDVPGDRTTIDLTEDENQYSIGNLK
PDTEYEVSLICPNYERISNPAKETFTTTGGGGSRLDAPSQIEVKDVTDTTALITWSPGER
IWMFTGCELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDTEYEVSLICPNYERISNPAK
ETFTTGGGGSGGGGSGGGGSEAHKSEIAHRYNDLGEQHFKGLVLIAFSQYLQKSSYDEHA
KLVQEVTDFAKTCVADESAANCDKSLHTLFGDKLCAIPNLRENYGELADCCTKQEPERNE
CFLQHKDDNPSLPPFERPEAEAMCTSFKENPTTFMGHYLHEVARRHPYFYAPELLYYAEQ
YNEILTQCCAEADKESCLTPKLDGVKEKALVSSVRQRMKCSSMQKFGERAFKAWAVARLS
QTFPNADFAEITKLATDLTKVNKECCHGDLLECADDRAELAKYMCENQATISSKLQTCCD
KPLLKKAHCLSEVEHDTMPADLPAIAADFVEDQEVCKNYAEAKDVFLGTFLYEYSRRHPD
YSVSLLLRLAKKYEATLEKCCAEANPPACYGTVLAEFQPLVEEPKNLVKTNCDLYEKLGE

YGFQNAILVRYTQKAPQVSTPTLVEAARNLGRVGTKCCTLPEDQRLPCVEDYLSAILNRV
CLLHEKTPVSEHVTKCCSGSLVERRPCFSALTVDETYVPKEFKAETFTFHSDICTLPEKE
KQIKKQTALAELVKHKPKATAEQLKTVMDDFAQFLDTCCKAADKDTCFSTEGPNLVTRSK
DALA

SEQ ID NO: 166
Clone 342 RDG to SDG mutant; mutation underlined
IEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDTE
YEVSLICRSGDMSSNPAKETFTT

SEQ ID NO: 167
309FGwt consensus
All strands are parent Tn3 strands; beta strand C is CELTYGI variant (SEQ ID NO: 14); AB, CD, EF loop are parent Tn3 loops
X1= Ser or Leu
X2= Asp or Glu
X3= His, Ile, Val, Phe or Trp
X4= Ala, Gly, Glu or Asp
X5= Glu, Leu, Gln, Ser, Asp or Asn
X6= Phe or Tyr
X7= Ile, Val, His, Glu or Asp
X8= Gly, Trp or Val
X9= Trp, Phe or Tyr
X10= Ser, Gln, Met or His
X11= Trp or His
X12= Arg or Ser
IEVKDVTDTTALITWX1DX2X3X4X5X6X7X8CELTYGIKDVPGDRTTIDLWX9HX10AX11YSIGNLKPDTEYE
VSLICRX12GDMSSNPAKETFTT
SEQ ID NO: 168
309FGwt consensus, BC loop
X1= Ser or Leu
X2= Asp or Glu
X3= His, Ile, Val, Phe or Trp
X4= Ala, Gly, Glu or Asp
X5= Glu, Leu, Gln, Ser, Asp or Asn
X6= Phe or Tyr
X7= Ile, Val, His, Glu or Asp
X8= Gly, Trp or Val
X1DX2X3X4X5X6X7X8

SEQ ID NO: 169
309FGwt consensus, DE loop
X9= Trp, Phe or Tyr
X10= Ser, Gln, Met or His
X11= Trp or His
WX9HX10AX11

SEQ ID NO: 170
309FGwt consensus, FG loop
X12= Arg or Ser
RX12GDMSSNPA

SEQ ID NO: 171
311 consensus; all strands are parent Tn3 strands; two beta strand C variants (SEQ ID NO: 13 and 14); CD loop is parent Tn3 loop
X1= Lys or Glu
X2= Thr or Ile
X3= Asn or Ala
X4= Ser, Leu, Ala, Phe or Tyr
X5= Tyr, Ala, Gly, Val, Ile or Ser (BC/N-term contact)
X6= Tyr, Ser, Ala or His

X7= Asn, Asp, His or Tyr
X8= Leu, Phe, His or Tyr
X9= His, Pro, Ser, Leu or Asp
X10= Gly, Phe, His or Tyr
X11= Ala or Thr
X12= Ser, Asn, Glu, Arg or Asp
X13= Ser, Gln, Thr, Asn or Ala
X14= Pro, Val, -, Ile or Ala (- no amino acid)
X15= - or Ile (- no amino acid)
X16= Glu or Lys
X17= Ser or Asn
IEVX1DVTDTTALITWX2X3RSX4X5X6X7X8X9X10CELX11YGIKDVPGDRTTIDLX12X13X14X15YVHYS
IGNLKPDTX16YEVSLICLTTDGTYX17NPAKETFTT
SEQ ID NO: 172
311 consensus; beta strand C in 311 family clones
X11= Ala or Thr
CELX11YGI

SEQ ID NO: 173
311 consensus; AB loop
X1= Lys or Glu
X1DVTDTT

SEQ ID NO: 174
311 consensus; BC loop
X2= Thr or Ile
X3= Asn or Ala
X4= Ser, Leu, Ala, Phe or Tyr X5= Tyr, Ala, Gly, Val, Ile or Ser (BC/N-term
contact)
X6= Tyr, Ser, Ala or His
X7= Asn, Asp, His or Tyr
X8= Leu, Phe, His or Tyr
X9= His, Pro, Ser, Leu or Asp
X10= Gly, Phe, His or Tyr
X2X3RSX4X5X6X7X8X9X10

SEQ ID NO: 175
311 consensus; DE loop
X12= Ser, Asn, Glu, Arg or Asp X13= Ser, Gln, Thr, Asn or Ala
X14= Pro, Val, -, Ile or Ala (- no amino acid)
X15= - or Ile (- no amino acid)
X12X13X14X15YVH

SEQ ID NO: 176
311 consensus; EF loop
X16= Glu or Lys
GNLKPDTX16

SEQ ID NO: 177
311 consensus; FG loop
X17= Ser or Asn
LTTDGTYX17NPA

SEQ ID NO: 178
BC9 NHT oligo; loop BC

Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G; V = A/C/G; M = A/C; K = G/T

ACCGCGCTGATTACCTGGNHTNHTSCGNHTGSTNHTNHTNHTGGCTGTGAACTGACCTAT
GGCATTAAA

SEQ ID NO: 179
BC11 NHT oligo; loop BC
Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G; V = A/C/G; M = A/C; K = G/T

ACCGCGCTGATTACCTGGNHTNHTBSTNHTNHTNHTNHTNHTNHTNHTGGCTGTGAACTG
ACCTATGGCATTAAA

SEQ ID NO: 180
BC12 NHT oligo; loop BC
Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G; V = A/C/G; M = A/C; K = G/T

ACCGCGCTGATTACCTGGNHTVMACCGNHTNHTNHTRRCRGCNHTVTTNHTGGCTGTGAA
CTGACCTATGGCATTAAA

SEQ ID NO: 181
DE NHT oligo; DE loop
Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G; V = A/C/G; M = A/C; K = G/T

CGATCGCACCACCATAGATCTGNHTNHTNHTNHTNHTNHTTATAGCATTGGTAACCTGAA
ACCG

SEQ ID NO: 182
FG9 NHT oligo; FG loop
Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G; V = A/C/G; M = A/C; K = G/T

GAATATGAAGTGAGCCTGATTTGCNHTAMSNHTNHTGGTNHTNHTNHTKCGAAAGAAACC
TTTACCACCGGTG

SEQ ID NO: 183
FG10 NHT oligo; FG loop
Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G; V = A/C/G; M = A/C; K = G/T

GAATATGAAGTGAGCCTGATTTGCNHTAMSNHTNHTNHTNHTRGCNHTCCGGCGAAAGAA
ACCTTTACCACCGGTG

SEQ ID NO: 184
FG11 NHT oligo; FG loop
Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G; V = A/C/G; M = A/C; K = G/T

GAATATGAAGTGAGCCTGATTTGCNHTAMSNHTNHTGGTNHTNHTAGCAACCCGGCGAAA
GAAACCTTTACCACCGGTG

SEQ ID NO: 185
BCX-DE bridge v2 oligo
CAGATCTATGGTGGTGCGATCGCCCGGCACATCTTTAATGCCATAGGTCAGTTCACA

SEQ ID NO: 186
DE-FGX bridge v2 oligo

GCAAATCAGGCTCACTTCATATTCGGTATCCGGTTTCAGGTTACCAATGCTAT

SEQ ID NO: 187
KpnI amp rev v2 oligo
CGGGTCGGTTGGGGTACCGCCACCGGTGGTAAAGGTTTCTTT

SEQ ID NO: 188
KpnI reverse v2 oligo
CGGGTCGGTTGGGGTA

SEQ ID NO: 189
BC library amp v2 oligo
GGCCCAGCCGGCCATGGCCGCCATTGAAGTGAAAGATGTGACCGATACCACCGCGCTGAT
TACCTGG

SEQ ID NO: 190
BC9 PCR oligo
Nucleotide codes: 1= codons for all 19aa(-cys); 2=codons for Ala/Pro 50/50;3= codons for Ala/Gly

ACCGCGCTGATTACCTGGTCT1213111GGCTGTGAACTGACCTATGGCATTAAAGATG

SEQ ID NO: 191
BC 9-loop NNK oligo
Nucleotide codes: K= 50%G/50%T

ACCGCGCTGATTACCTGGNNKNNKSMGNNKGSTNNKNNKNNKGGCTGTGAACTGACCTA
TGGCATTAAA

SEQ ID NO: 192
309 BC-loop NNKdope oligo
Nucleotide codes: 4= 70%G10%A10%C10%T; 5= 10%G,70%A,10%C,10%T; 6=10%G,10%A,70%C, 10%T; 7= 10%G,10%A,10%C,70%T; 8= 70%A15%C15%T; and K= 50%G/50%T

ACCGCGCTGATTACCTGG76K45K45K77K44K65K78T45K44KTGTGAACTGACCTA
TGGCATTAAA

SEQ ID NO: 193
DE PCR oligo
Nucleotide codes: 1= codons for all 19aa(-cys)

GATGTGCCGGGCGATCGCACCACCATAGATCTG111111TATAGCATTGGTAACCTGAAA
CCGG

SEQ ID NO: 194
Upstr BCloop Rev oligo
CCAGGTAATCAGCGCGGTGGTAT

SEQ ID NO: 195
BC shuffle rev oligo
CAGATCTATGGTGGTGCGATCGC

SEQ ID NO: 196
DE shuffle FWD oligo
TGTGAACTGACCTATGGCATTAAAGATGT

SEQ ID NO: 197
BC11-311Gly oligo

Nucleotide codes: 1=70%G, 10%A, 10%C, 10%T; 2=10%G, 70%A, 10%C, 10%T; 3=10%G, 10%A, 70%C, 10%T; 4=10%G, 10%A, 10%C, 70%T; 5=70%A, 15%C, 15%T; 6=15%A, 70%C, 15%T; 7=15%A, 15%C, 70%T; V=33%A, 33%C, 33%G.

ACCGCGCTGATTACCTGG26T25TV1T46T46T45T45T25T37T35TGGCTGTGAACTG
ACCTATGGCATTAAA

SEQ ID NO: 198
BC11-311NHT oligo

Nucleotide codes: 1=70%G, 10%A, 10%C, 10%T; 2=10%G, 70%A, 10%C, 10%T; 3=10%G, 10%A, 70%C, 10%T; 4=10%G, 10%A, 10%C, 70%T; 5=70%A, 15%C, 15%T; 6=15%A, 70%C, 15%T; 7=15%A, 15%C, 70%T; V=33%A, 33%C, 33%G; and H=33%A, 33%C, 33%T

ACCGCGCTGATTACCTGG26T25TV1T46T46T45T45T25T37T35TNHTTGTGAACTG
ACCTATGGCATTAAA

SEQ ID NO: 199
BC library amp K4E oligo
GGCCCAGCCGGCCATGGCCGCCATTGAAGTGGAAGATGTGACCGATACCACCGCGCTGAT
TACCTGG

SEQ ID NO: 200
Extended half-life HSA variant(C34S, L463N, K524L); mutations are underlined
DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAE
NCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEV
DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP
KLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTK
VHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPA
DLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKC
CAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVST
PTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVNHEKTPVSDRVTKCCTES
LVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQILKQTALVELVKHKPKAT
KEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL

SEQ ID NO: 201
311K4E_12-I variant monovalent construct (comprises GS linker and C34S HSA); linker and mutated serine are underlined
SQIEVEDVTDTTALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLK
PDTEYEVSLICLTTDGTYNNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKAL
VLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRE
TYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEI
ARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCAS
LQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAK
YICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEA
KDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVE
EPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPE
AKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEF
NAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKAD
DKETCFAEEGKKLVAASQAALGL

SEQ ID NO: 202
311K4E_12-I variant monovalent construct (comprises beta strand C CELTYG variant, all G linker, and C34S HSA); linker and mutated serine are underlined
SQIEVEDVTDTTALITWTNRSSYSNLHGCELTYGIKDVPGDRTTIDLNQPYVHYSIGNLK

PDTEYEVSLICLTTDGTYNNPAKETFTTGGGGGGGGGGDAHKSEVAHRFKDLGEENFKAL
VLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRE
TYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEI
ARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCAS
LQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAK
YICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEA
KDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVE
EPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPE
AKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEF
NAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKAD
DKETCFAEEGKKLVAASQAALGL

SEQ ID NO: 203
311K4E_12-I variant bivalent construct (comprises GS linkers and C34S HSA); linkers and mutated serine are underlined
SQIEVEDVTDTTALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLK
PDTEYEVSLICLTTDGTYNNPAKETFTTGGGGSGGGGSGGGGSRLDAPSQIEVEDVTDTT
ALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKPDTEYEVSLICL
TTDGTYNNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQS
PFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQ
EPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPEL
LFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAW
AVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSK
LKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEY
ARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCEL
FEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLS
VVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADIC
TLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKK
LVAASQAALGL

SEQ ID NO: 204
311K4E_12-I variant bivalent construct (comprises all G linkers and S34 HSA); linkers and mutated serine are underlined
SQIEVEDVTDTTALITWTNRSSYSNLHGCELTYGIKDVPGDRTTIDLNQPYVHYSIGNLK
PDTEYEVSLICLTTDGTYNNPAKETFTT**GGGGGGGGGGGGGGG**RLDAPSQIEVEDVTDTT
ALITWTNRSSYSNLHGCELAYGIKDVPGDRTTIDLNQPYVHYSIGNLKPDTEYEVSLICL
TTDGTYNNPAKETFTT**GGGGGGGGGG**DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQS
PFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQ
EPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPEL
LFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAW
AVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSK
LKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEY
ARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCEL
FEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLS
VVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADIC
TLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKK
LVAASQAALGL

SEQ ID NO: 205
309-3GS-309 bivalent construct
SQIEVKDVTDTTALITWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPD
TEYEVSLICYTDQEAGNPAKETFTTGGGGSGGGGSGGGGSRLDAPSQIEVKDVTDTTALI
TWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDTEYEVSLICYTDQEA
GNPAKETFTT

SEQ ID NO: 206
309-2GS-HSAC34S monovalent construct
SQIEVKDVTDTTALITWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPD
TEYEVSLICYTDQEAGNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKALVLI
AFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYG
EMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARR

HPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQK
FGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYIC
ENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDV
FLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQ
NLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKR
MPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAE
TFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKE
TCFAEEGKKLVAASQAALGL

SEQ ID NO: 207
309-3GS-309-2GS-HSAC34S bivalent construct
SQIEVKDVTDTTALITWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPD
TEYEVSLICYTDQEAGNPAKETFTTGGGGSGGGGSGGGGSRLDAPSQIEVKDVTDTTALI
TWSDEFGHYDGCELTYGIKDVPGDRTTIDLWWHSAWYSIGNLKPDTEYEVSLICYTDQEA
GNPAKETFTTGGGGSGGGGSDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHV
KLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNE
CFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKR
YKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLS
QRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCE
KPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGE
YKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQL
CVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKE
RQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQ
AALGL

SEQ ID NO: 208
342-3GS-342 bivalent construct
SQIEVKDVTDTTALITWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPD
TEYEVSLICRSGDMSSNPAKETFTTGGGGSGGGGSGGGGSRLDAPSQIEVKDVTDTTALI
TWSDDFGEYVWCELTYGIKDVPGDRTTIDLWYHHAHYSIGNLKPDTEYEVSLICRSGDMS
SNPAKETFTT

SEQ ID NO: 209
Gly-Ser linker module, **$(G_4X)_n$** where X=G, S, A, L, I, or V and n = 1-7; the $(G_4X)_n$ module wherein n=1 is shown
GGGGX

SEQ ID NO: 210
OppA signal peptide mutant L25/M
MTNITKRSLVAAGVLAALMAGNVAMA

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule encoding a Tn3 scaffold comprising a CD40L-specific monomer subunit, wherein the CD40L-specific monomer subunit comprises seven beta strands designated A, B, C, D, E, F, and G, and six loop regions designated AB, BC, CD, DE, EF, and FG, wherein:

(a) the AB loop comprises the amino acid sequence of SEQ ID NO: 4;

(b) the BC loop comprises the amino acid sequence of one of SEQ ID NOs: 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93 or 168 at the BC loop;

(c) the CD loop comprises the amino acid sequence of SEQ ID NO: 6;

(d) the DE loop comprises the amino acid sequence of one of SEQ ID NOs: 94, 95, 96, 97, 98 or 169;

(e) the EF loop comprises the amino acid sequence of SEQ ID NO: 8; and

(f) the FG loop comprises the amino acid sequence of one of SEQ ID NOs: 9, 99, 139 or 170.

2. The isolated nucleic acid of claim 1, wherein the Tn3 scaffold comprises two CD40L-specific monomer subunits connected in tandem by a peptide linker.

3. The isolated nucleic acid of claim 2, wherein the Tn3 scaffold binds CD40L and prevents binding of CD40L to CD40 and/or disrupts CD40 mediated signaling.

4. The isolated nucleic acid of claim 1, wherein:

(a) the BC loop comprises the amino acid sequence of SEQ ID NO:83, the DE loop comprises the amino acid sequence of SEQ ID NO:94, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(b) the BC loop comprises the amino acid sequence of SEQ ID NO:83, the DE loop comprises the amino acid sequence of SEQ ID NO:94, and the FG loop comprises the amino acid sequence of SEQ ID NO:99; or

(c) the BC loop comprises the amino acid sequence of SEQ ID NO:84, the DE loop comprises the amino acid sequence of SEQ ID NO:95, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(d) the BC loop comprises the amino acid sequence of SEQ ID NO:85, the DE loop comprises the amino acid sequence of SEQ ID NO:94, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(e) the BC loop comprises the amino acid sequence of SEQ ID NO:86, the DE loop comprises the amino acid sequence of SEQ ID NO:96, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(f) the BC loop comprises the amino acid sequence of SEQ ID NO:87, the DE loop comprises the amino acid sequence of SEQ ID NO:97, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(g) the BC loop comprises the amino acid sequence of SEQ ID NO:88, the DE loop comprises the amino acid sequence of SEQ ID NO:95, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(h) the BC loop comprises the amino acid sequence of SEQ ID NO:89, the DE loop comprises the amino acid sequence of SEQ ID NO:94, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(i) the BC loop comprises the amino acid sequence of SEQ ID NO:90, the DE loop comprises the amino acid sequence of SEQ ID NO:94, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(j) the BC loop comprises the amino acid sequence of SEQ ID NO:91, the DE loop comprises the amino acid sequence of SEQ ID NO:95, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(k) the BC loop comprises the amino acid sequence of SEQ ID NO:92, the DE loop comprises the amino acid sequence of SEQ ID NO:98, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139; or

(l) the BC loop comprises the amino acid sequence of SEQ ID NO:93, the DE loop comprises the amino acid sequence of SEQ ID NO:94, and the FG loop comprises the amino acid sequence of SEQ ID NO:9 or 139.

5. The isolated nucleic acid molecule of claim 1, wherein:

(a) the A beta strand consists of the amino acid sequence of SEQ ID NO:11;

(b) the B beta strand consists of the amino acid sequence of SEQ ID NO:12;

(c) the C beta strand consists of the amino acid sequence of SEQ ID NO:13 or 14;

(d) the D beta strand consists of the amino acid sequence of SEQ ID NO:15;

(e) the E beta strand consists of the amino acid sequence of SEQ ID NO:16;

(f) the F beta strand consists of the amino acid sequence of SEQ ID NO:17; and

(g) the G beta strand consists of the amino acid sequence of SEQ ID NO:18.

6. The isolated nucleic acid molecule of claim 1, wherein the Tn3 scaffold comprises the amino acid sequence of one of SEQ ID NOs: 134, 135, 144, 145, 166, 205, 206, 207 or 208.

7. The isolated nucleic acid molecule of claim 2, wherein the peptide linker comprises amino acid sequence $(G_mX)_n$, wherein:
   (a) X is Serine, Alanine, Glycine, Leucine, Isoleucine or Valine;
   (b) m and n are integers;
   (c) m is 1, 2, 3 or 4; and
   (d) n is 1, 2, 3, 4, 5, 6 or 7.

8. The isolated nucleic acid molecule of claim 7, wherein the peptide linker comprises the amino acid sequence of SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 142, or SEQ ID NO: 143.

9. An isolated nucleic acid molecule encoding a Tn3 scaffold fusion protein, wherein the fusion protein comprises a Tn3 scaffold protein and a heterologous moiety, wherein:

the Tn3 scaffold protein comprises a CD40L-specific monomer subunit, wherein the CD40L-specific monomer subunit comprises seven beta strands designated A, B, C, D, E, F, and G, and six loop regions designated AB, BC, CD, DE, EF, and FG, wherein the AB loop comprises SEQ ID NO; 4; the BC loop comprises the amino acid sequence as shown in one of SEQ ID NOs: 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93 or 168; the CD loop comprises the amino acid sequence as shown in SEQ ID NO: 6; the DE loop comprises the amino acid sequence as shown in one of SEQ ID NOs: 94, 95, 96, 97, 98, or 169; the EF loop comprises the amino acid sequence as shown in SEQ ID NO:8; and the FG loop comprises the amino acid sequence as shown in one of SEQ ID NOs: 9, 99, 139, or 170, and wherein the encoded Tn3 scaffold specifically binds to CD40L; and

wherein the heterologous moiety is biotin, an albumin, a human serum albumin (HSA), an HSA FcRn binding portion, an antibody, a domain of an antibody, an antibody fragment, a single chain antibody, a domain antibody, an albumin binding domain, an enzyme, a ligand, a receptor, a binding peptide, a non-FnIII scaffold, an epitope tag, or a cytokine.

10. The isolated nucleic acid molecule of claim 9, wherein the heterologous moiety is HSA.

11. The isolated nucleic acid molecule of claim 10, wherein the HSA comprises the amino acid sequence of one of SEQ ID NOs: 133 or 138.

12. The isolated nucleic acid molecule of claim 9, wherein the A beta strand consists of the amino acid sequence of SEQ ID NO:11, the B beta strand consists of the amino acid sequence of SEQ ID NO:12, the C beta strand consists of the amino acid sequence of SEQ ID NO:13 or 14, the D beta strand consists of the amino acid sequence of SEQ ID NO:15, the E beta strand consists of the amino acid sequence of SEQ ID NO:16, the F beta strand consists of the amino acid sequence of SEQ ID NO:17, and the G beta strand consists of the amino acid sequence of SEQ ID NO:18.

13. The isolated nucleic acid molecule of claim 9, wherein the Tn3 scaffold comprises two CD40L-specific monomer subunits connected in tandem by a peptide linker.

14. The isolated nucleic acid molecule of claim 13, wherein each of the two CD40L-specific monomer subunits comprise the amino acid sequence of one of SEQ ID NOs: 20, 22, 24, 26, 28, 30, 32, 34, 36, 40, 42, or 146.

15. The isolated nucleic acid molecule of claim 14, wherein the heterologous moiety is HSA or the HSA FcRn binding portion.

16. The isolated nucleic acid molecule of claim 15, wherein the Tn3 scaffold fusion protein comprises the amino acid sequence as shown in SEQ ID NO:135 or 145.

17. An expression vector comprising the nucleic acid of claim 1.

18. A host cell comprising the expression vector of claim 17.

19. A method of producing a Tn3 scaffold comprising: culturing the host cell of claim 18 under conditions in which the Tn3 scaffold encoded by the nucleic acid molecule is expressed.

20. The isolated nucleic acid molecule of claim 4, wherein the BC loop comprises the amino acid sequence of SEQ ID NO:86, the DE loop comprises the amino acid

sequence of SEQ ID NO:96, and the FG loop comprises the amino acid sequence of SEQ ID NO:139.

21. The isolated nucleic acid molecule of claim 14, wherein each of the two CD40L-specific monomer subunits comprise the amino acid sequence of SEQ ID NO:146.

22. The isolated nucleic acid molecule of claim 16, wherein the Tn3 scaffold fusion protein comprises the amino acid sequence as shown in SEQ ID NO: 145.