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~~ is 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, ~~both of which are herein incorporated by reference in their entireties~~.

[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.

[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~~., incorporated herein by reference~~.

[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

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~~, which are incorporated by reference in their entireties~~.

[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~~, both of which are incorporated by reference in their entireties~~. 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.

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.

~~**[0411]** All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.~~

EXAMPLES

~~**[0412]**~~**[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

~~**[0413]**~~**[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

~~[0414]~~[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

~~[0415]~~[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

~~[0416]~~[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.

~~[0417]~~[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

~~[0418]~~[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.

~~[0419]~~[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.

~~[0420]~~[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°C with shaking, phage were harvested by PEG precipitation from the overnight culture media.

~~[0421]~~[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.

~~[0422]~~[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.

~~[0423]~~[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.

~~[0424]~~[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 37°C, 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.

~~[0425]~~[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.

~~[0426]~~[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.

~~[0427]~~[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.

~~[0428]~~[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 37°C to an optical density (O.D.) of 0.5-0.8 then induced with 0.2 mM IPTG. After shaking at 37°C 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).

~~[0429]~~[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.

~~[0430]~~[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.

~~[0431]~~[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.

~~[0432]~~[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).

~~[0433]~~[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).

~~[0434]~~[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

~~[0435]~~[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

~~[0436]~~[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

~~[0437]~~[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.

~~[0438]~~[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 KpnI 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.

~~[0439]~~[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'-ACCGCGCTGATTACCTGGTCT1213111GGCTGTGA ACTGACCTATGGCATTAAAGATG	190
BC 9-loop NNK	5'-ACCGCGCTGATTACCTGGNNKNNKSMGNNKGSTN NKNNKNNKGGCTGTGAACTGACCTATGGCATTAAA-3'	191
309 BC-loop NNKdope	5'-ACCGCGCTGATTACCTGG76K45K45K77K44K65K78 T45K44KTGTGAACTGACCTATGGCATTAAA-3'	192
DE PCR	5'- GATGTGCCGGGCGATCGCACCACCATAGATCTG1 11111TATAGCATTGGTAACCTGAAACCGG-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

~~[0440]~~[0439] The NcoI-KpnI fragments were cloned into the phage display vector, and phage library generated as described in Example 1.

~~[0441]~~[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..

~~[0442]~~[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.

~~[0443]~~[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

~~[0444]~~[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.

~~[0445]~~[0444] Affinity measurements were performed on the ProteOn XPR36 protein interaction array system (Bio-Rad, Hercules, CA) with GLC sensor chip at 25°C. 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.

~~[0446]~~[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.

~~[0447]~~[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) ~~[0448]~~[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).

~~[0449]~~[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.

~~[0450]~~[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.

~~[0451]~~[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'- ACCGCGCTGATTACCTGG26T25TV1T46T46T45T45T25 T37T35TGGCTGTGAACTGACCTATGGCATTAAA-3'	197
BC11-311NHT	5'-ACCGCGCTGATTACCTGG26T25TV1T46T46T45T45T25 T37T35TNHTTGTGAACTGACCTATGGCATTAAA-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

~~[0452]~~[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.

~~[0453]~~[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.

~~[0454]~~[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

~~[0455]~~[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.

~~[0456]~~[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.

~~[0457]~~[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

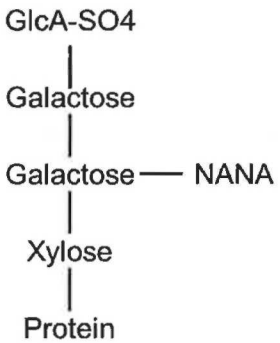
~~[0458]~~[0457] Tn3 constructs fused to HSA as outlined in FIG. 2A and 9A were expressed in mammalian 293F cell line by transient transfection. Tn3-HSA fusion expression constructs were generated based on an in-house generated mammalian expression vector. To increase product homogeneity, a mutant form 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).

~~[0459]~~[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.

~~[0460]~~[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.

~~[0461]~~[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:



~~[0462]~~[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

~~[0463]~~[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).

~~[0464]~~[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.

~~[0465]~~[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).

~~[0466]~~[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.

~~[0467]~~[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).

~~[0468]~~[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.

~~[0469]~~[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

~~[0470]~~[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.

~~[0471]~~[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).

~~[0472]~~[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).

~~[0473]~~[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).

~~[0474]~~[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.

~~[0475]~~[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.

~~[0476]~~[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).

~~[0477]~~[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.

~~[0478]~~[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).

~~[0479]~~[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.

~~[0480]~~[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).

~~[0481]~~[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).

~~[0482]~~[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.

~~[0483]~~[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.

~~[0484]~~[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.

~~[0485]~~[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.

~~[0486]~~[0485] One of the functional consequences of the CD40L:CD40 interaction is the up regulation of co-stimulatory molecules on B cells (Yellin *et 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).

~~[0487]~~[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.

~~[0488]~~[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.

~~[0489]~~[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).

~~[0490]~~[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.

~~[0491]~~[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.

~~[0492]~~[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.

~~[0493]~~[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.

~~[0494]~~[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).

~~[0495]~~[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.

~~[0496]~~[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

~~[0497]~~[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.

~~[0498]~~[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.

~~[0499]~~[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.

~~[0500]~~[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

~~[0501]~~[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.

~~[0502]~~[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.

~~[0503]~~[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

~~[0504]~~[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 0.5° were collected at crystal-to-detector distance of 300 mm and an exposure time of 0.8 seconds.

~~[0505]~~[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 1° were collected at crystal-to-detector distance of 300 mm and an exposure time of 0.8 seconds.

~~[0506]~~[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

~~[0507]~~[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.

~~[0508]~~[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.

~~[0509]~~[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.

~~[0510]~~[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.

~~[0511]~~[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.

~~[0512]~~[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=98.22°, resolution 1.9Å. The asymmetric unit contains two CD40 trimers, six 342 monomers, and six 311K4E-12 monomers.

~~[0513]~~[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=98.22°
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

~~[0514]~~[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.

~~[0515]~~[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.

~~[0516]~~[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.

~~[0517]~~[0516] The crystal structure of a 342 with CD40L is provided in Figure 20 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.

~~[0518]~~[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 21). The contacts for each of the separate scaffolds (as described above) are maintained.

~~[0519]~~[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.

~~[0520] All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.~~

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

WHAT IS CLAIMED IS:

1. 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 a CD40L epitope comprising amino acids located at positions 142 to 155, 200 to 230, or 247 to 251 of SEQ ID NO: 2.

2. The Tn3 scaffold of claim 1, comprising a single CD40L-specific monomer subunit.

3. The Tn3 scaffold of claim 1, comprising two CD40L-specific monomer subunits connected in tandem.

4. The Tn3 scaffold of claim 3, wherein the two CD40L-specific monomer subunits are directly connected.

5. The Tn3 scaffold of claim 3, wherein the two CD40L-specific monomer subunits are connected by a linker.

6. The Tn3 scaffold of claim 5, wherein the linker comprises a peptide linker.

7. The Tn3 scaffold of claim 6, wherein the peptide linker is a flexible peptide linker.

8. The Tn3 scaffold of claim 7, wherein the peptide linker comprises a $(G_mX)_n$ sequence wherein:

   (a) X is Serine (S), Alanine (A), Glycine (G), Leu (L), Isoleucine (I), or Valine (V);

   (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.

9. The Tn3 scaffold of claim 8, wherein the peptide linker comprises SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 142 or SEQ ID NO: 143.

10. The Tn3 scaffold of claim 3, wherein the binding of the scaffold to CD40L is improved over that of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

11. The Tn3 scaffold of claim 3, wherein the binding of the scaffold to CD40L improves the action on the target over that of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

12. The Tn3 scaffold of claim 10, wherein the improvement in binding to CD40L is an improvement in binding affinity, an improvement in binding avidity, or both.

13. The Tn3 scaffold of claim 10 wherein binding affinity for CD40L and protein stability are improved over those of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

14. The Tn3 scaffold of claim 10, wherein binding avidity for CD40L and protein stability are improved over those of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

15. The Tn3 scaffold of any one of claims 1-9, wherein at least one CD40L-specific monomer subunit is bound to a linker, or to a heterologous moiety.

16. The Tn3 scaffold of claim 15, wherein a linker or a heterologous moiety is conjugated to the N-terminus or the C-terminus of the scaffold.

~~17. The Tn3 scaffold of claim 15 or 16, wherein the linker comprises a peptide linker.~~

~~18. The Tn3 scaffold of claim 17, wherein the peptide linker is a flexible peptide linker.~~

~~19. The Tn3 scaffold of claim 17 or 18, wherein the peptide linker comprises a $(G_mX)_n$ sequence wherein:~~

~~(a) X is Serine (S), Alanine (A), Glycine (G), Leucine (L), Isoleucine (I), or Valine (V);~~

~~(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.~~

~~20. The Tn3 of claim 19, wherein the peptide linker comprises SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 142, or SEQ ID NO: 143.~~

~~21.~~17. The Tn3 scaffold of claim 15, wherein the linker comprises a functional moiety.

~~22.~~18. The Tn3 scaffold of claim ~~21~~17, wherein the functional moiety is an immunoglobulin or a fragment thereof.

~~23.~~19. The Tn3 scaffold of claim ~~22~~18, wherein the immunoglobulin or fragment thereof comprises an Fc domain.

~~24.~~20. The Tn3 scaffold of claim ~~23~~19, wherein the Fc domain fails to induce at least one FcγR-mediated effector function.

~~25.~~21. The Tn3 scaffold of claim ~~24~~20, wherein said at least one FcγR-mediated effector function is ADCC.

~~26.~~22. The Tn3 scaffold of claim 15, wherein at least one CD40L-specific monomer subunit is bound to a heterologous moiety.

~~27. The Tn3 scaffold of claim 15 or 26, wherein the 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.~~

~~28. The Tn3 scaffold of claim 27, wherein at least one CD40L-specific monomer subunit is conjugated to PEG.~~

~~29. The Tn3 scaffold of claim 27, wherein at least one CD40L-specific monomer subunit is conjugated to an albumin.~~

30. The Tn3 scaffold of claim 29, wherein the albumin is human serum albumin (HSA).

31. The Tn3 scaffold of claim 30, wherein said HSA is a variant HSA.

32. The Tn3 scaffold of claim 31, wherein the amino acid sequence of said variant HSA is SEQ ID NO: 133.

33. The Tn3 scaffold of claim 31, wherein said variant HSA has at least one improved property compared with a native HSA or a native HSA fragment.

34. The Tn3 scaffold of claim 33, wherein the improved property is an altered plasma half-life compared with the plasma half-life of a native HSA or a native HSA fragment.

35. The Tn3 scaffold of claim 34, wherein 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.

36. The Tn3 scaffold of claim 34, wherein 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.

37. The Tn3 scaffold of any one of claims 31 or 33-36, wherein the HSA variant comprises at least one amino acid substitution in HSA domain III.

38. The Tn3 scaffold of any one of claims 31 or 33-37, wherein 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, 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 said Tn3 scaffold has a plasma half-life longer than the plasma half-life of a Tn3 scaffold not conjugated to said HSA variant.

39. The Tn3 scaffold of claim 38, wherein the 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.

40. The Tn3 scaffold of any one of claims 31 or 33-38, wherein 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.

41. The Tn3 scaffold of claim 40, wherein 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 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.

42.23. The Tn3 scaffold of any one of claims 3-14, comprising at least two identical CD40L-specific monomer subunits.

~~43. The Tn3 scaffold of any one of claims 3-15, comprising at least two different CD40L-specific monomer subunits.~~

~~44. The Tn3 scaffold of any one of the preceding claims, wherein said scaffold is a CD40 receptor agonist.~~

~~45. The Tn3 scaffold of any one of the preceding claims, wherein said scaffold is a CD40 receptor antagonist.~~

~~46. The Tn3 scaffold of any one of claims 3-14, wherein at least two CD40L-specific monomer subunits specifically bind to the same CD40L epitope.~~

~~47.~~24. The Tn3 scaffold of any one of claims 3-14, wherein at least two CD40L-specific monomer subunits specifically bind to different CD40L epitopes.

~~48.~~25. The Tn3 scaffold of claim ~~47~~24, wherein the different CD40L epitopes are non-overlapping epitopes.

~~49.~~26. The Tn3 scaffold of claim ~~47~~24, wherein the different CD40L epitopes are overlapping epitopes.

~~50. The Tn3 scaffold of any one of the preceding claims, wherein the scaffold binds to at least two CD40L molecules.~~

~~51. The Tn3 scaffold of any one of the preceding claims, wherein a single CD40L-specific monomer subunit binds to at least two CD40L molecules.~~

~~52.~~27. The Tn3 scaffold of claim 1, wherein the beta strands of at least one CD40L-specific monomer subunit have at least 90% sequence identity to the beta strands of SEQ ID NO: 3.

~~53.~~28. The Tn3 scaffold of claim 1, wherein the A beta strand comprises SEQ ID NO: 11 except for at least one mutation.

~~54.~~29. The Tn3 scaffold of claim 1, wherein the B beta strand comprises SEQ ID NO: 12 except for at least one mutation.

~~55.~~30. The Tn3 scaffold of claim 1, wherein the C beta strand comprises 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.

~~56.~~31. The Tn3 scaffold of claim 1, wherein the D beta strand comprises SEQ ID NO: 15 except for at least one mutation.

~~57.~~32. The Tn3 scaffold of claim 1, wherein the E beta strand comprises SEQ ID NO: 16 except for at least one mutation.

~~58.~~33. The Tn3 scaffold of claim 1, wherein the F beta strand comprises SEQ ID NO: 17 except for at least one mutation, and wherein the cysteine in SEQ ID NO: 17 is not substituted.

~~59.~~34. The Tn3 scaffold of claim 1, wherein the G beta strand comprises SEQ ID NO: 18 except for at least one mutation.

~~60. The Tn3 scaffold of any one of the preceding claims, wherein the CD40L-specific monomer subunit comprises 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:~~

~~(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.~~

~~61. The Tn3 scaffold of claim 60, 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.~~

~~62. The Tn3 scaffold claim 60, 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,~~

63. The Tn3 scaffold of claim 60, 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.

64. The Tn3 scaffold of claim 60, wherein the sequence of the FG loop comprises a sequence selected from the group consisting of SEQ ID NOs: 9, 99, 139, and 170.

65. The Tn3 scaffold of claim 60, 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.

66. The Tn3 scaffold of claim 60, 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.

67. The Tn3 scaffold of claim 60, wherein the sequence of the FG loop comprises a sequence selected from the groups consisting of SEQ ID NOs: 129, 130, and 177.

68. The Tn3 scaffold of claim 60, wherein:

(a) 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;

(b) 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;

(c) 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;

(d) 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;

(e) 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;

(f) 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;

(g) 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;

(h) 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;

(i) 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;

(j) 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;

(k) 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; or,

(l) 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.

69. The Tn3 scaffold of claim 60, wherein:

(a) 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;

(b) 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;

(c) 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;

(d) 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;

(e) 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;

(f) 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;

(g) 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;

(h) 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;

(i) 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;

(j) 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;

(k) 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;

(l) 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;

(m) 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;

(n) 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;

(o) 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;

(p) 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;

(q) 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;

(r) 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; or,

(s) 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.

70. The Tn3 scaffold of claim 69, wherein the AB loop comprises SEQ ID NO: 136.

71. The Tn3 scaffold of claim 68, wherein 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.

72. The Tn3 scaffold of claim 60, wherein the CD40L-specific monomer subunit comprises 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).

73. The Tn3 scaffold of claim 69, wherein 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.

74. The Tn3 scaffold of claim 60, wherein the CD40L-specific monomer subunit comprises 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).

75. A Tn3 scaffold comprising a sequence selected from the group consisting of SEQ ID NOs: 134, 135, 205, 206, 207 and 208.

76. A Tn3 scaffold consisting of a sequence selected from the group consisting of SEQ ID NO: 134, 135, 205, 206, 207 and 208.

77. A Tn3 scaffold comprising a sequence selected from the group consisting of SEQ ID NOs: 201, 202, 203, and 204.

78. A Tn3 scaffold consisting of a sequence selected from the group consisting of SEQ ID NOs: 201, 202, 203, and 204.

79. The Tn3 scaffold of any one of the preceding claims, wherein said CD40L is human CD40L.

80. The Tn3 scaffold of claim 79, wherein said CD40L is membrane bound CD40L (SEQ ID NO: 1), soluble CD40L (SEQ ID NO: 2), or a fragment thereof.

81. A Tn3 scaffold of any one of the preceding claims, wherein the scaffold binds CD40L and prevents binding of CD40L to CD40.

82. A Tn3 scaffold of any one of the preceding claims, wherein the scaffold binds CD40L and disrupts CD40 mediating signaling.

83. A method of altering an activity in a CD40L expressing cell comprising contacting the cell with a Tn3 scaffold of any one of the preceding claims, wherein the Tn3 scaffold binds CD40L and prevents binding of CD40L to CD40.

84. A method of altering CD40 mediated signaling response comprising contacting a CD40L expressing cell with a Tn3 scaffold of any one of the preceding claims, wherein the Tn3 scaffold binds CD40L and disrupts CD40 mediated signaling.

85. The method of claim 84, wherein the CD40 mediated signaling response is a T-dependent immune response.

86. The Tn3 scaffold of any one of the preceding claims, wherein the 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.

87. The Tn3 scaffold of any one of the preceding claims, wherein a CD40L-specific monomer subunit 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.

88. The Tn3 scaffold of any one of claims 1-61, 65-67, 69, 70, 73, 74, 77, and 78, wherein a CD40L-specific monomer subunit interacts with CD40L amino acids E142, Y146, M148, N151, L155, R200, R203, and E230.

89. The Tn3 scaffold of any one of claims 1-64, 68, 71, 72, 75, and 76, wherein a CD40L-specific monomer subunit interacts with CD40L amino acids R203, I204, V247, H249, and T251.

90. The Tn3 scaffold of any one of claims 1-64, 68, 71, 72, 75, and 76, wherein a CD40L-specific monomer subunit interacts with CD40L amino acids S245, V247, H249, and T251.

91. The Tn3 scaffold of claim 88, wherein CD40L amino acids E142, Y146, M148, N151, L155 are located in a first CD40L molecule, and amino acids R200, R203, and E230 are located in a second CD40L molecule.

92. The Tn3 scaffold of claim 89, wherein CD40L amino acids R203 and I204 are located in a first CD40L molecule, and CD40L amino acids V247, H249, and T251 are located in a second CD40L molecule.

93. An isolated nucleic acid molecule encoding a Tn3 scaffold of any one of the preceding claims.

94. An expression vector comprising the nucleic acid of claim 93.

95. A host cell comprising the vector of claim 94.

96. A method of producing a Tn3 scaffold comprising culturing the host cell of claim 95 under conditions in which the Tn3 scaffold encoded by the nucleic acid molecule is expressed.

97. A composition comprising a Tn3 scaffold of any one of the preceding claims and a pharmaceutically acceptable excipient.

98. Use of an effective amount of a composition of claim 96 in the manufacture of a medicament for preventing, treating, ameliorating, or managing autoimmune disease in a patient in need thereof.

99. Use of a therapeutically effective amount of the composition of claim 93 in the manufacture of a medicament for reducing the frequency or quantity of corticosteroid administered to a patient with an autoimmune disease.

100. The use of any one of claims 98 or 99, wherein the autoimmune disease is selected from the group consisting of 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.

101. The use of any one of claims 98 or 99, wherein the autoimmune disease is Systemic Lupus Erythematosus (SLE).

102. The use of any one of claims 98 or 99, wherein said use further comprises an additional therapy, wherein said therapy is immunotherapy, biological therapy, chemotherapy, radiation therapy, or small molecule drug therapy.

103. 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.

104. The protein crystal of claim 103, wherein the asymmetric unit of the crystal comprises a trimer of CD40L and three molecules of Tn3 scaffold.

105. The protein crystal of claim 103, wherein the crystal diffract X-rays for a determination of structure coordinates to a resolution of a value equal to or less that 3.2 Å.

106. 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.

107. The protein crystal of claim 106, wherein the asymmetric unit of the crystal comprises one CD40L molecule and one Tn3 scaffold molecule.

108. The protein crystal of claim 106, wherein the crystal diffract X-rays for a determination of structure coordinates to a resolution of a value equal to or less that 2.7 Å.

109. 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.

110. The protein crystal of claim 109, wherein the asymmetric unit of the crystal comprises a one CD40L molecule and one Tn3 scaffold molecule.

111. The protein crystal of claim 109, wherein the crystal diffract X-rays for a determination of structure coordinates to a resolution of a value equal to or less that 2.8 Å.

112. 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.

113. The protein crystal of claim 112, wherein the asymmetric unit of the crystal comprises two CD40L trimers and six of each Tn3 scaffold molecule.

114. The protein crystal of claim 112, wherein the crystal diffract X-rays for a determination of structure coordinates to a resolution of a value equal to or less that 1.9Å

115. The protein crystal of any one of claims 103-114, wherein the protein is crystallized by sitting-drop vapor diffusion.

116. 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.

117. The method of claim 116, wherein the protein crystal is produced by sitting-drop vapor diffusion.

118. The method of claim 116, wherein the Tn3 scaffold comprises SEQ ID NO: 20, SEQ ID NO: 28, SEQ ID NO: 68 or SEQ ID NO: 146.

119. 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.

120. The machine-readable data storage medium of claim 119, wherein the Tn3 scaffold comprises SEQ ID NO: 20, SEQ ID NO: 28,SEQ ID NO: 68 or SEQ ID NO: 146.

121. The machine-readable data storage medium of claim 119, wherein the protein crystal is the crystal of claim 103, the crystal of claim 106, the crystal of claim 118, or any combination thereof.