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(54) Title: THERAPEUTIC TNFR-1B TARGETS AND USES THEREOF

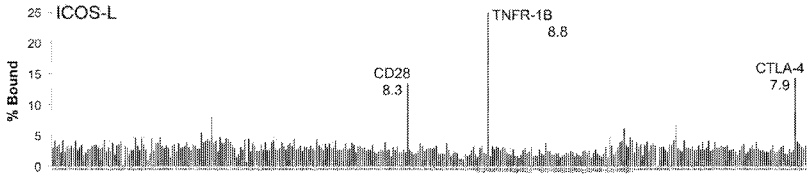

FIG. 1A

(57) Abstract: Disclosed are tumor necrosis factor receptor 1B (TNFR-1B) signaling targets and TNFR-1B mutants and their uses for treatment of diseases and disorders.

THERAPEUTIC TNFR-1B TARGETS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/243,688, filed on October 20, 2015, the contents of which are herein incorporated by reference in their entirety into the present application.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under grant numbers GM094662 and GM094665 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Throughout this application various publications are referred to in brackets. Full citations for these references may be found at the end of the specification. The disclosures of these publications are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

[0004] The present invention addresses the need for improved compounds for treating diseases or disorders such as rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, Crohn's disease, ulcerative colitis, inflammatory bowel disease, and other autoimmune and inflammatory diseases.

SUMMARY OF THE INVENTION

[0005] Methods are provided for screening for a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis, inflammatory bowel disease and inflammatory disease, the methods comprising testing the compound to determine if the compound modulates the interaction between one or more of TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and B7-1 and ISLR2, wherein a compound that is tested and determined to modulate the interaction between one or more of TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and B7-1 and ISLR2 is a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing

spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis, inflammatory bowel disease and inflammatory disease.

[0006] Methods are also provided for screening for a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease, the methods comprising testing a modified TNFR-1B compound or mutant to determine if the modified TNFR-1B compound or mutant has modified binding affinity and/or selectivity for one or more of ICOS-L, MadCAM-1 and ISLR2, wherein a modified TNFR-1B compound or mutant that is tested and determined to have modified binding affinity andor selectivity for one or more of ICOS-L, MadCAM-1 and ISLR2 is a candidate compound or mutant for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease.

[0007] Also provided are mutants of TNFR-1B that modulate the binding of TNFR-1B to one or more of TNFα, ICOS-L and MadCAM-1.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Fig. 1A. Identification of TNFR-1B ligands in a cell-cell based screen against Ig and TNFR superfamily members. Cells expressing ICOS-L were used to challenge a library consisting of ~400 Ig and TNFR superfamily members expressed as GFP fusions. Cell-cell complexes were analyzed by flow cytometery and the percent bound (GFP/mCherry double positive events divided by the total number of cells) was determined across all 400 targets. The numbers below each gene name are the statistical Z-scores, with a Z-score of 2.8 being equivalent to a P-value of 0.005. Z-scores were calculated by determining the average (μ) and std dev (σ) for the % bound across each 96-well plate and using the formula Abs $z = (x - \mu)/\sigma$. Besides two known receptors of ICOS-L, namely CD28 and CTLA-4, an additional interaction with TNFR-1B was identified.

[0009] Fig. 1B. Identification of TNFR-1B ligands in a cell-cell based screen against Ig and TNFR superfamily members. Screening the same library as in Fig. 1A with TNFR-1B-mCHERRY expressing cells identified ICOS-L, validating the original screen, as well as two additional interacting proteins, namely MadCAM-1 and ISLR2.

[0010] Fig. 1C. Identification of TNFR-1B ligands in a cell-cell based screen against Ig and TNFR superfamily members. Screening with B7-1 expressing cells identified known interactions with CD28, CTLA-4 and NGFR, and an interaction with ISR-L2.

[0011] Fig. 1D. Identification of TNFR-1B ligands in a cell-cell based screen against Ig and TNFR superfamily members. Screening with NGFR expressing cells identified the know interaction with B7-1, and interactions with ISLR and PTPR-F.

[0012] Fig. 2A. Validation of TNFR-1B interactions using full-length genes containing *bona fide* native transmembrane and cytoplasmic domains. A panel of genes expressed as GFP fusions in HEK 293 cells were challenged with full-length native gene-mCherry fusions of TNFR-1B query. Cell-cell complexes were analyzed by FACS to determine the percent bound calculated as the number of GFP & mCherry double positive events divided by the total number of cell events. Data shows the average percent bound for three independent experiments.

[0013] Fig. 2B. Validation of TNFR-1B interactions using full-length genes containing *bona fide* native transmembrane and cytoplasmic domains. A panel of genes expressed as GFP fusions in HEK 293 cells were challenged with full-length native gene-mCherry fusions of ICOS-L query. Cell-cell complexes were analyzed by FACS to determine the percent bound calculated as the number of GFP & mCherry double positive events divided by the total number of cell events. Data shows the average percent bound for three independent experiments.

[0014] Fig. 2C. Validation of TNFR-1B interactions using full-length genes containing *bona fide* native transmembrane and cytoplasmic domains. A panel of genes expressed as GFP fusions in HEK 293 cells were challenged with full-length native gene-mCherry fusions of MadCAM-1 query. Cell-cell complexes were analyzed by FACS to determine the percent bound calculated as the number of GFP & mCherry double positive events divided by the total number of cell events. Data shows the average percent bound for three independent experiments.

[0015] Fig. 3. ENBREL® protein binds to cells expressing TNF-alpha, ICOS-L, MadCAM-1 and ISLR2. ENBREL® protein was used to challenge cells expressing GFP fusions of CD28 (-control), TNF-alpha (+control), ICOS-L, MadCAM-1 or ISLR2. Bound ENBREL® was detected using a goat anti-human Alexa 594-labeled secondary antibody and samples were analyzed by flow cytometry. Data shows the geometric mean of FL4 (Alexa 594) for all live GFP positive cells. At low ENBREL® concentrations (2.5 ug ≈ 100

nM) binding was observed to TNF-alpha and ICOS-L, while binding was observed to MadCAM-1 and ISLR2 at higher ENBREL® concentrations (25 ug and 100 ug).

[0016] Fig. 4A-4C. Validation of ENBREL® and ICOS-L binding using a two different protein binding assays. A) A panel of 12 different mCherry constructs were transiently transfected into HEK 293 cells. Three days post transfection cells were challenged in parallel with either soluble Fc-fusion protein or microbeads coated with the same protein. The ICOS-L Fc (IgG2a) and control protein were expressed in HEK 293 cells using transient transfection and subsequently purified by Ni^{2+}-NTA and gel filtration chromatography. Anti-human-IgG Alexa-Fc 488 secondary antibody was used to detect soluble protein and microbeads were spiked with FITC Fc to label them green. Data show the percent of mCherry positive HEK cells bound to either protein or microbeads and are the average of two independent experiments. B) FACS scatter plots showing mCherry signal (y-axis) and Alexa 488 signal (x-axis) from one replicate of the soluble protein binding experiment (i.e. data in A represents two independent experiments). C) Same as in B but showing data from one replicate of the microbead binding experiment.

[0017] Fig. 5A-5E. Identification of TNFR-1B mutants with modulated affinities and selectivties for ligands. A) A panel of TNFR-1B mutants was generated by site-directed mutagenesis of surface accessible positions within the ectodomain of TNFR-1B. The TNFR-1B mutants were transiently expressed as mCherry fusions in HEK 293 and subsequently challenged with cells expressing GFP fusions of cell-surface displayed TNFα, ICOS-L or MadCAM-1. Data show the bound events as a fraction of all mCherry positive cells (mutant expression) and are the average of two independent experiments. B) The crystal structure of the TNFR-1B/ TNFα complex (PDB: 3ALQ) highlighting residues of interest identified in the mutant screen that effect binding of three TNFR-1B ligands (TNFα, ICOS-L and MadCAM-1). For comparison, the chart shows the average fraction bound (from the screen) for the mutants identified. C) The same as in B highlighting TNFR-1B mutants that effect binding of ICOS-L and MadCam-1 but not TNFα. D) The same as in B highlighting mutations that effect MadCAM1 binding only. E) The same as in B showing one mutant that effected TNFα and ICOS-L but not MadCAM1.

DETAILED DESCRIPTION OF THE INVENTION

[0018] The present invention provides a method for screening for a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis,

inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease, the method comprising testing the compound to determine if the compound modulates the interaction between one or more of TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and B7-1 and ISLR2, wherein a compound that is tested and determined to modulate the interaction between one or more of TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and B7-1 and ISLR2 is a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease.

[0019] Binding between TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and/or B7-1 and ISLR2 can be determined in the presence of the candidate compound and in the absence of the candidate compound, where a change in the binding between TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and/or B7-1 and ISLR2 in the presence of the candidate compound indicates that the candidate compound modulates the interaction between TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and/or B7-1 and ISLR2.

[0020] The compound can be, for example, a non-naturally occurring small molecule of 2,000 daltons or less, or an antibody or an antibody fragment.

[0021] Also provided is a method for screening for a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease, the method comprising testing a modified TNFR-1B compound or mutant to determine if the modified TNFR-1B compound or mutant has modified binding affinity for one or more of ICOS-L, MadCAM-1 and ISLR2, wherein a modified TNFR-1B compound or mutant that is tested and determined to have modified binding affinity for one or more of ICOS-L, MadCAM-1 and ISLR2 is a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease.

[0022] In some embodiments, the modified TNFR-1B compound or mutant recognizes TNF-alpha, but not one or more of ICOS-L, MadCAM-1 and ISLR2. In some embodiments, the modified TNFR-1B compound or mutant recognizes ICOS-L, but not one or more of TNF-alpha, MadCAM-1 and ISLR2. In some embodiments, the modified TNFR-1B compound or mutant recognizes MadCAM-1, but not one or more of TNF-alpha, ICOS-L, and ISLR2. In some embodiments, the modified TNFR-1B compound or mutant recognizes ISLR2, but not one or more of TNF-alpha, ICOS-L, and MadCAM-1. In some embodiments, the modified TNFR-1B compound or mutant recognizes ICOS-L and MadCAM-1, but not TNF-alpha or ISLR2. In some embodiments, the compound or mutant has enhanced affinities for all ligands compared to TNFR-1B. In some embodiments, the compound or mutant has enhanced affinities for some ligands compared to TNFR-1B. In some embodiments, the compound or mutant has reduced affinities for some ligands compared to TNFR-1B. In some embodiments, the compound or mutant has enhanced affinities for some ligands compared to TNFR-1B and reduced affinities for some ligands compared to TNFR-1B. In some embodiments, the compound or mutant recognizes only one ligand.

[0023] In one embodiment, the fusion protein that links the protein for tumor necrosis factor (TNF) receptor 1B (TNFR-1B) to the protein for Immunoglobulin (Ig)G1 Fc is encoded by the following nucleic acid sequence:

ATGGGCGTGCACGAGTGCCCCGCCTGGCTGTGGCTGCTGCTGAGCCTGCTGAGTCTACCTCTCGGCC
TGCCTGTGCTAGGCTTGCCCGCCCAGGTGGCATTTACACCCTACGCCCCGGAGCCCGGGAGCACATG
CCGGCTCAGAGAATACTATGACCAGACAGCTCAGATGTGCTGCAGCAAATGCTCGCCGGGCCAACAT
GCAAAAGTCTTCTGTACCAAGACCTCGGACACCGTGTGTGACTCCTGTGAGGACAGCACATACACCC
AGCTCTGGAACTGGGTTCCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAAAC
TCAAGCCTGCACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGC
AAGCAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCGCAAGTGCCGCCCGGGCTTCGGCGTGGCCAGAC
CAGGAACTGAAACATCAGACGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCCAACACGACTTC
ATCCACGGATATTTGCAGGCCCCACCAGATCTGTAACGTGGTGGCCATCCCTGGGAATGCAAGCATG
GATGCAGTCTGCACGTCCACGTCCCCCACCCGGAGTATGGCCCCAGGGGCAGTACACTTACCCCAGC
CAGTGTCCACACGATCCCAACACACGCAGCCAACTCCAGAACCCAGCACTGCTCCAAGCACCTCCTT
CCTGCTCCCAATGGGCCCCAGCCCCCCAGCTGAAGGGAGCACTGGCGACGAGCCCAAATCTTGTGAC
AAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCC
CCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGT
GAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAG

ACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACC
AGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGA
GAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGG
GAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCG
CCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTC
CGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTC
TTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCCC
CGGGTAAATAG (SEQ ID NO: 1).

[0024] In one embodiment, the amino acid sequence of the TNFR-1B Fc fusion is:

MGVHECPAWLWLLLSLLSLPLGLPVLGLPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQH
AKVFCTKTSDTVCDSCEDSTYTQLWNWVPECLSCGSRCSSDQVETQACTREQNRICTCRPGWYCALS
KQEGCRLCAPLRKCRPGFGVARPGTETSDVVCKPCAPGTFSNTTSSTDICRPHQICNVVAIPGNASM
DAVCTSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLLPMGPSPPAEGSTGDEPKSCD
KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV
FSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:2).

[0025] The nucleic acid sequence of the TNFR-1B construct used as the template for mutagenesis is:

ACCATGGGCGTGCACGAGTGCCCCGCCTGGCTGTGGCTGCTGCTGAGCCTGCTGAGTCTACCTCTCG
GCCTGCCTGTGCTAGGCTTGCCCGCCCAGGTGGCATTTACACCCTACGCCCCGGAGCCCGGGAGCAC
ATGCCGGCTCAGAGAATACTATGACCAGACAGCTCAGATGTGCTGCAGCAAATGCTCGCCGGGCCAA
CATGCAAAAGTCTTCTGTACCAAGACCTCGGACACCGTGTGTGACTCCTGTGAGGACAGCACATACA
CCCAGCTCTGGAACTGGGTTCCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGA
AACTCAAGCCTGCACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTG
AGCAAGCAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCGCAAGTGCCGCCCGGGCTTCGGCGTGGCCA
GACCAGGAACTGAAACATCAGACGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCCAACACGAC
TTCATCCACGGATATTTGCAGGCCCCACCAGATCTGTAACGTGGTGGCCATCCCTGGGAATGCAAGC
ATGGATGCAGTCTGCACGTCCACGTCCCCCACCCGGAGTATGGCCCCAGGGGCAGTACACTTACCCC
AGCCAGTGTCCACACGATCCCAACACACGCAGCCAACTCCAGAACCCAGCACTGCTCCAAGCACCTC
CTTCCTGCTCCCAATGGGCCCCAGCCCCCCAGCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTT
GGACTGATTGTGGGTGTGACAGCCTTGGGTCTACTAATAATAGGAGTGGTGAACTGTGTCATCATGA
CCCAGGTGAAAAAGAAGCCCTTGTGCCTGCAGAGAGAAGCCAAGGTGCCTCACTTGCCTGCCGATAA
GGCCCGGGGTACACAGGGCCCCGAGCAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGC
TCCCTGGAGAGCTCGGCCAGTGCGTTGGACAGAAGGGCGCCCACTCGGAACCAGCCACAGGCACCAG
GCGTGGAGGCCAGTGGGGCCGGGGAGGCCCGGGCCAGCACCGGGAGCTCAGATTCTTCCCCTGGTGG
CCATGGGACCCAGGTCAATGTCACCTGCATCGTGAACGTCTGTAGCAGCTCTGACCACAGCTCACAG

TGCTCCTCCCAAGCCAGCTCCACAATGGGAGACACAGATTCCAGCCCCTCGGAGTCCCCGAAGGACG
AGCAGGTCCCCTTCTCCAAGGAGGAATGTGCCTTTCGGTCACAGCTGGAGACGCCAGAGACCCTGCT
GGGGAGCACCGAAGAGAAGCCCCTGCCCCTTGGAGTGCCTGATGCTGGGATGAAGCCCAGTGGTGGC
GGAAGCGAGAACCTGTACTCCAGT (SEQ ID NO:3).

[0026] The amino acid sequence of the full-length TNFR-1B cDNA is: MAPVAVWAALAVGLELWAAAHALPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQHAKVFC TKTSDTVCDSCEDSTYTQLWNWVPECLSCGSRCSSDQVETQACTREQNRICTCRPGWYCALSKQEGC RLCAPLRKCRPGFGVARPGTETSDVVCKPCAPGTFSNTTSSTDICRPHQICNVVAIPGNASMDAVCT STSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLLPMGPSPPAEGSTGDFALPVGLIVGV TALGLLIIGVVNCVIMTQVKKKPLCLQREAKVPHLPADKARGTQGPEQQHLLITAPSSSSSSLESSA SALDRRAPTRNQPQAPGVEASGAGEARASTGSSDSSPGGHGTQVNVTCIVNVCSSSDHSSQCSSQAS STMGDTDSSPSESPKDEQVPFSKEECAFRSQLETPETLLGSTEEKPLPLGVPDAGMKPS (SEQ ID NO:4). Note that the numbering of the mutagenesis positions start after removal of the underlined signal peptide. Therefore, position 1 = L, position 2 = P, position 3 = A, etc.

[0027] The compound or mutant can also be a candidate for treating any disease or disorder mediated by TNF.

[0028] Also provided are mutants of TNFR-1B that modulates the binding of TNFR-1B to one or more of TNFα, ICOS-L and MadCAM-1. Such mutants include, for example, mutants K42D, T48A, N171D, S79D, R113D, L114A, R119D, K120D, D58A, R19D, S59D, L64D, R77A, S107D, R119A, K120A, R129A, V138D, K140A, I156D, I168D, N171A and M174D. Mutants K42D, T48A and N171D, for example, compared to TNFR-1B, have reduced binding to TNFα, ICOS-L and MadCAM-1. Mutants S79D, R113D, L114A, R119D and K120D, for example, compared to TNFR-1B, have reduce binding to ICOS-L and MadCAM-1, but not to TNFα. Mutant D58A, for example, compared to TNFR-1B, has reduced binding to TNFα and ICOS-L, but not to MadCAM-1. Mutants R19D, S59D, L64D, R77A, S107D, R119A, K120A, R129A, V138D, K140A, I156D, I168D, N171A and M174D, for example, compared to TNFR-1B, predominately have reduced binding to MadCAM-1.

[0029] Also provided are fusion proteins comprising mutants of TNFR-1B that modulate the binding of TNFR-1B to one or more of TNFα, ICOS-L and MadCAM-1, and an immunoglobulin Fc sequence. In an embodiment, the immunoglobulin is an IgG. In an embodiment, the IgG is an IgG1 or IgG2 or IgG3 or IgG4 or IgM. Preferably, the immunoglobulin is IgG1. Preferably, the Fc domain has the same sequence or 95% or greater sequence identity with a human IgG1 Fc domain. Immunoglobulin Fc sequences are

well known in the art. In an embodiment, the term "Fc sequence" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc sequence of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. In an embodiment, the C-terminal lysine of the Fc may be removed, for example, during production or purification, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody.

[0030] In a fusion protein, the presence of the Fc domain markedly increases the plasma half-life of the attached protein, which prolongs therapeutic activity. In addition, the Fc domain also enables the fusion protein to interact with Fc-receptors.

[0031] In an embodiment, the Fc domain is linked via a peptide linker that permits flexibility. In an embodiment, the linker is rigid. In an embodiment the linker is cleavable. Non-limiting examples of flexible linkers are Gn, and GGGGS, and (GGGGS)n where n = 2, 3, 4 or 5. Non-limiting examples of rigid linkers are (EAAAK)n, (XP)n. Non-limiting examples of cleavable linkers include disulfide links and protease cleavable linkers.

[0032] In an embodiment, the fusion protein described herein is recombinantly produced. In an embodiment, the fusion protein is produced in a eukaryotic expression system. In an embodiment, the fusion protein produced in the eukaryotic expression system comprises glycosylation at a residue on the Fc portion.

[0033] Also provided are pharmaceutical compositions comprising any of the fusion proteins disclosed herein and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art.

[0034] Also provided are methods for treating a subject with a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease, the methods comprising administering to the subject any of the fusion proteins disclosed herein in an amount effective to alleviate a sign or symptom of the disease or disorder.

[0035] This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specifics discussed

are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Introduction

[0036] Etanercept (ENBREL®) is a fusion protein that links the protein for tumor necrosis factor (TNF) receptor 1B (TNFR-1B) to the protein for Immunoglobulin (Ig)G1 Fc. ENBREL® is a leading anti-inflammatory drug. This disclosure describes interactions and pathways that are of immediate therapeutic importance and provides strategies for the design of ENBREL® variants with engineered selectivities, which recognize only a subset of its potential ligands, or exhibit increased or reduced affinities for all or subsets of its potential ligands, for the realization of more effective biologics with reduced side effects.

Methods and Results

[0037] Data are provided showing results from cell-cell Fluorescence-Activated Cell Sorting (FACS)-based screens utilizing a ~400 member secreted protein library, which contains most members of the human Immunoglobulin (Ig) superfamily and the human Tumor Necrosis Factor Receptor (TNFR) superfamily. In this library, all ectodomains were fused to the mouse PD-L1 transmembrane segment, with covalent linkage to cytoplasmic GFP as a proxy marker for expression. The human erythropoietin (EPO) signal sequence was used to direct secretion of all constructs. These screening efforts revealed a remarkable network of interactions, which demonstrate the coupling of a range of immune and neural regulatory pathways (Table 1). Screening with cells expressing ICOS-L resulted in the identification of two of its known binding partners, CD28 and CTLA-4 [1], as well as a novel interaction with TNFR-1B. Screening the library with cells expressing TNFR-1B resulted in the identification of three novel interacting proteins, ICOS-L, MadCAM-1 and ISLR2 (Fig. 1B), thus validating the TNFR-1B:ICOS-L interaction. The library was also screened with cells expressing MadCAM-1 in which only TNFR-1B was identified (again validating the initial screen, data not shown). Given the known interactions of ICOS-L with the CD28, CTLA-4 and ICOS immune receptors, the screen was expanded to include B7-1 and B7-2 (the ligands of CD28 and CTLA-4) as query molecules. These efforts identified ISLR2 as a novel B7-1 interacting protein and confirmed the previously reported B7-1:NGFR interaction (Fig. 1C). Importantly, most of the molecules identified in these new interactions possess additional previously defined interacting proteins, resulting in an

unanticipated network of interactions revealing previously unappreciated linkages between immune regulatory and neural development/function pathways (Table 1).

[0038] Further validation studies were performed for the TNFR-1B interactions. Cell-cell FACS assays using cells expressing full-length native versions (i.e., containing native transmembrane and cytoplasmic segments) of TNFR-1B, ICOS-L and MadCAM-1 were fully consistent with the original screen (Fig. 2). Furthermore, soluble ENBREL® protein binds to cells expressing all three of these novel ligands, as well as its well-characterized ligand TNF-alpha when expressed on cells (Fig. 3). Additionally, a soluble Fc-fusion construct of ICOS-L exhibited binding to cells expressing TNFR-1B, providing further validation of this interaction. These results indicate that the mechanism of ENBREL® is more complex than currently appreciated (i.e., TNF-alpha sequestration is not the sole contributing factor to the therapeutic function of ENBREL®) and provide the foundation for the generation of ENBREL® variants possessing modified affinities and selectivities with enhanced therapeutic potential and reduced side effects (e.g., the generation of TNFR-1B variants that solely recognize TNF-alpha, or mutants that recognize only one of the identified TNFR-1B-binding proteins (ICOS-L, MadCAM-1 or ISLR2) or mutants that exhibit increased affinities for some or all TNFR-1B-binding proteins, or mutants that exhibit reduced affinities for some ligands compared to TNFR-1B and enhanced affinities for some ligands compared to TNFR-1B).

[0039] Using a high-throughput cell-cell screen, ICOS-L, MadCAM-1 and ISLR2 were identified as three novel ligands of TNFR-1B. To further evaluate these interactions, two different *in vitro* assays that utilize purified protein reagents were used. The first was a soluble protein-binding assay in which Fc-fusion protein (IgG1 control, ENBREL® (a IgG1 fusion of TNFR-1B) or ICOS-L IgG1) was added directly to HEK 293 cells expressing a panel of cell surface receptors (Fig. 4A and 4B). The second was a microbead-binding assay in which the same Fc-fusion proteins were used to coat protein-A microspheres that were subsequently added to HEK 293 cells expressing the same panel of cell surface receptors (Fig. 4A and 4C). Soluble binding of ENBREL® was observed to cell surface expressed TNFα and to the newly identified target ICOS-L. Interestingly, when presented on microbeads, ENBREL® failed to bind to TNFα but showed improved binding to ICOS-L (compared to soluble protein). These data suggest that by modulating the valency of ENBREL®, a therapeutic derivative could be designed that affords enhanced recognition of ICOS-L. Binding was observed of soluble ENBREL® to cells expressing MadCAM-1 and

ISLR2 but only at a high concentration of ENBREL® (100μg) (Fig. 3). This suggests these interactions are either lower affinity or require a specific orientation that is only available in the context of cell surface expression. Indeed at lower ENBREL® concentrations (0.5 – 2.5 μg), binding was only observed to cells expressing ICOS-L and TNFα but not to cells expressing MadCAM-1 and increasing the avidity using microbeads did not rescue MadCAM-1 binding (Fig. 3 and 4). Soluble ICOS-L Fc-fusion protein bound to cells expressing all three of its previously reported receptors (CD28, CTLA-4 and ICOS), as well as to cells expressing TNFR-1B, further validating this interaction (Fig. 3 and 4). Unexpectedly, microbead-bound ICOS-L did not exhibit binding to cells expressing ICOS, and showed reduced binding to cells expressing CD28 and CTLA-4, but maintained binding to cells expressing TNFR-1B. This behavior indicates that these interactions are sensitive to specific orientations and overall orgnaizations, and support the design of deliberately engineered mutants with modified selectivity; i.e., reduced or enhanced affinities for all binding partners or a a subset of binding partners. This is demonstrated immiedately below.

[0040] It was also examined whether specific mutants of TNFR-1B could be identified that exhibited selective, enhanced and/or reduced recognition of its multiple ligands. A panel of 71 TNFR-1B point mutants was generated and screened for binding to TNFα, ICOS-L and MadCAM-1 using the cell-cell FACS assay (Fig. 5A). Three mutants (K42D, T48A and N171D) were identified that modulated binding to all three ligands (Fig. 5B); binding to TNFα and ICOS-L was reduced to ~50% of wild-type, while binding to MadCAM-1 was more significantly reduced. One mutant, D58A, showed reduced binding to TNFα and ICOS-L but not MadCAM-1 (Fig. 5E). However, five mutants (S79D, R113D, L114A, R119D, K120D) were identified that greatly reduced binding of TNFR-1B to ICOS-L and MadCAM-1, but which did not significantly modify recognition of TNFα (Fig. 5C). Two of the mutants R113D and L114A showed almost no binding to ICOS-L and MadCAM-1, while retaining near wild-type binding to TNFα. In addition, mutants were identified that predominately affected binding of MadCAM-1 (R19D, S59D, L64D, R77A, S107D, R119A, K120A, R129A, V138D, K140A, I156D, I168D, N171A, M174D) (Fig. 5D). This set of mutants, extending over much of the length of TNFR-1B, suggests that the MadCAM-1 recognition surface on TNFR-1B is more extensive than the recognition surfaces for ICOS-L and TNFα.

[0041] These data suggest that the binding sites for ICOS-L and MadCAM-1 on TNFR-1B overlap, at least in part, that of TNFα. They also demonstrate the feasibility of

generating TNFR-1B variants with engineered properties/selectivities (e.g., high selectivity for only TNFα, or other subsets of ligands with increased or decreased affinities).

Discussion

[0042] ICOS-L is the ligand for the inducible costimulatory molecule (ICOS), which controls a major T cell costimulatory pathway that represents a significant therapeutic target. ICOS-L is a major immune regulatory ligand expressed on monocytes, dendritic cells, and B cells, as well as other antigen presenting cells. Expression of ICOS-L on B-cells plays a significant role in the production of antibodies within the germinal center and is necessary for the development of rheumatoid arthritis [2, 3]. Expression of ICOS-L in dendritic cells has been increasingly associated with Crohn's disease and ulcerative colitis [4, 5]. Interestingly, in a subset of cell types (B-cells, monocytes, lung epithelial cells), TNF-alpha induces ICOS-L expression via activation of the NFKappaB pathway [6]. The present data demonstrating a direct interaction between ICOS-L and TNFR-1B suggests the presence of additional cross talk between the ICOS-L/ICOS and TNF-alpha/TNFR-1B pathways, which potentially impacts both therapeutic mechanisms and treatment strategies.

[0043] MadCAM-1, mucosal vascular addressin cell adhesion molecule 1, also known as addressin, is an endothelial cell adhesion molecule from the Ig superfamily that interacts preferentially with the leukocyte beta7 integrin LPAM-1 (alpha4/beta7), L-selectin, and VLA-4 (alpha4/beta1) on myeloid cells to direct leukocytes into mucosal and inflamed tissues [7]. MadCAM-1 expression is elevated in the intestinal tissue of both Crohn's disease and ulcerative colitis patients, but was more abundant and appeared in deeper tissues in patients with Crohn's disease [7]. The present results are the first indication of an association between MadCAM-1 and TNFR-1B. Notably, the ICOS-L [8-10] and MadCAM-1 [11] pathways are both themselves active targets for immunotherapy.

[0044] ISLR-2 (Immunoglobulin superfamily containing leucine-rich repeat protein 2) is believed to interact with TrkA and Ret receptor tyrosine kinases to regulate axonal extension, guidance and branching during neural development [13].

[0045] These interactions are of considerable clinical importance as soluble TNFR-1B is marketed as ENBREL®, a leading treatment for rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, and plaque psoriasis in adults, as well as juvenile idiopathic arthritis in children. The mechanism of action of ENBREL® is thought to be the targeting and binding of TNF, which results in the blockade of the TNF-mediated signaling pathways and an associated global inhibition of immune responsiveness [14, 15]. The identification of

these additional "off-target" interactions for TNFR-1B may provide new insights into the mechanisms of ENBREL® function, including its range of effective clinical indications and its considerable deleterious side effects, and offer the opportunity to develop "second generation ENBREL®s" with enhanced potency and reduced side effects. Furthermore, the involvement of both ICOS-L and MadCAM-1 in the onset of Crohn's disease, inflammatory bowel disease and ulcerative colitis suggests that development of new "ENBREL®" proteins with altered activity for one or both of these targets might create a therapeutic agent better suited to treat these diseases than the currently marketed ENBREL®, which has been proven less effective in their treatment of these particular diseases [12].

[0046] The present study identified new TNFR-1B interactors and demonstrated that TNFR-1B is a naturally occurring multi-specific receptor. This multi-specificity offers new opportunities for therapy (based, e.g., on the functional significance of interactions between TNFR-1B and TNFα, ICOS-L or MadCAM-1). The present approach provides the identification of new networks of interactions that impact this biology. These findings enable generation of TNFR-1B variants with novel properties and selectivities. This includes, but is not limited to, TNFR-1B variants which only recognize one ligand (e.g., TNFa, or MadCAM-1, or ICOS-L, or ISLR2), or with enhanced affinities for all ligands, or with reduced affinities for all ligands, or with enhanced affinities for some ligands (e.g., to provide more effective multi-specific reagents), or with reduced affinities for some ligands (e.g., to provide more effective multi-specific reagents). These variants can be identified by large-scale mutagenesis. Using single point mutants, it was demonstrated that considerable modulation of selectivity can be achieved (Fig. 5). Additional properties can be realized by incorporation of multiple point mutants and by a variety of selection strategies. Variants generated by manipulating valency can afford enhanced selectivity/avidity. This is based on the comparison of cell-cell vs microbead-cell data in Fig. 4. These altered biochemical properties can be translated into new therapeutic strategies and opportunities.

Table 1. Signaling networks defined by this disclosure.

Newly Discovered in this Disclosure	Previously Reported Interactions
TNFR-1B:ICOS-L	TNFR-1B:TNF-α ICOS-L:CD28; ICOS-L:CTLA-4; ICOS-L:ICOS
TNFR-1B:MadCAM-1	MadCAM-1:LPAM-1integrin; MadCAM-1:L-selectin; MadCAM-1:VLA-4
TNFR-1B:ISLR2	ISLR2:TrkA RTK; ISLR2:Ret RTK
B7-1:ISLR2	B7-1:CTLA-4; B7-1:CD28; B7-1:PD-L1; B7-1:NGFR

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What is claimed is:

1. A mutant of tumor necrosis factor receptor 1B (TNFR-1B) that modulates the binding of TNFR-1B to one or more of TNFα, ICOS-L and MadCAM-1, wherein the mutant is selected from the group consisting of mutants K42D, T48A, N171D, S79D, R113D, L114A, R119D, K120D, D58A, R19D, S59D, L64D, R77A, S107D, R119A, K120A, R129A, V138D, K140A, I156D, I168D, N171A and M174D.

2. The mutant of Claim 1, selected from the group consisting of mutants K42D, T48A and N171D that, compared to TNFR-1B, have reduced binding to TNFα, ICOS-L and MadCAM-1.

3. The mutant of Claim 1, selected from the group consisting of mutants S79D, R113D, L114A, R119D and K120D that, compared to TNFR-1B, have reduce binding to ICOS-L and MadCAM-1, but not to TNFα.

4. The mutant of Claim 1, which is mutant D58A that, compared to TNFR-1B, has reduced binding to TNFα and ICOS-L, but not to MadCAM-1.

5. The mutant of Claim 1, selected from the group consisting of mutants R19D, S59D, L64D, R77A, S107D, R119A, K120A, R129A, V138D, K140A, I156D, I168D, N171A and M174D that, compared to TNFR-1B, predominately reduce binding to MadCAM-1.

6. A fusion protein comprising the mutant of Claim 1 and an immunoglobulin Fc sequence.

7. The fusion protein of Claim 6, wherein the immunoglobulin is an IgG.

8. The fusion protein of Claim 6, wherein the immunoglobulin is IgG1.

9. A pharmaceutical composition comprising the fusion protein of Claim 6 and a pharmaceutically acceptable carrier.

10. A method for treating a subject with a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease, the method comprising administering to the subject the fusion protein of Claim 6 in an amount effective to alleviate a sign or symptom of the disease or disorder.

11. A method for screening for a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease, the method comprising testing the compound to determine if the compound modulates the interaction between one or more of TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and B7-1 and ISLR2, wherein a compound that is tested and determined to modulate the interaction between one or more of TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and B7-1 and ISLR2 is a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease.

12. The method of claim 11, wherein binding between TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and/or B7-1 and ISLR2 is determined in the presence of the candidate compound and in the absence of the candidate compound, and wherein a change in the binding between TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and/or B7-1 and ISLR2 in the presence of the candidate compound indicates that the candidate compound modulates the interaction between TNFR-1B and ICOS-L, TNFR-1B and MadCAM-1, TNFR-1B and ISLR2, and/or B7-1 and ISLR2.

13. The method of Claim 11 or 12, wherein the compound is a non-naturally occurring small molecule of 2,000 daltons or less.

14. The method of Claim 11 or 12, wherein the compound is an antibody or an antibody fragment.

15. A method for screening for a candidate compound for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease, the method comprising testing a modified TNFR-1B compound or mutant to determine if the modified TNFR-1B compound or mutant has modified binding affinity for one or more of ICOS-L, MadCAM-1 and ISLR2, wherein a modified TNFR-1B compound or mutant that is tested and determined to have modified binding affinity for one or more of ICOS-L, MadCAM-1 and ISLR2 is a candidate compound or mutant for treating a disease or disorder selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis, juvenile idiopathic arthritis, inflammation, autoimmune disease, immune disorder, Crohn's disease, ulcerative colitis and inflammatory bowel disease.

16. The method of Claim 15, wherein the modified TNFR-1B compound or mutant recognizes TNF-alpha, but not one or more of ICOS-L, MadCAM-1 and ISLR2.

17. The method of Claim 15, wherein the modified TNFR-1B compound or mutant recognizes ICOS-L, but not one or more of TNF-alpha, MadCAM-1 and ISLR2.

18. The method of Claim 15, wherein the modified TNFR-1B compound or mutant recognizes MadCAM-1, but not one or more of TNF-alpha, ICOS-L, and ISLR2.

19. The method of Claim 15, wherein the modified TNFR-1B compound or mutant recognizes ISLR2, but not one or more of TNF-alpha, ICOS-L, and MadCAM-1.

20. The method of Claim 15, wherein the modified TNFR-1B compound or mutant recognizes ICOS-L and MadCAM-1, but not TNF-alpha or ISLR2.

21. The method of any of Claims 11-15, wherein the compound or mutant recognizes only one ligand.

22. The method of any of Claims 11-15, wherein the compound or mutant has enhanced affinities for all ligands compared to TNFR-1B.

23. The method of any of Claims 11-15, wherein the compound or mutant has enhanced affinities for some ligands compared to TNFR-1B.

24. The method of any of Claims 11-15, wherein the compound or mutant has reduced affinities for some ligands compared to TNFR-1B.

25. The method of any of Claims 11-15, wherein the compound or mutant has reduced affinities for some ligands compared to TNFR-1B and enhanced affinities for some ligands compared to TNFR-1B.

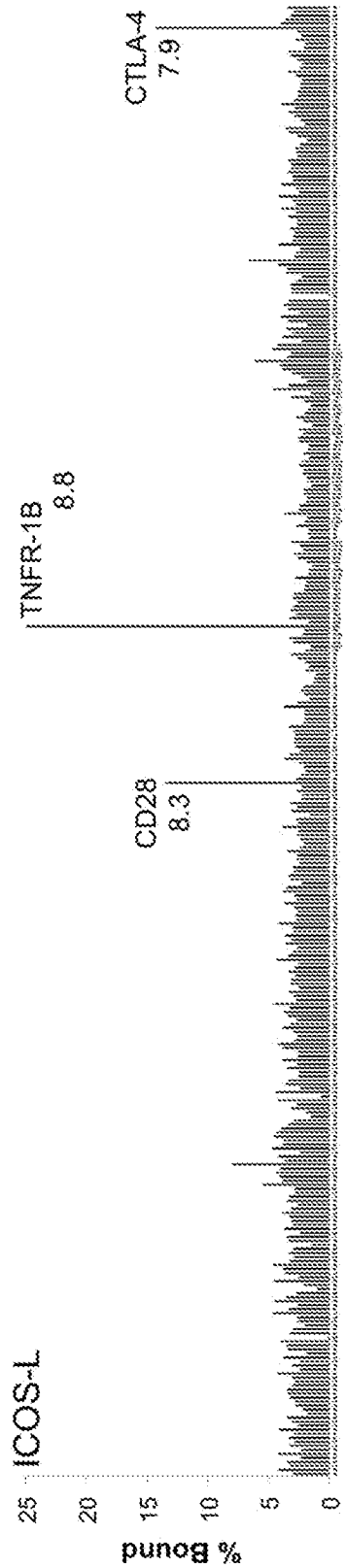

ICOS-L
CD28
8.3
TNFR-1B
8.8
CTLA-4
7.9
% Bound
0
5
10
15
20
25

FIG. 1A

TNFR-1B (ENBREL)
ICOS-L
6.7
MadCAM-1
5.6
ISLR2
2.8
% Bound
0
5
10
15
20
25

FIG. 1B

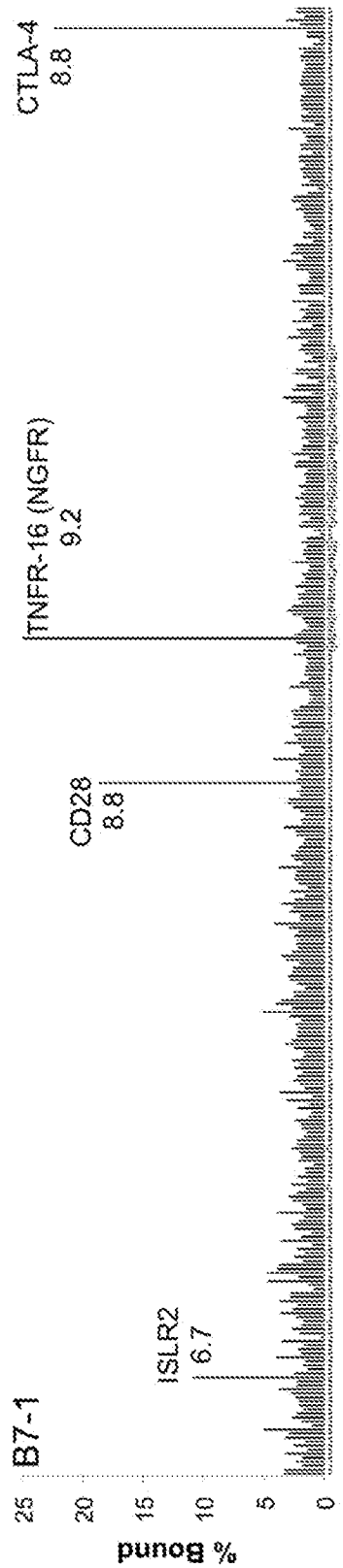

B7-1
ISLR2
6.7
CD28
8.8
TNFR-16 (NGFR)
9.2
CTLA-4
8.8
% Bound
25
20
15
10
5
0

FIG. 1C

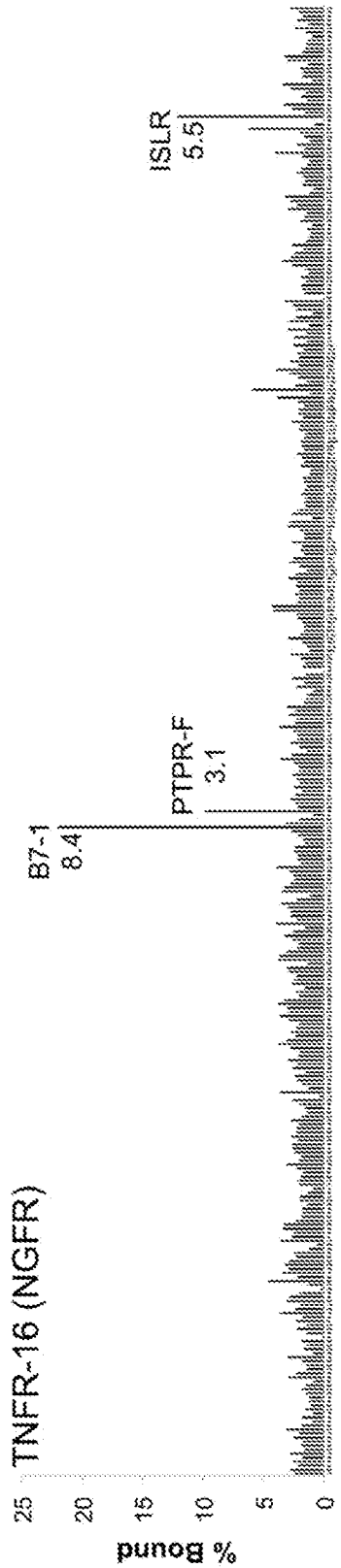

TNFR-16 (NGFR)
B7-1
8.4
PTPR-F
3.1
ISLR
5.5
% Bound
25
20
15
10
5
0

FIG. 1D

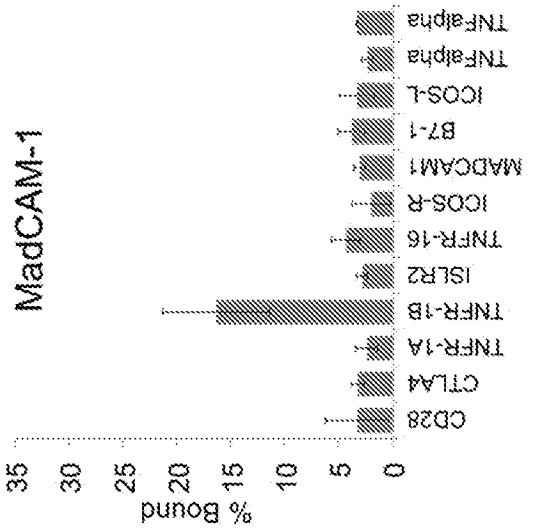


FIG. 2C

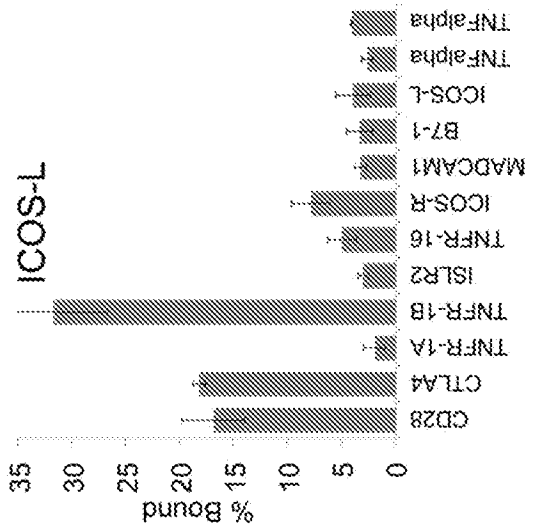


FIG. 2B

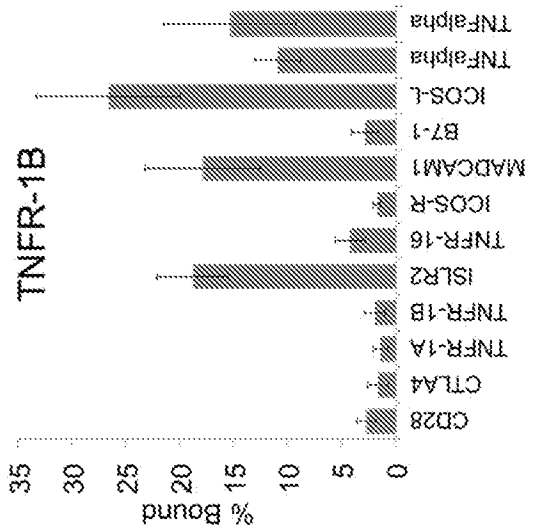


FIG. 2A

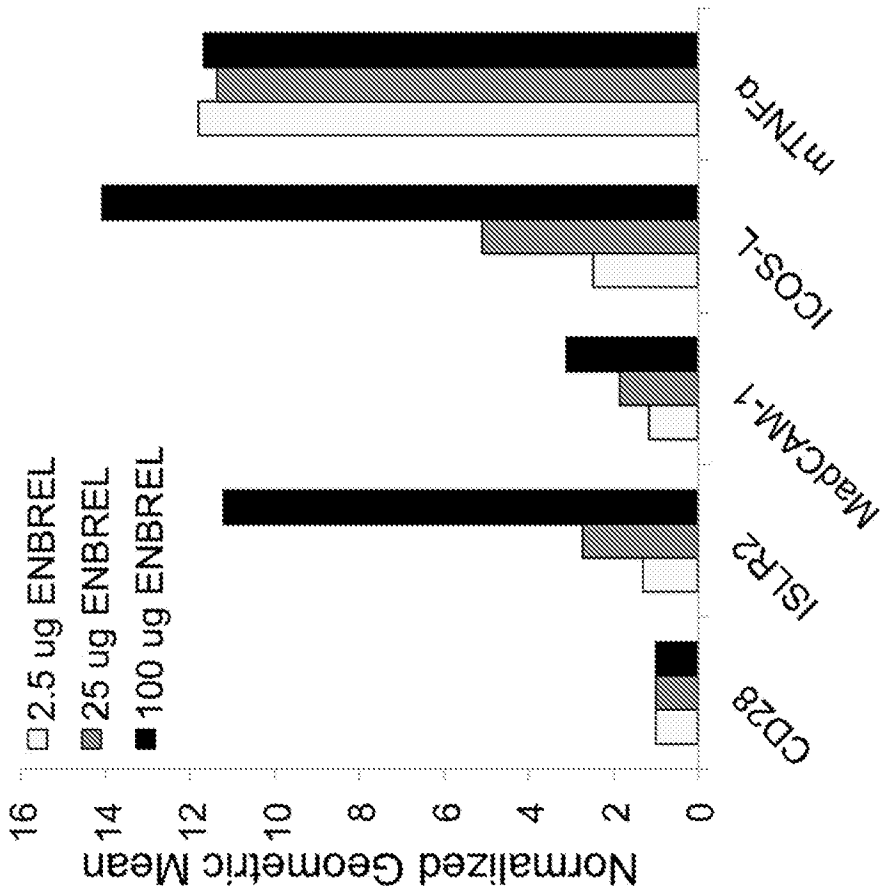

Normalized Geometric Mean
0
2
4
6
8
10
12
14
16
2.5 ug ENBREL
25 ug ENBREL
100 ug ENBREL
CD28
ISLR2
MadCAM-1
ICOS-L
mTNFa

FIG. 3

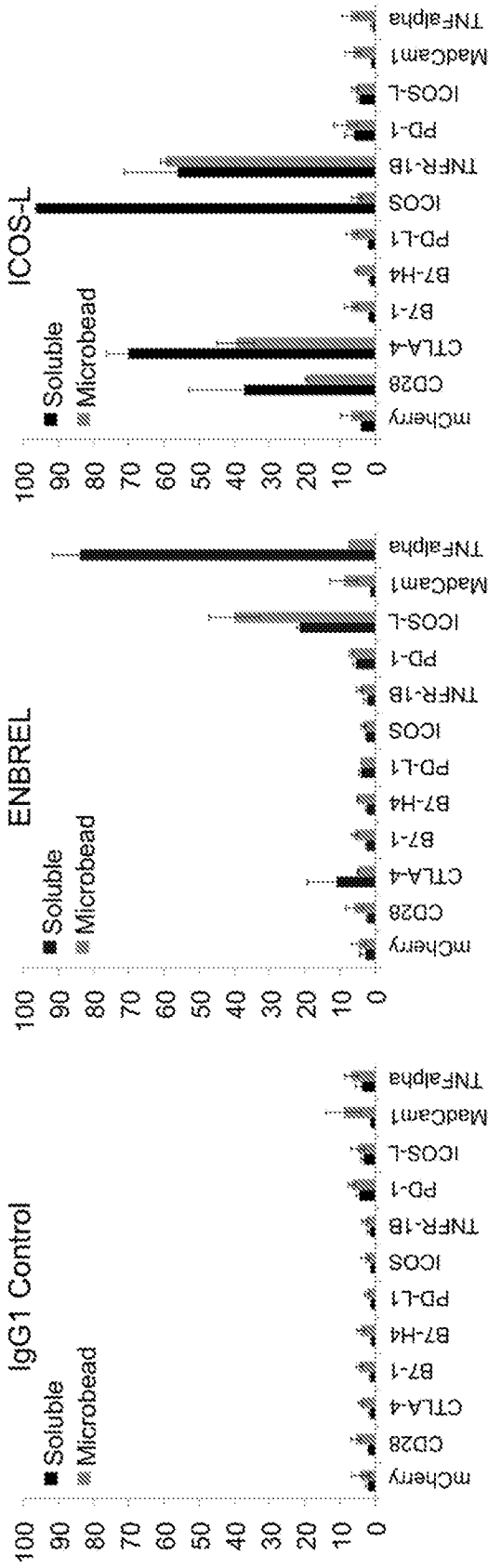

IgG1 Control
ICOS-L
ENBREL
Soluble
Microbead
100
90
80
70
60
50
40
30
20
10
0
mCherry
CD28
CTLA-4
B7-1
B7-H4
PD-L1
ICOS
TNFR-1B
PD-1
ICOS-L
MadCam1
TNFalpha

FIG. 4A

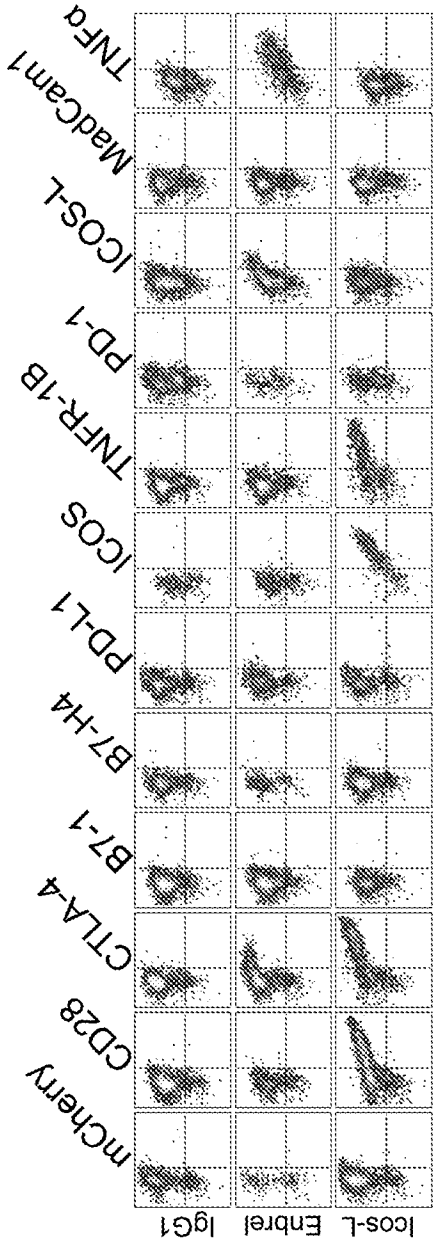

Icos-L
Enbrel
IgG1
mCherry
CD28
CTLA-4
B7-1
B7-H4
PD-L1
ICOS
TNFR-1B
PD-1
ICOS-L
MadCam1
TNFa

FIG. 4B

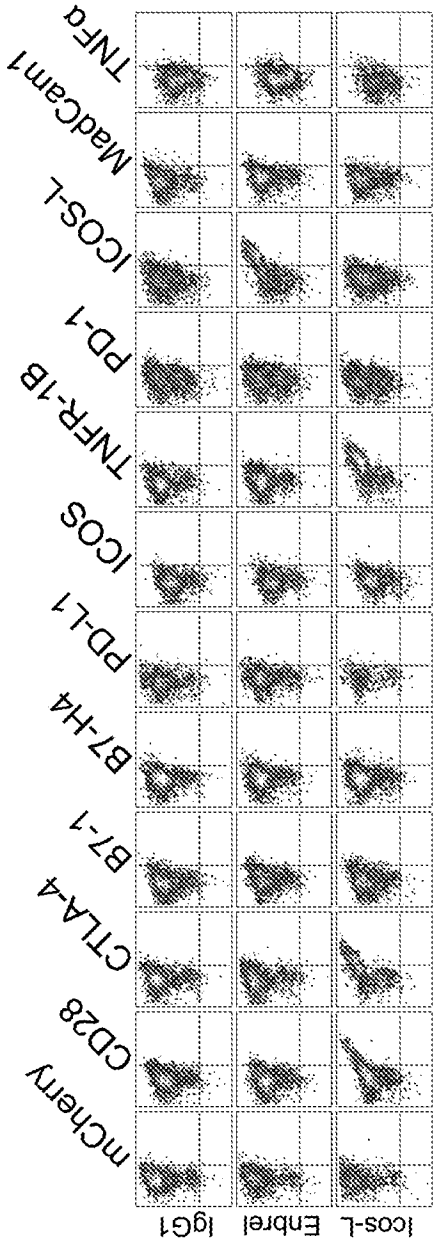

Icos-L
Enbrel
IgG1
mCherry
CD28
CTLA-4
B7-1
B7-H4
PD-L1
ICOS
TNFR-1B
PD-1
ICOS-L
MadCam1
TNFa

FIG. 4C

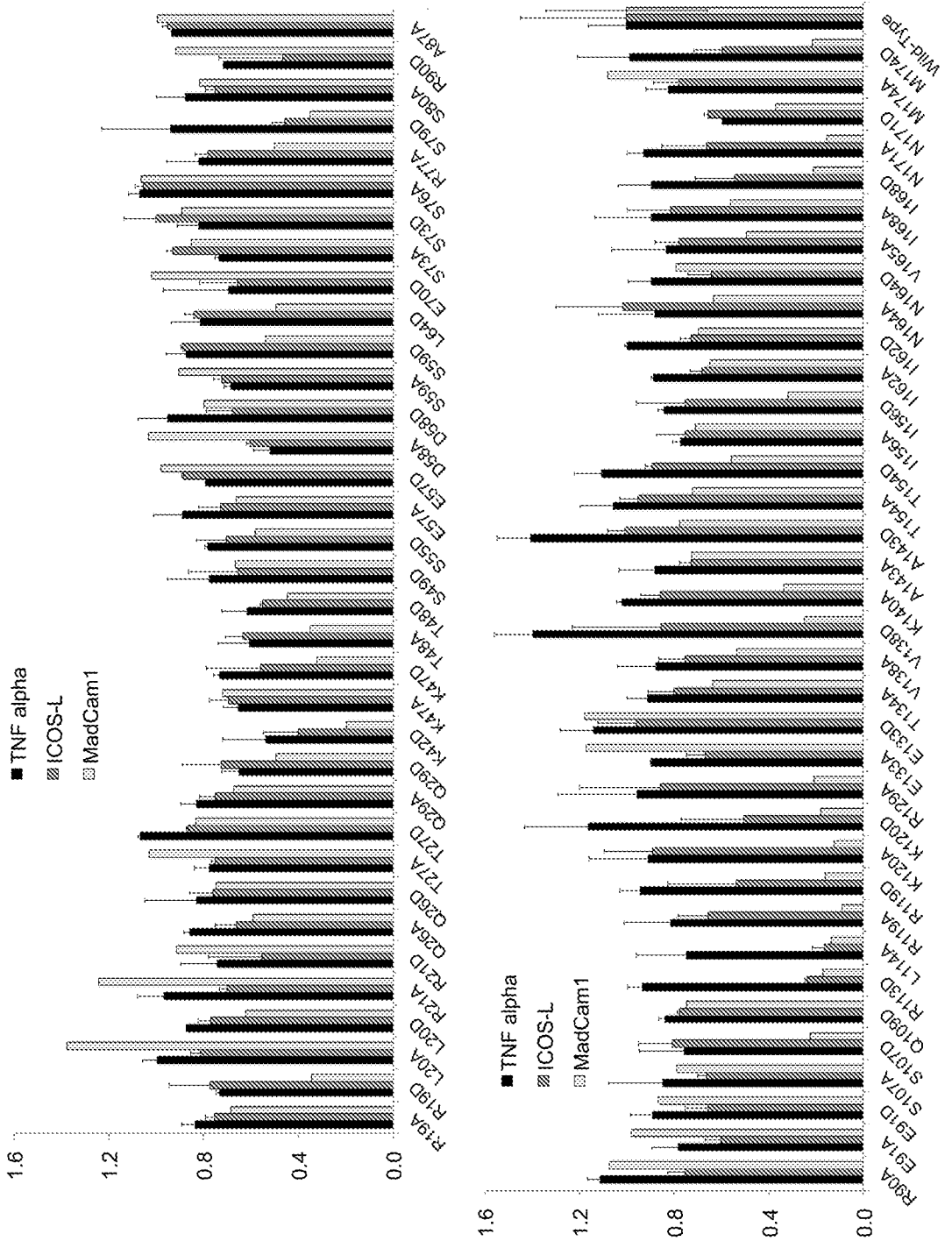
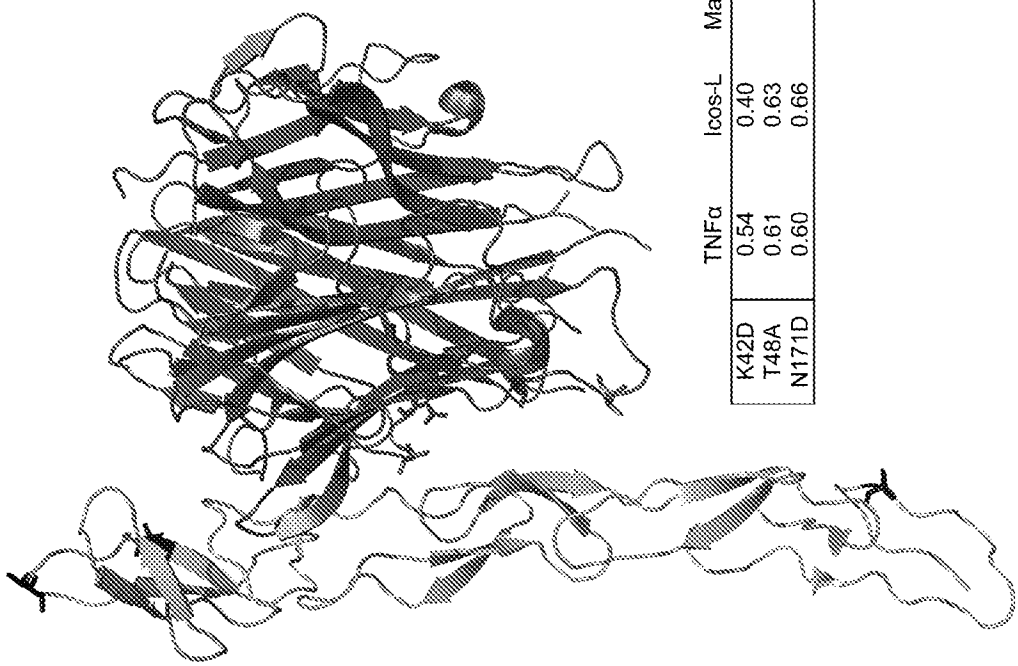
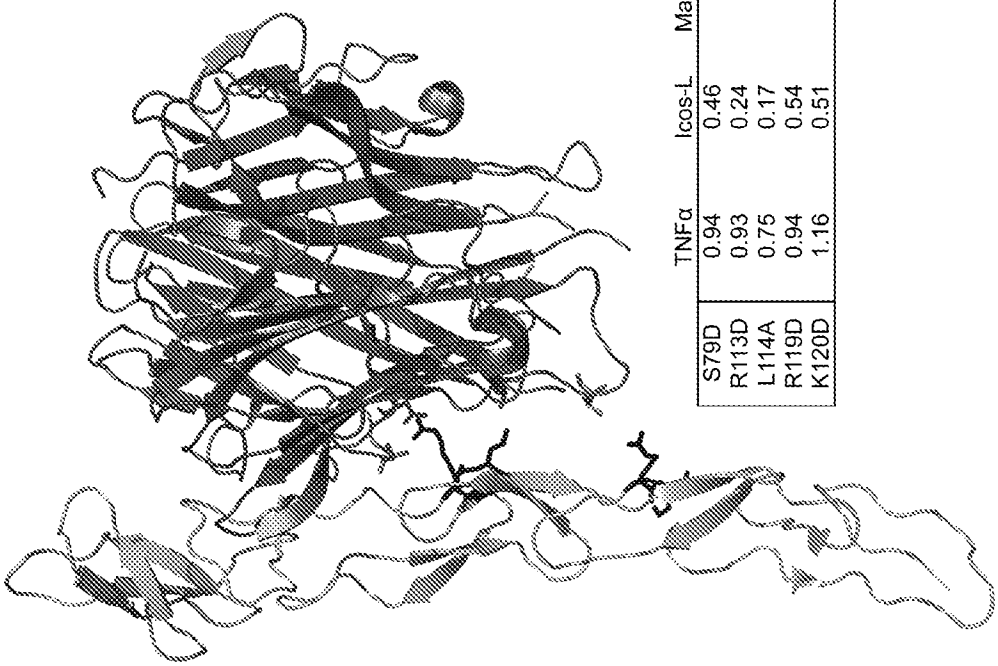

TNF alpha
ICOS-L
MadCam1
1.6
1.2
0.8
0.4
0.0
R19A
R19D
L20A
L20D
P21A
P21D
Q26A
Q26D
T27A
T27D
Q29A
Q29D
K42A
K47A
K47D
T48A
T48D
S49D
S55D
E57A
E57D
D58A
D58D
S59A
S59D
L64D
E70D
S73A
S73D
S76A
P77A
S79D
S80A
R90D
A87A
TNF alpha
ICOS-L
MadCam1
1.6
1.2
0.8
0.4
0.0
R90A
E91A
E91D
S107A
S107D
Q109D
R113D
L114A
R119A
R119D
K120A
K120D
R129A
E133A
E133D
T134A
V138A
V138D
K140A
A143A
A143D
T154A
T154D
I156A
I156D
I162A
I162D
N164A
N164D
V165A
I168A
I168D
N171A
N171D
M174A
M174D
Wild-Type

FIG. 5A



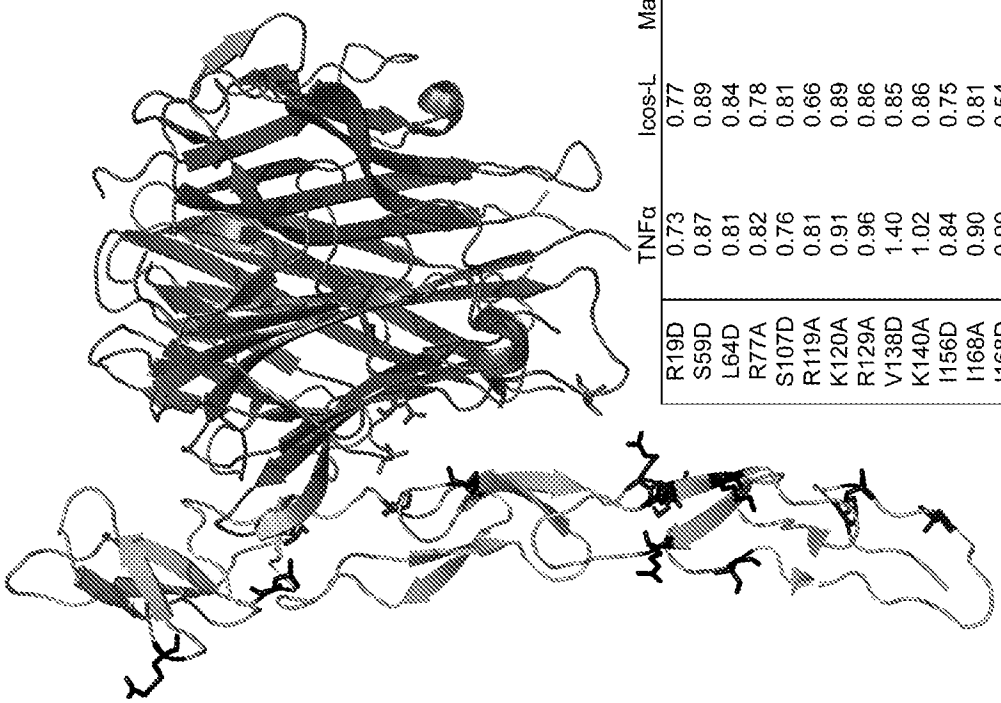
	TNFα	Icos-L	MadCam1
K42D	0.54	0.40	0.2
T48A	0.61	0.63	0.35
N171D	0.60	0.66	0.37

FIG. 5B



	TNFα	Icos-L	MadCam1
S79D	0.94	0.46	0.35
R113D	0.93	0.24	0.17
L114A	0.75	0.17	0.13
R119D	0.94	0.54	0.16
K120D	1.16	0.51	0.18

FIG. 5C



	TNFα	Icos-L	MadCam1
R19D	0.73	0.77	0.34
S59D	0.87	0.89	0.54
L64D	0.81	0.84	0.49
R77A	0.82	0.78	0.50
S107D	0.76	0.81	0.22
R119A	0.81	0.66	0.09
K120A	0.91	0.89	0.12
R129A	0.96	0.86	0.21
V138D	1.40	0.85	0.25
K140A	1.02	0.86	0.34
I156D	0.84	0.75	0.32
I168A	0.90	0.81	0.56
I168D	0.90	0.54	0.21
N171A	0.93	0.66	0.16
M174D	0.99	0.60	0.22

FIG. 5D

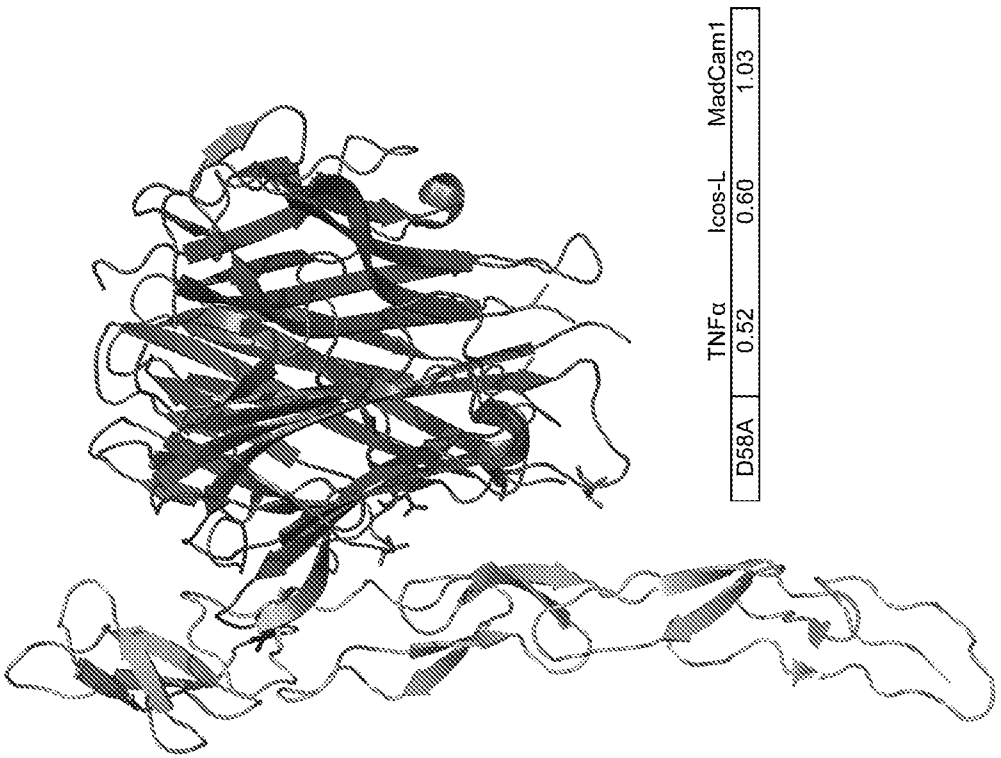


FIG. 5E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/057469

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/18; A61K 38/19; A61P 19/04; A61P 37/02; A61P 37/06 (2016.01)
CPC - A61K 38/1793; A61K 38/191; C07K 14/7151; C12N 15/1136; C12N 15/1138; G01N 33/5041 (2016.11)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - A61K 38/18; A61K 38/19; A61P 1/00; A61P 19/02; A61P 19/04; A61P 29/00; A61P 37/00; A61P 37/02; A61P 37/06
CPC - A61K 38/1793; A61K 38/191; C07K 14/7151; C12N 15/1136; C12N 15/1138; G01N 33/5041

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/9.2; 424/93.21; 435/7.21; 435/320.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, PubMed
Search terms used: TNFRSF1B TNF receptor 1B CD120b TBPII TNFR75 TNFBR TNFR1B TNFR2 TNFR80 p75 p75TNFR ICOS-L MadCAM-1 ISLR2 B7-1 TNFalpha assay screen test compound modulator

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2006/074370 A2 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES et al) 13 July 2006 (13.07.2006) entire document	1-20
A	US 2010/0150841 A1 (GOUKASSIAN et al) 17 June 2010 (17.06.2010) entire document	1-20
A	WO 2010/151671 A2 (CURNA, INC. et al) 29 December 2010 (29.12.2010) entire document	1-20
A	US 2008/0176796 A1 (BRADLEY et al) 24 July 2008 (24.07.2008) entire document	1-20
A	FAUSTMAN et al. "TNF receptor 2 pathway: drug target for autoimmune diseases," Nat Rev Drug Discov, 21 May 2010 (21.05.2010), Vol. 9, Pgs. 482-493. entire document	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 January 2017	24 JAN 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/057469

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NO:2 comprising amino acid substitutions K69D, T75A, N198D, S106D, R140D, L141A, R146D, K147D, D85A, R46D, S86D, L91D, R146A, K147A, R156A, V165D, K167A, I183D, N198A, and M201D were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/057469

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 21-25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.