(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 May 2016 (12.05.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/073875 A1

(51) International Patent Classification:
C07K 14/705 (2006.01) *C12N 15/62* (2006.01)
C07K 16/46 (2006.01)

(21) International Application Number:
PCT/US2015/059510

(22) International Filing Date:
6 November 2015 (06.11.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/076,120 6 November 2014 (06.11.2014) US
62/130,092 9 March 2015 (09.03.2015) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2016/073875 A1

(54) Title: CD8α AND T CELL RECEPTOR VARIANTS AND METHODS OF USING SAME IN MODULATING IMMUNE CELL RESPONSES

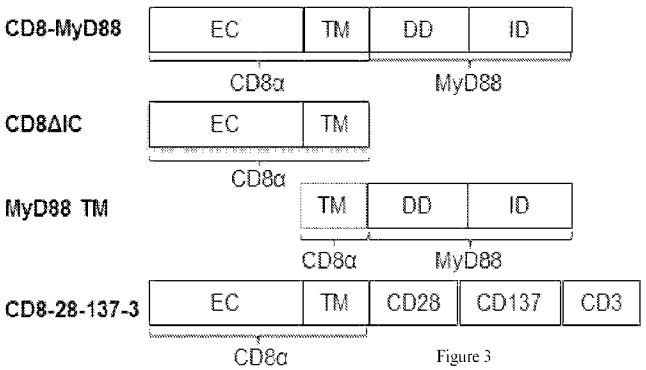


Figure 3

(57) Abstract: Novel costimulatory fusion proteins and DNA sequences that enhance T cell responses to weakly immunogenic and/or lowly expressed antigens and that confer T cell resistance against MDSC-mediated suppression are disclosed. The fusion proteins comprise portions of CD4, CD8α or the T cell receptor linked to a specific region of MyD88 or other signaling molecules. These fusion proteins and sequence variants thereof improve T cell activation and responsiveness. Also disclosed is the use of these molecules in host cells as a means to enhance and costimulate responses of immune cells including cytotoxic $CD8^+$ T cells and the use of these cells to treat cancer, infectious agents and other diseases.

CD8α AND T CELL RECEPTOR VARIANTS AND METHODS OF USING SAME IN MODULATING IMMUNE CELL RESPONSES

STATEMENT OF FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0001] This invention was made with government support under Grant Number CA140917 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0002] Recent research has shown that altering $CD8^+$ T cell responses in a subject can have a number of benefits. For example, ongoing clinical trials using infusions of tumor infiltrating lymphocytes or T cells engineered to express tumor-reactive T cell receptors (TCR) to destroy cancer have shown promise. However, these studies have also revealed subsets of patients in whom the T cell therapy is ineffective. Additional applications for augmented $CD8^+$ T cell responses are exemplified by the observation that many elderly and pediatric patients exhibit weak or no responses to vaccines such as the influenza vaccine, resulting in 50,000 deaths in the U.S. from influenza virus. Furthermore, the up-regulation of $CD8^+$ T cell responses can enhance vaccine efficacy and enhance the efficacy of T cell-based immunotherapies.

[0003] The type and intensity of $CD8^+$ T cell responses to any given antigen is governed by various molecular interactions that occur between T cells and antigen presenting cells. Such responses are largely the result of contact between TCRs on T cells and major histocompatibility complex (MHC) molecules on antigen presenting cells. Signaling by TCRs is modulated by the affinity of the TCR to the antigenic peptide presented on MHC I as well as the duration of the interaction between the TCR and the MHC-antigen (MHC/Ag). Thus, much attention has been focused on attempts to define high affinity peptides or to develop higher affinity TCRs.

[0004] The CD8α molecule serves as a TCR coreceptor and interacts with the α3 and α2 domain of the MHC I molecule and with HLA β_2 microglobulin. CD8α helps maintain TCR:MHC/Ag stability and keeps the TCR bound closely to the target cell during antigen-specific activation. Although CD8α interacts with CD8β, only the CD8α interacts with MHC I. Interaction between CD8α and MHC I contributes to cellular avidity in part by reducing the off rate of the TCR and promoting TCR clustering. The cytoplasmic domain of CD8α contains a $p56^{lck}$ binding domain that is important for TCR signal transduction.

[0005] Studies from various groups have demonstrated that stimulating Toll-like receptors (TLR) on T cells enhances a variety of T cell responses. For example, TLR2 engagement has been shown to decrease the TCR activation threshold and enhance T cell responses to suboptimal levels of Ags. TLR-stimulated T cells also exhibit increased IL-2 production, enhanced proliferation and survival, increased cytolytic activity, and enhanced antitumor activity in mice. However, it has been found that one limitation to stimulating TLRs on T cells is that the costimulatory effects require simultaneous TCR activation and that antitumor efficacy is limited in part by several factors, including low and transient TLR expression on T cells and the inability to localize sufficient TLR ligands at the tumor sites to costimulate T cells. Additionally, cancer cells, including melanoma, can express TLRs, and TLR engagement on cancer cells can induce the expression of various tumor growth factors.

[0006] Another limitation to achieving effective and durable T cell response includes the presence of myeloid derived suppressor cells (MDSCs). MDSCs represent a heterogeneous population of cells comprised of myeloid-cell progenitors and precursors of monocytic and granulocytic myeloid cells. MDSCs suppress T cell responses via: L-arginine depletion through arginase-1, inducible nitric oxide synthase (iNOS) activity, increased generation of reactive oxygen species (ROS), and production of TGF-β. A recent report emphasized a critical role of the cellular stress sensor C/EBP-homologous protein (Chop) in the inhibitory activity of MDSCs. In patients with advanced melanoma, circulating MDSCs correlate directly with low patient survival and inversely with functional TAg–specific T cells. In addition to blunting T cell activity, MDSCs play a critical role in inducing and maintaining Tregs in melanoma. Furthermore, factors produced by MDSCs (i.e. IL-6, TNF and IL-1β) promote tumor growth, therefore developing strategies to block their multi-factorial pro-tumor function has the potential to increase antitumor T cell activity.

BRIEF SUMMARY

[0007] As described in detail below, the inventors of the present application have found that MyD88 signaling directly within $CD8^+$ T cells reduces the T cell receptor (TCR) activation threshold to poorly immunogenic antigens and also augments responses to sub-optimal levels of antigen presentation. Concomitant activation of TCR and MyD88 signaling enhances T cell

proliferation, increases cytolytic activity, confers resistance to MDSC-mediated T cell suppression, reduces tumor growth kinetics and prolongs survival of tumor-bearing mice.

[0008] Based on these findings, the inventors prepared fusion proteins comprising molecules involved in T cell activation. In particular, the fusion proteins comprising portions of CD4, CD8α or the T cell receptor linked to a region of MyD88 or other signaling molecules were prepared. These fusion proteins, and sequence variants thereof (collectively, these fusion proteins and sequence variants are referred to herein as the "variants" of the invention), improve T cell activation and responsiveness. The expression of these variants is shown to have profound effects on T cell activation and responsiveness resulting in enhanced antitumor activity.

CD8α-MyD88

[0009] In a first embodiment, the invention is directed to CD8α fusion proteins, and polynucleotides encoding the same, comprising extracellular and transmembrane regions of CD8α linked to a region of MyD88 lacking the TIR domain. In one aspect of this embodiment, these regions are linked as: N-extracellular region of CD8α-transmembrane region of CD8α-region of MyD88 lacking the TIR domain-C. The CD8α portion of these fusion proteins may be from any mammalian CD8α, including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human.

[0010] In one aspect of this embodiment, the extracellular and transmembrane regions of mouse CD8α correspond to amino acids 1-217 of mouse CD8α (SEQ ID NO:16). In one aspect of this embodiment, the extracellular and transmembrane regions of human CD8α correspond to amino acids 1-203 of human CD8α (SEQ ID NO:12). In one aspect of this embodiment, the region of human MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24). In specific aspects of this embodiment, the invention includes the CD8α fusion proteins mCD8α-hMyD88, set forth in SEQ ID NO:17, and hCD8α-hMyD88, set forth in SEQ ID NO:14. In other specific aspects of this embodiment, the invention includes polynucleotides encoding mCD8α-hMyD88, set forth in SEQ ID NO:6, and hCD8α-hMyD88, set forth in SEQ ID NO:3.

CD8αTM-MyD88

[0011] In a second embodiment, the invention is directed to CD8α fusion proteins, and polynucleotides encoding the same, comprising the transmembrane region of CD8α linked to a region of MyD88 lacking the TIR domain. In one aspect of this embodiment, these regions are linked as: N-transmembrane region of CD8α-region of MyD88 lacking the TIR domain-C. The CD8α portion of these fusion proteins may be from any mammalian CD8α, including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human.

[0012] In one aspect of this embodiment, the transmembrane region of human CD8α corresponds to amino acids 128-210 of human CD8α (amino acids 1-83 of SEQ ID NO:18). In one aspect of this embodiment, the region of human MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24). In specific aspects of this embodiment, the invention includes the CD8α fusion protein hCD8αTM-hMyD88, set forth in SEQ ID NO:18. In other specific aspects of this embodiment, the invention includes the polynucleotide encoding hCD8αTM-hMyD88, set forth in SEQ ID NO:7.

CD8α-28-137-3

[0013] In a third embodiment, the invention is directed to CD8α fusion proteins, and polynucleotides encoding the same, comprising the extracellular and transmembrane regions of CD8α linked to the intracellular signaling domains of CD28, CD137 (4-1BB), and CD3ζ. The CD8α portion of these fusion proteins may be from any mammalian CD8α, including mouse and human. The CD28, CD137 (4-1BB), and CD3ζ intracellular signaling domains may also be from any mammalian source, including mouse and human.

[0014] In one aspect of this embodiment, the extracellular and transmembrane regions of mouse CD8α correspond to amino acids 1-217 of mouse CD8α (SEQ ID NO:16). In one aspect of this embodiment, the extracellular and transmembrane regions of human CD8α correspond to amino acids 1-203 of human CD8α (SEQ ID NO:12). In one aspect of this embodiment, the CD28, CD137 (4-1BB), and CD3ζ domains correspond to amino acids 218-417 of SEQ ID NO:19, where CD28 corresponds to amino acids 218-256; CD137 (4-1BB) corresponds to amino acids 259-305; CD3ζ corresponds to amino acids 308-417. In specific aspects of this

embodiment, the invention includes the CD8α fusion protein mCD8α-28-137-3, set forth in SEQ ID NO:19. In other specific aspects of this embodiment, the invention includes the polynucleotide encoding mCD8α-28-137-3, set forth in SEQ ID NO:8.

CD4-MyD88

[0015] In a fourth embodiment, the invention is directed to CD8α fusion proteins, and polynucleotides encoding the same, comprising extracellular and transmembrane regions of CD4 linked to a region of MyD88 lacking the TIR domain. In one aspect of this embodiment, these regions are linked as: N-extracellular region of CD4-transmembrane region of CD4-MyD88 lacking the TIR domain-C. The CD4 portion of these fusion proteins may be from any mammalian CD4, including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human.

[0016] In one aspect of this embodiment, the extracellular and transmembrane regions of mouse CD4 correspond to amino acids 1-417 of mouse CD4 (amino acids 1-417 of SEQ ID NO:21). In one aspect of this embodiment, the extracellular and transmembrane regions of human CD4 correspond to amino acids 1-418 of human CD4 (amino acids 1-418 of SEQ ID NO:20). In one aspect of this embodiment, the region of human MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24). In specific aspects of this embodiment, the invention includes the CD4 fusion proteins mCD4-hMyD88, set forth in SEQ ID NO:21, and hCD4-hMyD88, set forth in SEQ ID NO:20. In other specific aspects of this embodiment, the invention includes polynucleotides encoding mCD4-hMyD88, set forth in SEQ ID NO:10, and hCD4-hMyD88, set forth in SEQ ID NO:9.

TCR-MyD88

[0017] In a fifth embodiment, the invention is directed to TCR fusion proteins, and polynucleotides encoding the same, comprising a TCR linked to a region of MyD88 lacking the TIR domain. In one aspect of this embodiment, these elements are linked as: N-TCR-MyD88 lacking the TIR domain-C. The TCR portion of these fusion proteins may be from any mammalian TCR, including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human.

[0018] In one aspect of this embodiment, the TCR is the DMF5 TCR having the amino acid sequence of residues 1-603 of SEQ ID NO:22. In one aspect of this embodiment, the region of human MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24). In specific aspects of this embodiment, the invention includes the TCR fusion protein hTCR-hMyD88, set forth in SEQ ID NO:22. In other specific aspects of this embodiment, the invention includes the polynucleotide encoding hTCR-hMyD88, set forth in SEQ ID NO:11.

Sequence Variants

[0019] Each of the embodiments and aspects of the invention includes sequence variants of the fusion proteins and the polynucleotides encoding the fusion proteins wherein the length of each peptide region or domain comprising a fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained. In certain aspects, sequence variants have at least 75% of the activity of the specific fusion protein upon which they are based.

[0020] In one aspect of this embodiment, the sequence variant is a sequence variant wherein the length of at least one peptide region or domain comprising the fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained.

[0021] Each of the embodiments and aspects of the invention also includes sequence variants of the fusion proteins, and polynucleotides encoding the same, having at least 80% sequence identity with a specific fusion protein defined herein, over the entire length of that specific fusion protein. In certain aspects, these sequence variants will have at least 75% of the activity of the specific fusion protein upon which they are based.

Cells

[0022] In a sixth embodiment, the invention is directed to isolated populations of cells expressing at least one variant of the invention. In one aspect of this embodiment, the at least one variant is selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-

hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof. In another aspect of this embodiment, the at least one variant is encoded by a polynucleotide sequence selected from the group consisting of the polynucleotide sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88), and sequence variants thereof.

[0023] In one aspect of this embodiment, the sequence variant is a sequence variant wherein the length of at least one peptide region or domain comprising the fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained.

[0024] In one aspect of this embodiment, the sequence variant has at least 80% sequence identity with a specific fusion protein defined herein, over the entire length of that specific fusion protein.

[0025] In one aspect of this embodiment, the cells are selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells and other peripheral blood mononuclear cells (PBMC) or other primary or established cell lines including the so-called universal donor cells.

[0026] In one aspect of this embodiment, the cells are engineered to express the fusion proteins and sequence variants via viral-mediated gene integration. However, other means of gene integration or protein expression such as nucleofection or transient expression of DNA, RNA or proteins are also suitable.

Methods of Treatment

[0027] In a seventh embodiment, the invention is directed to methods of treating a subject having cancer, comprising administering to a subject having cancer a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention.

[0028] In one aspect of this embodiment, the at least one variant is selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof. In another aspect of this embodiment, the at least one variant is encoded by a polynucleotide sequence selected from the group consisting of the polynucleotide sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88), and sequence variants thereof.

[0029] In one aspect of this embodiment, the sequence variant is a sequence variant wherein the length of at least one peptide region or domain comprising the fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained.

[0030] In one aspect of this embodiment, the sequence variant has at least 80% sequence identity with a specific fusion protein defined herein, over the entire length of that specific fusion protein.

[0031] In one aspect of this embodiment, the cells are selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells and other peripheral blood mononuclear cells (PBMC), tumor infiltrating lymphocytes or other primary or established cell lines including the so-called universal donor cells.

[0032] In an eighth embodiment, the invention is directed to methods of treating a subject having an infectious disease, comprising administering to a subject having an infectious disease a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention.

[0033] In one aspect of this embodiment, the at least one variant is selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof. In another aspect of this embodiment, the at least one variant is encoded by a polynucleotide sequence selected from the group consisting of the polynucleotide sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88), and sequence variants thereof.

[0034] In one aspect of this embodiment, the sequence variant is a sequence variant wherein the length of at least one peptide region or domain comprising the fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained.

[0035] In one aspect of this embodiment, the sequence variant has at least 80% sequence identity with a specific fusion protein defined herein, over the entire length of that specific fusion protein.

[0036] In one aspect of this embodiment, the cells are selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells and other peripheral blood mononuclear cells (PBMC), tumor infiltrating lymphocytes or other primary or established cell lines including the so-called universal donor cells.

[0037] In one aspect of this embodiment, the infectious disease is caused by a bacterium, a virus or a fungus.

[0038] In an ninth embodiment, the invention is directed to methods of treating a subject having an autoimmune disorder, comprising administering to a subject having an autoimmune disorder a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention.

[0039] In one aspect of this embodiment, the at least one variant is selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14),

hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof. In another aspect of this embodiment, the at least one variant is encoded by a polynucleotide sequence selected from the group consisting of the polynucleotide sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88), and sequence variants thereof.

[0040] In one aspect of this embodiment, the sequence variant is a sequence variant wherein the length of at least one peptide region or domain comprising the fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained.

[0041] In one aspect of this embodiment, the sequence variant has at least 80% sequence identity with a specific fusion protein defined herein, over the entire length of that specific fusion protein.

[0042] In one aspect of this embodiment, the cells are selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells and other peripheral blood mononuclear cells (PBMC), tumor infiltrating lymphocytes or other primary or established cell lines including the so-called universal donor cells.

[0043] In one aspect of this embodiment, the autoimmune disorder is selected from the group consisting of lupus, arthritis, Type I diabetes, multiple sclerosis, Alopecia areata, and Celiac disease.

Methods of Enhancing Antigen Recognition

[0044] In a tenth embodiment, the invention is directed to methods of conferring T cell resistance against MDSC-mediated suppression. These methods comprise expressing at least one variant of the present invention in a T cells.

[0045] In one aspect of this embodiment, the at least one variant is selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof. In another aspect of this embodiment, the at least one variant is encoded by a polynucleotide sequence selected from the group consisting of the polynucleotide sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88), and sequence variants thereof.

[0046] In one aspect of this embodiment, the sequence variant is a sequence variant wherein the length of at least one peptide region or domain comprising the fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained.

[0047] In one aspect of this embodiment, the sequence variant has at least 80% sequence identity with a specific fusion protein defined herein, over the entire length of that specific fusion protein.

[0048] In an eleventh embodiment, the invention is directed to methods of enhancing immune cell recognition of an antigen. These methods comprise expressing at least one variant of the present invention in an immune cell.

[0049] In one aspect of this embodiment, the at least one variant is selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof. In another aspect of this embodiment, the at least one variant is encoded by a polynucleotide sequence selected from the group consisting of the polynucleotide

sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88), and sequence variants thereof.

[0050] In one aspect of this embodiment, the sequence variant is a sequence variant wherein the length of at least one peptide region or domain comprising the fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained.

[0051] In one aspect of this embodiment, the sequence variant has at least 80% sequence identity with a specific fusion protein defined herein, over the entire length of that specific fusion protein.

[0052] In one aspect of this embodiment, the immune cells are selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells and other peripheral blood mononuclear cells (PBMC), tumor infiltrating lymphocytes or other primary or established cell lines including the so-called universal donor cells.

[0053] In certain aspects of this embodiment, the antigen is present at a low concentration *in vitro* or *in vivo*, or the antigen is a weakly antigenic antigen, or both.

[0054] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described herein, which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that any conception and specific embodiment disclosed herein may be readily utilized as a basis for modifying or designing other formulations for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that such equivalent formulations do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features which are believed to be characteristic of the invention, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It

is to be expressly understood, however, that any description, figure, example, etc. is provided for the purpose of illustration and description only and is by no means intended to define the limits the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0055] **Figure 1** is an illustration of the physical interaction between T cell receptors and MHC molecules.

[0056] **Figure 2** is a graph showing IFN-γ production by T cells retrovirally transduced with different CD8α variants or a GFP vector control after co-culturing of the T cells with melanoma tumor cells for 24 hours.

[0057] **Figure 3** is a schematic of the CD8α variants of the invention. EC, extracellular domain of CD8α; TM, transmembrane domain of CD8α; DD, death domain of MyD88; ID, intermediate domain of MyD88; CD28, intracellular domain of CD28; CD137, intracellular domain of CD137(4-1BB); CD3, intracellular domain of CD3ζ.

[0058] **Figures 4A-4B.** CD8α expression and transduction efficiency in HEK and $CD8^+$ T cells. **Fig. 4A** - HEK cells remained untransfected or were transfected with mCD8α-hMyD88. **Fig. 4B -** mouse $CD8^+$ pmel T cells were transduced with mCD8α-hMyD88 and transfection efficiency, based on % of GFP+ T cells, was determined 48 hours after transduction.

[0059] **Figure 5.** MyD88 signaling activates NF-κB. HEK-Blue reporter cells were transiently transfected with the constructs. SEAP activity was determined as a readout for NF-κB activation at 48 hours, measured by change in absorbance at 620 nm. LPS serves as a positive control, pMIG is the empty vector control.

[0060] **Figures 6A-6B**. mCD8α-hMyD88 expression in mouse CD8 T cells augments T cell proliferation and IFN-γ production in response to stimulation with varying concentrations of tumor antigen. Proliferation was determined by ^{3}H-thymidine incorporation and IFN-γ production by ELISA. The average (counts per minute) of triplicate wells ($\pm$SD) is shown after 48 hours of stimulation with peptide-pulsed splenocytes. *$P<0.05$ vs mCD8α-28-137-3; ANOVA.

[0061] **Figure 7**. mCD8α–hMyD88 expression in $CD8^+$ T cells augments T cell proliferation in response to activation with titrating numbers of tumor cells. The average CPM (counts per

minute) of triplicate wells (+SD) is shown after 72 hours of stimulation with the indicated number of B16 cells. **P<0.01 vs mCD8α-137-28-3; ANOVA.

[0062] **Figure 8.** MyD88 signaling in T cells alters cytokine secretion in response to tumor antigen on tumor cells. Transduced pmel T cells were co-cultured with irradiated B16-F1 tumor cells. Supernatant was collected at 24 and the levels of the various factors were evaluated using Milliplex Cytokine Array.

[0063] **Figure 9**. MyD88 signaling in T cells increases cytokine secretion in response to tumor antigen on tumor cells. Transduced pmel T cells were co-cultured with irradiated B16-F1 tumor cells. Supernatant was collected at 24 hours and diluted 100-fold before conducting an ELISA. *P<0.05 vs mCD8α-28-137-3; ANOVA.

[0064] **Figure 10**. CD8-MyD88 signaling. $CD8^+$ pmel T cells were transduced with CD8α-MyD88, CD8αΔIC or pMIG control vector. T cells were stimulated in at a 1 to 1 ratio with B16 tumor cells for 10 and 30 minutes, then fixed in 4% PFA. The 0 time point indicates no B16 were added. Cells were permeabilizied and stained for the indicated phosphorylated proteins, p-p38, p-JNK, p-ERK1/2, which are activated in response to TCR signaling.

[0065] **Figure 11**. mCD8α-hMyD88-transduced T cells exhibit enhanced cytotoxicity against B16 melanoma *in vitro*. Cytotoxicity was evaluated at the indicated effector (T cells) to target (B16 melanoma) ratios over the course of 4 hours. *P<0.05 vs mCD8α-28-137-3; ANOVA.

[0066] **Figure 12**. $CD8^+$ T cells expressing mCD8α-hMyD88 resist MDSC-mediated suppression and exhibit increased proliferation. $CD8^+$ T cells engineered to express mCD8α-hMyD88 or control GFP were co-cultured with MDSCs at the indicated ratios. The average CPM (counts per minute) of triplicate wells (±SD) is shown after 72 hrs. *P<0.05; T-test.

[0067] **Figures 13A-13B**. Tumor-bearing mice treated with mCD8α-hMyD88 T cells exhibit enhanced antitumor responses and prolonged survival.

[0068] **Figures 14A-14B.** mCD8α-hMyD88 expression in tumor reactive T cells enhances antitumor responses and prolongs mouse survival in the absence of any support therapy. Mice treated with mCD8α-hMyD88 T cells exhibited significantly enhanced antitumor responses (**Fig. 14A**) beginning on day 23 (one-way ANOVA; p<0.01) and overall prolonged survival (**Fig. 14B**; Wilcoxon, p<0.05) as compared with mice untreated mice or mice treated with control pMIG pmel T cells.

[0069] **Figure 15**. T cells expressing MyD88-linked to a tumor-reactive TCR showed enhanced T cell proliferation.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0070] Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found, for example, in Benjamin Lewin, Genes VII, published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew et al. (eds.); The Encyclopedia of Molecular Biology, published by Blackwell Publishers, 1994 (ISBN 0632021829); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and other similar technical references.

[0071] As used herein, "a" or "an" may mean one or more. As used herein when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more. Furthermore, unless otherwise required by context, singular terms include pluralities and plural terms include the singular.

[0072] As used herein, "about" refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term "about" generally refers to a range of numerical values (e.g., +/- 5-10% of the recited value) that one of ordinary skill in the art would consider equivalent to the recited value (e.g., having the same function or result). In some instances, the term "about" may include numerical values that are rounded to the nearest significant figure.

II. The Present Invention

[0073] The intensity of $CD8^+$ T cell responses to any given antigen is governed by various molecular interactions that occur between T cells and antigen presenting cells. Such interactions include the interaction between T cell receptors (TCRs) expressed by T cells and MHC molecules expressed by antigen presenting cells, as shown in Figure 1.

[0074] Signaling generated by the TCR is modulated in large part by the interaction between the CD8 co-receptor on $CD8^+$ T cells and the MHC protein on the antigen-presenting cells. As

can been seen in Figure 1, the CD8 co-receptor is composed of two subunits: CD8α and the CD8β, with only the CD8α subunit interacting directly with MHC I, via the α3 domain of the protein.

[0075] Data presented herein and published reports have also demonstrated that stimulating Toll-like receptor (TLR)–MyD88 signaling directly within $CD8^+$ and $CD4^+$ T cells reduces the T cell receptor (TCR) activation threshold to poorly immunogenic antigens and also augments responses to sub-optimal levels of immunodominant antigen presentation. Concomitant activation of TCR and TLR signaling enhances T cell proliferation, increases cytolytic activity, and prolongs survival of effector T cells in tumor-bearing mice. Intriguingly, immune cells from elderly individuals exhibit reduced expression levels of TLRs and are generally hyporesponsive to TLR stimulation.

[0076] Together with extensive studies conducted by the inventor, these observations have led to the development of the CD4, CD8 and TCR variants described herein, polypeptides involved in T cell activation that have improved T cell activation and responsiveness.

[0077] In particular, it has been found that altering the amino acid sequence of the CD8α subunit and the T cell receptor to include a specified region of human MyD88 protein can have profound effects on T cell activation and responsiveness. This was accomplished by creating synthetic genes that fuse the extracellular and transmembrane regions of CD8α or the TCR to a specific a region of MyD88. $CD8^+$ T cells were then engineered to express these genes and the CD8α-MyD88 fusion proteins were found to exhibit significantly improved responses over other CD8α vectors as measured by IFN-γ production, T cell proliferation and enhanced cytotoxicity. Thus, changes to the amino acid sequence of the CD8α subunit were found modulate the affinity of CD8 for MHC I and in turn, the nature of the $CD8^+$ T cell response to a selected antigen. Some of these same results were found when TCR-MyD88 fusion proteins were prepared and tested. This surprising discovery serves as a basis for the novel polypeptides, cells lines, and methods reported and claimed herein.

[0078] Some novel features and useful applications of the present invention include:

- CD8α variants of the invention can be linked together with the soluble TCR (sTCR) or membrane-bound TCR to enhance the TCR affinity to peptide MHC.

- CD8α variants of the invention can be expressed on T cells to enhance endogenous TCR signals to any given antigen.
- CD8α variants of the invention can be co-expressed along with transgenic tumor-reactive TCRs (or TCRs specific against other antigens such viral or other intracellular pathogenic antigens). In addition to augmenting T cell activity, the CD8α variants could be used to reduce PD1 and CTLA4-mediated immune suppression by overriding those negative signals (by virtue of the ability of MyD88 to enhance TCR signaling).
- Mutations in the CD8α or CD8β subunits that alter affinity to MHC I can serve as biomarkers to predict patient T cell responses to vaccines or responses by gene modified T cells. Because mutations can either reduce or enhance CD8 affinity, specific mutations could predict both strong and weak responses. For example, patients with K273A and S53N mutations would be expected to show stronger responses.

[0079] Current strategies to enhance T cell responses include:

- antibody-based approaches to prevent T cells from becoming deactivated or prevent their death;
- T cells engineered to express high-affinity TCRs; and
- T cells engineered to expressed chimeric antigen receptors (CAR) that endow T cells with an ability to recognize tumor antigens expressed on the tumor cells surface.

The last two approaches are expensive, time- and labor-intensive processes. The use of high-affinity TCRs could result in the killing of non-cancerous tissues and CAR T cells can only target one antigen. Thus, there is a need for new strategies to enhance T cell responses. The approach described herein avoids the need to identify high-affinity TCRs. Moreover, the CD8α variants of the invention offer the possibility of enhancing the response of T cells carrying low-affinity TCRs, by exploiting the ability of CD8α to amplify TCR signals. Although CAR T cells can only target one antigen, the CD8α variants of the invention could be used as a single platform to enhance the TCR or CAR response against any antigen.

- Vaccine based approaches, such as with dendritic cells, modified tumor cells, tumor lysates, peptide or protein-based vaccines, bacterial or viral vaccines modified to express tumor antigens and other forms of vaccines intended to elicit T cell response.
- Tumor infiltrating lymphocytes for immunotherapy

The last two approaches could all benefit from enhancing T cell responses with MyD88 with the invention described herein.

[0080] Thus, provided herein are CD8α variants, including but not limited to CD8α-MyD88 fusion proteins and sequence variants thereof, that when introduced into T cells, can serve as TCR co-receptors that augment T cell responses to antigen stimulation. Also provided herein are TCR variants, including but not limited to TCR-MyD88 fusion proteins and sequence variants thereof. These TCR variants can be introduced into T cells to augment T cell responses to weakly immunogenic antigens and/or to further potentiate T cell responses to antigen stimulation.

[0081] The present invention is also directed to cells lines expressing the variants and to methods for using the variants and cell lines *in vitro* and *in vivo*.

Fusion Proteins

[0082] In initial studies it was found that fusing CD8α to the full-length MyD88 protein (or overexpressing MyD88 without CD8α) led to the death of T cells. Without wishing to be bound by theory, it is speculated that this killing occurred in response to chronic and enhanced MyD88 signaling. Because the MyD88 protein contains a region called the TIR domain (Toll/IL-1 receptor domain), which interacts with toll-like receptors (TLR) and IL-1 receptors (IL-1R), it was speculated that removing the TIR domain from MyD88 could prevent clustering to TLRs and IL-1Rs, and thus result in MyD88 activation only upon TCR signaling (or encounter with MHC antigen).

[0083] It was further considered that due to the fact that physical interactions between CD8 and MHC I occur through the extracellular domains of CD8, the intracellular portion of the molecule may be less important could potentially be replaced by MyD88 lacking the TIR domain.

[0084] As reported below, fusion proteins comprising the extracellular and transmembrane regions of CD8α linked to MyD88 lacking the TIR domain demonstrated excellent properties with respect to increasing T cell proliferation, T cell cytokine and chemokine production, and T cell cytotoxic activity in T cells in which they were expressed. The present invention is directed, in part, to these molecules.

CD8α-MyD88

[0085] The present invention thus includes murine and human CD8α variants. In a first aspect the CD8α variants include CD8α-MyD88 fusion proteins comprising extracellular and transmembrane regions of CD8α linked to a region of MyD88 lacking the TIR domain. In a preferred embodiment, these regions are linked as: N-extracellular region of CD8α-transmembrane region of CD8α-MyD88 lacking the TIR domain-C. The CD8α portion of these fusion proteins may be from any mammalian CD8α, including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human. The extracellular and transmembrane regions of mouse CD8α generally correspond to amino acids 1-217 of mouse CD8α (SEQ ID NO:16; mCD8αΔIC). The extracellular and transmembrane regions of human CD8α generally correspond to amino acids 1-203 of human CD8α (SEQ ID NO:12; hCD8αΔIC). The region of human MyD88 lacking the TIR domain generally corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24). Non-limiting examples of these CD8α variants include mCD8α-hMyD88 (SEQ ID NO:17) and hCD8α-hMyD88 (SEQ ID NO:14).

[0086] The invention also includes CD8α variants comprising native, full-length CD8α linked to a region of MyD88 lacking the TIR domain. In a preferred embodiment, these regions are linked as: N-CD8α-MyD88 lacking the TIR domain-C. The CD8α portion of these fusion proteins may be any mammalian CD8α, including mouse (SEQ ID NO:15) and human (SEQ ID NO:12). The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human. The region of human MyD88 lacking the TIR domain generally corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24).

[0087] Each of these CD8α variants may also include more than one of the MyD88 regions lacking the TIR domain linked in tandem, and these MyD88 regions can appear on the N-terminus, the C-terminus, or both the N- and C-termini of the CD8α portion of the fusion proteins.

CD8αTM-MyD88

[0088] In a second aspect, the CD8α variants include CD8α-MyD88 fusion proteins comprising only the transmembrane region of CD8α linked to a region of MyD88 lacking the TIR domain. In a preferred embodiment, these regions are linked as: N-transmembrane region of CD8α-MyD88 lacking the TIR domain-C. The CD8α portion of these fusion proteins may be from any mammalian CD8α, including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human. The transmembrane region of human CD8α generally corresponds to amino acids 128-210 of human CD8α (amino acids 1-83 of SEQ ID NO:18). The region of human MyD88 lacking the TIR domain generally correspond to amino acids 1-155 of human MyD88 (SEQ ID NO:24). A non-limiting example of this CD8α variant includes hCD8αTM-hMyD88 (SEQ ID NO:18).

[0089] Each of these CD8α variants may also include more than one of the MyD88 regions lacking the TIR domain linked in tandem, and these MyD88 regions can appear on the N-terminus, the C-terminus, or both the N- and C-termini of the CD8α portion of the fusion proteins.

CD8α-28-137-3

[0090] In a third aspect, the CD8α variants include CD8α fusion proteins comprising the extracellular and transmembrane regions of CD8α linked to the intracellular signaling domains of a traditional 3rd generation CAR: human CD28, CD137 (4-1BB), CD3ζ. In a preferred embodiment, these elements are linked as: N-extracellular region of CD8α-transmembrane region of CD8α-CD28-CD137-CD3ζ-C. The CD8α portion of these fusion proteins may be from any mammalian CD8α, including mouse and human. The CD28, CD137 (4-1BB), and CD3ζ intracellular signaling domains may also be from any mammalian source, including mouse and human. The extracellular and transmembrane regions of mouse CD8α generally correspond to amino acids 1-217 of mouse CD8α (SEQ ID NO:16; mCD8αΔIC). The extracellular and transmembrane regions of human CD8α generally correspond to amino acids 1-203 of human CD8α (SEQ ID NO:12; hCD8αΔIC). Human CD28, CD137 (4-1BB), and CD3ζ generally correspond to amino acids 218-417 of SEQ ID NO:19, where CD28 generally corresponds to amino acids 218-256; CD137 (4-1BB) generally corresponds to amino acids 259-305; CD3ζ

generally corresponds to amino acids 308-417. A non-limiting example of this CD8α variant includes mCD8α-28-137-3 (SEQ ID NO:19), also referred to herein in some instances as mCD8α-137-28-3.

[0091] Each of these CD8α variants may also include more than one of the CD28, CD137 (4-1BB), and CD3ζ intracellular signaling domains linked in tandem, and these intracellular signaling domains can appear on the N-terminus, the C-terminus, or both the N- and C-termini of the CD8α portion of the fusion proteins.

CD4-MyD88

[0092] The present invention also includes murine and human CD4 variants. The CD4 variants include CD4-MyD88 fusion proteins comprising extracellular and transmembrane regions of CD4 linked to a region of MyD88 lacking the TIR domain. In a preferred embodiment, these regions are linked as: N-extracellular region of CD4-transmembrane region of CD4-MyD88 lacking the TIR domain-C. The CD4 portion of these fusion proteins may be from any mammalian CD4, including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human. The extracellular and transmembrane regions of mouse CD4 generally correspond to amino acids 1-417 of mouse CD4 (amino acids 1-417 of SEQ ID NO:21). The extracellular and transmembrane regions of human CD4 generally correspond to amino acids 1-418 of human CD4 (amino acids 1-418 of SEQ ID NO:20). The region of human MyD88 lacking the TIR domain generally corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24). Non-limiting examples of these CD4 variants include mCD4-hMyD88 (SEQ ID NO:21) and hCD4-hMyD88 (SEQ ID NO:20).

[0093] The invention also includes CD4 variants comprising native, full-length CD4 linked to a region of MyD88 lacking the TIR domain. In a preferred embodiment, these regions are linked as: N-CD4-MyD88 lacking the TIR domain-C. The CD4 portion of these fusion proteins may be any mammalian CD4 , including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human. The region of human MyD88 lacking the TIR domain generally corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24).

[0094] Each of these CD4 variants may also include more than one of the MyD88 regions lacking the TIR domain linked in tandem, and these MyD88 regions can appear on the N-terminus, the C-terminus, or both the N- and C-termini of the CD4 portion of the fusion proteins.

TCR-MyD88

[0095] The present invention also includes murine and human TCR variants. The TCR variants include TCR-MyD88 fusion proteins comprising TCRs linked to a region of MyD88 lacking the TIR domain. In a preferred embodiment, these elements are linked as: N-TCR-MyD88 lacking the TIR domain-C. The TCR portion of these fusion proteins may be from any mammalian TCR, including mouse and human. The MyD88 portion of these fusion proteins may also be from any mammalian source, including mouse and human. In one example, the TCR is the DMF5 TCR. It comprises amino acids 1-603 of SEQ ID NO:22. The region of human MyD88 lacking the TIR domain generally corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24). Non-limiting examples of these TCR variants include hTCR-hMyD88 (SEQ ID NO:22).

Linkers and Spacers

[0096] As indicated herein, the variants of the invention include fusion proteins comprised of peptide domains and regions from molecules such as CD8α, CD4, MyD88, TCR CD28, CD137 (4-1BB), and CD3ζ. When these domains and regions are linked in the context of a fusion protein, a short linker or spacer may be desirable to enhance formation of a proper three-dimensional structure or shape. The variants of the invention may thus have short peptide linkers or spacers of up to 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acids positioned between the functional peptide domains and regions of the fusions proteins.

Sequence Variants

Variations in Length

[0097] It will be apparent that the different peptide domains and regions that comprise the variants of the invention can have some amino acid variability without adversely affecting the activity of the variant. For example, the peptide domains and regions can vary somewhat in their length. Thus, while the mouse CD8α-hMyD88 fusion protein is said to comprise amino acids 1-217 of mouse CD8α (SEQ ID NO:15), the length of this portion of the fusion protein may be extended or reduced by a number of amino acids, e.g., to be amino acids 1-210 of SEQ ID NO:15, or amino acids 1-210 of SEQ ID NO:15, or amino acids 10-215 of SEQ ID NO:15, or amino acids 8-222 of SEQ ID NO:15, as only a few examples.

[0098] Thus the invention includes sequence variants wherein, for each of the fusion proteins of the invention, the length of the peptide domains and regions used in the fusion proteins can individually be increased or decreased on the amino terminus, carboxy terminus, or both ends, by up to 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 or 0 amino acids based on the native sequence of the polypeptide from which the domain or region is obtained. These sequence variants, in which the peptide domains and regions differ in length from a specific variant defined herein, will have at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the activity of the specific variant upon which they are based. The cytoplasmic domains will generally have 20-1000 amino acids depending on the number of domains and the specific domains to be used. The transmembrane domain will generally have 20-60 amino acids. The extracellular domain will generally have 50-5000 amino acids depending on the number of domains and the specific domains to be used.

Variations in Sequence

[0099] It will also be apparent that the variants of the invention can have variability in their amino acid composition without adversely affecting the activity of the variant. For example, the variants can have amino acid additions (conservative and/or non-conservative), deletions and/or substitutions, and any combination thereof. Thus the invention also includes sequence variants having at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 85%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%

sequence identity with a specific variant defined herein, over the entire length of that specific variant. These sequence variants will have at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the activity of the specific variant upon which they are based.

[00100] As shown in Example 1 below, variations in the amino acid composition of the variants described herein can be expected to alter or change various attributes and characteristics of immune cells expressing the variants, including their responses when contacting antigen-presenting cells. For example, a K73A mutation in mouse CD8α resulted in a CD8α molecule with higher affinity than the native version of the protein. T cells expressing such "high affinity" CD8α (i.e., CD8α bearing the K73A mutation) showed a higher level of activation that T cells expressing other CD8α variants.

Polynucleotide Sequences

[00101] The invention also encompasses the polynucleotide sequences encoding each of the fusion proteins and sequence variants of the invention. Specific polynucleotide sequences encompassed within the scope of the invention include the polynucleotide sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88). The skilled artisan will understand that due to the redundancy of the genetic code, there are a large number of different polynucleotide sequences that encode a single polypeptide. The invention includes each polynucleotide sequence encoding a fusion protein or sequence variant of the invention. Thus the invention includes polynucleotide sequences encoding mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and each of the sequence variants thereof encompassed within the scope of the invention.

Cells

[00102] The present invention also encompasses cells engineered to produce the variants (i.e., the fusion proteins and sequence variants) of the invention. The identity of such cells is only limited by the ability of the cell to produce the variant. In preferred aspects, the cell can both produce the variant and express it on the surface of the cell. The cells include mammalian cells, such as human or mouse cells, insect cells, and prokaryotic cells, such as bacterial cells.

[00103] The variants include fusion proteins mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof. In another aspect, the variants are encoded by a polynucleotide sequence selected from the polynucleotide sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88), and sequence variants thereof.

[00104] In a particular aspect of the invention, these variant-expressing cells may be used in the methods of medical treatment, as discussed herein. Variant-expressing cells used in such methods may be derived from cells autologous, syngeneic or allogeneic to the individual being treated, with the selection dependent on the disease to be treated and the means available to do so. Suitable populations of cells that may be used in the methods include any immune cells with cytolytic activity, such as T cells. Exemplary sub-populations of T cells include, but are not limited to those expressing CD4 such as $CD4^+$ T cells, those expressing CD8 such as $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells and other peripheral blood mononuclear cells (PBMC) or other primary or established cell lines including the so-called universal donor cells. The cells may also be isolated from any source, including the blood, lymph node or spleen of a subject, or from a tumor explant of a subject or intratumoral T cells of a subject.

[00105] Cells may be engineered to express one or more than one of the variants of the invention. Thus, the invention encompasses populations of cells expressing at least one, two,

three or more of the variants of the invention, as well as populations of cells expressing one or more of the variants of the invention.

[00106] Cells may be engineered to express the variants of the invention by means readily known to the skilled artisan. Generally, a polynucleotide vector is constructed that encodes the fusion protein or sequence variant and the vector is transfected into a population of cells, such as T cells. The cells are then grown under conditions promoting expression of the fusion protein or sequence variant by the cells. Successful transfection (or transduction which refers to viral-mediated gene integration) and cell-surface display of polypeptides is conducted via conventional means, some of which are disclosed in the Examples herein.

[00107] In one aspect, T cells may be engineered to produce the variants by first constructing a retroviral vector encoding a selected fusion protein or sequence variant. An exemplary retroviral vector includes, but is not limited to, the vector backbone pMSGV1-CD8-28BBZ, which is derived from pMSGV (murine stem cell virus-based splice-gag vector). However, other means of gene integration or protein expression, such as nucleofection or transient expression of DNA, RNA or proteins, are also suitable. DNA sequencing can be used to confirm proper construction of the vector before transfection of T cells. Retroviral transduction may be performed using known techniques, such as that of Johnson et al. (Blood 114, 535-546 (2009)). The surface expression of fusion proteins and sequence variants on transduced T cells may be determined, for example, by flow cytometry after staining cells with labeled antibodies.

[00108] Immune cells expressing the variants of the present invention have improved attributes in comparison to native immune cells that do not express the variants. For example, expression of the variants in T cells confers T cell resistance to MDSC-mediated suppression. Further, expressing the variants in certain immune cells enhances the ability of those cells to recognize low concentrations of antigens and/or recognize weakly immunogenic antigens. Therefore, the present invention includes methods of conferring resistance in T cells to MDSC-mediated suppression, comprising expressing at least one variant of the present invention in a T cell or population of T cells.

[00109] The present invention also includes methods of enhancing the ability of an immune cell to recognize low concentrations of a selected antigen comprising expressing at least one variant of the present invention in an immune cell or population of immune cells. The present

invention further includes methods of enhancing the ability of an immune cell to recognize a weakly immunogenic antigen comprising expressing at least one variant of the present invention in an immune cell or population of immune cells. The immune cells include, but are not limited to, T cells (e.g., $CD4^+$ and $CD8^+$ T cells), natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells and other peripheral blood mononuclear cells (PBMC) or other primary or established cell lines including the so-called universal donor cells.

Cell Administration

[00110] Populations of variant-expressing cells may be formulated for administered to a subject using techniques known to the skilled artisan. Formulations comprising variant-expressing cells may include pharmaceutically acceptable excipient(s). Excipients included in the formulations will have different purposes depending, for example, on the nature of the variant being expressed (e.g., CD8α-MyD88 or TCR-MyD88), the subpopulation of T cells used, and the mode of administration. Examples of generally used excipients include, without limitation: saline, buffered saline, dextrose, water-for-infection, glycerol, ethanol, and combinations thereof, stabilizing agents, solubilizing agents and surfactants, buffers and preservatives, tonicity agents, bulking agents, and lubricating agents. The formulations comprising populations of variant-expressing T cells will typically have been prepared and cultured in the absence of any non-human components, such as animal serum (e.g., bovine serum albumin).

[00111] A formulation may include one population of variant-expressing cells, or more than one, such as two, three, four, five, six or more different populations of variant-expressing cells. The different populations of variant-expressing cells can vary based on the identity of the variant, the identity of the subpopulation of T cells, or a combination thereof.

[00112] The formulations comprising population(s) of variant-expressing cells may be administered to a subject using modes and techniques known to the skilled artisan. Exemplary modes include, but are not limited to, intravenous injection. Other modes include, without limitation, intratumoral, intradermal, subcutaneous (s.c., s.q., sub-Q, Hypo), intramuscular (i.m.), intraperitoneal (i.p.), intra-arterial, intramedulary, intracardiac, intra-articular (joint),

intrasynovial (joint fluid area), intracranial, intraspinal, and intrathecal (spinal fluids). Any known device useful for parenteral injection or infusion of the formulations can be used to effect such administration.

[00113] The formulations comprising population(s) of variant-expressing cells that are administered to a subject comprise a number of variant-expressing cells that is effective for the treatment of the specific indication or disease. Thus, therapeutically-effective amounts of formulations comprising population(s) of variant-expressing cells are administered to subjects when the methods of the present invention are practiced. In general, formulations are administered that comprise between about 1 x 10^3 and about 1 x 10^{10} variant-expressing cells. In most cases, the formulation will comprise between about 1 x 10^3 and about 1 x 10^8 variant-expressing cells, from about 5 x 10^5 to about 5 x 10^8 variant-expressing cells, or from about 1 x 10^6 to about 1 x 10^7 variant-expressing cells. However, the number of variant-expressing cells administered to a subject will vary between wide limits, depending upon the location, source, identity, extent and severity of the disease, the age and condition of the individual to be treated, etc. A physician will ultimately determine appropriate dosages to be used.

Methods

Treatment and Prevention of Cancer

[00114] $CD8^+$ T cells recognize peptides presented on MHC I molecules. Because all nucleated cells including cancer cells express MHC I, virtually any type of cancer can be detected and destroyed by cytotoxic T cells. In some cases, tumor cells can reduce MHC I expression and evade T cell recognition. However, expressing CD8α-MyD88 in T cells offers the advantage of enhancing T cell responses even in the face of low antigen levels or weakly immunogenic antigens.

[00115] $CD4^+$ T cells on the other hand recognize peptides presented in the context of an MHC II molecule. Like CD8, CD4 serves as a TCR co-receptor. It is therefore possible that expressing either CD8α-MyD88 or CD4-MyD88 (in either $CD8^+$ T cells or $CD4^+$ T cells, respectively) will augment their responses. Examples of CD4-MyD88 variants are provided herein. It is also possible that since CD8 localizes to the immunological synapse, the area where the TCRs and TCR signaling molecules localize to interact with the MHC on target cells,

MyD88 brought to the immunological synapse by any means could also enhance TCR signals. For example, fusing the transmembrane domain of CD8 (or CD4 or any other TCR-signaling related molecule) to the MyD88 can be used to augment T cell responses through localization of MyD88.

[00116] The present invention thus encompasses methods of treating a subject having cancer, comprising administering to a subject having cancer a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention. The invention also encompasses methods of treating a subject having cancer, comprising administering to a subject having cancer a therapeutically-effective amount of a formulation comprising at least one population of cells expressing at least one variant of the invention and an excipient.

[00117] The present invention also encompasses methods of preventing cancer in a subject, comprising administering to a subject at risk of developing cancer a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention. The invention further encompasses methods of preventing cancer in a subject, comprising administering to a subject at risk of developing cancer a therapeutically-effective amount of a formulation comprising at least one population of cells expressing at least one variant of the invention and an excipient.

[00118] The term "cancer" is intended to be broadly interpreted and it encompasses all aspects of abnormal cell growth and/or cell division. Examples include: carcinoma, including but not limited to adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, anaplastic carcinoma, large cell carcinoma, small cell carcinoma, and cancer of the skin, breast, prostate, bladder, vagina, cervix, uterus, liver, kidney, pancreas, spleen, lung, trachea, bronchi, colon, small intestine, stomach, esophagus, gall bladder; sarcoma, including but not limited to chondrosarcoma, Ewing's sarcoma, malignant hemangioendothelioma, malignant schwannoma, osteosarcoma, soft tissue sarcoma, and cancers of bone, cartilage, fat, muscle, vascular, and hematopoietic tissues; lymphoma and leukemia, including but not limited to mature B cell neoplasms, such as chronic lymphocytic leukemia/small lymphocytic lymphoma, B-cell prolymphocytic leukemia, lymphomas, and plasma cell neoplasms, mature T cell and natural killer (NK) cell neoplasms, such as T cell prolymphocytic leukemia, T cell large granular lymphocytic leukemia, aggressive NK cell leukemia, and adult T cell leukemia/lymphoma,

Hodgkin lymphomas, and immunodeficiency-associated lymphoproliferative disorders; germ cell tumors, including but not limited to testicular and ovarian cancer; blastoma, including but not limited to hepatoblastoma, medulloblastoma, nephroblastoma, neuroblastoma, pancreatoblastoma, leuropulmonary blastoma and retinoblastoma. The term also encompasses benign tumors.

Treatment and Prevention of Infectious Disease

[00119] Infectious agents such as viruses and certain bacteria and fungi become internalized by the host cell or by immune cells called professional antigen-presenting cells. The cells process parts of the infectious agents and present them on the cell surface in the context of MHC I, which can then be recognized by T cells. Expressing CD8α-MyD88 in T cells offers the advantage of enhancing T cell responses to any antigen presented on MHC molecules regardless of the source of the antigen. This strategy can be used to augment the immune responses of immunosuppressed individuals, including but not limited to: elderly individuals, patients infected with human immunodeficiency virus (HIV), patients who have been treated with therapies that suppress T cell responses (i.e. steroid therapies, cancer chemotherapies, radiation therapies).

[00120] The present invention thus encompasses methods of treating a subject having an infectious disease, comprising administering to a subject having an infectious disease a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention. The invention also encompasses methods of treating a subject having an infectious disease comprising administering to a subject having an infectious disease a therapeutically-effective amount of a formulation comprising at least one population of cells expressing at least one variant of the invention and an excipient.

[00121] In addition, the present invention encompasses methods of preventing an infectious disease in a subject, comprising administering to a subject at risk of developing an infectious disease a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention. The invention further encompasses methods of preventing an infectious disease in a subject comprising administering to a subject at risk of developing an infectious disease a therapeutically-effective amount of a formulation comprising at least one population of cells expressing at least one variant of the invention and an excipient.

[00122] The identity of the infectious disease is limited only in that antigen-presenting cells have the ability to present antigens derived from the causative agent of the infectious disease on the cell surface in the context of MHC I. Infectious diseases caused by viruses, bacteria and fungi are encompassed within the scope of the invention.

Treatment and Prevention of Autoimmune Disease

[00123] The present invention encompasses methods of treating a subject having an autoimmune disorder by targeting for destruction autoreactive cells. Thus included in the invention are methods of treating a subject having an autoimmune disorder, comprising administering to a subject having an autoimmune disorder a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention. The invention also encompasses methods of treating a subject having an autoimmune disorder, comprising administering to a subject having an autoimmune disorder a therapeutically-effective amount of a formulation comprising at least one population of cells expressing at least one variant of the invention and an excipient.

[00124] The present invention also encompasses methods for preventing an autoimmune disorder by targeting for destruction autoreactive cells. Thus included in the invention are methods of preventing an autoimmune disorder in a subject, comprising administering to a subject at risk for developing an autoimmune disorder a therapeutically-effective amount of a population of cells expressing at least one variant of the present invention. The invention also encompasses methods of preventing an autoimmune disorder in a subject, comprising administering to a subject at risk of developing an autoimmune disorder a therapeutically-effective amount of a formulation comprising at least one population of cells expressing at least one variant of the invention and an excipient.

[00125] The identity of the autoimmune disorder is not limited. However, non-limiting examples include lupus, arthritis, Type I diabetes, multiple sclerosis, Alopecia areata, Celiac disease.

Enhancing Antigen Recognition

[00126] The present invention encompasses methods of altering the activity of immune cells, such as T cells, through the engineering of the immune cells to express one or more of the variants of the present invention. For example, expression of the variants in immune cells can provide improvements in the ability of the immune cell to recognize an antigen (e.g., antigens present at a low concentration in a subject or weakly antigenic antigens), including recognition of an antigen in the context of MHC I presentation; activate an effector cell; be activated by an effector cell; remain associated with an antigen-presenting cell; avoid suppression by effector cells, among other examples.

[00127] Thus included in the invention are methods of conferring T cell resistance against MDSC-mediated suppression. These methods comprise expressing at least one variant of the present invention in a T cells. In specific aspects, the variants are selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof.

[00128] In a related aspect, the invention includes methods of enhancing immune cell recognition of an antigen. These methods comprise expressing at least one variant of the present invention in an immune cell. In specific aspects, the variants are selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22), and sequence variants thereof. In certain aspects of this embodiment, the antigen is present at a low concentration *in vitro* or *in vivo*, or the antigen is a weakly antigenic antigen, or both.

[00129] The immune cells include, but are not limited to, $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells and other peripheral blood mononuclear cells (PBMC), tumor infiltrating lymphocytes or other primary or established cell lines including the so-called universal donor cells.

[00130] As used herein, the terms "treat", "treating", and "treatment" have their ordinary and customary meanings, and include one or more of: blocking, ameliorating, or decreasing in severity and/or frequency a symptom of cancer or an infection in a subject, and/or inhibiting the growth, division, spread, or proliferation of cancer cells, bacterial cells or a virus, or progression of cancer (e.g., emergence of new tumors), a bacterial infection or a viral infection in a subject. Treatment means blocking, ameliorating, decreasing, or inhibiting by about 1% to about 100% versus a subject in which the methods of the present invention have not been practiced. Preferably, the blocking, ameliorating, decreasing, or inhibiting is about 100%, 99%, 98%, 97%, 96%, 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5% or 1% versus a subject in which the methods of the present invention have not been practiced.

[00131] As used herein, the terms "prevent", "preventing" and "prevention" have their ordinary and customary meanings, and include one or more of, stopping, averting, avoiding, alleviating or blocking development of cancer or an infection in a subject, and/or stopping, averting, avoiding, alleviating or blocking the growth, division, spread, or proliferation of cancer cells, bacterial cells or a virus, or progression of cancer (e.g., emergence of new tumors), a bacterial infection or a viral infection in a subject. Prevention means stopping by at least about 95% versus a subject to which the prevention has not been administered. Preferably, the stopping is about 100%, about 99%, about 98%, about 97%, about 96% or about 95%. The results of the prevention may be permanent or may continue for a period of days (such as 1, 2, 3, 4, 5, 6 or 7 days), weeks (such as 1, 2, 3 or 4 weeks) or months (such as 1, 2, 3, 4, 5, 6 or more months).

[00132] Administration frequencies of the formulations comprising populations of variant-expressing cells will vary depending on factors that include the disease or condition being treated, the identity of the variant of the variant-expressing cells, and the mode of administration. Each formulation may be independently administered 4, 3, 2 or once daily, every other day, every third day, every fourth day, every fifth day, every sixth day, once weekly, every eight days, every nine days, every ten days, bi-weekly, monthly and bi-monthly.

[00133] The duration of treatment will be based on the disease or condition being treated and will be best determined by the attending physician. However, continuation of treatment is contemplated to last for a number of days, weeks, or months.

[00134] The invention also provides a kit comprising one or more containers filled with one or more populations of variant-expressing cells. The kit may also include instructions for use. Associated with the kit may further be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

III. Examples

Example 1

[00135] To explore the possibility that altering CD8α affinity to the MHC could increase T cell responses, three mouse CD8α variants were prepared. The first variant was a mouse CD8α construct having a K73A mutation ("high affinity CD8α"; SEQ ID NO:23). The second variant was a mouse CD8α construct comprising the extracellular and transmembrane portion of mouse CD8α linked to the 4-1BB-CD28-CD3 intracellular activation domain (mCD8α-28-137-3; SEQ ID NO:19). The third variant was a mouse CD8α construct in which the intracellular domain was deleted (mCD8αΔIC; SEQ ID NO:16). Melanoma-specific $CD8^+$ T cells were engineered via retroviral transduction to express one of these three CD8α variants or a GFP vector control. Approximately 50-60% of $CD8^+$ T cells expressed the vector as indicated by GFP expression (data not shown). IFN-γ production (a measure of T cell activation) was examined after co-culturing T cells with melanoma tumor cells for 24 hours. Upon stimulation, T cells expressing high affinity CD8α (i.e., CD8α bearing the K73A mutation) showed a higher level of activation that T cells expressing the other CD8α variants (Figure 2).

mCD8α (SEQ ID NO:23):

MASPLTRFLSLNLLLLGESIILGSGEAKPQAPELRIFPKKMDAELGQKVDLVCEVLGSVS
QGCSWLFQNSSS**A**LPQPTFVVYMASSHNKITWDEKLNSSKLFSAMRDTNNKYVLTLNK
FSKENEGYYFCSVISNSVMYFSSVVPVLQKVNSTTTKPVLRTPSPVHPTGTSQPQRPEDC
RPRGSVKGTGLDFACDIYIWAPLAGICVALLLSLIITLICYHRSRKRVCKCPRPLVRQEGK
PRPSEKIV

Example 2

Materials and Methods

[00136] Mice and cell lines. C57BL6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), housed in the University of Maryland, Baltimore, specific pathogen-free animal facility and used as recipients for adoptive immunotherapy. Experiments were reviewed and approved by the University of Maryland, Baltimore, Institutional Animal Care and Use Committee.

[00137] The mouse melanoma B16 cell line (ATCC, Manassas, VA) was maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO brand; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA), 2 mM L-glutamine (GIBCO brand; Invitrogen) and 1% penicillin-streptomycin (GIBCO brand; Invitrogen). The Phoenix Ecotropic or Amphtropic packaging cell lines were purchased from Orbigen (San Diego, CA, USA) and maintained in D10 medium containing DMEM, 10% FBS, 1% sodium pyruvate, 2 mM L-glutamine and 1% penicillin-streptomycin.

[00138] Construction of CD8 and TCR Variants. Figure 3 shows a schematic representation of the vector constructs, and the order of placement of components in-frame from the 5' to the 3' ends. The CD8α-MyD88 variants were designed by fusing the murine or human CD8α sequence to the human MyD88 death and intermediate domain sequences. The Toll-Interleukin receptor (TIR) domain of the MyD88 molecule was excluded to eliminating binding to endogenous receptors. The CD8α-28-137-3 variants were composed of extracellular murine or human CD8α linked to the intracellular signaling domains of a traditional 3rd generation CAR: CD28, CD137 (4-1BB), CD3ζ. This acts as a control to compare the level of T cell activation with the MyD88 signaling construct. The CD8ΔIC variants are comprised of extracellular and hinge domains of murine or human CD8α and do not contain any intracellular signaling moieties. The MyD88 TM variants are comprised of the death and intermediate domains of MyD88 fused to the transmembrane portion of murine or human CD8α molecules to control for overexpression of MyD88 at the membrane. Genes were and cloned into the pMIG-w vector, which contains a GFP reported downstream of an IRES sequence. The construct was transfected into HEK293 cells and analyzed for CD8α expression via flow cytometry.

[00139] T cell receptor (TCR) variants were also produced that comprise of DMF5, a human TCR specific for the MART-1_{26-35} peptide from the *MART-1* melanoma antigen presented by MHC I, fused to human MyD88 lacking the TIR domain.

[00140] Construction of retroviral vectors. The retroviral vector backbone pMSGV1-CD8-28BBZ (Hughes M.S. et al., Transfer of a T-cell receptor (TCR) gene derived from a patient with a marked antitumor response conveys highly active T-cell effector functions. *Hum Gene Ther* 2005 Apr;16(4):457-72) was a kind gift from Dr. Richard Morgan (National Cancer Institute) and is derived from pMSGV (murine stem cell virus-based splice-gag vector).

[00141] Mouse CD8α (Genbank NM_001081110.2), human CD8α (Genbank NM_001768.6) and human MyD88 (Genbank NM_001172567.1) were used in the production of retroviral vectors encoding the CD8α variants. The MyD88 nucleotide sequences used refer to a region lacking the Toll/IL-1 receptor (TIR) domain (NM_001172567.1, nucleotides #224-688). The extracellular and transmembrane region of human CD8α (NM_001768.6, nucleotides #890-1498) or mouse CD8α (NM_001081110.2, nucleotides #247-777) were also used.

[00142] For construction of the CD4-MyD88 variants, retroviral vectors encoding human (Genbank NM_000616) or mouse (Genbank NM_013488) CD4 linked to MyD88 lacking the TIR domain were prepared.

[00143] For construction of the TCR-MyD88 variant, human MyD88 (lacking the TIR domain) was cloned downstream of the human DMF5 TCRαβ chain (TCR alpha chain - Genbank Accession (3QEU_D) and GI (GI:339717586); TCR beta chain - Genbank Accession (3QEU_E) and GI:339717587; see also Johnson et al., *Journal of Immunology*. 2006; 177(9):6548-59)) in the retroviral vector. The DMF5 vector was provided by Dr. Laura Johnson (NCI, Bethesda, MD; Johnson, LA et al. *Blood* 2009;114:535-546; Johnson, LA et al. *J. Immunol.* 2006;177:6548-6559) .

[00144] The construct sequences were confirmed by DNA sequencing and are as follows:

Human CD8α full sequence (hCD8α): gi:225007534:ref:NM_001768.6 (SEQ ID NO:1):
ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCC
AGGCCGAGCCAGTTCCGGGTGTCGCCGCTGGATCGGACCTGGAACCTGGGCGAGAC
AGTGGAGCTGAAGTGCCAGGTGCTGCTGTCCAACCCGACGTCGGGCTGCTCGTGGCT
CTTCCAGCCGCGCGGCGCCGCCGCCAGTCCCACCTTCCTCCTATACCTCTCCCAAAA
CAAGCCCAAGGCGGCCGAGGGGCTGGACACCCAGCGGTTCTCGGGCAAGAGGTTGG
GGGACACCTTCGTCCTCACCCTGAGCGACTTCCGCCGAGAGAACGAGGGCTACTATT

TCTGCTCGGCCCTGAGCAACTCCATCATGTACTTCAGCCACTTCGTGCCGGTCTTCCT
GCCAGCGAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCA
TCGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGC
GCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGATATCTACATCTGGGCGCCCTTG
GCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTTATCACCCTTTACTGCAACCACA
GGAACCGAAGACGTGTTTGCAAATGTCCCCGGCCTGTGGTCAAATCGGGAGACAAG
CCCAGCCTTTCGGCGAGATACGTC

Human CD8α without the intracellular signaling domain (hCD8αΔIC) (SEQ ID NO:2):
ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCC
AGGCCGAGCCAGTTCCGGGTGTCGCCGCTGGATCGGACCTGGAACCTGGGCGAGAC
AGTGGAGCTGAAGTGCCAGGTGCTGCTGTCCAACCCGACGTCGGGCTGCTCGTGGCT
CTTCCAGCCGCGCGGCGCCGCCGCCAGTCCCACCTTCCTCCTATACCTCTCCCAAAA
CAAGCCCAAGGCGGCCGAGGGGCTGGACACCCAGCGGTTCTCGGGCAAGAGGTTGG
GGGACACCTTCGTCCTCACCCTGAGCGACTTCCGCCGAGAGAACGAGGGCTACTATT
TCTGCTCGGCCCTGAGCAACTCCATCATGTACTTCAGCCACTTCGTGCCGGTCTTCCT
GCCAGCGAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCA
TCGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGC
GCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGATATCTACATCTGGGCGCCCTTG
GCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTTATCACC

Human CD8α-MyD88ΔTIR where MyD88 lacks the TIR domain (hCD8α-hMyD88) (SEQ ID NO:3):
The underlined section denotes the MyD88 sequence.
ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCC
AGGCCGAGCCAGTTCCGGGTGTCGCCGCTGGATCGGACCTGGAACCTGGGCGAGAC
AGTGGAGCTGAAGTGCCAGGTGCTGCTGTCCAACCCGACGTCGGGCTGCTCGTGGCT
CTTCCAGCCGCGCGGCGCCGCCGCCAGTCCCACCTTCCTCCTATACCTCTCCCAAAA
CAAGCCCAAGGCGGCCGAGGGGCTGGACACCCAGCGGTTCTCGGGCAAGAGGTTGG
GGGACACCTTCGTCCTCACCCTGAGCGACTTCCGCCGAGAGAACGAGGGCTACTATT
TCTGCTCGGCCCTGAGCAACTCCATCATGTACTTCAGCCACTTCGTGCCGGTCTTCCT
GCCAGCGAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCA
TCGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGC
GCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGATATCTACATCTGGGCGCCCTTG
GCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTTATCACCATGGCTGCAGGAGGT
CCCGGCGCGGGGTCTGCGGCCCCGGTCTCCTCCACATCCTCCCTTCCCCTGGCTGCTC
TCAACATGCGAGTGCGGCGCCGCCTGTCTCTGTTCTTGAACGTGCGGACACAGGTGG
CGGCCGACTGGACCGCGCTGGCGGAGGAGATGGACTTTGAGTACTTGGAGATCCGG
CAACTGGAGACACAAGCGGACCCCACTGGCAGGCTGCTGGACGCCTGGCAGGGACG
CCCTGGCGCCTCTGTAGGCCGACTGCTCGAGCTGCTTACCAAGCTGGGCCGCGACGA
CGTGCTGCTGGAGCTGGGACCCAGCATTGAGGAGGATTGCCAAAAGTATATCTTGA
AGCAGCAGCAGGAGGAGGCTGAGAAGCCTTTACAGGTGGCCGCTGTAGACAGCAGT
GTCCCACGGACAGCAGAGCTGGCGGGCATCACCACACTTGATGACCCCCTGGGG

Mouse CD8α full sequence (mCD8α): gi:126722839:ref:001081110.2 (SEQ ID NO:4):
ATGGCCTCACCGTTGACCCGCTTTCTGTCGCTGAACCTGCTGCTGCTGGGTGAGTCG
ATTATCCTGGGGAGTGGAGAAGCTAAGCCACAGGCACCCGAACTCCGAATCTTTCC
AAAGAAAATGGACGCCGAACTTGGTCAGAAGGTGGACCTGGTATGTGAAGTGTTGG
GGTCCGTTTCGCAAGGATGCTCTTGGCTCTTCCAGAACTCCAGCTCCAAACTCCCCC
AGCCCACCTTCGTTGTCTATATGGCTTCATCCCACAACAAGATAACGTGGGACGAGA
AGCTGAATTCGTCGAAACTGTTTTCTGCCATGAGGGACACGAATAATAAGTACGTTC
TCACCCTGAACAAGTTCAGCAAGGAAAACGAAGGCTACTATTTCTGCTCAGTCATCA
GCAACTCGGTGATGTACTTCAGTTCTGTCGTGCCAGTCCTTCAGAAAGTGAACTCTA
CTACTACCAAGCCAGTGCTGCGAACTCCCTCACCTGTGCACCCTACCGGGACATCTC
AGCCCCAGAGACCAGAAGATTGTCGGCCCCGTGGCTCAGTGAAGGGGACCGGATTG
GACTTCGCCTGTGATATTTACATCTGGGCACCCTTGGCCGGAATCTGCGTGGCCCTTC
TGCTGTCCTTGATCATCACTCTCATCTGCTACCACAGGAGCCGAAAGCGTGTTTGCA
AATGTCCCAGGCCGCTAGTCAGACAGGAAGGCAAGCCCAGACCTTCAGAGAAAATT
GTGTAA

Mouse CD8α without the intracellular signaling domain (mCD8αΔIC) (SEQ ID NO:5):
ATGGCCTCACCGTTGACCCGCTTTCTGTCGCTGAACCTGCTGCTGCTGGGTGAGTCG
ATTATCCTGGGGAGTGGAGAAGCTAAGCCACAGGCACCCGAACTCCGAATCTTTCC
AAAGAAAATGGACGCCGAACTTGGTCAGAAGGTGGACCTGGTATGTGAAGTGTTGG
GGTCCGTTTCGCAAGGATGCTCTTGGCTCTTCCAGAACTCCAGCTCCAAACTCCCCC
AGCCCACCTTCGTTGTCTATATGGCTTCATCCCACAACAAGATAACGTGGGACGAGA
AGCTGAATTCGTCGAAACTGTTTTCTGCCATGAGGGACACGAATAATAAGTACGTTC
TCACCCTGAACAAGTTCAGCAAGGAAAACGAAGGCTACTATTTCTGCTCAGTCATCA
GCAACTCGGTGATGTACTTCAGTTCTGTCGTGCCAGTCCTTCAGAAAGTGAACTCTA
CTACTACCAAGCCAGTGCTGCGAACTCCCTCACCTGTGCACCCTACCGGGACATCTC
AGCCCCAGAGACCAGAAGATTGTCGGCCCCGTGGCTCAGTGAAGGGGACCGGATTG
GACTTCGCCTGTGATATTTACATCTGGGCACCCTTGGCCGGAATCTGCGTGGCCCTTC
TGCTGTCCTTGATCATCACTCTCATC

Mouse CD8α-human MyD88ΔTIR where MyD88 lacks TIR domain (mCD8α-hMyD88) (SEQ ID NO:6):
The underlined section denotes the MyD88 sequence.
ATGGCCTCACCGTTGACCCGCTTTCTGTCGCTGAACCTGCTGCTGCTGGGTGAGTCG
ATTATCCTGGGGAGTGGAGAAGCTAAGCCACAGGCACCCGAACTCCGAATCTTTCC
AAAGAAAATGGACGCCGAACTTGGTCAGAAGGTGGACCTGGTATGTGAAGTGTTGG
GGTCCGTTTCGCAAGGATGCTCTTGGCTCTTCCAGAACTCCAGCTCCAAACTCCCCC
AGCCCACCTTCGTTGTCTATATGGCTTCATCCCACAACAAGATAACGTGGGACGAGA
AGCTGAATTCGTCGAAACTGTTTTCTGCCATGAGGGACACGAATAATAAGTACGTTC
TCACCCTGAACAAGTTCAGCAAGGAAAACGAAGGCTACTATTTCTGCTCAGTCATCA

GCAACTCGGTGATGTACTTCAGTTCTGTCGTGCCAGTCCTTCAGAAAGTGAACTCTA
CTACTACCAAGCCAGTGCTGCGAACTCCCTCACCTGTGCACCCTACCGGGACATCTC
AGCCCCAGAGACCAGAAGATTGTCGGCCCCGTGGCTCAGTGAAGGGGACCGGATTG
GACTTCGCCTGTGATATTTACATCTGGGCACCCTTGGCCGGAATCTGCGTGGCCCTTC
TGCTGTCCTTGATCATCACTCTCATCATGGCTGCAGGAGGTCCCGGCGCGGGGTCTG
CGGCCCCGGTCTCCTCCACATCCTCCCTTCCCCTGGCTGCTCTCAACATGCGAGTGCG
GCGCCGCCTGTCTCTGTTCTTGAACGTGCGGACACAGGTGGCGGCCGACTGGACCGC
GCTGGCGGAGGAGATGGACTTTGAGTACTTGGAGATCCGGCAACTGGAGACACAAG
CGGACCCCACTGGCAGGCTGCTGGACGCCTGGCAGGGACGCCCTGGCGCCTCTGTA
GGCCGACTGCTCGAGCTGCTTACCAAGCTGGGCCGCGACGACGTGCTGCTGGAGCT
GGGACCCAGCATTGAGGAGGATTGCCAAAAGTATATCTTGAAGCAGCAGCAGGAGG
AGGCTGAGAAGCCTTTACAGGTGGCCGCTGTAGACAGCAGTGTCCCACGGACAGCA
GAGCTGGCGGGCATCACCACACTTGATGACCCCCTGGGG

Human CD8α TM-MyD88 (hCD8αTM-hMyD88) (SEQ ID NO:7): This vector lacks the extracellular part of human CD8α but contains the CD8α transmembrane hinge domain, followed by the human MyD88ΔTIR intracellular signaling sequence (underlined).
TTCGTGCCGGTCTTCCTGCCAGCGAAGCCCACCACGACGCCAGCGCCGCGACCACCA
ACACCGGCGCCCACCATCGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGTGCCG
GCCAGCGGCGGGGGGCGCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGATATCT
ACATCTGGGCGCCCTTGGCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTTATCAC
CCTTTACTGCAACCACAGGAACATGGCTGCAGGAGGTCCCGGCGCGGGGTCTGCGG
CCCCGGTCTCCTCCACATCCTCCCTTCCCCTGGCTGCTCTCAACATGCGAGTGCGGCG
CCGCCTGTCTCTGTTCTTGAACGTGCGGACACAGGTGGCGGCCGACTGGACCGCGCT
GGCGGAGGAGATGGACTTTGAGTACTTGGAGATCCGGCAACTGGAGACACAAGCGG
ACCCCACTGGCAGGCTGCTGGACGCCTGGCAGGGACGCCCTGGCGCCTCTGTAGGC
CGACTGCTCGAGCTGCTTACCAAGCTGGGCCGCGACGACGTGCTGCTGGAGCTGGG
ACCCAGCATTGAGGAGGATTGCCAAAAGTATATCTTGAAGCAGCAGCAGGAGGAGG
CTGAGAAGCCTTTACAGGTGGCCGCTGTAGACAGCAGTGTCCCACGGACAGCAGAG
CTGGCGGGCATCACCACACTTGATGACCCCCTGGGG

Mouse CD8α-human CD28-human 41BB-human CD3zeta (mCD8α-28-137-3; in some instances this same construct is term mCD8α-137-28-3) (SEQ ID NO:8):
This vector contains extracellular mouse CD8α and transmembrane hinge domain followed by the human intracellular signaling sequences that activate CD28, CD137 and the CD3 zeta chain. The CD28 is bold, the 41BB is double underlined and the CD3zeta is underlined with dotted line. Linkers are shown with wavy underlines.
ATGGCCTCACCGTTGACCCGCTTTCTGTCGCTGAACCTGCTGCTGCTGGGTGAGTCG
ATTATCCTGGGGAGTGGAGAAGCTAAGCCACAGGCACCCGAACTCCGAATCTTTCC

AAAGAAAATGGACGCCGAACTTGGTCAGAAGGTGGACCTGGTATGTGAAGTGTTGG
GGTCCGTTTCGCAAGGATGCTCTTGGCTCTTCCAGAACTCCAGCTCCAAACTCCCCC
AGCCCACCTTCGTTGTCTATATGGCTTCATCCCACAACAAGATAACGTGGGACGAGA
AGCTGAATTCGTCGAAACTGTTTTCTGCCATGAGGGACACGAATAATAAGTACGTTC
TCACCCTGAACAAGTTCAGCAAGGAAAACGAAGGCTACTATTTCTGCTCAGTCATCA
GCAACTCGGTGATGTACTTCAGTTCTGTCGTGCCAGTCCTTCAGAAAGTGAACTCTA
CTACTACCAAGCCAGTGCTGCGAACTCCCTCACCTGTGCACCCTACCGGGACATCTC
AGCCCCAGAGACCAGAAGATTGTCGGCCCCGTGGCTCAGTGAAGGGGACCGGATTG
GACTTCGCCTGTGATATTTACATCTGGGCACCCTTGGCCGGAATCTGCGTGGCCCTTC
TGCTGTCCTTGATCATCACTCTCATC**AGGAGTAAGAGGAGCAGGCTCCTGCACAG**
TGACTACATGAACATGACTCCCCGCCGCCCCGGGCCCACCCGCAAGCATTACC
AGCCCTATGCCCCACCACGCGACTTCGCAGCCTATCGCTCCCGTTTCTCTGTTGTT
AAACGGGGCAGAAAGAAGCTCCTGTATATATTCAAACAACCATTTATGAGACCAGT
ACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAG
GAGGATGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAG
CAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGA
TGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGG
AAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGG
CCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGG
CCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCA
GGCCCTGCCCCCTCGCTAA

Human CD4-MyD88ΔTIR where MyD88 lacks the TIR domain (hCD4-hMyD88) (SEQ ID NO:9):

This vector contains extracellular human CD4 (double underline) and transmembrane hinge domain (wavy underline) followed by human MyD88 lacking the TIR domain (single underline).

ATGAACCGGGGAGTCCCTTTTAGGCACTTGCTTCTGGTGCTGCAACTGGCGCTCCTC
CCAGCAGCCACTCAGGGAAAGAAAGTGGTGCTGGGCAAAAAAGGGGATACAGTGG
AACTGACCTGTACAGCTTCCCAGAAGAAGAGCATACAATTCCACTGGAAAAACTCC
AACCAGATAAAGATTCTGGGAAATCAGGGCTCCTTCTTAACTAAAGGTCCATCCAAG
CTGAATGATCGCGCTGACTCAAGAAGAAGCCTTTGGGACCAAGGAAACTTTCCCCTG
ATCATCAAGAATCTTAAGATAGAAGACTCAGATACTTACATCTGTGAAGTGGAGGA
CCAGAAGGAGGAGGTGCAATTGCTAGTGTTCGGATTGACTGCCAACTCTGACACCC
ACCTGCTTCAGGGGCAGAGCCTGACCCTGACCTTGGAGAGCCCCCCTGGTAGTAGCC
CCTCAGTGCAATGTAGGAGTCCAAGGGGTAAAAACATACAGGGGGGGAAGACCCTC
TCCGTGTCTCAGCTGGAGCTCCAGGATAGTGGCACCTGGACATGCACTGTCTTGCAG
AACCAGAAGAAGGTGGAGTTCAAAATAGACATCGTGGTGCTAGCTTTCCAGAAGGC
CTCCAGCATAGTCTATAAGAAAGAGGGGGAACAGGTGGAGTTCTCCTTCCCACTCGC
CTTTACAGTTGAAAAGCTGACGGGCAGTGGCGAGCTGTGGTGGCAGGCGGAGAGGG

CTTCCTCCTCCAAGTCTTGGATCACCTTTGACCTGAAGAACAAGGAAGTGTCTGTAA
AACGGGTTACCCAGGACCCTAAGCTCCAGATGGGCAAGAAGCTCCCGCTCCACCTC
ACCCTGCCCCAGGCCTTGCCTCAGTATGCTGGCTCTGGAAACCTCACCCTGGCCCTT
GAAGCGAAAACAGGAAAGTTGCATCAGGAAGTGAACCTGGTGGTGATGAGAGCCA
CTCAGCTCCAGAAAAATTTGACCTGTGAGGTGTGGGGACCCACCTCCCCTAAGCTGA
TGCTGAGTTTGAAACTGGAGAACAAGGAGGCAAAGGTCTCGAAGCGGGAGAAGGC
GGTGTGGGTGCTGAACCCTGAGGCGGGGATGTGGCAGTGTCTGCTGAGTGACTCGG
GACAGGTCCTGCTGGAATCCAACATCAAGGTTCTGCCCACATGGTCCACCCCGGTGC
AGCCAATGGCCCTGATTGTGCTGGGGGGCGTCGCCGGCCTCCTGCTTTTCATTGGGC
TAGGCATCTTCTTCATGGCTGCAGGAGGTCCCGGCGCGGGGTCTGCGGCCCCGGTCT
CCTCCACATCCTCCCTTCCCCTGGCTGCTCTCAACATGCGAGTGCGGCGCCGCCTGTC
TCTGTTCTTGAACGTGCGGACACAGGTGGCGGCCGACTGGACCGCGCTGGCGGAGG
AGATGGACTTTGAGTACTTGGAGATCCGGCAACTGGAGACACAAGCGGACCCCACT
GGCAGGCTGCTGGACGCCTGGCAGGGACGCCCTGGCGCCTCTGTAGGCCGACTGCT
CGAGCTGCTTACCAAGCTGGGCCGCGACGACGTGCTGCTGGAGCTGGGACCCAGCA
TTGAGGAGGATTGCCAAAAGTATATCTTGAAGCAGCAGCAGGAGGAGGCTGAGAAG
CCTTTACAGGTGGCCGCTGTAGACAGCAGTGTCCCACGGACAGCAGAGCTGGCGGG
CATCACCACACTTGATGACCCCCTGGGGTGA

Mouse CD4-human MyD88ΔTIR where MyD88 lacks the TIR domain (mCD4-hMyD88) (SEQ ID NO:10):

This vector contains extracellular mouse CD4 (double underline) and transmembrane hinge domain (wavy underline) followed by human MyD88 lacking the TIR domain (single underline).

ATGTGCCGAGCCATCTCTCTTAGGCGCTTGCTGCTGCTGCTGCTGCAGCTGTCACAA
CTCCTAGCTGTCACTCAAGGGAAGACGCTGGTGCTGGGGAAGGAAGGGGAATCAGC
AGAACTGCCCTGCGAGAGTTCCCAGAAGAAGATCACAGTCTTCACCTGGAAGTTCTC
TGACCAGAGGAAGATTCTGGGGCAGCATGGCAAAGGTGTATTAATTAGAGGAGGTT
CGCCTTCGCAGTTTGATCGTTTTGATTCCAAAAAAGGGGCATGGGAGAAAGGATCGT
TTCCTCTCATCATCAATAAACTTAAGATGGAAGACTCTCAGACTTATATCTGTGAGC
TGGAGAACAGGAAAGAGGAGGTGGAGTTGTGGGTGTTCAAAGTGACCTTCAGTCCG
GGTACCAGCCTGTTGCAAGGGCAGAGCCTGACCCTGACCTTGGATAGCAACTCTAA
GGTCTCTAACCCCTTGACAGAGTGCAAACACAAAAAGGGTAAAGTTGTCAGTGGTT
CCAAAGTTCTCTCCATGTCCAACCTAAGGGTTCAGGACAGCGACTTCTGGAACTGCA
CCGTGACCCTGGACCAGAAAAAGAACTGGTTCGGCATGACACTCTCAGTGCTGGGTT
TTCAGAGCACAGCTATCACGGCCTATAAGAGTGAGGGAGAGTCAGCGGAGTTCTCC
TTCCCACTCAACTTTGCAGAGGAAAACGGGTGGGGAGAGCTGATGTGGAAGGCAGA
GAAGGATTCTTTCTTCCAGCCCTGGATCTCCTTCTCCATAAAGAACAAAGAGGTGTC
CGTACAAAAGTCCACCAAAGACCTCAAGCTCCAGCTGAAGGAAACGCTCCCACTCA
CCCTCAAGATACCCCAGGTCTCGCTTCAGTTTGCTGGTTCTGGCAACCTGACTCTGA
CTCTGGACAAAGGGACACTGCATCAGGAAGTGAACCTGGTGGTGATGAAAGTGGCT
CAGCTCAACAATACTTTGACCTGTGAGGTGATGGGACCTACCTCTCCCAAGATGAGA
CTGACCCTGAAGCAGGAGAACCAGGAGGCCAGGGTCTCTGAGGAGCAGAAAGTAGT

TCAAGTGGTGGCCCCTGAGACAGGGCTGTGGCAGTGTCTACTGAGTGAAGGTGATA
AGGTCAAGATGGACTCCAGGATCCAGGTTTTATCCAGAGGGGTGAACCAGACAGTG
TTCCTGGCTTGCGTGCTGGGTGGCTCCTTCGGCTTTCTGGGTTTCCTTGGGCTCTGCA
TCCTCTGCATGGCTGCAGGAGGTCCCGGCGCGGGGTCTGCGGCCCCGGTCTCCTCCA
CATCCTCCCTTCCCCTGGCTGCTCTCAACATGCGAGTGCGGCGCCGCCTGTCTCTGTT
CTTGAACGTGCGGACACAGGTGGCGGCCGACTGGACCGCGCTGGCGGAGGAGATGG
ACTTTGAGTACTTGGAGATCCGGCAACTGGAGACACAAGCGGACCCCACTGGCAGG
CTGCTGGACGCCTGGCAGGGACGCCCTGGCGCCTCTGTAGGCCGACTGCTCGAGCTG
CTTACCAAGCTGGGCCGCGACGACGTGCTGCTGGAGCTGGGACCCAGCATTGAGGA
GGATTGCCAAAAGTATATCTTGAAGCAGCAGCAGGAGGAGGCTGAGAAGCCTTTAC
AGGTGGCCGCTGTAGACAGCAGTGTCCCACGGACAGCAGAGCTGGCGGGCATCACC
ACACTTGATGACCCCCTGGGG TGA

Human DMF5 TCR-MyD88 (hTCR-hMyD88) (SEQ ID NO:11):

The DMF5 T cell receptor, which recognizes the 27–35 nonameric and 26–35 decameric peptide epitopes from the *MART-1* melanoma antigen presented by MHC I, is fused to human MyD88 lacking the TIR domain. DMF5 was kindly provided by Dr. Laura Johnson at the National Cancer Institute and is the same sequence used in clinical trials registered at www.ClinicalTrials.gov as NCI-07-C-0174 and NCI-07-C-0175 (Johnson, LA et al. *Blood* 2009;114:535-546; Johnson, LA et al. *J. Immunol.* 2006;177:6548-6559). TCRαβ sequence (single underline) is linked to the MyD88 sequence (double underline).

CCGCCATGATGAAATCCTTGAGAGTTTTACTAGTGATCCTGTGGCTTCAGTTGAGCT
GGGTTTGGAGCCAACAGAAGGAGGTGGAGCAGAATTCTGGACCCCTCAGTGTTCCA
GAGGGAGCCATTGCCTCTCTCAACTGCACTTACAGTGACCGAGGTTCCCAGTCCTTC
TTCTGGTACAGACAATATTCTGGGAAAAGCCCTGAGTTGATAATGTTCATATACTCC
AATGGTGACAAAGAAGATGGAAGGTTTACAGCACAGCTCAATAAAGCCAGCCAGTA
TGTTTCTCTGCTCATCAGAGACTCCCAGCCCAGTGATTCAGCCACCTACCTCTGTGCC
GTGAACTTCGGAGGAGGAAAGCTTATCTTCGGACAGGGAACGGAGTTATCTGTGAA
ACCCAATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAG
TGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAG
TAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGA
CTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAA
CGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGAAAGTTC
CTGTGATGTCAAGCTGGTCGAGAAAAGCTTTGAAACAGATACGAACCTAAACTTTCA
AAACCTGTCAGTGATTGGGTTCCGAATCCTCCTCCTGAAGGTGGCCGGGTTTAATCT
GCTCATGACGCTGCGGCTGTGGTCCAGCAGAGCCAAAAGAGAGGGCAGAGGAAGTC
TTCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCTATGAGAATCAGGCTCCTGT
GCTGTGTGGCCTTTTCTCTCCTGTGGGCAGGTCCAGTGATTGCTGGGATCACCCAGG
CACCAACATCTCAGATCCTGGCAGCAGGACGGCGCATGACACTGAGATGTACCCAG
GATATGAGACATAATGCCATGTACTGGTATAGACAAGATCTAGGACTGGGGCTAAG
GCTCATCCATTATTCAAATACTGCAGGTACCACTGGCAAAGGAGAAGTCCCTGATGG

TTATAGTGTCTCCAGAGCAAACACAGATGATTTCCCCCTCACGTTGGCGTCTGCTGT
ACCCTCTCAGACATCTGTGTACTTCTGTGCCAGCAGCCTAAGTTTCGGCACTGAAGC
TTTCTTTGGACAAGGCACCAGACTCACAGTTGTAGAGGACCTGAACAAGGTGTTCCC
ACCCGAGGTCGCTGTGTTTGAGCCATCAGAAGCAGAGATCTCCCACACCCAAAAGG
CCACACTGGTGTGCCTGGCCACAGGCTTCTTCCCTGACCACGTGGAGCTGAGCTGGT
GGGTGAATGGGAAGGAGGTGCACAGTGGGGTCAGCACGGACCCGCAGCCCCTCAA
GGAGCAGCCCGCCCTCAATGACTCCAGATACTGCCTGAGCAGCCGCCTGAGGGTCT
CGGCCACCTTCTGGCAGAACCCCCGCAACCACTTCCGCTGTCAAGTCCAGTTCTACG
GGCTCTCGGAGAATGACGAGTGGACCCAGGATAGGGCCAAACCCGTCACCCAGATC
GTCAGCGCCGAGGCCTGGGGTAGAGCAGACTGTGGCTTTACCTCGGTGTCCTACCAG
CAAGGGGTCCTGTCTGCCACCATCCTCTATGAGATCCTGCTAGGGAAGGCCACCCTG
TATGCTGTGCTGGTCAGCGCCCTTGTGTTGATGGCCATGGTCAAGAGAAAGGATTTC
GGATCCATGGCTGCAGGAGGTCCCGGCGCGGGGTCTGCGGCCCCGGTCTCCTCCAC
ATCCTCCCTTCCCCTGGCTGCTCTCAACATGCGAGTGCGGCGCCGCCTGTCTCTGTTC
TTGAACGTGCGGACACAGGTGGCGGCCGACTGGACCGCGCTGGCGGAGGAGATGGA
CTTTGAGTACTTGGAGATCCGGCAACTGGAGACACAAGCGGACCCCACTGGCAGGC
TGCTGGACGCCTGGCAGGGACGCCCTGGCGCCTCTGTAGGCCGACTGCTCGAGCTGC
TTACCAAGCTGGGCCGCGACGACGTGCTGCTGGAGCTGGGACCCAGCATTGAGGAG
GATTGCCAAAAGTATATCTTGAAGCAGCAGCAGGAGGAGGCTGAGAAGCCTTTACA
GGTGGCCGCTGTAGACAGCAGTGTCCCACGGACAGCAGAGCTGGCGGGCATCACCA
CACTTGATGACCCCCTGGGG

Amino acid sequences:

hCD8α (SEQ ID NO:12):
MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQ
PRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYYFCSAL
SNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRPVVKSGDKPSLSARYV

hCD8αΔIC (SEQ ID NO:13):
MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQ
PRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYYFCSAL
SNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDIYIWAPLAGTCGVLLLSLVIT

hCD8α-hMyD88; MyD88 lacks the TIR domain (SEQ ID NO:14):
The underlined section denotes the MyD88 sequence.

MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQ
PRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYYFCSAL
SNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDIYIWAPLAGTCGVLLLSLVITMAAGGPGAGSAAPVSSTSSLPLAALNMRVRRRLSLF
LNVRTQVAADWTALAEEMDFEYLEIRQLETQADPTGRLLDAWQGRPGASVGRLLELLT
KLGRDDVLLELGPSIEEDCQKYILKQQQEEAEKPLQVAAVDSSVPRTAELAGITTLDDPL
G

mCD8α (SEQ ID NO:15):
MASPLTRFLSLNLLLLGESIILGSGEAKPQAPELRIFPKKMDAELGQKVDLVCEVLGSVS
QGCSWLFQNSSSKLPQPTFVVYMASSHNKITWDEKLNSSKLFSAMRDTNNKYVLTLNK
FSKENEGYYFCSVISNSVMYFSSVVPVLQKVNSTTTKPVLRTPSPVHPTGTSQPQRPEDC
RPRGSVKGTGLDFACDIYIWAPLAGICVALLLSLIITLICYHRSRKRVCKCPRPLVRQEGK
PRPSEKIV

mCD8αΔIC (SEQ ID NO:16):
MASPLTRFLSLNLLLLGESIILGSGEAKPQAPELRIFPKKMDAELGQKVDLVCEVLGSVS
QGCSWLFQNSSSKLPQPTFVVYMASSHNKITWDEKLNSSKLFSAMRDTNNKYVLTLNK
FSKENEGYYFCSVISNSVMYFSSVVPVLQKVNSTTTKPVLRTPSPVHPTGTSQPQRPEDC
RPRGSVKGTGLDFACDIYIWAPLAGICVALLLSLIITLI

mCD8α-hMyD88; MyD88 lacks the TIR domain (SEQ ID NO:17):
The underlined section denotes the MyD88 sequence.
MASPLTRFLSLNLLLLGESIILGSGEAKPQAPELRIFPKKMDAELGQKVDLVCEVLGSVS
QGCSWLFQNSSSKLPQPTFVVYMASSHNKITWDEKLNSSKLFSAMRDTNNKYVLTLNK
FSKENEGYYFCSVISNSVMYFSSVVPVLQKVNSTTTKPVLRTPSPVHPTGTSQPQRPEDC
RPRGSVKGTGLDFACDIYIWAPLAGICVALLLSLIITLIMAAGGPGAGSAAPVSSTSSLPL
AALNMRVRRRLSLFLNVRTQVAADWTALAEEMDFEYLEIRQLETQADPTGRLLDAWQ
GRPGASVGRLLELLTKLGRDDVLLELGPSIEEDCQKYILKQQQEEAEKPLQVAAVDSSVP
RTAELAGITTLDDPLG

hCD8αTM-hMyD88 (SEQ ID NO:18):
CD8α transmembrane hinge domain, followed by the human MyD88ΔTIR intracellular signaling sequence (underlined).
FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAP
LAGTCGVLLLSLVITLYCNHRNMAAGGPGAGSAAPVSSTSSLPLAALNMRVRRRLSLFL
NVRTQVAADWTALAEEMDFEYLEIRQLETQADPTGRLLDAWQGRPGASVGRLLELLTK
LGRDDVLLELGPSIEEDCQKYILKQQQEEAEKPLQVAAVDSSVPRTAELAGITTLDDPLG

mCD8α-28-137-3 (and mCD8α-137-28-3) (SEQ ID NO:19):
Extracellular mouse CD8α and transmembrane hinge domain, followed by the human intracellular signaling sequences that activate CD28 (bold), CD137 (double underlined) and the CD3 zeta chain (dotted line). Linkers are shown with wavy underlines.
MASPLTRFLSLNLLLLGESIILGSGEAKPQAPELRIFPKKMDAELGQKVDLVCEVLGSVS
QGCSWLFQNSSSKLPQPTFVVYMASSHNKITWDEKLNSSKLFSAMRDTNNKYVLTLNK
FSKENEGYYFCSVISNSVMYFSSVVPVLQKVNSTTTKPVLRTPSPVHPTGTSQPQRPEDC
RPRGSVKGTGLDFACDIYIWAPLAGICVALLLSLIITLI**RSKRSRLLHSDYMNMTPRRPG**
PTRKHYQPYAPPRDFAAYRSRFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP
EEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK
PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH
MQALPPR

hCD4-hMyD88 (SEQ ID NO:20):
Human CD4 extracellular (double underline) and transmembrane hinge domains (wavy underline) followed by human MyD88 lacking the TIR domain (single underline).
MNRGVPFRHLLLVLQLALLPAATQGKKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQ
IKILGNQGSFLTKGPSKLNDRADSRRSLWDQGNFPLIIKNLKIEDSDTYICEVEDQKEEVQ
LLVFGLTANSDTHLLQGQSLTLTLESPPGSSPSVQCRSPRGKNIQGGKTLSVSQLELQDS
GTWTCTVLQNQKKVEFKIDIVVLAFQKASSIVYKKEGEQVEFSFPLAFTVEKLTGSGEL
WWQAERASSSKSWITFDLKNKEVSVKRVTQDPKLQMGKKLPLHLTLPQALPQYAGSG
NLTLALEAKTGKLHQEVNLVVMRATQLQKNLTCEVWGPTSPKLMLSLKLENKEAKVS
KREKAVWVLNPEAGMWQCLLSDSGQVLLESNIKVLPTWSTPVQPMALIVLGGVAGLLL
FIGLGIFFMAAGGPGAGSAAPVSSTSSLPLAALNMRVRRRLSLFLNVRTQVAADWTALA
EEMDFEYLEIRQLETQADPTGRLLDAWQGRPGASVGRLLELLTKLGRDDVLLELGPSIEE
DCQKYILKQQQEEAEKPLQVAAVDSSVPRTAELAGITTLDDPLG

mCD4-hMyD88 (SEQ ID NO:21):
Mouse CD4 extracellular (double underline) and transmembrane hinge domains (wavy underline) followed by human MyD88 lacking the TIR domain (single underline).
MCRAISLRRLLLLLLQLSQLLAVTQGKTLVLGKEGESAELPCESSQKKITVFTWKFSDQR
KILGQHGKGVLIRGGSPSQFDRFDSKKGAWEKGSFPLIINKLKMEDSQTYICELENRKEE
VELWVFKVTFSPGTSLLQGQSLTLTLDSNSKVSNPLTECKHKKGKVVSGSKVLSMSNLR
VQDSDFWNCTVTLDQKKNWFGMTLSVLGFQSTAITAYKSEGESAEFSFPLNFAEENGW
GELMWKAEKDSFFQPWISFSIKNKEVSVQKSTKDLKLQLKETLPLTLKIPQVSLQFAGSG
NLTLTLDKGTLHQEVNLVVMKVAQLNNTLTCEVMGPTSPKMRLTLKQENQEARVSEE
QKVVQVVAPETGLWQCLLSEGDKVKMDSRIQVLSRGVNQTVFLACVLGGSFGFLGFLG
LCILCMAAGGPGAGSAAPVSSTSSLPLAALNMRVRRRLSLFLNVRTQVAADWTALAEE

MDFEYLEIRQLETQADPTGRLLDAWQGRPGASVGRLLELLTKLGRDDVLLELGPSIEED
CQKYILKQQQEEAEKPLQVAAVDSSVPRTAELAGITTLDDPLG

hTCR-hMyD88 (SEQ ID NO:22):
TCRαβ sequence (single underline) is linked to the MyD88 sequence (double underline).
MMKSLRVLLVILWLQLSWVWSQQKEVEQNSGPLSVPEGAIASLNCTYSDRGSQSFFWY
RQYSGKSPELIMFIYSNGDKEDGRFTAQLNKASQYVSLLIRDSQPSDSATYLCAVNFGGG
KLIFGQGTELSVKPNIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDSDVYITD
KTVLDMRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTFFPSPESSCDVKLVEKSFET
DTNLNFQNLSVIGFRILLLKVAGFNLLMTLRLWSSRAKREGRGSLLTCGDVEENPGPMRI
RLLCCVAFSLLWAGPVIAGITQAPTSQILAAGRRMTLRCTQDMRHNAMYWYRQDLGLG
LRLIHYSNTAGTTGKGEVPDGYSVSRANTDDFPLTLASAVPSQTSVYFCASSLSFGTEAF
FGQGTRLTVVEDLNKVFPPEVAVFEPSEAEISHTQKATLVCLATGFFPDHVELSWWVNG
KEVHSGVSTDPQPLKEQPALNDSRYCLSSRLRVSATFWQNPRNHFRCQVQFYGLSENDE
WTQDRAKPVTQIVSAEAWGRADCGFTSVSYQQGVLSATILYEILLGKATLYAVLVSALV
LMAMVKRKDFGSMAAGGPGAGSAAPVSSTSSLPLAALNMRVRRRLSLFLNVRTQVAA
DWTALAEEMDFEYLEIRQLETQADPTGRLLDAWQGRPGASVGRLLELLTKLGRDDVLL
ELGPSIEEDCQKYILKQQQEEAEKPLQVAAVDSSVPRTAELAGITTLDDPLGGS

Human MyD88 (SEQ ID NO:24):
MAAGGPGAGSAAPVSSTSSLPLAALNMRVRRRLSLFLNVRTQVAADWTALAEEMDFE
YLEIRQLETQADPTGRLLDAWQGRPGASVGRLLELLTKLGRDDVLLELGPSIEEDCQKYI
LKQQQEEAEKPLQVAAVDSSVPRTAELAGITTLDDPLGHMPERFDAFICYCPSDIQFVQE
MIRQLEQTNYRLKLCVSDRDVLPGTCVWSIASELIEKRCRRMVVVVSDDYLQSKECDFQ
TKFALSLSPGAHQKRLIPIKYKAMKKEFPSILRFITVCDYTNPCTKSWFWTRLAKALSLP

[00145] Transfection of HEK cells. That the mouse CD8α variant could be properly expressed in the cell membrane was confirmed using in human endothelial kidney cells (HEK; ATCC, Manassas, VA). HEK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO brand; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA), 2 mM L-glutamine (GIBCO brand; Invitrogen) and 1% penicillin-streptomycin (GIBCO brand; Invitrogen). The day before transfection, the cells were trypsinized and counted. 0.5 x 10^5 cells/ml were plated at a cell density of 50% confluency. Cells were transfected with 2.5 μg of DNA encoding mCD8α in 100 μl of Opti-MEM® I Reduced Serum Media without serum and 0.5 μl of Lipofectamine LTX (LIFE Technologies, Grand Island, NY) according to the manufacturer's protocol. After a 30

minute incubation, 100 µl of the DNA- Lipofectamine LTX Reagent complexes were added directly to each well containing cells and mixed gently by rocking the plate back and forth. 48 hours after transfection the expression of CD8α was determined by flow cytometry.

[00146] Retroviral transduction of T cells. Spleen and lymph node–derived mouse pmel T cells (Jackson Laboratory) were activated using plate bound anti-CD3 (5ug/ml) and anti-CD28 Ab (2.5ug/ml) (BD Biosciences, Franklin Lakes, NJ, USA) for two days while T cell receptors specific for the mouse $gp100_{25-33}$ antigen expression were activated by adding 1 ug/ml of $gp100_{25-33}$ peptide. Two days later cells were collected for retroviral transduction. For transduction, 24-well non-tissue culture treated plates (BD Biosciences, Franklin Lakes, NJ, USA) were coated with 0.5 ml per well of 10 µg/ml recombinant human fibronectin fragment (RetroNectin; Takara, Otsu, Shiga, Japan) overnight at 4 °C. After incubation, wells were blocked with 1 ml of Hank's balanced salt solution (GIBCO brand; Invitrogen) plus 2.5% human AB serum for 30 min at RT, and washed with Hank's balanced salt solution plus 2.5% N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (GIBCO brand; Invitrogen).

[00147] Transductions were conducted as previously described (Johnson et al. *Blood* 114, 535-546 (2009)). Briefly, approximately 2.5 ml of retroviral supernatant were added to each coated well followed by centrifugation at 2000g for 2 h at 32 °C. 1.5 ml of viral supernatant was removed and 1×10^6 (0.5 ml) activated T cells were added to each well in the presence of 100 U/ml IL-2. Plates were centrifugated at 1000g for 10 min, and then incubated overnight at 37 °C. After transduction, cells were washed and maintained in the presence of IL-2 (100U/ml) and used in experiments five days after transduction. The transduction efficiencies are determined by evaluating the percentage of T cells positive for green fluorescent protein (GFP+), as the MGSV vector used contains a gene that encodes GFP. The plasmid is referred to as pMIG and was purchased from Addgene (Cambridge, MA). The successful transduction of T cells was confirmed by the expression of GFP which is located downstream of the CD8, CD4 and TCR constructs.

[00148] For transduction of human T cells, peripheral blood mononuclear cells (PBMC) from healthy donors were purchased from Biological Specialty Corp (Colmar, PA, USA), and isolated by Ficoll-Pague (GE Healthcare, Piscataway, NJ, USA) density gradient centrifugation. Isolated PBMC were cultured at 3×10^6 per well in 24-well tissue culture plates in AIM V medium

(GIBCO brand; Invitrogen) supplemented with 5% human AB serum (Sigma-Aldrich), 1% MEM non-essential amino acids, 1% penicillin-streptomycin and 100 U/ml recombinant human IL-2 (BioLegend, San Diego, CA, USA), and activated with 50 ng/ml OKT3 (eBioscience, San Diego, CA, USA). Two days later, cells were collected for retroviral transduction.

[00149] For transduction, 24-well non-tissue culture treated plates (BD Biosciences, Franklin Lakes, NJ, USA) were coated with 0.5 ml per well of 10 μg/ml recombinant human fibronectin fragment (RetroNectin; Takara, Otsu, Shiga, Japan) overnight at 4 °C. After incubation, wells were blocked with 1 ml of Hank's balanced salt solution (GIBCO brand; Invitrogen) plus 2.5% human AB serum for 30 min at RT, and washed with Hank's balanced salt solution plus 2.5% N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (GIBCO brand; Invitrogen).

[00150] Transductions were conducted as previously described (Johnson et al. *Blood* 114, 535-546 (2009)). Briefly, approximately 2.5 ml of retroviral supernatant were added to each coated well followed by centrifugation at 2000g for 2 h at 32 °C. 1.5 ml of viral supernatant was removed and 1×10^6 (0.5 ml) activated PBMC were added to each well in the presence of 100 U/ml IL-2. Plates were centrifuged at 1000g for 10 min, and then incubated overnight at 37 °C. After transduction, cells were washed and maintained in the presence of IL-2 (100U/ml) and used in experiments five days after transduction. The surface expression of DMF5 on transduced human T cells was determined by flow cytometry after staining cells with CD8 and MART-1 MHC tetramers.

[00151] NF-κB activation assay. TLR4-expressing HEK-Blue cells were cultured in DMEM 10% FBS 1% PenStrep 1X HEK-Blue Solution (InvivoGen). Cells were plated at $1x10^6$ cells per well in a 6-well plate in antibiotic-free media and cultured at 37°C/5%CO_2 overnight. Cells were transfected with Lipofectamine 2000 (Invitrogen) with 4 μg DNA. 24 hours later, cells were collected and aliquoted at 50,000 cells per well in a 96-well plate in quadruplicate. 50 ug/mL of LPS (Invitrogen) was used as a positive control. Cell supernatant was collected after 24 hours and combined with QUANTI-Blue reagent (InvivoGen). Absorbance was measured at 620 nm after 3 hours. These cells were used to evaluate the ability of the different CD8 constructs to activate NF-kB and used as a surrogate for CD8 function.

[00152] T cell proliferation assay, cytokine and chemokine production assay. Three to five days after transduction, 1×10^5 T cells were cultured in 96-well round-bottom plates with B16

tumor cells or with mouse splenocytes pulsed with $gp100_{25-33}$ peptide. 1×10^5 effector T cells and 1×10^5 tumor cells were co-cultured in 200 µl of culture volume in 96-well round-bottom plates for 72 h. Sixteen hours before harvesting, 0.5 µCi of 3H-thymidine was added to each well prior to measuring thymidine uptake using a 1450 LSC & luminescence counter (PerkinElmer, Waltham, MA, USA). Cytokine and chemokine production levels were measured from culture supernatants collected 48 hours after stimulation using a Cytokine/Chemokine kit (Millipore, Billerica, MA, USA) according to manufacturer's instructions. For studies examining the costimulatory effects of MyD88 when fused to a tumor-reactive T cell receptor (TCR), human T cells transduced with the 1×10^5 effector T cells DMF5 or DMF5-MyD88 were co-cultured with 1×10^5 human Malm-3M melanoma tumor cells in 96-well round-bottom plates in 200 µl of culture volume in 96-well round-bottom plates for 72 h. Sixteen hours before harvesting, 0.5 µCi of 3H-thymidine was added to each well prior to measuring thymidine uptake using a 1450 LSC & luminescence counter (PerkinElmer, Waltham, MA, USA).

[00153] Cytotoxicity assay. Cytotoxic activity against tumor target cells was measured using a standard ^{51}Cr release assay. Target cells were labeled with 200 µCi of ^{51}Cr for 2 h at 37 °C, washed 3 times, and pulsed with anti-human antibodies for 1 h at 37 °C. 1×10^4 labeled target cells were then co-cultured with decreasing numbers of effector T cells at the indicated effector to target (E:T) ratios in 200 µl of culture volume in 96-well round-bottom plates. Target cells incubated in media alone were used to determined spontaneous ^{51}Cr release, and maximal release was determined by incubating labeled target cells in 10% Triton X-100. After 5 hours at 37 °C, 50 µl of supernatant was collected and ^{51}Cr radioactivity was measured in a 1450 LSC & luminescence counter. The mean percentage of specific lysis was calculated according to the following equation: % specific lysis = (test release – spontaneous release) / (maximal release – spontaneous release) × 100. All tests were performed in triplicate wells and results are shown as mean ±SD. In other experiments, a non-radioactive cytotoxicity kit was used (Promega, Madison, WI).

[00154] MDSC suppression assays. Myeloid-derived suppressor cells (MDSC; as characterized be expression of CD11b+Gr1+) were collected from the blood of mice with established B16-GMCSF melanoma tumors. MDSCs were irradiated (10,000 rads; gamma-radiation source) and co-cultured with T cells engineered to express CD8α-MyD88 or a control

vector (GFP) in 96-well round bottom plates. 1×10^5 T cells were cultured with 1×10^5 or 2.5×10^5 MDSCs for 48 hours. Sixteen hours before harvesting, 0.5 μCi of 3H-thymidine was added to each well prior to measuring thymidine uptake using a 1450 LSC & luminescence counter (PerkinElmer, Waltham, MA, USA).

Results

[00155] Experiment 2A. CD8α expression was confirmed in CD8α-transfected HEK cells (which do not express endogenous CD8α). As shown in **Figure 4A,** CD8α was detected on virtually 100% of HEK cells, indicating the successful expression and translocation of CD8α to the cell surface. The transduction efficiency in mouse $CD8^+$ T cells was also confirmed by flow cytometry. The data in **Figure 4B** demonstrates an average $CD8^+$ T cell transduction efficiency of approximately 50% (of all $CD8^+$ T cells).

[00156] Experiment 2B. The ability of MyD88 to induce signaling in HEK-Blue cells was determined. As can be seen from **Figure 5**, HEK-Blue cells expressing mCD8α fused to hMyD88 (lacking the TIR domain) (CD8-MyD88) or expressing MyD88 linked to the transmembrane hinge domain of mCD8α (MyD88TM) exhibit higher levels of NK-κB activation in comparison to cells expressing an mCD8α variant having only the extracellular region and transmembrane hinge domain of mCD8α (CD8ΔIC) or the extracellular region and transmembrane hinge domain of CD8α linked to 28-137-3 (CD8-28-137-3). The levels of NK-κB activation by CD8ΔIC or CD8-28-137-3 were similar to cells engineered with control vectors (pMIG) or untransduced cells (WT).

[00157] Experiment 2B. The ability of MyD88 to induce signaling in HEK-Blue cells was determined. As can be seen from **Figure 5**, HEK-Blue cells expressing mCD8α fused to hMyD88 (lacking the TIR domain) (CD8-MyD88) or expressing MyD88 linked to the transmembrane hinge domain of mCD8α exhibit higher levels of NK-κB activation in comparison to cells expressing an mCD8α variant having only the transmembrane hinge domain of mCD8α (CD8ΔIC) or the CD8α linked to 28-137-3 (CD8-28-137-3). The levels of NK-κB activation by CD8ΔIC or CD8-28-137-3 were similar to cells engineered with control vectors (pMIG) or untransduced cells (WT).

[00158] Experiment 2C. The ability of each CD8α construct to alter TCR-mediated $CD8^+$ T cell proliferation and cytokine production was examined. $CD8^+$ T cells from TCR transgenic mice, specific for the $gp100_{25-33}$ antigen, were engineered to express mCD8α-hMyD88, mCD8αΔIC, mCD8α-28-137-3, or GFP control (pMIG). Forty-eight hours after transduction, T cells were stimulated with splenocytes pulsed with the varying concentrations of the $gp100_{25-33}$ peptide, representing a melanoma tumor antigen. T cells expressing mCD8α-hMyD88 exhibited greater proliferation (**Figure 6A**) and IFN-gamma production (**Figure 6B**), than did control cells at exceedingly lower concentrations of tumor antigen. Furthermore, it is important to note that at higher antigen concentrations (2.5μg/ml), mCD8αΔIC–expressing T cells or mCD8α-28-137-3–expressing T cells exhibited reduced proliferation or a leveling off of IFN-gamma production while mCD8α-hMyD88 maintained a high proliferative capacity and IFN-gamma production. T cells engineered to express mCD8αΔIC, mCD8α-28-137-3, and GFP all demonstrated similar proliferation. The fact that mCD8α-MyD88 T cells did not proliferate or produce IFN-γ in the absence of antigen is very important as it indicates that the costimulatory effects of MyD88 occur in a TCR– and tumor antigen–specific manner.

[00159] Experiment 2D. To determine how the different CD8α constructs altered the capacity of $CD8^+$ T cells to proliferate in response to stimulation with tumor cells, pmel T cells were engineered to express mCD8α-hMyD88, mCD8αΔIC, mCD8α-28-137-3 (mCD8α-137-28-3), or GFP control (pMIG) and co-cultured with varying numbers of mouse B16 melanoma cells. As shown in **Figure 7**, mCD8α-hMyD88-transfected pmel T cells exhibited greater proliferation than did control cells when co-cultured with all but the lowest number of B16 cells. Proliferation occurred in a B16 cell number–dependent fashion indicating that the T cell response was in fact due the presence of tumor antigen. T cells engineered to express mCD8αΔIC, mCD8α-137-28-3 and GFP all demonstrated similar proliferative capacity.

[00160] Experiment 2E. Mouse T cells were transduced with mCD8α-hMyD88, mCD8αΔIC, mCD8α-137-28-3 (CD8-28-1BB-3ζ), or GFP control (pMIG) as indicated in **Figure 8**. T cells were co-cultured with B16 cells for 24 hours. The levels of the various factors shown in **Figure 8** were evaluated using a Milliplex Cytokine Array. mCD8α-hMyD88 T cells exhibited an increased production of IFNγ, IL-2, GM-CSF and TNFα as compared with T cells expressing the control vector (pMIG) and mCD8αΔIC (lacking intracellular signaling domains). Importantly,

mCD8α-hMyD88 T cells demonstrated increased levels of these same cytokines over mCD8α-28-137-3. In contrast, mCD8α-28-137-3 showed increased levels of IL-3 as compared with mCD8α-hMyD88. These data highlight specific distinctions in the ability of MyD88 to activate T cells as compared with 28-137-3 signaling. mCD8α-hMyD88 was as effective at inducing the expression of IL-17, MIP-1A, IP-10 and MIP-1B as were mCD8αΔIC and mCD8α-28-137-3, suggesting that the elevated levels of other factors did not occur in a non-specific manner. Only mCD8α-hMyD88 induced IL-1α. It is worth noting that overexpressing CD8α also resulted in increased cytokine secretion relative to control T cells (transduced with pMIG). This highlights the potential to use CD8α overexpression as an alternate approach to further potentiate T cell responses; albeit in the absence of an activating intracellular signaling domain CD8α overexpression alone is considerably weaker than mCD8α-hMyD88. That mCD8α-hMyD88 T cells increased IFN-γ production was confirmed by ELISA in separate experiments, **Figure 9**.

[00161] Experiment 2F. CD8-MyD88 signaling. $CD8^+$ pmel T cells were transduced with CD8α-MyD88, CD8αΔIC or pMIG control vector. T cells were stimulated in at a 1 to 1 ratio with B16 tumor cells for 10 and 30 minutes, then fixed in 4% PFA. The 0 time point indicates no B16 were added. Cells were permeabilized and stained for the indicated phosphorylated proteins, p-p38, p-JNK, p-ERK1/2 which are activated in response to TCR signaling. As shown in **Figure 10**, these results indicate that CD8α-MyD88 enhances signaling in part by increasing the expression levels of these proteins, sustaining the overall duration of signaling above control cells and by increasing the number of responding cells.

[00162] Experiment 2G. The ability of mCD8α-hMyD88 to augment T cell cytotoxicity was examined. mCD8α-hMyD88 expression significantly enhanced the killing of mouse melanoma cells as compared with T cells expressing mCD8αΔIC or mCD8α-28-137-3, **Figure 11**.

[00163] Experiment 2H. It has been demonstrated that tumor reactive T cells engineered to secrete a ligand that activates toll-like receptor 5 (TLR5) enhanced the ability of T cells to destroy a melanoma tumor in mice (Geng et al., *Can Res*. 2015; 75:1959-1971). The increased antitumor activity was associated with a reduced number of myeloid derived suppressor cells (MDSC) in tumors and spleens. MDSCs derived from mice treated with TLR5 ligand-secreting T cells also demonstrated phenotypic changes including increased levels of major histocompatibility complex (MHC) I and MHC II as well as increased expression levels of the

costimulatory molecule CD86 which could potentiate antitumor T cell activity. Furthermore, TLR engagement on T cells alters the cytokine and chemokine profile *in vitro* and *in vivo* and these changes were associated with reduced numbers of and phenotypic alterations in myeloid derived suppressor cells (MDSC). MDSCs are potent inhibitors of antitumor CD8 and CD4 T cell responses. TLR stimulation requires MyD88 signaling. Therefore, the ability of mCD8α-hMyD88 expression in $CD8^+$ T cells to alter cellular responses to MDSC-mediated suppression was evaluated. As shown in **Figure 12**, MDSCs suppressed the proliferation of GFP control T cells in an MDSC number-dependent fashion. At a ratio of 1 T cell to 2.5 MDSCs, T cells were suppressed nearly 40%. In sharp contrast, $CD8^+$ T cells expressing CD8α-hMyD88 were not only resistant to MDSC-mediated suppression, such T cells demonstrated a significant increase in proliferation at the ratios of T cell to MDSC examined as compared with mCD8α-hMyD88 T cells in the absence of MDSCs. Therefore, in addition to augmenting responses to weak tumor antigens and antigens expressed at low density, mCD8α-hMyD88 expression offers the advantage of further potentiating T cell responses by overcoming MDSC-mediated suppression.

[00164] Experiment 2I. The antitumor activity of pmel $CD8^+$ T cells engineered to express GFP (which serves as a transduction control), CD28-CD137-CD3zeta, or MyD88 was tested in mice with established B16 melanoma tumors. When tumors reached a size of approximately $30mm^2$, mice were intravenously injected with ~2.5 x 10^6 T cells and tumor growth was measured over the course of several weeks. Mice treated with mCD8α-hMyD88 T cells exhibited delayed tumor growth kinetics (**Figure 13A**) and prolonged mouse survival (**Figure 13B**) as compared with mice treated with control GFP or mCD8α-28-137-3 T cells. No significant differences were detected between mice treated with GFP and mCD8α-28-137-3 T cells.

[00165] Experiment 2J. A further *in vivo* experiment confirmed that mCD8α-hMyD88 expression in tumor reactive T cells enhances antitumor responses and prolongs mouse survival in the absence of any support therapy. C57BL6 mice were injected s.c. with B16 melanoma tumor cells in the rear flank. Eight days after tumor cell injection, mice received by tail i.v. injection ~10^6 pmel T cells engineered to express mCD8α-hMyD88 or control pMIG vector or mice remained untreated. No support therapy (i.e., IL-2, immune adjuvants or checkpoint blockade Ab) was provided. Mice treated with mCD8α-hMyD88 T cells exhibited significantly enhanced antitumor responses (**Figure 14A**) beginning on day 23 (one-way ANOVA; $p<0.01$)

and overall prolonged survival (**Figure 14B**; Wilcoxon, $p<0.05$) as compared with mice untreated mice or mice treated with control pMIG pmel T cells. Untreated mice had a median survival of 24 d; pMIG T cell–treated mice: 23.5 d; mCD8α-hMyD88–treated mice: 31 d. The error bars represent the standard deviation from the mean of eight mice.

[00166] Experiment 2K. The ability for MyD88 signaling to enhance TCR-induced proliferation when linked directly to the TCR was examined. DMF5, a TCR specific for the MART-1_{26-35} peptide from the *MART-1* melanoma antigen presented by MHC I, was fused to MyD88 lacking the TIR domain. The DMF5 TCR was kindly provided by Dr. Laura Johnson at the National Cancer Institute and is the same sequence used in clinical trials registered at www.ClinicalTrials.gov as NCI-07-C-0174 and NCI-07-C-0175 (Johnson, LA et al. *Blood* 2009;114:535-546; Johnson, LA et al. *J. Immunol.* 2006;177:6548-6559). Transduction efficiencies were similar between DMF5 and DMF5-MyD88. As shown in **Figure 15**, linking MyD88 to TCR enhanced T cell proliferation, suggesting that recruiting MyD88 to the TCR signaling complex through different means including, but not restricted to, CD8α or TCR can enhance T cell responses.

[00167] While the invention has been described with reference to certain particular embodiments thereof, those skilled in the art will appreciate that various modifications may be made without departing from the spirit and scope of the invention. The scope of the appended claims is not to be limited to the specific embodiments described.

REFERENCES

[00168] All patents and publications mentioned in this specification are indicative of the level of skill of those skilled in the art to which the invention pertains. Each cited patent and publication is incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

1. A fusion protein comprising extracellular and transmembrane regions of CD8α linked to a region of MyD88 lacking the TIR domain.

2. The fusion protein of claim 1, wherein the extracellular and transmembrane regions of CD8α correspond to amino acids 1-217 of mouse CD8α (SEQ ID NO:16) and the region of MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24).

3. The fusion protein of claim 1, wherein the extracellular and transmembrane regions of CD8α correspond to amino acids 1-203 of human CD8α (SEQ ID NO:12) and the region of MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24).

4. The fusion protein of claim 1, wherein the fusion protein comprises mCD8α-hMyD88 as set forth in SEQ ID NO:17 or hCD8α-hMyD88 as set forth in SEQ ID NO:14.

5. A fusion protein comprising the transmembrane region of CD8α linked to a region of MyD88 lacking the TIR domain.

6. The fusion protein of claim 5, wherein the transmembrane region of CD8α corresponds to amino acids 1-83 of SEQ ID NO:18 and the region of MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24).

7. The fusion protein of claim 5, wherein the fusion protein comprises hCD8αTM-hMyD88 as set forth in SEQ ID NO:18.

8. A fusion protein comprising the extracellular and transmembrane regions of CD8α linked to the intracellular signaling domains of CD28, CD137 (4-1BB), and CD3ζ.

9. The fusion protein of claim 8, wherein the extracellular and transmembrane regions of CD8α correspond to amino acids 1-217 of mouse CD8α (SEQ ID NO:16) and the CD28, CD137 (4-1BB), and CD3ζ domains correspond to amino acids 218-417 of SEQ ID NO:19.

10. The fusion protein of claim 8, wherein the extracellular and transmembrane regions of CD8α correspond to amino acids 1-203 of human CD8α (SEQ ID NO:12) and the CD28, CD137 (4-1BB), and CD3ζ domains correspond to amino acids 218-417 of SEQ ID NO:19.

11. The fusion protein of claim 8, wherein the fusion protein comprises mCD8α-28-137-3 as set forth in SEQ ID NO:19.

12. A fusion protein comprising extracellular and transmembrane regions of CD4 linked to a region of MyD88 lacking the TIR domain.

13. The fusion protein of claim 12, wherein the extracellular and transmembrane regions of CD4 correspond to amino acids 1-417 of mouse CD4 (amino acids 1-417 of SEQ ID NO:21) and the region of MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24).

14. The fusion protein of claim 12, wherein the extracellular and transmembrane regions of CD4 correspond to amino acids 1-418 of human CD4 (amino acids 1-418 of SEQ ID NO:20) and the region of MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24).

15. The fusion protein of claim 12, wherein the fusion protein comprises mCD4-hMyD88 as set forth in SEQ ID NO:21 or hCD4-hMyD88 as set forth in SEQ ID NO:20.

16. A fusion protein comprising a T cell receptor linked to a region of MyD88 lacking the TIR domain.

17. The fusion protein of claim 16, wherein the TCR is the DMF5 TCR having the amino acid sequence of residues 1-603 of SEQ ID NO:22 and the region of MyD88 lacking the TIR domain corresponds to amino acids 1-155 of human MyD88 (SEQ ID NO:24).

18. The fusion protein of claim 16, wherein the fusion protein comprises hTCR-hMyD88 as set forth in SEQ ID NO:22.

19. A sequence variant of a fusion protein of any one of claims 1-18, wherein the length of at least one peptide region or domain comprising the fusion protein individually varies on the amino terminus, carboxy terminus, or both ends, by up to 10 amino acids based on the native sequence of the polypeptide from which the peptide region or domain is obtained.

20. A sequence variant of a fusion protein of any one of claims 1-18, wherein the sequence variant has at least 80% sequence identity with the fusion protein over the entire length of the fusion protein.

21. An isolated population of cells expressing at least one fusion protein of any one of claims 1-18.

22. The isolated population of cells of claim 21, wherein the isolated population of cells expresses at least one fusion protein selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22).

23. The isolated population of cells of claim 21, wherein the isolated population of cells is selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells, and peripheral blood mononuclear cells (PBMC).

24. An isolated population of cells expressing at least one sequence variant of claim 19.

25. The isolated population of cells of claim 24, wherein the isolated population of cells expresses at least one fusion protein selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22).

26. An isolated population of cells expressing at least one sequence variant of claim 20.

27. The isolated population of cells of claim 26, wherein the isolated population of cells expresses at least one fusion protein selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22).

28. A method of treating a subject having cancer, comprising administering to a subject having cancer a therapeutically-effective amount of at least one population of cells as defined in claim 21.

29. A method of treating a subject having cancer, comprising administering to a subject having cancer a therapeutically-effective amount of at least one population of cells as defined in claim 22.

30. A method of treating a subject having cancer, comprising administering to a subject having cancer a therapeutically-effective amount of at least one population of cells as defined in claim 24.

31. A method of treating a subject having cancer, comprising administering to a subject having cancer a therapeutically-effective amount of at least one population of cells as defined in claim 26.

32. A method of treating a subject having an infectious disease, comprising administering to a subject having an infectious disease a therapeutically-effective amount of at least one population of cells as defined in claim 21.

33. A method of treating a subject having an infectious disease, comprising administering to a subject having an infectious disease a therapeutically-effective amount of at least one population of cells as defined in claim 22.

34. A method of treating a subject having an infectious disease, comprising administering to a subject having an infectious disease a therapeutically-effective amount of at least one population of cells as defined in claim 24.

35. A method of treating a subject having an infectious disease, comprising administering to a subject having an infectious disease a therapeutically-effective amount of at least one population of cells as defined in claim 26.

36. The method of claim 32, wherein the infectious disease is caused by a bacterium, a virus or a fungus.

37. The method of claim 33, wherein the infectious disease is caused by a bacterium, a virus or a fungus.

38. The method of claim 34, wherein the infectious disease is caused by a bacterium, a virus or a fungus.

39. The method of claim 35, wherein the infectious disease is caused by a bacterium, a virus or a fungus.

40. A method of treating a subject having an autoimmune disorder, comprising administering to a subject having an autoimmune disorder a therapeutically-effective amount of at least one population of cells as defined in claim 21.

41. A method of treating a subject having an autoimmune disorder, comprising administering to a subject having an autoimmune disorder a therapeutically-effective amount of at least one population of cells as defined in claim 22.

42. A method of treating a subject having an autoimmune disorder, comprising administering to a subject having an autoimmune disorder a therapeutically-effective amount of at least one population of cells as defined in claim 24.

43. A method of treating a subject having an autoimmune disorder, comprising administering to a subject having an autoimmune disorder a therapeutically-effective amount of at least one population of cells as defined in claim 26.

44. A method of conferring T cell resistance against myeloid derived suppressor cells (MDSC)-mediated suppression, comprising expressing at least one fusion protein of any one of claims 1-18 in a T cell.

45. The method of claim 44, wherein the fusion protein is selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22).

46. A method of conferring T cell resistance against MDSC-mediated suppression, comprising expressing at least one sequence variant of claim 19 in a T cell.

47. A method of conferring T cell resistance against MDSC-mediated suppression, comprising expressing at least one sequence variant of claim 20 in a T cell.

48. A method of enhancing immune cell recognition of an antigen, comprising expressing at least one fusion protein of any one of claims 1-18 in an immune cell.

49. The method of claim 48, wherein the fusion protein is selected from the group consisting of mCD8α-hMyD88 (SEQ ID NO:17), hCD8α-hMyD88 (SEQ ID NO:14), hCD8αTM-hMyD88 (SEQ ID NO:18), mCD8α-28-137-3 (SEQ ID NO:19), mCD4-hMyD88 (SEQ ID NO:21), hCD4-hMyD88 (SEQ ID NO:20) and hTCR-hMyD88 (SEQ ID NO:22).

50. The method of claim 48, wherein the immune cell is selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells, and peripheral blood mononuclear cells (PBMC).

51. The method of claim 48, wherein the antigen is present at a low concentration *in vitro* or *in vivo*.

52. The method of claim 48, wherein the antigen is a weakly antigenic antigen.

53. A method of enhancing immune cell recognition of an antigen, comprising expressing at least one sequence variant of claim 19 in an immune cell.

54. The method of claim 53, wherein the immune cell is selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells, and peripheral blood mononuclear cells (PBMC).

55. The method of claim 53, wherein the antigen is present at a low concentration *in vitro* or *in vivo*.

56. The method of claim 53, wherein the antigen is a weakly antigenic antigen.

57. A method of enhancing immune cell recognition of an antigen, comprising expressing at least one sequence variant of claim 20 in an immune cell.

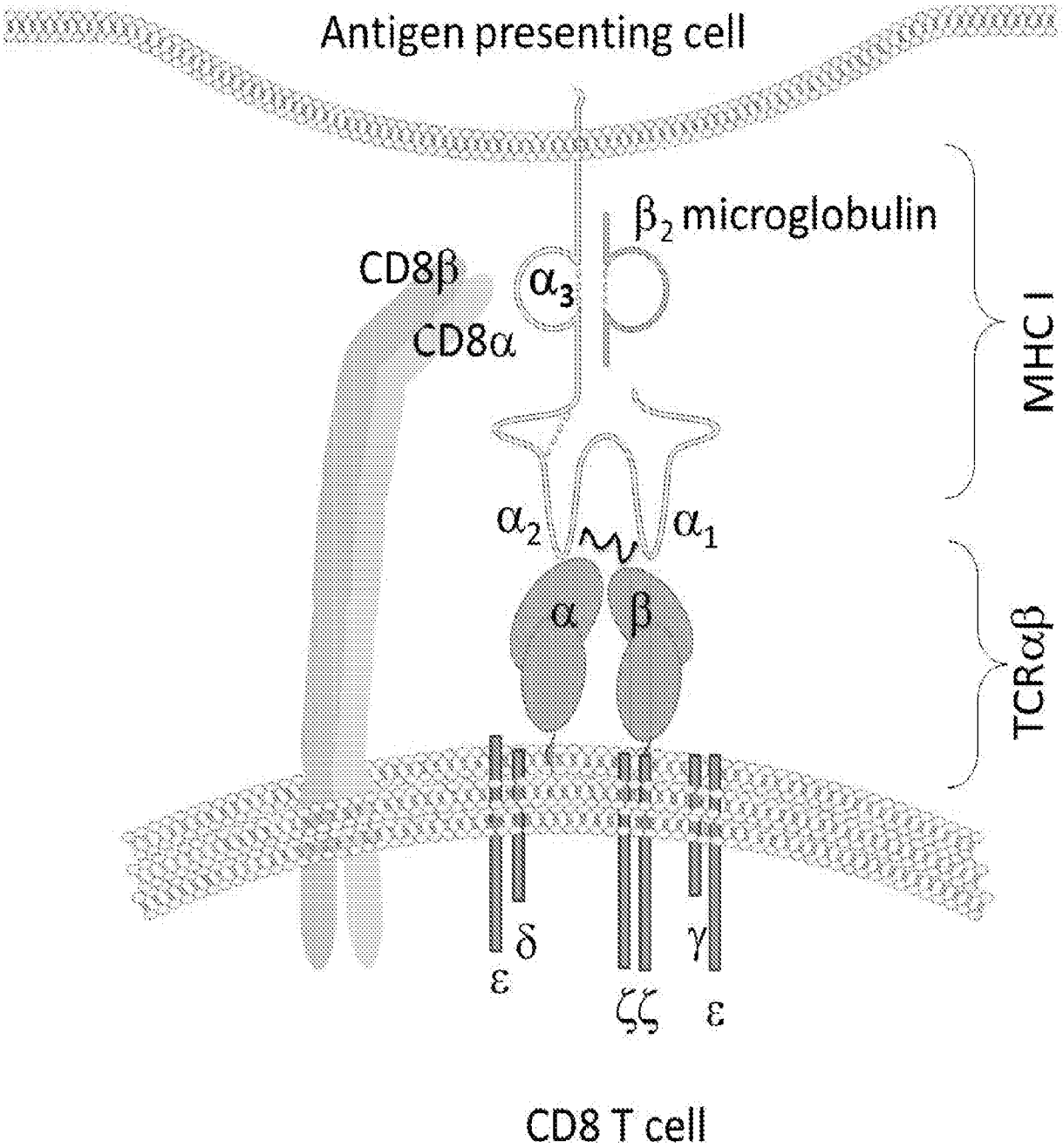
58. The method of claim 57, wherein the immune cell is selected from the group consisting of $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), natural killer cells (NK), neutrophils, macrophages, dendritic cells, mast cells, basophils, B cells, and peripheral blood mononuclear cells (PBMC).

59. The method of claim 57, wherein the antigen is present at a low concentration *in vitro* or *in vivo*.

60. The method of claim 57, wherein the antigen is a weakly antigenic antigen.

61. A polynucleotide sequence selected from the group consisting of the polynucleotide sequences set forth in SEQ ID NO:6 (mCD8α-hMyD88), SEQ ID NO:3 (hCD8α-hMyD88), SEQ ID NO:7 (hCD8αTM-hMyD88), SEQ ID NO:8 (mCD8α-28-137-3), SEQ ID NO:10 (mCD4-hMyD88), SEQ ID NO:9 (hCD4-hMyD88) and SEQ ID NO:11 (hTCR-hMyD88), and sequence variants thereof.

Figure 1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β2 microglobulin
CD8β
α3
CD8α
MHC I
α2
α1
α
β
TCRαβ
ε
δ
γ
ζζ
ε
CD8 T cell

Figure 2

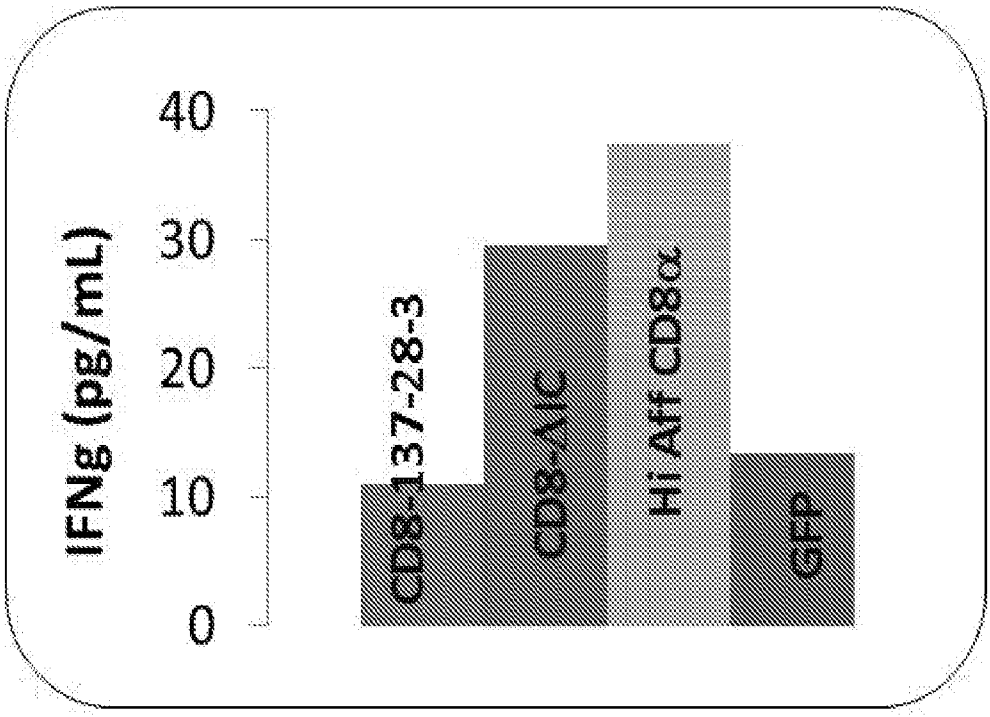
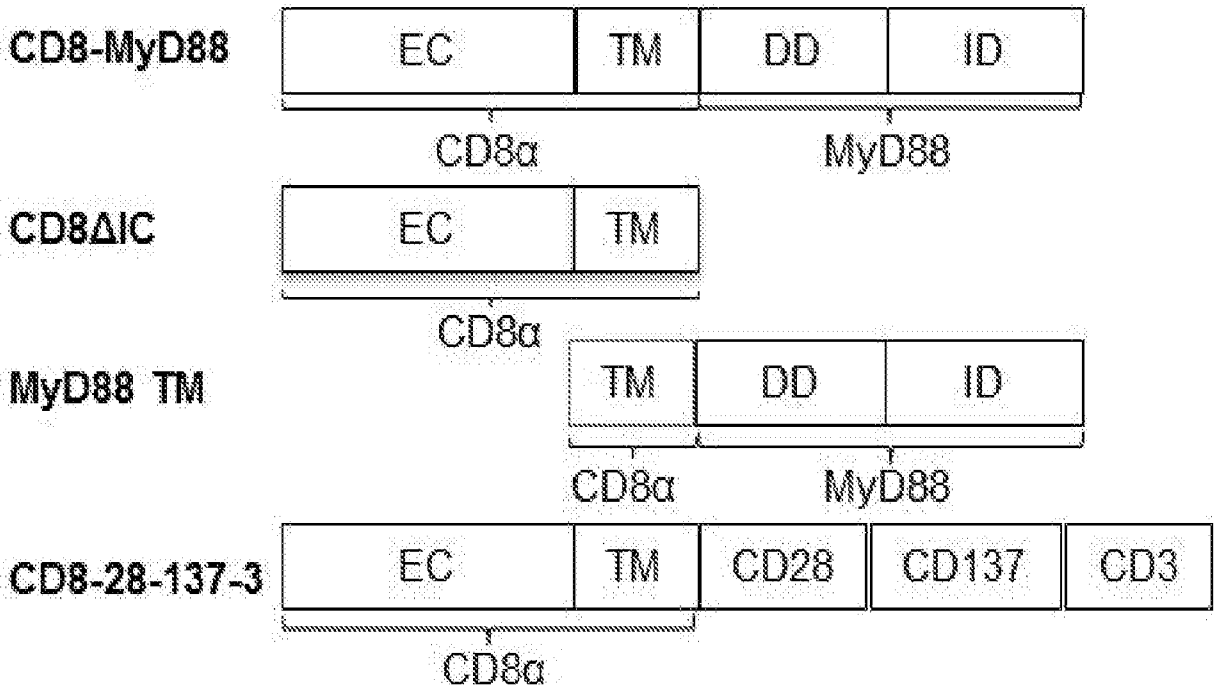
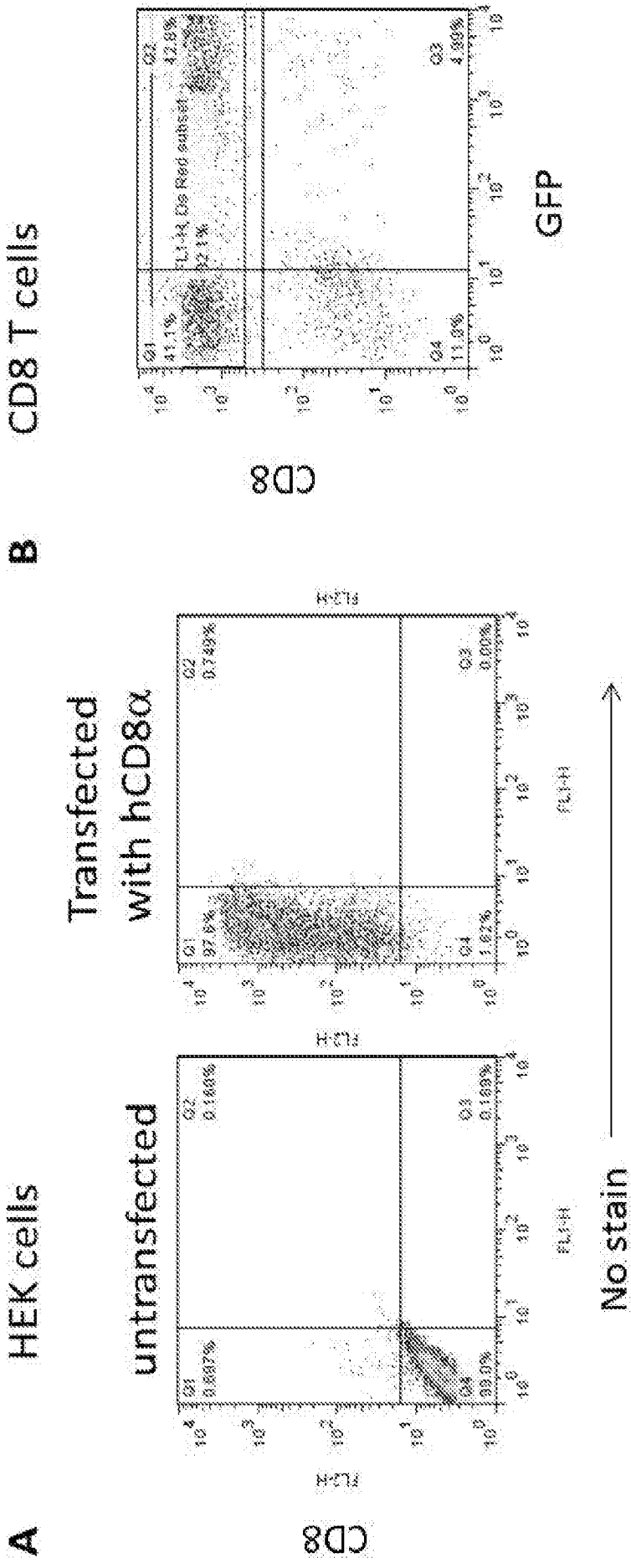


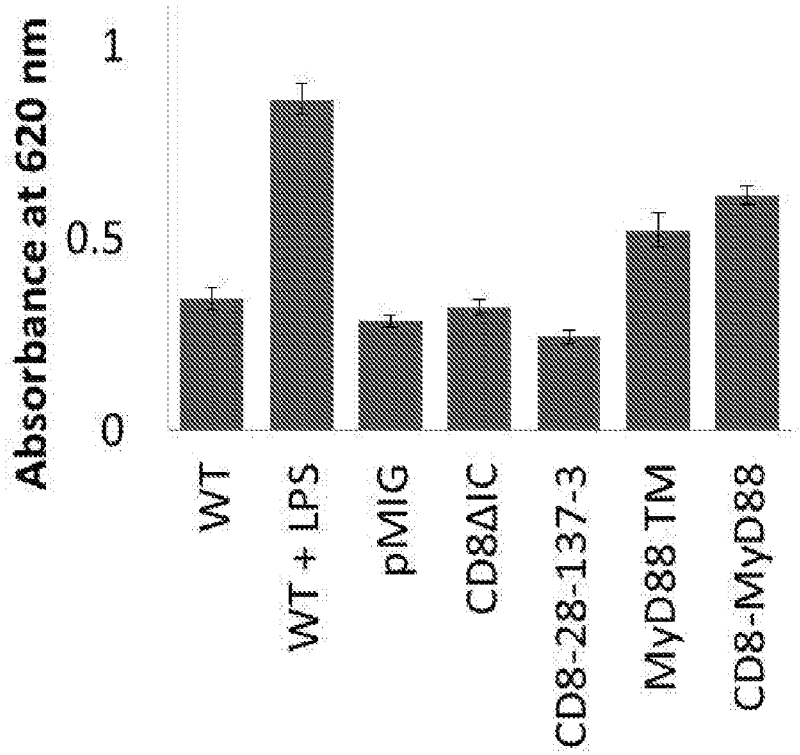
Figure 3

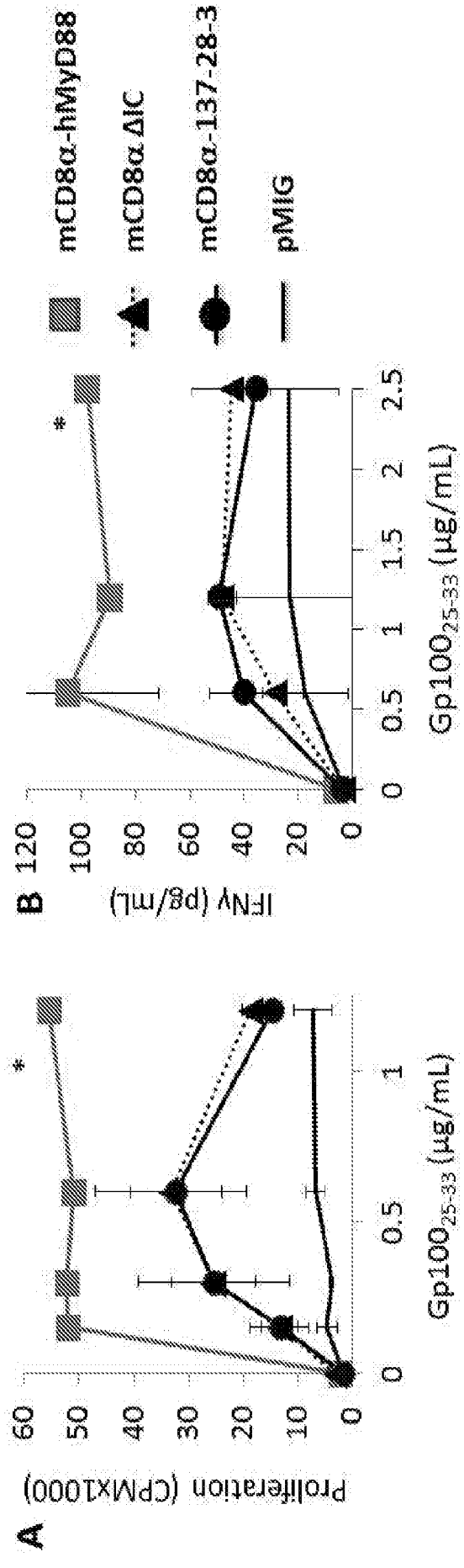

CD8-MyD88
EC
TM
DD
ID
CD8α
MyD88
CD8ΔIC
EC
TM
CD8α
MyD88 TM
TM
DD
ID
CD8α
MyD88
CD8-28-137-3
EC
TM
CD28
CD137
CD3
CD8α



Figures 4A-4B

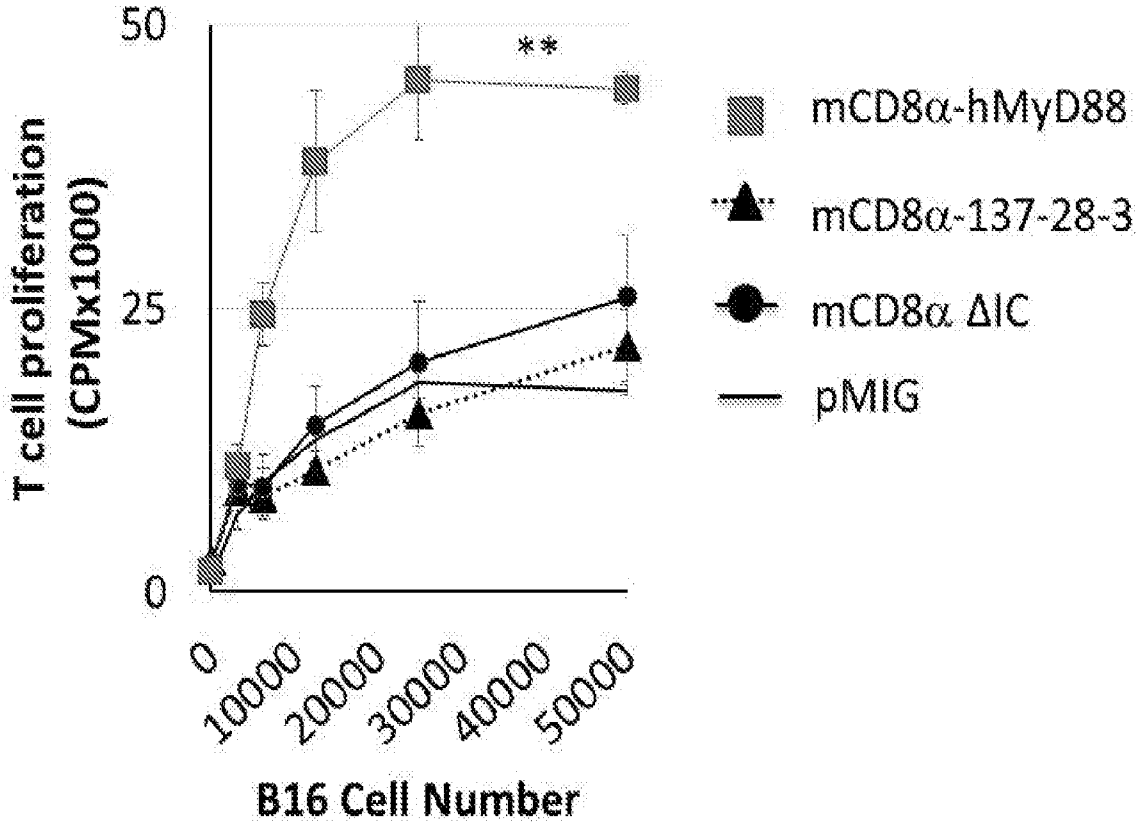
Figure 5


Absorbance at 620 nm
1
0.5
0
WT
WT + LPS
pMIG
CD8ΔIC
CD8-28-137-3
MyD88 TM
CD8-MyD88



Figures 6A-6B

Figure 7


50
25
0
T cell proliferation
(CPMx1000)
**
0
10000
20000
30000
40000
50000
B16 Cell Number
mCD8α-hMyD88
mCD8α-137-28-3
mCD8α ΔIC
pMIG

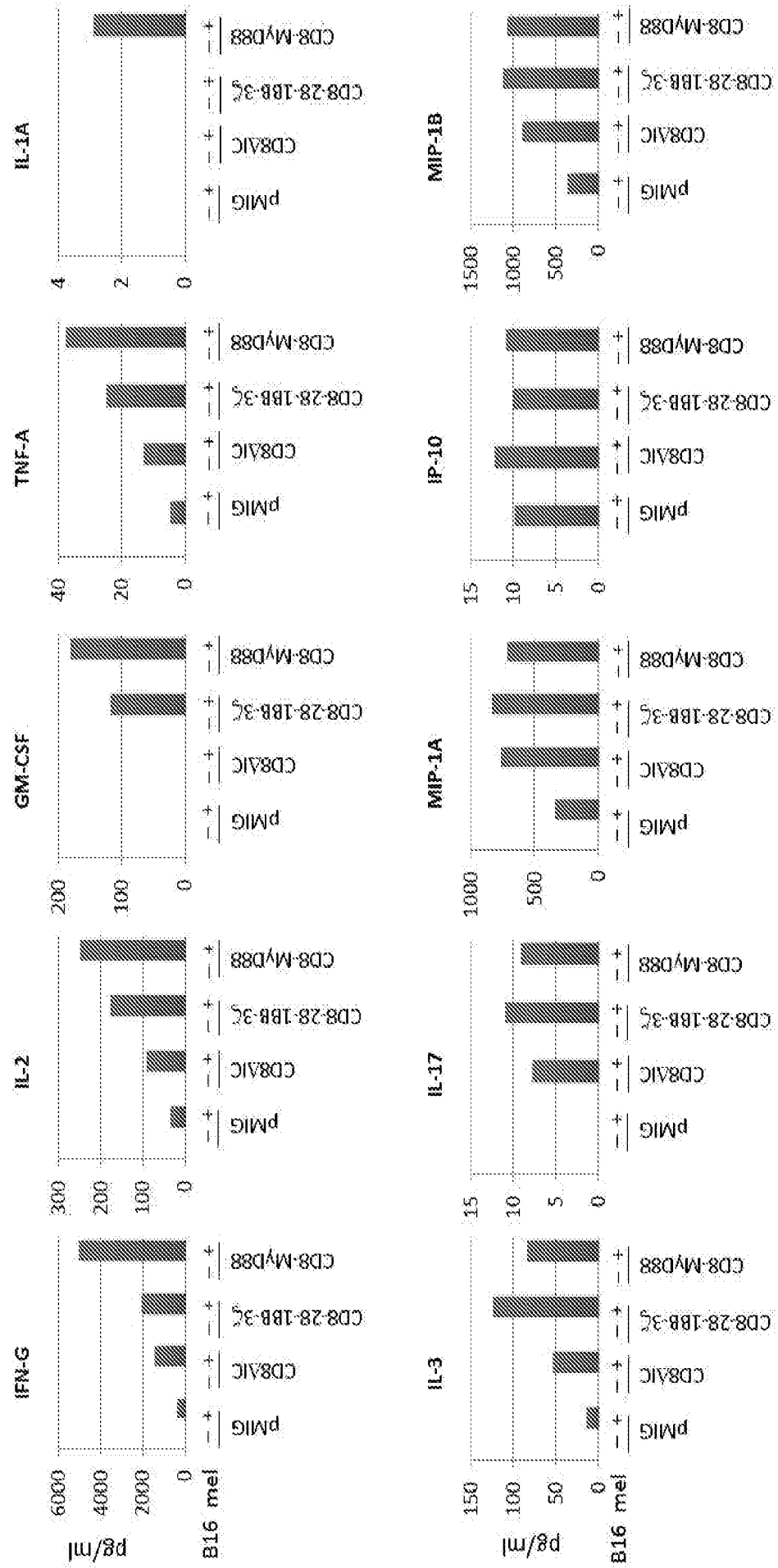

IFN-G
IL-2
GM-CSF
TNF-A
IL-1A
IL-3
IL-17
MIP-1A
IP-10
MIP-1B
pg/ml
B16 mel
pMIG
CD8AIC
CD8-28-1BB-3ζ
CD8-MyD88

Figure 8

Figure 9

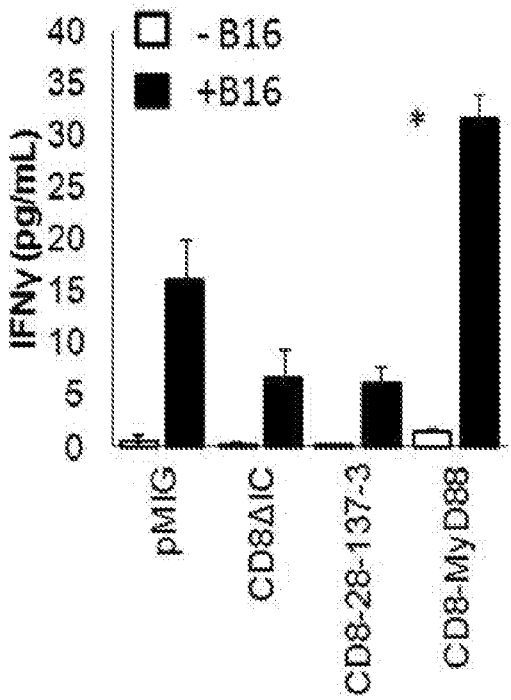

40
35
30
25
20
15
10
5
0
IFNγ (pg/mL)
- B16
+B16
*
pMIG
CD8ΔIC
CD8-28-137-3
CD8-MyD88

Figure 10

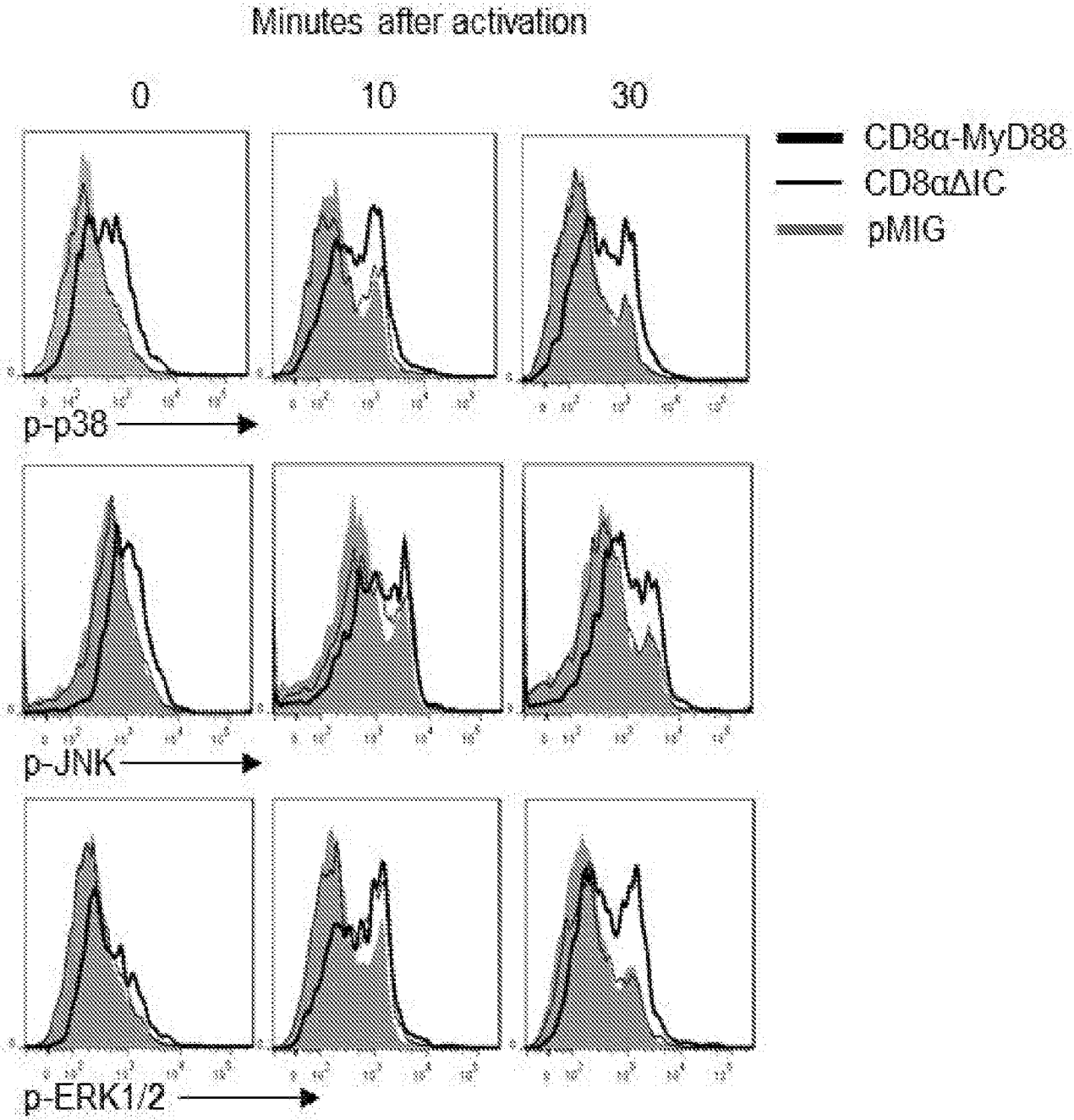

Minutes after activation
0
10
30
CD8α-MyD88
CD8αΔIC
pMIG
p-p38
p-JNK
p-ERK1/2

Figure 11

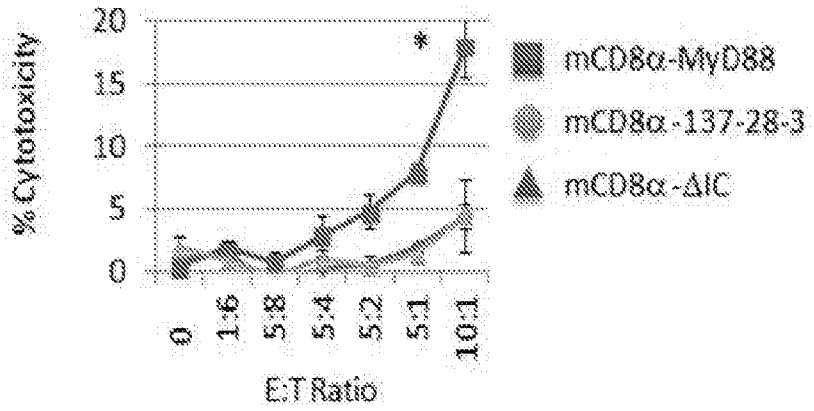


Figure 12

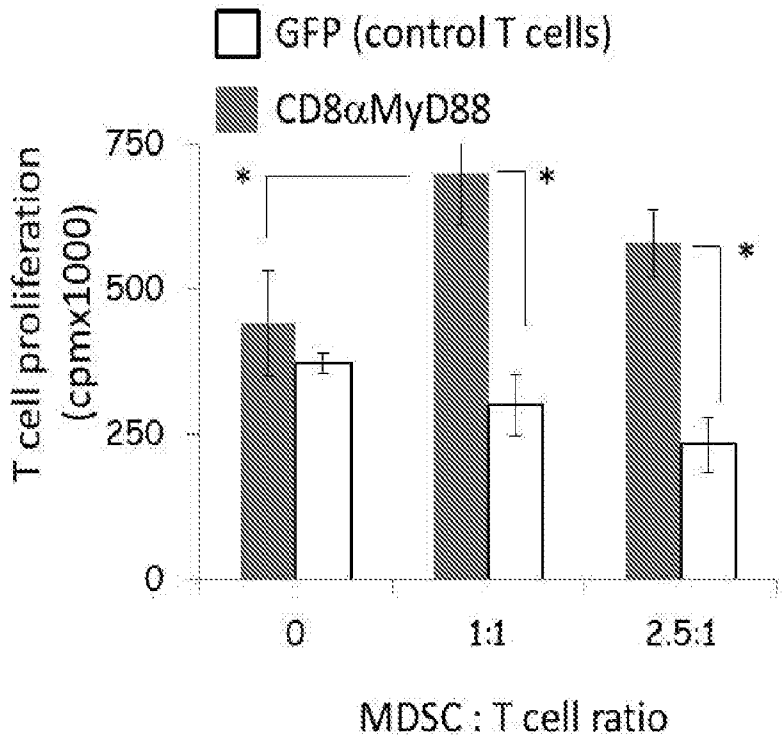


Figure 13A

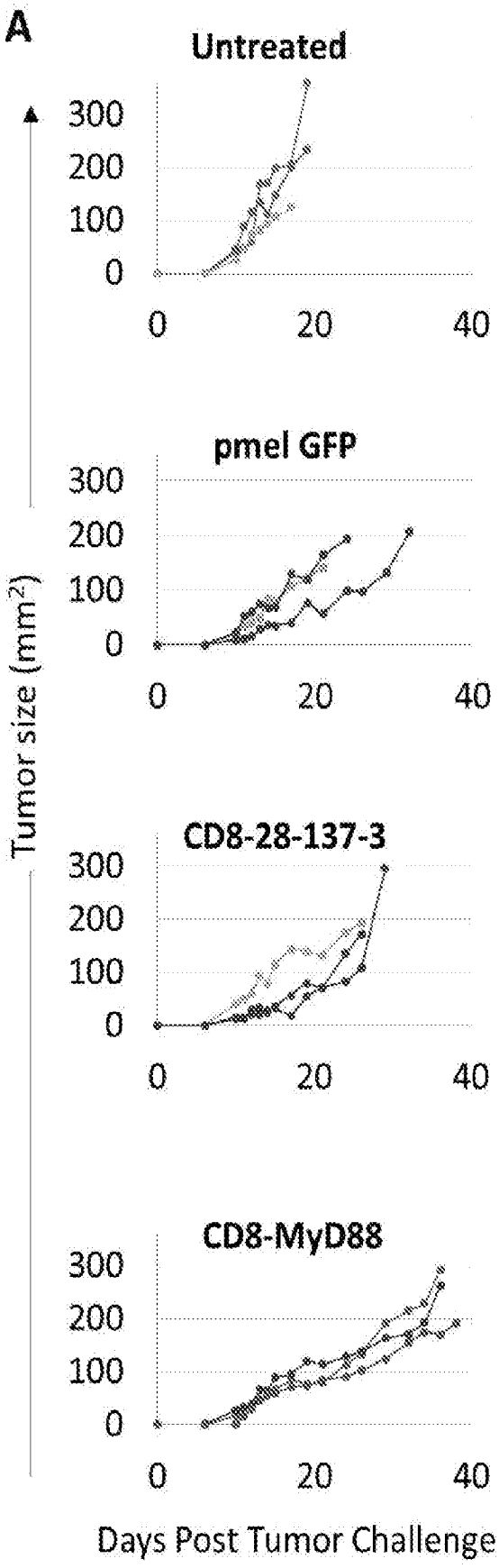
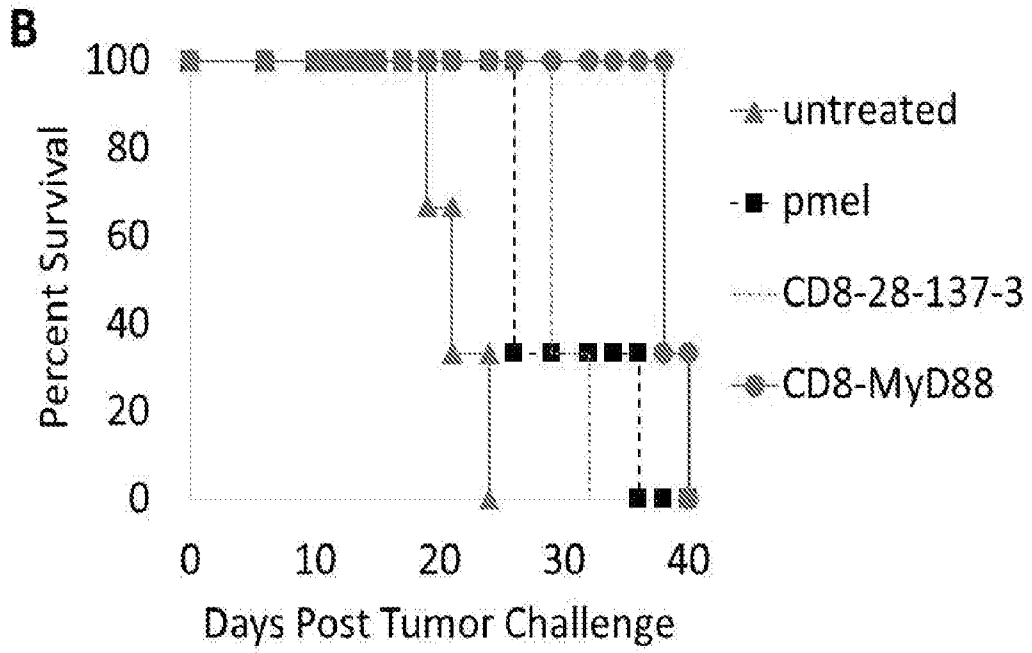

A
Untreated
pmel GFP
CD8-28-137-3
CD8-MyD88
300
200
100
0
0
20
40
Tumor size (mm2)
Days Post Tumor Challenge

Figure 13B


B
100
80
60
40
20
0
Percent Survival
0
10
20
30
40
Days Post Tumor Challenge
untreated
pmel
CD8-28-137-3
CD8-MyD88

Figures 14A-14B

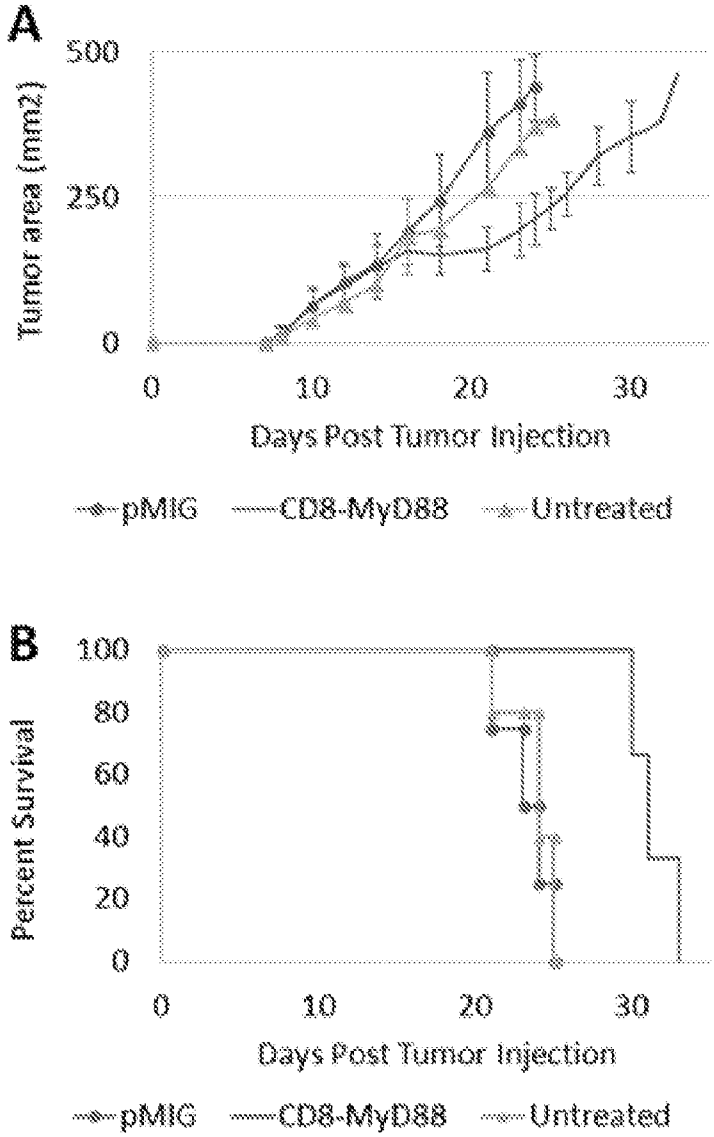
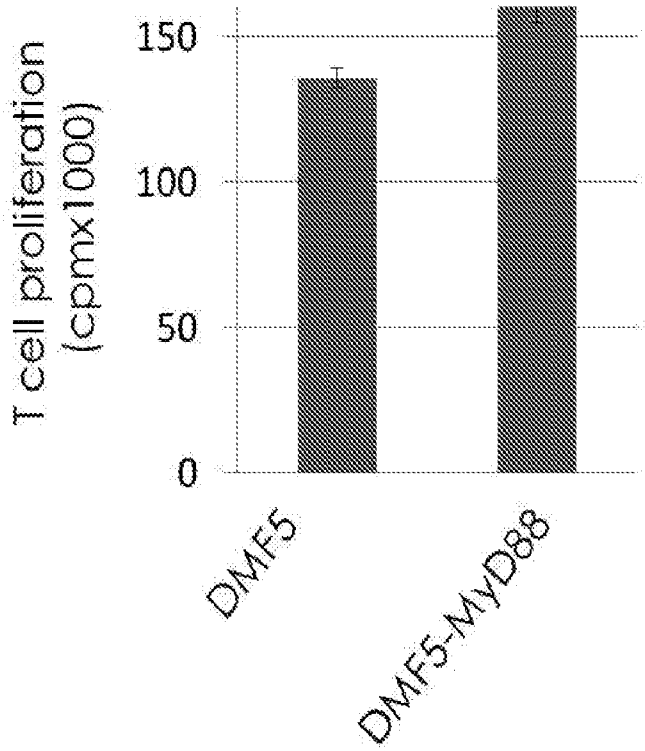


Figure 15



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/59510

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/705, 16/46; C12N 15/62 (2016.01)
CPC - C12N 5/0637, 15/62; C07K 14/705
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(8) Classification(s): C07K 14/705, 16/46; C12N 15/62 (2016.01)
CPC Classification(s): C12N 5/0637, 15/62; C07K 14/705, 16/46

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, RU, AT, CH, TH, BR, PH, SE, NO, DK, FI, BE, NL, LU, Other Countries (INPADOC)); Google; Google Scholar; Google Patents; PubMed; EBSCO; The Lens.org; NCBI BLAST; European Nucleotide Archive; Search Terms: chimeric, fusion, chimeric antigen receptor, MyD88, CD8, CD3, CD28, CD137, Death Domain, SEQ ID Nos.14,16-19, 24

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2013/142034 A1 (THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES) September 26, 2013; paragraphs [0009], [0021], [0025], [0026], [0063]-[0066], [0091], ; Table 1; SEQ ID Nos. 19, 23	8-11, 19-60
Y	US 5712149 (ROBERTS, MR) January 27, 1998; column 6, lines 47-61; column 17, line 67, to column 18, line 2; Figure 1C	8-11, 19-60
Y	(SHORE, DA et al.) The Crystal Structure of CD8αβ in Complex with YTS156.7.7 Fab and Interaction with Other CD8 Antibodies Define the Binding Mode of CD8αβ to MHC Class I. Journal of Molecular Biology. 31 December 2008, Vol. 384, No. 5; pages 1190-1202; DOI: 10.1016/j.jmb.2008.09.069	9, 11
Y	(NORMENT, AM et al.) Alternatively Spliced mRNA Encodes a Secreted Form of Human CD8 Alpha: Characterization of the Human CD8 Alpha Gene. Journal of Immunology. 1 May 1989, Vol. 142, No. 9; pages 3312-3319	10
A	XU, Y et al. Tumor-specific dendritic cells generated by genetic redirection of Toll-like receptor signaling against the tumor-associated antigen, erbB2. Cancer Gene Therapy. 2007, Vol. 14, pages 773–780; doi:10.1038/sj.cgt.7701073	1-7, 19-60
A	ZHONG, X-S et al. Chimeric Antigen Receptors Combining 4-1BB and CD28 Signaling Domains Augment PI3kinase/AKT/Bcl-XL Activation and CD8+ T Cell–mediated Tumor Eradication. Molecular Therapy. February 2010, Vol. 18, No. 2, page 413–420; doi:10.1038/mt.2009.210	1-7, 19-60

☒ 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
17 March 2016 (17.03.2016)	30 MAR 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas 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/US15/59510

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/011987 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) January 16, 2014	1-7, 19-60
A	WO 2014/039961 A1 (UNIVERSITY OF MIAMI) March 13, 2014	1-07, 19-60

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/59510

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.:
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:

-***-Please See Supplemental Page-***-

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.:
Group I: Claims 1-11, 19-60 (directed toward a fusion protein comprising extracellular and transmembrane regions of CD8a.)

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.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US15/59510

-***-Continued from Box No. III: Observations Where Unity of Invention Is Lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-11, 19 (in-part), 20 (in-part), 21 (in-part), 22 (in-part), 23 (in-part), 24 (in-part), 25 (in-part), 26 (in-part), 27 (in-part), 28 (in-part), 29 (in-part), 30 (in-part), 31 (in-part), 32 (in-part), 33 (in-part), 34 (in-part), 35 (in-part), 36 (in-part), 37 (in-part), 38 (in-part), 39 (in-part), 40 (in-part), 41 (in-part), 42 (in-part), 43 (in-part), 44 (in-part), 45 (in-part), 46 (in-part), 47 (in-part), 48 (in-part), 49 (in-part), 50 (in-part), 51 (in-part), 52 (in-part), 53 (in-part), 54 (in-part), 55 (in-part), 56 (in-part), 57 (in-part), 58 (in-part), 59 (in-part) and 60 (in-part) are directed toward a fusion protein comprising extracellular and transmembrane regions of CD8a.

Group II: Claims 12-18, 19 (in-part), 20 (in-part), 21 (in-part), 22 (in-part), 23 (in-part), 24 (in-part), 25 (in-part), 26 (in-part), 27 (in-part), 28 (in-part), 29 (in-part), 30 (in-part), 31 (in-part), 32 (in-part), 33 (in-part), 34 (in-part), 35 (in-part), 36 (in-part), 37 (in-part), 38 (in-part), 39 (in-part), 40 (in-part), 41 (in-part), 42 (in-part), 43 (in-part), 44 (in-part), 45 (in-part), 46 (in-part), 47 (in-part), 48 (in-part), 49 (in-part), 50 (in-part), 51 (in-part), 52 (in-part), 53 (in-part), 54 (in-part), 55 (in-part), 56 (in-part), 57 (in-part), 58 (in-part), 59 (in-part) and 60 (in-part) are directed toward a fusion protein comprising extracellular and transmembrane regions of CD4 linked to a region of MyD88 lacking the TIR domain.

Groups III+: Claim 61 is directed toward a polynucleotide sequence.

The polynucleotide sequence can be searched to the extent that it encompasses SEQ ID NO: 6 (mCD8-alpha-hMyD88) (Mouse CD8a without the intracellular signaling domain amino acid sequence). It is believed that Claim 61 (in-part) encompasses this first named invention and thus this claim will be searched without fee to the extent that it encompasses SEQ ID NO: 6 (mCD8-alpha-hMyD88) (Mouse CD8a without the intracellular signaling domain amino acid sequence). Applicant is invited to elect additional polynucleotide sequence(s) with specified SEQ ID NO: for each, or specified substitution(s) at specified site(s) of a SEQ ID NO: for variants thereof, to be searched. Additional polynucleotide sequence(s) and/or variant(s) thereof will be searched upon the payment of additional fees. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An Exemplary Election would be: SEQ ID NO: 3 (hCD8alpha-hMyD88) (Human CD8a-MyD88, where MyD88 lacks the TIR domain, artificial DNA sequence).

The inventions listed as Groups I-III+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include SEQ ID NO: 16 (Mouse CD8a without the intracellular signaling domain, artificial amino acid sequence), which is not present in any of Groups II or III+, the special technical features of Group II include SEQ ID NO: 21 (Mouse CD4-human MyD88, where MyD88 lacks the TIR domain, artifical amino acid sequence), which is not present in any of Groups I or III+, the special technical features of Groups III+ include SEQ ID NO: 6 (mCD8-alpha-hMyD88) (Mouse CD8a without the intracellular signaling domain amino acid sequence), which is not present in either of Groups I or II.

No technical features are shared between the nucleic acid sequences and variants thereof of Groups III+ and, accordingly, these groups lack unity a priori.

Groups I-III+ share the technical features including: a fusion construct comprising a region of MyD88 lacking the TIR domain. Groups I and II share the technical features including: a fusion protein comprising extracellular and transmembrane regions linked to a region of MyD88 lacking the TIR domain; SEQ ID NO: 24; an isolated population of cells expressing at least one fusion protein; a method of treating a subject having cancer, comprising administering to a subject having cancer a therapeutically-effective amount of at least one population of cells; method of treating a subject having an infectious disease, comprising administering to a subject having an infectious disease a therapeutically-effective amount of at least one population of cells; a method of treating a subject having an autoimmune disorder, comprising administering to a subject having an autoimmune disorder a therapeutically-effective amount of at least one population of cells; a method of conferring T cell resistance against myeloid derived suppressor cells (MDSC)-mediated suppression, comprising expressing at least one fusion protein in a T cell; a method of enhancing immune cell recognition of an antigen, comprising expressing at least one fusion protein in an immune cell.

However, these shared technical features are previously disclosed by WO 2014/039961 A1 (UNIVERSITY OF MIAMI) (hereinafter 'Miami') and further in view of the publication entitled 'Molecular Characterization And Modular Analysis Of Human MyD88: UniProt Accession Q99836' by Hardiman, et al. (hereinafter 'Hardiman'), the publication entitled 'Redirection of Regulatory T Cells With Predetermined Specificity for the Treatment of Experimental Colitis in Mice' by Elinav, et al. (hereinafter 'Elinav') and US 2008/0026986 A1 by Wang, et al. (hereinafter 'Wang').

Miami discloses a fusion construct (a fusion protein and nucleic acid encoding said protein (a fusion construct); abstract) comprising extracellular (comprising an LMP1 N-terminal region (comprising extracellular); paragraph [0009]; figure 1; wherein the N-terminal region includes extracellular loops) and transmembrane regions (paragraph [0009]) linked to a region of MyD88 lacking the TIR domain (paragraphs [00023], [00027]); an isolated population of cells (an isolated cell line; paragraph [00020]) expressing at least one fusion protein (paragraph [0005]); a method of treating a subject having cancer (paragraph [0005]), comprising administering to a subject having cancer (paragraph [0005]) a therapeutically-effective amount (a safe and effective amount (a therapeutically-effective amount); paragraph [00021]) of at least one population of cells (paragraph [0005]); a method of treating a subject having an infectious disease (a method of treating a subject having an infection (an infectious disease); paragraph [0005]), comprising administering to a subject having an infectious disease (comprising administering to a subject having an infection (an infectious disease); paragraph [0005]) a therapeutically-effective amount (a safe and effective amount (a therapeutically-effective amount); paragraph [00021]) of at least one population of cells (paragraph [0005]); and a method of enhancing immune cell recognition of an antigen (a method of activating antigen presenting cells; paragraph [0005]), comprising expressing at least one fusion protein in an immune cell (paragraph [0005]).

Miami does not disclose SEQ ID NO: 24; a method of treating a subject having an autoimmune disorder; a method of conferring T cell resistance against myeloid derived suppressor cells (MDSC)-mediated suppression, comprising expressing at least one fusion protein in a T cell.

-***-Continued on Next Supplemental Page-***-

-***-Continued from Previous Supplemental Page:

Hardiman discloses a human MyD88 amino acid sequence (a human MyD88 amino acid sequence; page 1) comprising SEQ ID NO: 24 (comprising the disclosed amino acid sequence (comprising SEQ ID NO: 24); page 11, wherein the sequence disclosed is 100% identical to SEQ ID NO: 24 of the instant PCT application).

Elinav discloses mice comprising regulatory T cells (abstract), wherein the T cells express a chimeric receptor (abstract) comprising a CD28 signaling domain (abstract, page 1, column 2, paragraph 3 to page 2, column 1, paragraph 1); wherein activation of the receptors suppressed autoimmune disease (wherein activation of the receptors suppressed colitis (autoimmune disease); abstract).

Wang discloses wherein CD8 regulatory T cells suppress host immune responses (abstract); including wherein myeloid suppressor cells convert nonsuppressive Treg cells into suppressive Treg cells (paragraph [0156]), and wherein activation of MyD88 reverses the suppression (abstract).

It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Miami, for utilizing a known sequence for human MyD88, such as the sequence disclosed by Hardiman, for the region of MyD88 lacking the TIR domain disclosed by Miami, for producing a chimeric molecule, including a region of MyD88 lacking the TIR domain useful in the treatment of human subjects. Additionally, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Miami, for providing the MyD88-comprising fusion construct, as disclosed by Miami, to a T regulatory cell population derived from a patient with (MDSC)-mediated suppression, for reversing suppression, based upon the previous disclosure of Wang, for enabling the activation of the immune system, thereby treating a disease or disorder, as disclosed by Miami. Moreover, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Miami, for further providing a CD28 domain in the fusion constructs disclosed by Miami, for providing T regulatory cells with a construct that provides an active CD28 domain, which enables the inhibition of an autoimmune disorder, such as colitis, as disclosed by Wang, for exapnding the range of disorders treatable utilizing fusion constructs comprising the fusion protein previously disclosed by Miami.

Since none of the special technical features of the Groups I-III+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Miami, Hardiman, Elinav and Wang references, unity of invention is lacking.