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DESCRIPTION

Description

FIELD OF THE INVENTION

[0001] This invention relates generally to bifunctional molecules including (a) a TGFβRII or fragment thereof capable of binding TGFβ and (b) an antibody, or antigen binding fragment thereof, that binds to Programmed Death Ligand 1 (PD-L1), uses of such molecules (e.g., for treating cancer), and methods of making such molecules.

BACKGROUND

[0002] In cancer treatment, it has long been recognized that chemotherapy is associated with high toxicity and can lead to emergence of resistant cancer cell variants. Even with targeted therapy against overexpressed or activated oncoproteins important for tumor survival and growth, cancer cells invariably mutate and adapt to reduce dependency on the targeted pathway, such as by utilizing a redundant pathway. Cancer immunotherapy is a new paradigm in cancer treatment that instead of targeting cancer cells, focuses on the activation of the immune system. Its principle is to rearm the host's immune response, especially the adaptive T cell response, to provide immune surveillance to kill the cancer cells, in particular, the minimal residual disease that has escaped other forms of treatment, hence achieving long-lasting protective immunity.

[0003] FDA approval of the anti-CTLA-4 antibody ipilimumab for the treatment of melanoma in 2011 ushered in a new era of cancer immunotherapy. The demonstration that anti-PD-1 or anti-PD-L1 therapy induced durable responses in melanoma, kidney, and lung cancer in clinical trials further signify its coming of age (Pardoll, D.M., Nat Immunol. 2012; 13: 1129-32). However, ipilimumab therapy is limited by its toxicity profile, presumably because anti-CTLA-4 treatment, by interfering with the primary T cell inhibitory checkpoint, can lead to the generation of new autoreactive T cells. While inhibiting the PD-L1/PD-1 interaction results in dis-inhibiting existing chronic immune responses in exhausted T cells that are mostly antiviral or anticancer in nature (Wherry, E.J., Nat Immunol. 2011; 12:492-9), anti-PD-1 therapy can nevertheless sometimes result in potentially fatal lung-related autoimmune adverse events. Despite the promising clinical activities of anti-PD1 and anti-PD-L1 so far, increasing the therapeutic index, either by increasing therapeutic activity or decreasing toxicity, or both, remains a central goal in the development of immunotherapeutic.

[0004] WO 2011/109789 discloses compositions and methods for targeted immunomodulatory antibodies and fusion proteins.

SUMMARY OF THE INVENTION

[0005] The present invention is based on the discovery that a bifunctional protein containing at least portion of TGFβ Receptor II (TGFβRII) that is capable of binding TGFβ and antibody or antigen-binding fragment that binds to human protein Programmed Death Ligand 1 (PD-L1) can be an effective anti-tumor and anti-cancer therapeutic. The protein can exhibit a synergistic effect in cancer treatment, as compared to the effect of administering the two agents separately. The invention is defined by the claims.

[0006] Accordingly, in a first aspect, the present invention features a protein including (a) human TGFβRII, or a fragment thereof capable of binding TGFβ (*e.g.,* a soluble fragment); and (b) an antibody, or an antigen-binding fragment thereof, that binds PD-L1 and comprises: (i) a heavy chain variable region including an HVR-H1, HVR-H2, and HVR-H3 having the amino acid sequences of SYIMM, SIYPSGGITFYADTVKG, and IKLGTVTTVDY, respectively, and (ii) a light chain variable region including an HVR-L1, HVR-L2, and HVR-L3 having the amino acid sequences of TGTSSDVGGYNYVS, DVSNRPS, and SSYTSSSTRV, respectively..

[0007] In a related aspect, the invention features a polypeptide including (a) at least a variable domain of a heavy chain of an antibody that binds PD-L1 (*e.g.*, amino acids 1-120 of SEQ ID NO: 2); and (b) human TGFβRII, or a soluble fragment thereof capable of binding TGFβ (*e.g.,* a human TGFβRII extra-cellular domain (ECD), amino acids 24-159 of SEQ ID NO: 9, or any of those described herein). The polypeptide may further include an amino acid linker connecting the C-terminus of the variable domain to the N-terminus of the human TGFβRII or soluble fragment thereof capable of binding TGFβ. The polypeptide may include the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence substantially identical to SEQ ID NO: 3. The antibody fragment may be an scFv, Fab, $F(ab')_2$, or Fv fragment.

[0008] In certain embodiments, the protein or polypeptide includes an antibody or antigen-binding fragment thereof that includes SEQ ID NO: 2 and human TGFβRII. The antibody may optionally include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0009] In certain embodiments, the protein or polypeptide includes an antibody or antigen-binding fragment thereof that includes SEQ ID NO: 2 and a fragment of human TGFβRII capable of binding TGFβ (e.g., a soluble fragment). The antibody may optionally include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0010] In certain embodiments, the protein or polypeptide includes an antibody or antigen-binding fragment thereof that includes SEQ ID NO: 2 and a human TGFβRII ECD. The antibody may include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0011] In certain embodiments, the protein or polypeptide includes an antibody or antigen-binding fragment thereof that includes amino acids 1-120 of SEQ ID NO: 2 and human TGFβRII. The antibody may include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0012] In certain embodiments, the protein or polypeptide includes an antibody or antigen-binding fragment thereof that includes amino acids 1-120 of SEQ ID NO: 2 and a fragment of human TGFβRII capable of binding TGFβ (e.g., a soluble fragment). The antibody may include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0013] In certain embodiments, the protein or polypeptide includes an antibody or antigen-binding fragment thereof that includes amino acids 1-120 of SEQ ID NO: 2 and a human TGFβRII ECD. The antibody may include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0014] In certain embodiments, the protein or polypeptide includes an antibody or antigen-binding fragment thereof that includes the hypervariable regions present in SEQ ID NO: 2 and human TGFβRII. The antibody may include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0015] In certain embodiments, the protein or polypeptide includes an antibody or antigen-binding fragment thereof that includes the hypervariable regions present in SEQ ID NO: 2 and a fragment of human TGFβRII capable of binding TGFβ (e.g., a soluble fragment). The antibody may include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0016] In certain embodiments, the protein or polypeptide includes an antibody or antigen-

binding fragment thereof that includes the hypervariable regions present in SEQ ID NO: 2 and a human TGFβRII ECD. The antibody may include a modified constant region (e.g., including a C-terminal Lys→Ala substitution, a mutation of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) sequence to Ala-Thr-Ala-Thr (SEQ ID NO: 20), or a hybrid constant region including an IgG1 hinge region and an IgG2 CH2 domain).

[0017] The invention also features a nucleic acid that includes a nucleotide sequence that encodes a polypeptide described above (e.g., human TGFBRII or a fragment thereof and a variable domain of a heavy chain of an antibody which, when combined with the polypeptide, forms an antigen-binding site that binds PD-L1). In certain embodiments, the nucleic acid further includes a second nucleotide sequence encoding at least a variable domain of a light chain of an antibody which, when combined with the polypeptide, forms an antigen-binding site that binds PD-L1 (*e.g.*, including amino acids 1-110 of SEQ ID NO: 1). The second nucleotide sequence may encode the amino acid sequence of SEQ ID NO: 1 (secreted anti-PD-L1 lambda light chain) or an amino acid sequence substantially identical to SEQ ID NO: 1.

[0018] The invention also features a cell comprising on the same or on different nucleic acids a first nucleotide sequence encoding a polypeptide comprising (a) at least a variable domain of a heavy chain of an antibody that binds human PD-L1; and (b) human TGFβ3RII, or a soluble fragment thereof capable of binding TGFβ, wherein the heavy chain variable domain of the antibody includes an HVR-H1, HVR-H2, and HVR-H3 having the amino acid sequences of SYIMM, SIYPSGGITFYADTVKG, and IKLGTVTTVDY, respectively; and a second nucleotide sequence encoding at least a variable domain of a light chain of an antibody which, when combined with the polypeptide, forms an antigen-binding site that binds PD-L1, wherein the light chain variable domain of the antibody includes an HVR-L1, HVR-L2, and HVR-L3 having the amino acid sequences of TGTSSDVGGYNYVS, DVSNRPS, and SSYTSSSTRV, respectively.

[0019] The invention also features a method of producing a protein including (a) the extracellular domain of the human TGFβRII, or a fragment thereof capable of binding TGFβ (*e.g.,* a soluble fragment), and (b) an antibody, or an antigen-binding fragment thereof, that binds human PD-L1 and comprises: (i) a heavy chain variable region including an HVR-H1, HVR-H2, and HVR-H3 having the amino acid sequences of SYIMM, SIYPSGGITFYADTVKG, and IKLGTVTTVDY, respectively, and (ii) a light chain variable region including an HVR-L1, HVR-L2, and HVR-L3 having the amino acid sequences of TGTSSDVGGYNYVS, DVSNRPS, and SSYTSSSTRV, respectively. The method includes maintaining a cell described under conditions that permit expression of the protein. The method may further include harvesting the protein.

[0020] Disclosed herein are proteins including the polypeptide described above and at least a variable domain of a light chain of an antibody which, when combined with the polypeptide, forms an antigen-binding site that binds PD-L1. The protein may include (a) two polypeptides, each having an amino acid sequence consisting of the amino acid sequence of SEQ ID NO: 3, and (b) two additional polypeptides each having an amino acid sequence consisting of the

amino acid sequence of SEQ ID NO: 1.

[0021] The invention also features a protein including (a) human TGFβRII, or a fragment thereof capable of binding TGFβ (*e.g.,* a soluble fragment); and (b) an antibody, or an antigen-binding fragment thereof, that binds PD-L1 and comprises: (i) a heavy chain variable region including an HVR-H1, HVR-H2, and HVR-H3 having the amino acid sequences of SYIMM, SIYPSGGITFYADTVKG, and IKLGTVTTVDY, respectively, and (ii) a light chain variable region including an HVR-L1, HVR-L2, and HVR-L3 having the amino acid sequences of TGTSSDVGGYNYVS, DVSNRPS, and SSYTSSSTRV, respectively; for use in methods of inhibiting tumour growth in a patient, and/or for use in methods of treating cancer in a patient, said methods comprising administering the protein to the patient. Methods may further include administration of radiation or administration of a chemotherapeutic, a biologic, or a vaccine.

[0022] Disclosed herein are proteins for use in promoting local depletion of TGFβ at a tumor, or for use in inhibiting SMAD3 phosphorylation in a cell (*e.g.,* a tumor cell or an immune cell). Methods of promoting local depletion of TGFβ are also disclosed, said methods comprising administering a protein described above, where the protein binds TGFβ in solution, binds PD-L1 on a cell surface, and carries the bound TGFβ into the cell (*e.g.,* a cancer cell). Similarly, methods of inhibiting SMAD3 phosphorylation in a cell (e.g., a cancer cell or an immune cell), comprising exposing a cell in the tumor microenvironment to a protein described above are disclosed herein.

[0023] The cancer or tumor may be selected from the group consisting of colorectal, breast, ovarian, pancreatic, gastric, prostate, renal, cervical, myeloma, lymphoma, leukemia, thyroid, endometrial, uterine, bladder, neuroendocrine, head and neck, liver, nasopharyngeal, testicular, small cell lung cancer, non-small cell lung cancer, melanoma, basal cell skin cancer, squamous cell skin cancer, dermatofibrosarcoma protuberans, Merkel cell carcinoma, glioblastoma, glioma, sarcoma, mesothelioma, and myelodisplastic syndromes.

[0024] All references in the subsequent description to methods of treatment are made solely to facilitate the disclosure of compounds, pharmaceutical compositions and/or medicaments of the invention, for use in methods of treatment of the human or animal body by therapy. The invention is defined by the claims.

[0025] The term "TGFβRII" or "TGFβ Receptor II" means a polypeptide having the wild-type human TGFβ Receptor Type 2 Isoform A sequence (*e.g.*, the amino acid sequence of NCBI Reference Sequence (RefSeq) Accession No. NP_001020018 (SEQ ID NO: 8)), or a polypeptide having the wild-type human TGFβ Receptor Type 2 Isoform B sequence (*e.g.*, the amino acid sequence of NCBI RefSeq Accession No. NP_003233 (SEQ ID NO: 9)) or having a sequence substantially identical the amino acid sequence of SEQ ID NO: 8 or of SEQ ID NO: 9. The TGFβRII may retain at least 0.1%, 0.5%, 1%, 5%, 10%, 25%, 35%, 50%, 75%, 90%, 95%, or 99% of the TGFβ-binding activity of the wild-type sequence. The polypeptide of expressed TGFβRII lacks the signal sequence.

[0026] The term "fragment of TGFβRII capable of binding TGFβ" means any portion of NCBI RefSeq Accession No. NP_001020018 (SEQ ID NO: 8) or of NCBI RefSeq Accession No. NP_003233 (SEQ ID NO: 9), or a sequence substantially identical to SEQ ID NO: 8 or SEQ ID NO: 9 that is at least 20 (*e.g.,* at least 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 175, or 200) amino acids in length that retains at least some of the TGFβ-binding activity *(e.g.,* at least 0.1%, 0.5%, 1%, 5%, 10%, 25%, 35%, 50%, 75%, 90%, 95%, or 99%) of the wild-type receptor or of the corresponding wild-type fragment. Typically such fragment is a soluble fragment. An exemplary such fragment is a TGFβRII extra-cellular domain having the sequence of SEQ ID NO: 10.

[0027] The term "substantially identical" means a polypeptide exhibiting at least 50%, desirably 60%, 70%, 75%, or 80%, more desirably 85%, 90%, or 95%, and most desirably 99% amino acid sequence identity to a reference amino acid sequence. The length of comparison sequences will generally be at least 10 amino acids, desirably at least 15 contiguous amino acids, more desirably at least 20, 25, 50, 75, 90, 100, 150, 200, 250, 300, or 350 contiguous amino acids, and most desirably the full-length amino acid sequence.

[0028] The term "patient" means either a human or non-human animal *(e.g.,* a mammal).

[0029] The term "treating" a disease, disorder, or condition *(e.g.,* a cancer) in a patient means reducing at least one symptom of the disease, disorder, or condition by administrating a therapeutic agent to the patient.

[0030] The term "cancer" means a collection of cells multiplying in an abnormal manner.

[0031] Other embodiments and details of the invention are presented herein below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032]

FIG. 1A is a schematic drawing of an anti-PD-L1/TGFβ Trap molecule comprising one anti-PD-L1 antibody fused to two extracellular domain (ECD) of TGFβ Receptor II via a $(Gly_4Ser)_4Gly$ linker. **FIG. 1B** is a photograph of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of anti-PD-L1/TGFβ Trap under non-reducing and reducing conditions.

FIG. 2 is photograph of an SDS-PAGE gel showing analysis of extent of clipping of anti-PD-L1/TGFβ Trap expressed by clone 02B15 at various population doubling levels. Anti-PD-L1/TGFβ Trap from clone 02B15 after a single protein A chromatography step was analyzed by SDS-PAGE under reducing conditions. Lanes 1 and 10, See Blue Plus 2 MW Standard; lane 2, purified anti-PD-L1/TGFβ Trap reference; lane 3, clone 02B15 at PDL0; lane 4, clone 02B15 at PDL30; lane 5, clone 02B15 at PDL60; and lane 6, clone 02B15 at PDL90. (PDL, population doubling level).

FIG. 3 is a graph showing FACS analysis of anti-PD-L1/TGFβ Trap binding to HEK cells transfected to express human PD-L1.

FIG. 4 is a graph showing the ability of anti-PD-L1/TGFβ Trap to inhibit TGFβ-induced phosphorylation of SMAD3 using a pSMAD3-luciferase reporter cell line (filled circle: anti-PD-L1; X: anti-PD-L1(mut); filled square: anti-PD-L1/TGFβ Trap; filled triangle: anti-PD-L1(mut)/TGFβ Trap; +: anti-TGFβ antibody 1D11; star: TGFβ RII-Fc).

FIGS. 5A and **5B** are graphs showing pharmacokinetics of intravenously administered anti-PD-L1/TGFβ Trap and related proteins in mice.

FIG. 6A is a graph showing PD-L1 target-mediated endocytosis of anti-PD-L1/ TGFβ Trap. **FIG. 6B** is a graph showing PD-L1 target-mediated endocytosis of anti-PD-L1. **FIG. 6C** is a graph showing percent internalization of anti-PD-L1/ TGFβ Trap and anti-PD-L1 bound on HEK/PD-L1 cells.

FIGS. 7A-7C are graphs showing anti-tumor efficacy of anti-PD-L1/TGFβ Trap and related proteins in the EMT-6 breast carcinoma subcutaneous model (Example 7). **FIG. 7A** shows tumor growth curves of average tumor volumes of surviving mice in different treatment groups (star: Group 1: filled circle: Group 2; filled triangle: Group 3; filled square: Group 4; open square: Group 5; filled square/dashed line: Group 6; filled square/stippled line: Group 7). **FIG. 7B** shows tumor growth curves of individual tumor volumes in different treatment groups. **FIG. 7C** is a Kaplan-Meier plot of percent survival in different treatment groups (symbols as in 7A).

FIG. 8 is a graph showing anti-tumor efficacy of anti-PD-L1/TGFβ Trap and related proteins in the MC38 colorectal carcinoma subcutaneous tumor model (Example 8; star: Group 1; filled circle: Group 2; filled circle/dashed line: Group 3; filled triangle: Group 4; filled triangle/dashed line: Group 5; filled square: Group 6; filled square/dashed line: Group 7).

FIG. 9 is a graph showing anti-tumor efficacy of anti-PDL1/TGFβ Trap and related proteins in an orthotopic EMT-6 breast cancer model (Example 9; star: Group 1; filled circle/dashed line: Group 2; filled triangle: Group 3; filled triangle/dashed line: Group 4; filled diamond: Group 5).

FIG. 10 is a graph showing anti-tumor efficacy of anti-PDL1/TGFβ Trap and related proteins in an intramuscular MC38 colorectal carcinoma model (Example 10; star: Group 1; filled circle: Group 2; filled circle/dashed line: Group 3: filled diamond/dashed line: Group 4; filled square: Group 5; filled square/dashed line: Group 6; filled diamond: Group 7).

Fig. 11 is a graph showing anti-tumor efficacy of anti-PD-L1/TGF-β Trap and the combination of anti-PD-L1 and TGFβ Trap control administered to give equivalent *in vivo* exposure in an orthotopic EMT-6 breast tumor model (Example 11; star: Group 1; filled square: Group 2; open square: Group 3; filled diamond: Group 4; open diamond: Group 5).

FIGS. 12A-12C are graphs showing anti-tumor efficacy of anti-PD-L1/TGF-β Trap and the combination of anti-PD-L1 and TGFβ Trap control administered to give equivalent *in vivo* exposure in an intramuscular MC38 colorectal carcinoma model (Example 12). **FIG. 12A** shows tumor growth curves of mice treated with both intermediate and low doses of the

proteins (star: Group 1; filled squares: Group 2; open squares: Group 3; filled diamonds: Group 4; open diamonds Group 5). **FIG. 12B** (star: Group 1; filled square: Group 2; filled diamond: Group 4; *: $p < 0.0001$ compared to Group 1; **: $p < 0.0001$ compared to Group 2) and **12C** (star: Group 1; filled square: Group3; filled diamond: Group 5; *: $p < 0.0001$ compared to Group 1; **: $p < 0.0001$ compared to Group 3) show statistical analysis of tumor growth curves of mice treated with intermediate and low doses of the proteins, respectively

FIGS. 13A-13B are graphs showing anti-tumor efficacy of anti-PD-L1(YW)/TGF-β Trap and related proteins in an orthotopic EMT-6 breast tumor model (Example 13; star: Group 1; filled circle: Group 2; filled triangle: Group 3; filled square: Group 4; filled diamond: Group 5). **FIG. 13A** shows tumor growth curves of mice in different treatment groups. **FIG. 13B** is a Kaplan-Meier plot of percent survival in different treatment groups.

FIGS. 14A-14B are graphs showing anti-tumor efficacy of anti-PD-L1(YW)/TGF-β Trap and related proteins based on (A) tumor volumes and (B) tumor weights, in an intramuscular MC38 colorectal carcinoma model (Example 14; star: Group 1; filled circle: Group 2; filled triangle: Group 3; filled square: Group 4; filled diamond: Group 5).

FIG. 15 is a graph comparing the anti-tumor efficacy of an anti-PD-1 antibody treatment with and without TGFβ Trap control in an orthotopic EMT-6 breast tumor model (Example 15; star: Group 1; filled square: Group 2; filled inverted triangle: Group 3; open inverted triangle: Group 4).

FIG. 16 is a graph comparing the anti-tumor efficacy of an anti-PD-1 antibody treatment with and without TGFβ Trap control in an intramuscular MC38 colorectal tumor model (Example 16; star: Group 1; filled square: Group 2; filled inverted triangle: Group 3; open inverted triangle: Group 4).

FIG. 17 is a graph comparing the anti-tumor efficacy of an anti-LAG3 or anti-TIM3 antibody treatment with and without TGFβ Trap control in an orthotopic EMT-6 breast tumor model (Example 17; star: Group 1; filled square: Group 2; filled triangle: Group 3; filled inverted triangle: Group 4; open triangle: Group 5; open inverted triangle: Group 6).

FIG. 18 is a graph comparing the anti-tumor efficacy of an anti-LAG3 or anti-TIM3 antibody treatment with and without TGFβ Trap control in an intramuscular MC38 colorectal tumor model (Example 18; star: Group 1; filled square: Group 2; filled triangle: Group 3; filled inverted triangle: Group 4; open triangle: Group 5; open inverted triangle: Group 6).

DETAILED DESCRIPTION

[0033] The current invention permits localized reduction in TGFβ in a tumor microenvironment by capturing the TGFβ using a soluble cytokine receptor (TGFβRII) tethered to an antibody moiety targeting PD-L1found on the exterior surface of certain tumor cells or immune cells.

This bifunctional molecule, sometimes referred to in this document as an "antibody-cytokine trap," is effective precisely because the anti-PD-L1 antibody and cytokine trap are physically linked. The resulting advantage (over, for example, administration of the antibody and the receptor as separate molecules) is partly because cytokines function predominantly in the local environment through autocrine and paracrine functions. The antibody moiety directs the cytokine trap to the tumor microenvironment where it can be most effective, by neutralizing the local immunosuppressive autocrine or paracrine effects. Furthermore, in cases where the target of the antibody is internalized upon antibody binding, an effective mechanism for clearance of the cytokine/cytokine receptor complex is provided. Antibodymediated target internalization has been shown for PD-L1. This is a distinct advantage over using an anti-TGFβ antibody because first, an anti-TGFβ antibody might not be completely neutralizing; and second, the antibody can act as a carrier extending the half-life of the cytokine, and antibody/cytokine complexes often act as a circulating sink that builds up and ultimately dissociates to release the cytokine back in circulation (Montero-Julian et al., Blood. 1995; 85:917-24). The use of a cytokine trap to neutralize the ligand can also be a better strategy than blockading the receptor with an antibody, as in the case of CSF-1. Because CSF-1 is cleared from the circulation by receptor-mediated endocytosis, an anti-CSF-1 receptor antibody blockade caused a significant increase in circulating CSF-1 concentration (Hume et al., Blood. 2012;119:1810-20)

[0034] Indeed, as described below, treatment with the anti-PD-L1/TGFβ Trap elicits a synergistic anti-tumor effect due to the simultaneous blockade of the interaction between PD-L1 on tumor cells and PD-1 on immune cells, and the neutralization of TGFβ in the tumor microenvironment. As demonstrated in the following examples, anti-PDL1/TGFβ Trap has efficacy superior to that of the single agent anti-PD-L1 or TGFβ Trap control. Without being bound by theory, this presumably is due to a synergistic effect obtained from simultaneous blocking the two major immune escape mechanisms, and in addition, the targeted depletion of the TGFβ in the tumor microenvironment by a single molecular entity. This depletion is achieved by (1) anti-PD-L1 targeting of tumor cells; (2) binding of the TGFβ autocrine/paracrine in the tumor microenvironment by the TGFβ Trap; and (3) destruction of the bound TGFβ through the PD-L1 receptor-mediated endocytosis. The aforementioned mechanisms of action cannot be achieved by the combination therapy of the two single agents anti-PD-L1 and TGFβ Trap. Furthermore, the TGFβRII fused to the C-terminus of Fc (fragment of crystallization of IgG) was several-fold more potent than the TGFβRII-Fc that places the TGFβRII at the N-terminus of Fc (see Example 3). The superb efficacy obtained with anti-PDL1/TGFβ Trap also allays some concerns that the TGFβRII does not trap TGFβ2. As pointed out by Yang et al., Trends Immunol. 2010; 31:220-227, although some tumor types do secrete TGFβ2 initially, as the tumor progresses, the TGFβ in the tumor microenvironment is predominantly secreted by myeloid-derived suppressor cells, which secrete TGFβ1. In addition to showing great promise as an effective immuno-oncology therapeutic, treatment with soluble TGFβRII can potentially reduce the cardiotoxicity concerns of TGFβ targeting therapies, especially the TGFβRI kinase inhibitors. This is because of the important roles TGFβ2 plays in embryonic development of the heart as well as in repair of myocardial damage after ischemia and reperfusion injury (Roberts et al., J Clin Invest. 1992; 90:2056-62).

TGFβ as a cancer target

[0035] TGFβ had been a somewhat questionable target in cancer immunotherapy because of its paradoxical roles as the molecular Jekyll and Hyde of cancer (Bierie et al., Nat Rev Cancer. 2006; 6:506-20). Like some other cytokines, TGFβ activity is developmental stage and context dependent. Indeed TGFβ can act as either a tumor promoter or a tumor suppressor, affecting tumor initiation, progression and metastasis. The mechanisms underlying this dual role of TGFβ remain unclear (Yang et al., Trends Immunol. 2010; 31:220-227). Although it has been postulated that Smad-dependent signaling mediates the growth inhibition of TGFβ signaling, while the Smad independent pathways contribute to its tumor-promoting effect, there are also data showing that the Smad-dependent pathways are involved in tumor progression (Yang et al., Cancer Res. 2008; 68:9107-11).

[0036] Both the TGFβ ligand and the receptor have been studied intensively as therapeutic targets. There are three ligand isoforms, TGFβ1, 2 and 3, all of which exist as homodimers. There are also three TGFβ receptors (TGFβR), which are called TGFβR type I, II and III (López-Casillas et al., J Cell Biol. 1994; 124:557-68). TGFβRI is the signaling chain and cannot bind ligand. TGFβRII binds the ligand TGFβ1 and 3, but not TGFβ2, with high affinity. The TGFβRII/TGFβ complex recruits TGFβRI to form the signaling complex (Won et al., Cancer Res. 1999; 59:1273-7). TGFβRIII is a positive regulator of TGFβ binding to its signaling receptors and binds all 3 TGFβ isoforms with high affinity. On the cell surface, the TGFβ/TGFβRIII complex binds TGFβRII and then recruits TGFβRI, which displaces TGFβRIII to form the signaling complex.

[0037] Although the three different TGFβ isoforms all signal through the same receptor, they are known to have differential expression patterns and non-overlapping functions in vivo. The three different TGF-β isoform knockout mice have distinct phenotypes, indicating numerous non-compensated functions (Bujak et al., Cardiovasc Res. 2007; 74:184-95). While TGFβ1 null mice have hematopoiesis and vasculogenesis defects and TGFβ3 null mice display pulmonary development and defective palatogenesis, TGFβ2 null mice show various developmental abnormalities, the most prominent being multiple cardiac deformities (Bartram et al., Circulation. 2001; 103:2745-52; Yamagishi et al., Anat Rec. 2012; 295:257-67). Furthermore, TGFβ is implicated to play a major role in the repair of myocardial damage after ischemia and reperfusion injury. In an adult heart, cardiomyocytes secrete TGFβ, which acts as an autocrine to maintain the spontaneous beating rate. Importantly, 70-85% of the TGFβ secreted by cardiomyocytes is TGFβ2 (Roberts et al., J Clin Invest. 1992; 90:2056-62). In summary, given the predominant roles of TGFβ1 and TGFβ2 in the tumor microenvironment and cardiac physiology, respectively, a therapeutic agent that neutralizes TGFβ1 but not TGFβ2 could provide an optimal therapeutic index by minimizing the cardiotoxicity without compromising the anti-tumor activity. This is consistent with the findings by the present inventors, who observed a lack of toxicity, including cardiotoxicity, for anti-PD-L1/TGFβ Trap in monkeys.

[0038] Therapeutic approaches to neutralize TGFβ include using the extracellular domains of TGFβ receptors as soluble receptor traps and neutralizing antibodies. Of the receptor trap approach, soluble TGFβRIII may seem the obvious choice since it binds all the three TGFβ ligands. However, TGFβRIII, which occurs naturally as a 280-330 kD glucosaminoglycan (GAG)-glycoprotein, with extracellular domain of 762 amino acid residues, is a very complex protein for biotherapeutic development. The soluble TGFβRIII devoid of GAG could be produced in insect cells and shown to be a potent TGFβ neutralizing agent (Vilchis-Landeros et al, Biochem J 355:215, 2001). The two separate binding domains (the endoglin-related and the uromodulin-related) of TGFβRIII could be independently expressed, but they were shown to have affinities 20 to 100 times lower than that of the soluble TGFβRIII, and much diminished neutralizing activity (Mendoza et al., Biochemistry. 2009; 48:11755-65). On the other hand, the extracellular domain of TGFβRII is only 136 amino acid residues in length and can be produced as a glycosylated protein of 25-35 kD. The recombinant soluble TGFβRII was further shown to bind TGFβ1 with a K_D of 200 pM, which is fairly similar to the K_D of 50 pM for the full length TGFβRII on cells (Lin et al., J Biol Chem. 1995; 270:2747-54). Soluble TGFβRII-Fc was tested as an anti-cancer agent and was shown to inhibit established murine malignant mesothelioma growth in a tumor model (Suzuki et al., Clin Cancer Res. 2004; 10:5907-18). Since TGFβRII does not bind TGFβ2, and TGFβRIII binds TGFβ1 and 3 with lower affinity than TGFβRII, a fusion protein of the endoglin domain of TGFβRIII and extracellular domain of TGFβRII was produced in bacteria and was shown to inhibit the signaling of TGFβ1 and 2 in cell based assays more effectively than either TGFβRII or RIII (Verona et al., Protein Eng Des Sel. 2008; 21:463-73). Despite some encouraging anti-tumor activities in tumor models, to our knowledge no TGFβ receptor trap recombinant proteins have been tested in the clinic.

[0039] Still another approach to neutralize all three isoforms of the TGFβ ligands is to screen for a pan-neutralizing anti-TGFβ antibody, or an anti-receptor antibody that blocks the receptor from binding to TGFβ1, 2 and 3. GC1008, a human antibody specific for all isoforms of TGFβ, was in a Phase I/II study in patients with advanced malignant melanoma or renal cell carcinoma (Morris et al., J Clin Oncol 2008; 26:9028 (Meeting abstract)). Although the treatment was found to be safe and well tolerated, only limited clinical efficacy was observed, and hence it was difficult to interpret the importance of anti-TGFβ therapy without further characterization of the immunological effects (Flavell et al., Nat Rev Immunol. 2010; 10:554-67). There were also TGFβ-isoform-specific antibodies tested in the clinic. Metelimumab, an antibody specific for TGFβ1 was tested in Phase 2 clinical trial as a treatment to prevent excessive post-operative scarring for glaucoma surgery; and Lerdelimumab, an antibody specific for TGFβ2, was found to be safe but ineffective at improving scarring after eye surgery in a Phase 3 study (Khaw et al., Ophthalmology 2007; 114:1822-1830). Anti-TGFβRII antibodies that block the receptor from binding to all three TGFβ isoforms, such as the anti-human TGFβRII antibody TR1 and antimouse TGFβRII antibody MT1, have also shown some therapeutic efficacy against primary tumor growth and metastasis in mouse models (Zhong et al., Clin Cancer Res. 2010; 16:1191-205). To date, the vast majority of the studies on TGFβ targeted anticancer treatment, including small molecule inhibitors of TGFβ signaling that often are quite toxic, are mostly in the preclinical stage and the anti-tumor efficacy obtained has been limited (Calone et al., Exp Oncol. 2012; 34:9-16; Connolly et al., Int J Biol Sci. 2012;

8:964-78).

[0040] The antibody-TGFβ trap of the invention is a bifunctional protein containing at least portion of a human TGFβ Receptor II (TGFβRII) that is capable of binding TGFβ. In one embodiment, the TGFβ trap polypeptide is a soluble portion of the human TGFβ Receptor Type 2 Isoform A (SEQ ID NO: 8) that is capable of binding TGFβ. In a further embodiment, TGFβ trap polypeptide contains at least amino acids 73-184 of SEQ ID NO:8. In yet a further embodiment, the TGFβ trap polypeptide contains amino acids 24-184 of SEQ ID NO:8. In another embodiment, the TGFβ trap polypeptide is a soluble portion of the human TGFβ Receptor Type 2 Isoform B (SEQ ID NO: 9) that is capable of binding TGFβ. In a further embodiment, TGFβ trap polypeptide contains at least amino acids 48-159 of SEQ ID NO:9. In yet a further embodiment, the TGFβ trap polypeptide contains amino acids 24-159 of SEQ ID NO:9. In yet a further embodiment, the TGFβ trap polypeptide contains amino acids 24-105 of SEQ ID NO:9.

Immune Checkpoint Dis-inhibition

[0041] The approach of targeting T cell inhibition checkpoints for dis-inhibition with therapeutic antibodies is an area of intense investigation (for a review, see Pardoll, Nat Rev Cancer. 2012; 12:253-264). The antibody moiety targets PD-L1, the counter-receptor on antigen presenting cells and tumor cells (which co-opt this counter-receptor for their own immune evasion).

[0042] The invention contemplates antibody TGFβ traps that target, through their antibody moiety or antigen binding fragment thereof, T cell inhibition checkpoints for dis-inhibition. To that end the present inventors have tested the anti-tumor efficacy of combining a TGFβ trap with antibodies targeting various T cell inhibition checkpoint receptor proteins, such as anti-PD-1, anti-PD-L1, anti-TIM-3 and anti-LAG3. The experimental results are further detailed in Examples 7-18. The present inventors found that combining a TGFβ trap with an anti-PD-L1 antibody exhibited remarkable anti-tumor activity beyond what was observed with the monotherapies. In contrast, none of the other combinations with antibodies to the targets listed above showed any superior efficacy. In particular, one may have expected that a combination treatment of a TGFβ trap with an anti-PD-1 antibody would demonstrate similar activity to the one observed with anti-PD-L1, as PD-1 / PD-L1 are cognate receptors that bind to each other to effect the immune checkpoint inhibition. However, this is not what the present inventors have found.

Anti-PD-L1 Antibodies

[0043] Antibody moieties of proteins of the invention may comprise any anti-PD-L1 antibody, or antigen-binding fragment thereof, described in the art that comprises: (i) a heavy chain variable region including an HVR-H1, HVR-H2, and HVR-H3 having the amino acid sequences

of SYIMM, SIYPSGGITFYADTVKG, and IKLGTVTTVDY, respectively, and (ii) a light chain variable region including an HVR-L1, HVR-L2, and HVR-L3 having the amino acid sequences of TGTSSDVGGYNYVS, DVSNRPS, and SSYTSSSTRV, respectively. Antibodies can be monoclonal, chimeric, humanized, or human. Antibody fragments include Fab, F(ab')2, scFv and Fv fragments, which are described in further detail below.

[0044] Exemplary antibodies may comprise variable region heavy chain framework sequences juxtaposed between the HVRs according to the formula: (HC-FR1)-(HVR-H1)-(HC-FR2)-(HVR-H2)-(HC-FR3)-(HVR-H3)-(HC-FR4). For example, in one aspect, the framework sequences may be derived from human consensus framework sequences or human germline framework sequences.

[0045] In another aspect, at least one of the framework sequences is the following:

HC-FR1 is EVQLLESGGGLVQPGGSLRLSCAASGFTFS;

HC-FR2 is WVRQAPGKGLEWVS;

HC-FR3 is RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR;

HC-FR4 is WGQGTLVTVSS.

[0046] Exemplary antibodies may also comprise variable region light chain framework sequences juxtaposed between the HVRs according to the formula: (LC-FR1MHVR-L1)-(LC-FR2)-(HVR-L2)-(LC-FR3)-(HVR-L3)-(LC-FR4).

[0047] For example, in one aspect, the light chain framework sequences may be derived from human consensus framework sequences or human germline framework sequences.

[0048] In another aspect, the light chain framework sequences are lambda light chain sequences.

[0049] In a further aspect, at least one of the framework sequence is the following:

LC-FR1 is QSALTQPASVSGSPGQSITISC;

LC-FR2 is WYQQHPGKAPKLMIY;

LC-FR3 is GVSNRFSGSKSGNTASLTISGLQAEDEADYYC;

LC-FR4 is FGTGTKVTVL.

[0050] In a further aspect, the heavy chain variable region includes one or more framework

sequences juxtaposed between the HVRs as: (HC-FR1)-(HVR-H1)-(HC-FR2)-(HVR-H2)-(HC-FR3)-(HVR-H3)-(HC-FR4), and the light chain variable regions include one or more framework sequences juxtaposed between the HVRs as: (LC-FR1 HVR-L1)-(LC-FR2)-(HVR-L2)-(LC-FR3)-(HVR-L3)-(LC-FR4).

[0051] In a still further aspect, the heavy chain variable region polypeptide, antibody, or antibody fragment further includes at least a C_H1 domain.

[0052] In a more specific aspect, the heavy chain variable region polypeptide, antibody, or antibody fragment further includes a C_H1, a C_H2, and a C_H3 domain.

[0053] In a still further aspect, the variable region light chain, antibody, or antibody fragment further includes a C_L domain.

[0054] In a still further aspect, the antibody further includes a C_H1, a C_H2, a C_H3, and a C_L domain.

[0055] In a still further specific aspect, the antibody further includes a human or murine constant region.

[0056] In a still further aspect, the human constant region is selected from the group consisting of IgG1, IgG2, IgG2, IgG3, IgG4.

[0057] In a still further specific aspect, the human or murine constant region is IgG1.

[0058] The invention features an anti-PD-L1 antibody including a heavy chain and a light chain variable region sequence, where:

1. (a) the heavy chain includes an HVR-H1, an HVR-H2, and an HVR-H3, having at least 80% overall sequence identity to SYIMM, SIYPSGGITFYADTVKG, and IKLGTVTTVDY, respectively, and
2. (b) the light chain includes an HVR-L1, an HVR-L2, and an HVR-L3, having at least 80% overall sequence identity to TGTSSDVGGYNYVS, DVSNRPS, and SSYTSSSTRV, respectively.

[0059] In a still further embodiment, the invention features an anti-PD-L1 antibody including a heavy chain and a light chain variable region sequence, where:

1. (a) the heavy chain sequence has at least 85% sequence identity to the heavy chain sequence:
   EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYIMMVWRQAPGKGLEWVS SIYPSGGITF YADWKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARIKLGTVTTVDYWGQGTLVT

VSS, and

2. (b) the light chain sequence has at least 85% sequence identity to the light chain sequence:
QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYDVSN
RPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTRVFGTGTKVTVL.

[0060] In a specific aspect, the sequence identity is 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%.

[0061] Antibodies disclosed herein may bind to human, mouse, or cynomolgus monkey PD-L1. In a specific aspect the antibody may be capable of blocking the interaction between human, mouse, or cynomolgus monkey PD-L1 and the respective human, mouse, or cynomolgus monkey PD-1 receptors.

[0062] Antibodies disclosed herein may bind to human PD-L1 with a K_D of 5×10^{-9} M or less, preferably with a K_D of 2×10^{-9} M or less, and even more preferred with a K_D of 1×10^{-9} M or less.

[0063] Antibodies disclosed herein may bind to a functional epitope including residues Y56 and D61 of human PD-L1.

[0064] The functional epitope may further include E58, E60, Q66, R113, and M115 of human PD-L1.

[0065] Specifically, antibodies disclosed herein may bind to a conformational epitope, including residues 54-66 and 112-122 of human PD-L1.

[0066] Disclosed herein are pharmaceutical compositions comprising proteins and polypeptides including any of the above described anti-PD-L1 antibodies in combination with at least one pharmaceutically acceptable carrier.

[0067] In a still further embodiment, the invention features an isolated nucleic acid encoding a light chain or a heavy chain variable region sequence of an anti-PD-L1 antibody, wherein:

1. (a) the heavy chain includes an HVR-H1, an HVR-H2, and an HVR-H3 sequence having at least 80% sequence identity to SYIMM, SIYPSGGITFYADTVKG, and IKLGTVTTVDY, respectively, or
2. (b) the light chain includes an HVR-L1, an HVR-L2, and an HVR-L3 sequence having at least 80% sequence identity to TGTSSDVGGYNYVS, DVSNRPS, and SSYTSSSTRV, respectively.

[0068] In a further aspect of the disclosure, the nucleic acid sequence for the heavy chain is:

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atggagttgc ctgttaggct gttggtgctg atgttctgga ttcctgctag ctccagcgag      60
gtgcagctgc tggaatccgg cggaggactg gtgcagcctg gcggctccct gagactgtct     120
tgcgccgcct ccggcttcac cttctccagc tacatcatga tgtgggtgcg acaggccccc     180
ggcaagggcc tggaatgggt gtcctccatc taccctccg gcggcatcac cttctacgcc      240
gacaccgtga agggccggtt caccatctcc cgggacaact ccaagaacac cctgtacctg     300
cagatgaact ccctgcgggc cgaggacacc gccgtgtact actgcgcccg gatcaagctg     360
ggcaccgtga ccaccgtgga ctactggggc cagggcaccc tggtgacagt gtcctccgcc     420
tccaccaagg gcccatcggt cttccccctg gcaccctcct ccaagagcac ctctgggggc     480
acagcggccc tgggctgcct ggtcaaggac tacttccccg aaccggtgac ggtgtcgtgg     540
aactcaggcg ccctgaccag cggcgtgcac accttcccgg ctgtcctaca gtcctcagga     600
ctctactccc tcagcagcgt ggtgaccgtg ccctccagca gcttgggcac ccagacctac     660
atctgcaacg tgaatcacaa gcccagcaac accaaggtgg acaagaaagt tgagcccaaa     720
tcttgtgaca aaactcacac atgcccaccg tgcccagcac ctgaactcct gggggggaccg    780
tcagtcttcc tcttcccccc aaaacccaag gacaccctca tgatctcccg gacccctgag     840
gtcacatgcg tggtggtgga cgtgagccac gaagaccctg aggtcaagtt caactggtac     900
gtggacggcg tggaggtgca taatgccaag acaaagccgc gggaggagca gtacaacagc     960
acgtaccgtg tggtcagcgt cctcaccgtc ctgcaccagg actggctgaa tggcaaggag    1020
tacaagtgca aggtctccaa caaagccctc ccagccccca tcgagaaaac catctccaaa    1080
gccaaagggc agccccgaga accacaggtg tacaccctgc ccccatcacg ggatgagctg    1140
accaagaacc aggtcagcct gacctgcctg gtcaaaggct tctatcccag cgacatcgcc    1200
gtggagtggg agagcaatgg gcagccggag aacaactaca agaccacgcc tcccgtgctg    1260
gactccgacg gctccttctt cctctatagc aagctcaccg tggacaagag caggtggcag    1320
caggggaacg tcttctcatg ctccgtgatg catgaggctc tgcacaacca ctacacgcag    1380
aagagcctct ccctgtcccc gggtaaa                                        1407
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and the nucleic acid sequence for the light chain is:

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atggagttgc ctgttaggct gttggtgctg atgttctgga ttcctgcttc cttaagccag      60
tccgccctga cccagcctgc ctccgtgtct ggctcccctg gccagtccat caccatcagc      120
tgcaccggca cctccagcga cgtgggcggc tacaactacg tgtcctggta tcagcagcac      180
cccggcaagg cccccaagct gatgatctac gacgtgtcca accggccctc cggcgtgtcc      240
aacagattct ccggctccaa gtccggcaac accgcctccc tgaccatcag cggactgcag      300
gcagaggacg aggccgacta ctactgctcc tcctacacct cctccagcac cagagtgttc      360
ggcaccggca caaaagtgac cgtgctgggc cagcccaagg ccaacccaac cgtgacactg      420
ttccccccat cctccgagga actgcaggcc aacaaggcca ccctggtctg cctgatctca      480
gatttctatc caggcgccgt gaccgtggcc tggaaggctg atggctcccc agtgaaggcc      540
ggcgtggaaa ccaccaagcc ctccaagcag tccaacaaca aatacgccgc ctcctcctac      600
ctgtccctga cccccgagca gtggaagtcc caccggtcct acagctgcca ggtcacacac      660
gagggctcca ccgtggaaaa gaccgtcgcc cccaccgagt gctca                      705
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[0069] Further exemplary anti-PD-L1 antibodies that can be used in an anti-PD-L1/TGFβ Trap are described in US patent application publication US 2010/0203056. In one aspect of the disclosure, the antibody moiety is YW243.55S70. In another aspect of the disclosure, the antibody moiety is MPDL3280A.

[0070] In a further aspect of the disclosure features an anti-PD-L1 antibody moiety including a

heavy chain and a light chain variable region sequence, where:

1. (a) the heavy chain sequence has at least 85% sequence identity to the heavy chain sequence:
   EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYY
   ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSS (SEQ ID NO:12),
   and
2. (b) the light chain sequence has at least 85% sequence identity to the light chain sequence:
   DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSG
   SGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGQGTKVEIKR (SEQ ID NO:13).

[0071] The sequence identity may be 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0072] A further aspect of the disclosure features an anti-PD-L1 antibody moiety including a heavy chain and a light chain variable region sequence, where:

1. (a) the heavy chain variable region sequence is:
   EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYY
   ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSS (SEQ ID NO:12)
   , and
2. (b) the light chain variable region sequence is:
   DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSG
   SGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGQGTKVEIKR (SEQ ID NO:13).

[0073] A further aspect of the disclosure an anti-PD-L1 antibody moiety including a heavy chain and a light chain variable region sequence, where:

1. (a) the heavy chain variable region sequence is:
   EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYYADSV
   KGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSA (SEQ ID NO:14),
   and
2. (b) the light chain variable region sequence is:
   DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSG
   SGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGQGTKVEIKR (SEQ ID NO:13).

[0074] Yet further exemplary anti-PD-L1 antibodies that can be used in an anti-PD-L1/TGFβ Trap are described in US patent publication US 7,943,743.

[0075] In one aspect of the disclosure, the anti-PD-L1 antibody is MDX-1105.

[0076] In yet a further aspect of the disclosure, the anti-PD-L1 antibody is MEDI-4736.

Constant region

[0077] The proteins and peptides as disclosed herein can include a constant region of an immunoglobulin or a fragment, analog, variant, mutant, or derivative of the constant region. In preferred aspects of the disclosure, the constant region is derived from a human immunoglobulin heavy chain, for example, IgG1, IgG2, IgG3, IgG4, or other classes. In one aspect of the disclosure, the constant region includes a CH2 domain. In another aspect of the disclosure, the constant region includes CH2 and CH3 domains or includes hinge-CH2-CH3. Alternatively, the constant region can include all or a portion of the hinge region, the CH2 domain and/or the CH3 domain.

[0078] In one aspect of the disclosure, the constant region contains a mutation that reduces affinity for an Fc receptor or reduces Fc effector function. For example, the constant region can contain a mutation that eliminates the glycosylation site within the constant region of an IgG heavy chain. In some aspects of the disclosure, the constant region contains mutations, deletions, or insertions at an amino acid position corresponding to Leu234, Leu235, Gly236, Gly237, Asn297, or Pro331 of IgG1 (amino acids are numbered according to EU nomenclature). In a particular aspect of the disclosure, the constant region contains a mutation at an amino acid position corresponding to Asn297 of IgG1. In alternative aspects of the disclosure, the constant region contains mutations, deletions, or insertions at an amino acid position corresponding to Leu281, Leu282, Gly283, Gly284, Asn344, or Pro378 of IgG1.

[0079] In some aspects of the disclosure, the constant region contains a CH2 domain derived from a human IgG2 or IgG4 heavy chain. Preferably, the CH2 domain contains a mutation that eliminates the glycosylation site within the CH2 domain. In one aspect of the disclosure, the mutation alters the asparagine within the Gln-Phe-Asn-Ser (SEQ ID NO: 15) amino acid sequence within the CH2 domain of the IgG2 or IgG4 heavy chain. Preferably, the mutation changes the asparagine to a glutamine. Alternatively, the mutation alters both the phenylalanine and the asparagine within the Gln-Phe-Asn-Ser (SEQ ID NO: 15) amino acid sequence. In one aspect of the disclosure, the Gln-Phe-Asn-Ser (SEQ ID NO: 15) amino acid sequence is replaced with a Gln-Ala-Gln-Ser (SEQ ID NO: 16) amino acid sequence. The asparagine within the Gln-Phe-Asn-Ser (SEQ ID NO: 15) amino acid sequence corresponds to Asn297 of IgG1.

[0080] In another aspect of the disclosure, the constant region includes a CH2 domain and at least a portion of a hinge region. The hinge region can be derived from an immunoglobulin heavy chain, e.g., IgG1, IgG2, IgG3, IgG4, or other classes. Preferably, the hinge region is derived from human IgG1, IgG2, IgG3, IgG4, or other suitable classes. More preferably the hinge region is derived from a human IgG1 heavy chain. In one aspect of the disclosure the cysteine in the Pro-Lys-Ser-Cys-Asp-Lys (SEQ ID NO: 17) amino acid sequence of the IgG1 hinge region is altered. In a preferred aspect of the disclosure the Pro-Lys-Ser-Cys-Asp-Lys (SEQ ID NO: 17) amino acid sequence is replaced with a Pro-Lys-Ser-Ser-Asp-Lys (SEQ ID NO: 18) amino acid sequence. In one aspect of the disclosure, the constant region includes a CH2 domain derived from a first antibody isotype and a hinge region derived from a second antibody isotype. In a specific aspect of the disclosure, the CH2 domain is derived from a human IgG2 or IgG4 heavy chain, while the hinge region is derived from an altered human IgG1 heavy chain.

[0081] The alteration of amino acids near the junction of the Fc portion and the non-Fc portion can dramatically increase the serum half-life of the Fc fusion protein (PCT publication WO 01/58957). Accordingly, the junction region of a protein or polypeptide of the present disclosure can contain alterations that, relative to the naturally-occurring sequences of an immunoglobulin heavy chain and erythropoietin, preferably lie within about 10 amino acids of the junction point. These amino acid changes can cause an increase in hydrophobicity. In one aspect of the disclosure, the constant region is derived from an IgG sequence in which the C-terminal lysine residue is replaced. Preferably, the C-terminal lysine of an IgG sequence is replaced with a non-lysine amino acid, such as alanine or leucine, to further increase serum half-life. In another aspect of the disclosure, the constant region is derived from an IgG sequence in which the Leu-Ser-Leu-Ser (SEQ ID NO: 19) amino acid sequence near the C-terminus of the constant region is altered to eliminate potential junctional T-cell epitopes. For example, in one aspect of the disclosure, the Leu-Ser-Leu-Ser amino acid sequence is replaced with an Ala-Thr-Ala-Thr (SEQ ID NO: 20) amino acid sequence. In other aspect of the disclosure, the amino acids within the Leu-Ser-Leu-Ser (SEQ ID NO: 19) segment are replaced with other amino acids such as glycine or proline. Detailed methods of generating amino acid substitutions of the Leu-Ser-Leu-Ser (SEQ ID NO: 19) segment near the C-terminus of an IgG1, IgG2, IgG3, IgG4, or other immunoglobulin class molecule have been described in U.S. Patent Publication No. 2003/0166877.

[0082] Suitable hinge regions for antibodies of the disclosure can be derived from IgG1, IgG2, IgG3, IgG4, and other immunoglobulin classes. The IgG1 hinge region has three cysteines, two of which are involved in disulfide bonds between the two heavy chains of the immunoglobulin. These same cysteines permit efficient and consistent disulfide bonding formation between Fc portions. Therefore, a preferred hinge region of the present disclosure is derived from IgG1, more preferably from human IgG1. In some aspects of the disclosure, the first cysteine within the human IgG1 hinge region is mutated to another amino acid, preferably serine. The IgG2 isotype hinge region has four disulfide bonds that tend to promote oligomerization and possibly incorrect disulfide bonding during secretion in recombinant systems. A suitable hinge region can be derived from an IgG2 hinge; the first two cysteines are

each preferably mutated to another amino acid. The hinge region of IgG4 is known to form interchain disulfide bonds inefficiently. However, a suitable hinge region for the present disclosure can be derived from the IgG4 hinge region, preferably containing a mutation that enhances correct formation of disulfide bonds between heavy chain-derived moieties (Angal S, et al. (1993) Mol. Immunol., 30:105-8).

[0083] In accordance with the present disclosure, the constant region can contain CH2 and/or CH3 domains and a hinge region that are derived from different antibody isotypes, i.e., a hybrid constant region. For example, in one aspect of the disclosure, the constant region contains CH2 and/or CH3 domains derived from IgG2 or IgG4 and a mutant hinge region derived from IgG1. Alternatively, a mutant hinge region from another IgG subclass is used in a hybrid constant region. For example, a mutant form of the IgG4 hinge that allows efficient disulfide bonding between the two heavy chains can be used. A mutant hinge can also be derived from an IgG2 hinge in which the first two cysteines are each mutated to another amino acid. Assembly of such hybrid constant regions has been described in U.S. Patent Publication No. 2003/0044423.

[0084] In accordance with the present disclosure, the constant region can contain one or more mutations described herein. The combinations of mutations in the Fc portion can have additive or synergistic effects on the prolonged serum half-life and increased in vivo potency of the bifunctional molecule. Thus, in one exemplary aspect of the disclosure, the constant region can contain (i) a region derived from an IgG sequence in which the Leu-Ser-Leu-Ser (SEQ ID NO: 19) amino acid sequence is replaced with an Ala-Thr-Ala-Thr (SEQ ID NO: 20) amino acid sequence; (ii) a C-terminal alanine residue instead of lysine; (iii) a CH2 domain and a hinge region that are derived from different antibody isotypes, for example, an IgG2 CH2 domain and an altered IgG1 hinge region; and (iv) a mutation that eliminates the glycosylation site within the IgG2-derived CH2 domain, for example, a Gln-Ala-Gln-Ser (SEQ ID NO: 16) amino acid sequence instead of the Gln-Phe-Asn-Ser (SEQ ID NO: 15) amino acid sequence within the IgG2-derived CH2 domain.

Antibody fragments

[0085] The proteins and polypeptides of the disclosure can also include antigen-binding fragments of antibodies. Exemplary antibody fragments include scFv, Fv, Fab, $F(ab')_2$, and single domain VHH fragments such as those of camelid origin.

[0086] Single-chain antibody fragments, also known as single-chain antibodies (scFvs), are recombinant polypeptides which typically bind antigens or receptors; these fragments contain at least one fragment of an antibody variable heavy-chain amino acid sequence (V_H) tethered to at least one fragment of an antibody variable light-chain sequence (V_L) with or without one or more interconnecting linkers. Such a linker may be a short, flexible peptide selected to assure that the proper three-dimensional folding of the V_L and V_H domains occurs once they

are linked so as to maintain the target molecule binding-specificity of the whole antibody from which the single-chain antibody fragment is derived. Generally, the carboxyl terminus of the V_L or V_H sequence is covalently linked by such a peptide linker to the amino acid terminus of a complementary V_L and V_H sequence. Single-chain antibody fragments can be generated by molecular cloning, antibody phage display library or similar techniques. These proteins can be produced either in eukaryotic cells or prokaryotic cells, including bacteria.

[0087] Single-chain antibody fragments contain amino acid sequences having at least one of the variable regions or CDRs of the whole antibodies described in this specification, but are lacking some or all of the constant domains of those antibodies. These constant domains are not necessary for antigen binding, but constitute a major portion of the structure of whole antibodies. Single-chain antibody fragments may therefore overcome some of the problems associated with the use of antibodies containing part or all of a constant domain. For example, single-chain antibody fragments tend to be free of undesired interactions between biological molecules and the heavy-chain constant region, or other unwanted biological activity. Additionally, single-chain antibody fragments are considerably smaller than whole antibodies and may therefore have greater capillary permeability than whole antibodies, allowing single-chain antibody fragments to localize and bind to target antigen-binding sites more efficiently. Also, antibody fragments can be produced on a relatively large scale in prokaryotic cells, thus facilitating their production. Furthermore, the relatively small size of single-chain antibody fragments makes them less likely than whole antibodies to provoke an immune response in a recipient.

[0088] Fragments of antibodies that have the same or comparable binding characteristics to those of the whole antibody are also disclosed. Such fragments may contain one or both Fab fragments or the $F(ab')_2$ fragment. The antibody fragments may contain all six CDRs of the whole antibody, although fragments containing fewer than all of such regions, such as three, four or five CDRs, are also functional.

Protein production

[0089] The antibody-cytokine trap proteins are generally produced recombinantly, using mammalian cells containing a nucleic acid engineered to express the protein. Although one example of a suitable cell line and protein production method is described in Examples 1 and 2, a wide variety of suitable vectors, cell lines and protein production methods have been used to produce antibody-based biopharmaceuticals and could be used in the synthesis of these antibody-cytokine trap proteins..

Therapeutic indications

[0090] The anti-PD-L1/TGFβ Trap proteins described in the application can be used to treat

cancer or reduce tumor growth in a patient. Exemplary cancers include colorectal, breast, ovarian, pancreatic, gastric, prostate, renal, cervical, myeloma, lymphoma, leukemia, thyroid, endometrial, uterine, bladder, neuroendocrine, head and neck, liver, nasopharyngeal, testicular, small cell lung cancer, non-small cell lung cancer, melanoma, basal cell skin cancer, squamous cell skin cancer, dermatofibrosarcoma protuberans, Merkel cell carcinoma, glioblastoma, glioma, sarcoma, mesothelioma, and myelodisplastic syndromes.

[0091] The cancer or tumor to be treated with an anti-PD-L1/ TGFβ Trap may be selected based on the expression or elevated expression of PD-L1 and TGFβ in the tumor, the correlation of their expression levels with prognosis or disease progression, and preclinical and clinical experience on the sensitivity of the tumor to treatments targeting PD-L1 and TGFβ. Such cancers or tumors include but are not limited to colorectal, breast, ovarian, pancreatic, gastric, prostate, renal, cervical, bladder, head and neck, liver, non-small cell lung cancer, melanoma, Merkel cell carcinoma, and mesothelioma.

Pharmaceutical compositions

[0092] The present invention also features pharmaceutical compositions that contain a therapeutically effective amount of a protein described herein. The composition can be formulated for use in a variety of drug delivery systems. One or more physiologically acceptable excipients or carriers can also be included in the composition for proper formulation. Suitable formulations for use in the present invention are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed., 1985. For a brief review of methods for drug delivery, see, e.g., Langer (Science 249:1527-1533, 1990).

[0093] The pharmaceutical compositions are intended for parenteral, intranasal, topical, oral, or local administration, such as by a transdermal means, for therapeutic treatment. The pharmaceutical compositions can be administered parenterally (e.g., by intravenous, intramuscular, or subcutaneous injection), or by oral ingestion, or by topical application or intraarticular injection at areas affected by the vascular or cancer condition. Additional routes of administration include intravascular, intra-arterial, intratumor, intraperitoneal, intraventricular, intraepidural, as well as nasal, ophthalmic, intrascleral, intraorbital, rectal, topical, or aerosol inhalation administration. Thus, the disclosure provides compositions for parenteral administration that comprise the above mention agents dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, e.g., water, buffered water, saline, PBS, and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. The disclosure also provides compositions for oral delivery, which may contain inert ingredients such as binders or fillers for the formulation of a tablet, a capsule, and the like. Furthermore, this disclosure provides compositions for local administration, which may contain inert ingredients such as solvents or emulsifiers for the formulation of a cream, an ointment, and the like.

[0094] These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as-is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably between 5 and 9 or between 6 and 8, and most preferably between 7 and 8, such as 7 to 7.5. The resulting compositions in solid form may be packaged in multiple single dose units, each containing a fixed amount of the above-mentioned agent or agents, such as in a sealed package of tablets or capsules. The composition in solid form can also be packaged in a container for a flexible quantity, such as in a squeezable tube designed for a topically applicable cream or ointment.

[0095] The optimal dose of the antibody- TGFØ trap is based on the percent receptor occupancy by the antibody moiety to achieve maximal therapeutic effect because the cytokine trap is used in a large excess. For example, the therapeutic dose for a monoclonal antibody targeting a cellular receptor is determined such that the trough level is around 10 to 100 µg/ml, *i.e.,* 60 to 600 nM (for antibody with a dissociation constant (K_D) of 6 nM, this trough level would ensure that between 90 to 99% of the target receptors on the cells are occupied by the antibody). This is in large excess of cytokines, which are typically present in pg to ng/ml in circulation.

[0096] The optimal dose of antibody-TGFβ trap polypeptide will depend on the disease being treated, the severity of the disease, and the existence of side effects. The optimal dose can be determined by routine experimentation. For parenteral administration a dose between 0.1 mg/kg and 100 mg/kg, alternatively between 0.5 mg/kg and 50 mg/kg, alternatively, between 1 mg/kg and 25 mg/kg, alternatively between 2 mg/kg and 10 mg/kg, alternatively between 5 mg/kg and 10 mg/kg is administered and may be given, for example, once weekly, once every other week, once every third week, or once monthly per treatment cycle.

EXAMPLES

[0097] The invention now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects of the present disclosure.

EXAMPLE 1 - DNA construction and protein expression

[0098] Anti-PD-L1/TGFβ Trap is an anti-PD-L1 antibody-TGFβ Receptor II fusion protein. The light chain of the molecule is identical to the light chain of the anti-PD-L1 antibody (SEQ ID NO: 1). The heavy chain of the molecule (SEQ ID NO:3) is a fusion protein comprising the heavy chain of the anti-PD-L1 antibody (SEQ ID NO:2) genetically fused to via a flexible $(Gly_4Ser)_4Gly$ linker (SEQ ID NO: 11) to the N-terminus of the soluble TGFβ Receptor II (SEQ ID NO: 10). At

the fusion junction, the C-terminal lysine residue of the antibody heavy chain was mutated to alanine to reduce proteolytic cleavage. For expression of anti-PD-L1/TGFβ Trap, the DNA encoding the anti-PD-L1 light chain (SEQ ID NO:4) and the DNA encoding the anti-PD-L1/TGFβ Receptor II (SEQ ID NO:5) in either the same expression vector or separate expression vectors were used to transfect mammalian cells using standard protocols for transient or stable transfection. Conditioned culture media were harvested and the anti-PD-L1/TGFβ Trap fusion protein was purified by standard Protein A Sepharose chromatography. The purified protein comprising one anti-PD-L1 antibody and two soluble TGFβ Receptor II molecules (FIG. 1A) has an estimated molecular weight (MW) of about 190 kilodaltons on size exclusion chromatography and SDS-polyacrylamide electrophoresis under non-reducing conditions. Under reducing conditions, the light and heavy chains have apparent MW of 28 and 75 kilodaltons, respectively (FIG. 1B).

[0099] The anti-PD-L1(mut)/TGFβ Trap fusion protein, which contains an analogous heavy chain fusion polypeptide (SEQ ID NO:7) and a light chain with the mutations A31G, D52E, R99Y in the variable region that abrogate the binding to PD-L1 (SEQ ID NO:6), was similarly prepared. It was used in subsequent experiments as a TGFβ Trap control.

EXAMPLE 2 - Production of anti-PD-L1/TGFβ Trap as a biotherapeutic

[0100] The anti-PD-L1/TGFβ Trap produced by transient transfection of human embryonic kidney 293 (HEK) cells was found to contain varying degrees of a clipped species, which appeared as a faint band with an apparent MW of about 60 kD on SDS-PAGE under reducing conditions (FIG. 1B). This band was confirmed to be the heavy chain of the anti-PD-L1/TGFβ Trap cleaved at a site in the N-terminal portion of TGFβRII close to the fusion junction.

[0101] Stable clones expressing anti-PD-L1/TGFβ Trap were generated in the CHO-S host cell line, which was pre-adapted for growth in serum-free media in suspension culture. Cells were transfected with an expression vector containing a gene encoding the anti-PD-L1-TGFβRII protein and a glutamine synthetase selection marker. Subsequent selection of stable integrants was made with L-methionine sulfoximine (MSX). Anti-PD-L1/TGFβ Trap expressing cell lines were generated using a minipool approach, followed by the deposition of single cells into 384-well plates, using a Beckton-Dickinson fluorescence activated cell sorter (FACS Aria II). Growth, productivity, and protein quality were evaluated in a generic platform fed-batch assay. Based on these analyses, 14 clones were selected as lead candidates for further studies. A stability study with the clones was carried out to ~90 PDL (population doubling level) from research cell banks established during scale up of clones. At the conclusion of mini-pool development it was discovered that the heavy chain-linker-TGFβRII subunit underwent clipping, as was seen in transient expression. All clones in the stability study produced the clipped species, although it was shown in the protein A-purified material that the percent clipped species relative to the intact subunit varied with each clone. In addition, an improved purification process consisting a protein A chromatography followed by strong cation exchange was developed to reduce co-purification of the clipped species. Even with the improved

process, purified material with the required final levels of clipped species of <5% could only be achieved using clones producing low levels of clipping. Based on these combined analyses, clone 02B15 was selected as the final candidate clone. Analysis of anti-PD-L1/TGFβ Trap expressed by this clone at zero PDL, thirty PDL, sixty PDL and ninety PDL shows that the percentage of clipping did not increase with population doubling levels (FIG. 2).

EXAMPLE 3 - Fluorescence-activated cell sorting (FACS) analysis of binding of anti-PD-L1/TGFβ Trap and controls to human PD-L1 on cells

[0102] The binding of anti-PD-L1 antibody and fusion proteins on HEK cells stably transfected to express human PD-L1 was studied using the following procedure.

[0103] The following exemplary procedure was used determine PD-L1 binding by FACS:

1. a. 50 µl serial dilutions of test samples were set up in FACS buffer.
2. b. 50 µl of HEK cells stably transfected to express human PD-L1 at 5 $\times 10^6$ cells/ml were dispensed to the wells with test samples and mixed.
3. c. Plate(s) were incubated in the dark on ice for 1 hour.
4. d. Cells were pelleted at 300x g for 5 minutes.
5. e. Supernatant was decanted and cells were resuspended in 300 µl FACS buffer and re-pelleted at 300x g for 5.
6. f. Sample rinse was repeated.
7. g. Cells were resuspended in 100 µl FACS buffer containing DyLight 488 conjugated whole IgG Goat Anti-Human IgG, Fcy (1:300 diluted).
8. h. Plate(s) was incubated in the dark on ice for 45 minutes.
9. i. Cells were pelleted at 300x g for 5.
10. j. Supernatant was decanted and cells were resuspended in 300 µl FACS buffer and re-pelleted at 300x g for 5 minutes
11. k. Sample rinse was repeated and cells were finally resuspended in 200 µl FACS buffer.
12. l. Data was acquired on FACS Caliber and was analyzed using Microsoft Excel. EC50 was calculated using non-linear regression (Sigmoidal dose-response) with Graphpad PrismS.

[0104] As shown in Fig. 3, FACS analysis showed that the anti-PD-L1/TGFβ Trap fusion protein retains similar binding affinity as the positive control anti-PD-L1 antibody on HEK cells stably transfected to express human PD-L1 (HEK/PD-L1 cells). The EC50's for anti-PD-L1/TGFβ Trap and anti-PD-L1 are 0.116 µg/ml (0.64 nM) and 0.061 µg/ml (0.41 nM), respectively. The observed MFI (mean fluorescent intensity) was specific to binding to human PD-L1 since no MFI was observed on the parental HEK cells that were not transfected. The anti-PD-L1(mut)/TGFβ Trap negative control did not show any binding to the HEK cells stably transfected to express human PD-L1.

EXAMPLE 4 - Determination of ability of anti-PD-L1/TGFβ Trap to inhibit TGFβ induced phosphorylation of SMAD3

[0105] The ability of anti-PD-L1/TGFβ Trap to neutralize TGFβ was determined using 4T1 cells carrying a SMAD3-luciferase reporter. In the assay detailed below, inhibition of TGFβ-induced phosphorylation of SMAD3 was measured using a luciferase reporter under the control of the SMAD3 promoter.

[0106] An exemplary assay to evaluate potency to inhibit TGFβ-induced reporter activity was performed as follows.

1. 1. One day prior to the study, 4T1 cells carrying SMAD3-luciferase reporter were fed.
2. 2. On day 0, cells were plated in a Biocoat 96-well plate at a concentration of 5×10^4 cells/well in 100 µl of fresh media and incubated overnight at 37°C and 5% CO_2.
3. 3. On day 1:
    1. i. 50 µl of fresh complete media containing indicated concentration of anti-PD-L1/TGFβ trap samples to be tested or its controls was added to the wells and incubated for one hour. All samples were tested in triplicates.
    2. ii. 50 µl of fresh complete media containing 20 ng/ml human TGFβ was added to each well and samples were incubated overnight (final concentration in the well is 5 ng/ml).
4. 4. On day 2:
    1. i. 100 µl culture supernatant was removed and 100 µl fresh complete media, containing 150 µg/ml D-Luciferin was added, and samples were incubated for at least five minutes.
    2. ii. Luminescence was measured using Envision 2104 plate reader by recording CPM.
5. 5. Data was analyzed using MS Excel or Graphpad prism 5. Luciferase activity was recorded as CPM. Inhibitory Activity of (%) was calculated using the following equation:
6. 6. Nonlinear regression fit was carried out using Sigmoidal dose-response (variable slope) of Graphpad prism 5. IC50 values were calculated.

[0107] FIG. 4 shows that anti-PD-L1/TGFβ Trap inhibits TGFβ-induced pSMAD3 reporter activity in a dose dependent manner. The fact that the anti-PD-L1(mut)/TGFβ Trap control had comparable potency and IC50 (concentration required to inhibit 50% of the maximal activity) plus the fact that the anti-PD-L1 antibody had no effect showed that this inhibition of signaling is independent of anti-PD-L1 activity. Surprisingly, anti-PD-L1/TGFβ Trap was several-fold more potent than TGFβRII-Fc (R&D Systems), which places the TGFβRII at the N-terminus instead of the C-terminus of the fusion protein. It is also noteworthy that anti-PD-L1/TGFβ Trap is significantly more potent than 1D11 (GC1008), the anti-TGFβ antibody that was tested in

patients with advanced malignant melanoma or renal cell carcinoma (Morris et al., J Clin Oncol 2008; 26:9028 (Meeting abstract)). In this assay, 1D11 and TGFβRII-Fc showed similar activity.

EXAMPLE 5 - Pharmacokinetic (PK) analysis in mice

[0108] Eighteen male C57BL/6 mice, 5-6 weeks old, were randomly assigned to 3 groups (N=6/group), and each group received one of the three proteins (anti-PD-L1/TGFβ Trap, anti-PD-L1(mut)/TGFβ Trap, and anti-PD-L1). Mouse body weight was recorded before dosing. After a brief warm-up under a heating lamp, each mouse received 120 µg of protein in 200 µl intravenously (IV) via the tail vein regardless of its body weight. Each group dosed with the same protein was further divided into 2 subgroups (n=3). Blood samples were alternately taken from each of two subgroups, *i.e.* one subgroup was withdrawn for blood samples at 1h, 24h, 72h, and 168h, whereas another subgroup was for blood samples at 7h, 48h, 120h, and 240h. At each time point, approximate 50 µl of blood samples were collected from each mouse via tail vein using a heparinized micro glass capillary (100 µl in capacity). The blood sample was then transferred to a tube pre-coated with Li-Heparin and kept at 4 °C. Within 10 min of collection, the blood samples were spun at 14,000 rpm for 10 min. At least 20 µl of plasma sample was transferred into a new set of pre-labeled tubes and stored at -20 °C until the day of analysis.

[0109] The ELISA to measure total human IgG used goat anti-Human IgG (H+L) (heavy and light chains) (Jackson ImmunoResearch Laboratories) coated wells for capture and peroxidase-AffiniPure mouse anti-Human IgG, F(ab')2 (Jackson ImmunoResearch Laboratories) for detection. The ELISA to measure fully functional anti-PD-L1 antibody and/or fusion protein used PD-L1-Fc (extracellular domain of human PD-L1 fused to Fc) coated wells (coated at 1.25 µg/ml) for capture and peroxidase-AffiniPure mouse anti-Human IgG, F(ab')2 for detection. The ELISA to measure fully functional anti-PD-L1 and intact TGFβRII used PD-L1-Fc coated wells for capture and biotinylated anti-human TGFβRII (R&D Systems) for detection.

[0110] FIG. 5A shows that the anti-PD-L1/TGFβ Trap fusion protein had a PK profile very similar to that of the anti-PD-L1 antibody. For example, as measured by the total human IgG ELISA, the serum concentrations at the 168 hr time point of anti-PD-L1/TGFβ Trap and anti-PD-L1 were 16.8 and 16.2 µg/ml, respectively, and the respective area under the curve (AUC) from 0 to 168 hr were 4102 and 3841 hr-µg/ml. Similarly, when the serum concentrations were measured by the total functional anti-PD-L1 ELISA, the serum concentrations at the 168 hr time point of anti-PD-L1/TGFβ Trap and anti-PD-L1 were 9.5 and 11.1 µg/ml, respectively, and the respective AUC from 0 to 168 hr were 3562 and 3086 hr-µg/ml. The serum concentration of intact anti-PD-L1/TGFβ Trap fusion protein was determined by the ELISA, which detects fully functional anti-PD-L1 and the fused TGFβRII. In this case, the serum concentration of anti-PD-L1/TGFβ Trap was 5.9 µg/ml at the 168 hr time point and the AUC (0 to 168 hr) was 2656 hr-µg/ml, which were somewhat lower than those from the fully functional anti-PD-L1 ELISA, presumably due to degradation of the TGFβRII moiety after receptor-mediated endocytosis.

Antibody binding to PD-L1 has been shown to result in PD-L1-mediated endocytosis, and an antibody-X fusion protein is known to undergo degradation of the X moiety after receptor-mediated endocytosis (Gillies et al., Clin Cancer Res. 2002; 8:210-6). This is supported by the finding in FIG. 5 that when the antibody moiety does not bind PD-L1, as in the anti-PD-L1(mut)/TGFβ Trap control, the exposure is about 3 times higher, with a serum concentration of 53 µg/ml at the 168 hr time point and AUC(0 to 168 hr) of 9585 hr-µg/ml, suggesting that at least part of the clearance is receptor-mediated.

[0111] In order to confirm the ~3-fold difference in exposure between anti-PD-L1/TGFβ Trap and anti-PD-L1(mut)/TGFβ Trap, the pharmacokinetics experiment was repeated and the concentrations of the intact fusion proteins in the serum samples were determined. Mice (B6.129S2 female mice, 8 wks old, Jackson Lab) were injected with anti-PD-L1/TGFβ Trap or anti-PD-L1(mut)/TGFβ Trap (164 µg/mouse). The serum concentrations of the two fusion proteins were measured by an ELISA using anti-human IgG Fab (Jackson Immunoresearch, West Grove, PA) for capture and biotinylated anti-human TGFβRII (R&D Systems, Minneapolis, MN) and peroxidase-conjugated streptavidin (Zymed/ThermoFisher Scientific, Grand Island, NY) to detect intact anti-PD-L1/TGFβ Trap proteins. The serum concentrations of the intact fusion proteins at various time points were shown in the Table below and plotted in FIG. 5B. The total area under the curve (AUC) up to 336 hr is 11781 hr-µg/ml for anti-PD-L1/TGFβ Trap and 35575 hr-µg/ml for anti-PD-L1(mut)/TGFβ Trap (Table 1), therefore confirming the three-fold higher exposure of the Trap control molecule.

Table 1. Exposures of anti-PD-L1/TGFβ Trap and the anti-PD-L1(mut)/TGFβ Trap control as determined by the area under the curve (AUC) in the pharmacokinetics graph in Fig. 5B.

	AUC (h*µg/ml)	
Time (h)	**Anti-PD-L1/TGFβ Trap**	**Anti-PD-L1(mut)/TGFβ Trap**
7	72	173
24	1161	2789
48	1306	3511
72	1113	2968
120	2327	5192
168	2014	5225
240	2159	7530
336	1629	8188
total	**11781**	**35575**

EXAMPLE 6 - PD-L1 target-mediated endocytosis of anti-PD-L1/ TGFβ Trap

[0112] Receptor-mediated endocytosis was studied using the Alexa Fluor 488 quenching techniques according to manufacturer's protocol (Life Technologies, Carlsbad, CA). Briefly,

HEK cells expressing PD-L1 (HEK/PD-L1 cells) were incubated with 10 µg/ml Alexa Fluor 488-conjugated anti-PD-L1/TGFβ Trap on ice for about 1 hr and washed 4 times with cold media. Washed cells were then pulsed at 37 °C for 0.25, 0.5, 0.75, 1, 1.5, 2, 3 and 4 hr to allow internalization. Cell samples at each time point were then divided into two portions. One portion was incubated on ice and total fluorescence from the Alexa Fluor 488-conjugated anti-PD-L1/TGFβ Trap bound on the cell surface and internalized was measured; the other portion was incubated with anti-Alexa Fluor 488 at 4 °C for about an hour and the non-quenchable fluorescence from the internalized Alexa Fluor 488-conjugated anti-PD-L1/TGFβ Trap was measured. A graph showing a time course of the non-quenchable and total mean fluorescence intensity (MFI) of anti-PD-L1/TGFβ Trap at 37 °C is shown in FIG. 6A. The receptor-mediated internalization kinetics is very similar to that of the anti-PD-L1 antibody, which is shown in FIG. 6B. The percentage of receptor-mediated internalization of anti-PD-L1/TGFβ Trap and anti-PD-L1 on HEK/PD-L1 cells at various time points at 37 °C is shown in FIG. 6C, using the following formula to account for the fact that the quenching by the anti-Alexa Fluor 488 is not 100%:

EXAMPLE 7 - Anti-PD-L1/TGFβ Trap demonstrated a superior anti-tumor effect that is synergistic of anti-PD-L1 and TGFβ Trap activities in the EMT-6 (breast carcinoma) subcutaneous model

[0113] 8-12 week old female Jh (Igh-J^{tm1Dhu}) Balb/C mice (Taconic Farms, Hudson, NY) were inoculated with 0.5 × 10^6 viable EMT6 cells in 0.1 ml PBS on the right flanks subcutaneously. About five days later, when tumors reached an average size of 20-30 mm^3, mice were sorted into groups (N=10) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated (Day 0). Group 1 received 400 µg of isotype antibody control three times weekly (or "eod" (every other day); Group 2 received 400 µg of anti-PD-L1 antibody three times weekly; Group 3 received 164 µg of anti-PD-L1(mut)/TGFβ Trap three times weekly; Group 4 received 492 µg of anti-PD-L1/TGFβ Trap three times weekly; Group 5 received 492 µg of anti-PD-L1/TGFβ Trap twice weekly (equimolar to 400 µg of anti-PD-L1 antibody); Group 6 received 164 µg of anti-PD-L1/TGFβ Trap three times weekly; and Group 7 received 55 µg of anti-PD-L1/TGFβ Trap three times weekly. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula: tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0114] All the treatments were well tolerated. The inhibition of tumor growth by the various treatments is shown in FIG. 7A, which showed the average tumor volumes of the surviving mice, and FIG. 7B, which showed the individual tumor volume of the surviving mice, noting that

mice with tumors over 2500 mm^3 had to be euthanized. Anti-PD-L1/TGFβ Trap demonstrated potent anti-tumor efficacy, achieving T/C ratios of 0.30, 0.40, and 0.44 for the high (492 µg, Group 4), medium (164 µg, Group 6), and low (55 µg, Group 7) dose groups, respectively on Day 28.). While the anti-PD-L1 antibody (Group 2, T/C =0.87, $p>0.05$, on Day 16, the last day for which the average tumor volume of all the mice were available, *i.e.,* before mice with tumors over 2500 mm^3 were euthanized) or the TGFβ Trap control (Group 2, T/C =0.97 on Day 16, $p>0.05$) alone had marginal efficacy in this model, combining the two agents in a single molecule resulted in profound synergistic anti-tumor effect. This is evident in the median survival times observed for the 492 µg dose (58 and greater than 80 days, respectively, for three times weekly dosing and twice weekly dosing) and 164 µg dose (35 days) of the fusion protein (log rank test: $p<0.0001$) (FIG. 7C). Importantly, anti-PD-L1/TGFβ Trap at the medium dose of 164 µg (Group 6), with a median survival of 35 days, was far more efficacious than the same dose of anti-PD-L1(mut)/TGFβ Trap (Group 3) or three times the equivalent dose of anti-PD-L1 (Group 2), both of which yielded a median survival of 22 days, respectively (log rank test: $p<0.0001$). This synergistic anti-tumor activity is especially striking because the exposure of the TGFβ Trap moiety of the 164 µg dose of PD-L1(mut)/TGFβ Trap should be about 3 times higher than that of the 164 µg dose of PD-L1/TGFβ Trap due to receptor-mediated clearance of the latter (see Examples 5 and 6). It is remarkable that tumors in mice which received the high dose of anti-PD-L1/TGFβ Trap continued to regress after dosing was stopped on Day 18 (3 of 10 from Group 4 and 6 of 10 from Group 5 with complete regressions at day 78), demonstrating the long-lasting immunologic anti-tumor effect of targeting the two immunosuppressive mechanisms simultaneously (FIG. 7C). It is also noteworthy that the efficacy for Group 4 is not any better than that of Group 5, suggesting that the dose of 492 µg administered twice weekly was near the saturating dose, or was a more optimal dosing regimen than the 492 µg administered three times weekly.

[0115] The protective effect of the anti-tumor immunity elicited by the anti-PD-L1/TGFβ Trap treatment was evident when the mice with tumors in complete regression were challenged with 25,000 viable EMT6 cells injected subcutaneously. While all ten naive mice in a control group developed tumors to an average tumor volume of 726 mm^3 by Day 18 post challenge, none of the eleven mice previously treated with PD-E1/TGFβ Trap (three from Group 4, six from Group 5, and one each from Groups 6 and 7) showed any sign of tumor growth.

EXAMPLE 8 - Anti-PD-L1/TGF-β Trap showed profound synergistic anti-tumor activity in the MC38 (colorectal carcinoma) subcutaneous tumor model.

[0116] 8-12 week old female B6.129S2-Ighm^{tm1Cgn}/J mice (Jackson Laboratory, Bar Harbor, ME) were injected with 0.5×10^6 viable MC38 tumor cells in 0.1 ml PBS subcutaneously into the right flank. About eight days later, when average tumor size reached about 80-100 mm^3, mice were sorted into groups (N=10) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated (Day 0). Group 1 received 400 µg of isotype

antibody control; Group 2 received 400 µg of anti-PD-L1 antibody; Group 3 received 133 µg of anti-PD-L1 antibody; Group 4 received 492 µg of anti-PD-L1(mut)/TGFβ Trap; Group 5 received 164 µg of anti-PD-L1(mut)/TGFβ Trap; Group 6 received 492 µg of anti-PD-L1/TGFβ Trap; and Group 7 received 164 µg of anti-PD-L1/TGFβ Trap. The treatment was administered three times weekly for two weeks. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula: tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0117] All the treatments were well tolerated. The inhibition of tumor growth by the various treatments is shown in FIG. 8. On day 19 of the study, anti-PD-L1/TGFβ Trap demonstrated potent dose-dependent anti-tumor efficacy, achieving T/C ratios of 0.18 ($p<0.001$) and 0.38 ($p<0.001$) for the high (492 µg, Group 6) and low (164 µg, Group 7) dose groups, respectively. On the other hand, neither anti-PD-L1 or anti-PD-L1(mut)/TGFβ Trap showed any anti-tumor activity at all. Therefore, a profound syngergistic anti-tumor activity was obtained when the anti-PD-L1 antibody and the TGFβ Trap moiety were combined into one molecule to target these two immunosuppressive mechanisms simultaneously.

EXAMPLE 9 - Anti-PDL1/TGFβ Trap was effective in the EMT-6 orthotopic model of metastatic breast cancer.

[0118] 8-12 week old female Jh (Igh-J^{tm1Dhu}) Balb/C mice (Taconic Farms, Hudson, NY) were inoculated with 0.25×10^6 viable EMT6 cells in 0.1 ml PBS into the right mammary pad. About a week later, when average tumor size reached about 50 mm^3, mice were sorted into groups (N=10) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated (Day 0). Group 1 received 133 µg of isotype antibody control; Group 2 received 133 µg of anti-PD-L1 antibody; Group 3 received 164 µg of anti-PD-L1(mut)/TGFβ Trap; Group 4 received 164 µg of anti-PD-L1/TGFβ Trap; and Group 5 received a combination of 133 µg of anti-PD-L1 and 164 µg of anti-PD-L1(mut)/TGFβ Trap. Treatment was repeated on Days 0, 2, 4, 7, 9, 11 (*i.e.* 3 times weekly for two weeks). Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula: tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0119] All treatments were well tolerated. The inhibition of tumor growth by the various treatments is shown in FIG. 9. Anti-PD-L1/TGFβ Trap demonstrated potent anti-tumor efficacy,

achieving T/C ratio of 0.03 ($p<0.001$) on Day 21. On the other hand, equimolar doses of anti-PD-L1 or anti-PD-L1(mut)/TGFβ Trap were less efficacious, giving T/C ratios of 0.31 ($p<0.001$ vs. Group 1; $p<0.001$ vs. Group 4) and 0.68 ($p<0.001$ vs. Group 1; $p<0.001$ vs. Group 4), respectively. The combination therapy of equimolar doses of anti-PD-L1 and anti-PD-L1(mut)/TGFβ Trap achieved almost identical anti-tumor efficacy as the fusion protein, although the exposure of the TGFβ Trap of the fusion protein (Group 4) was estimated to be about 3-fold lower than that of the anti-PD-L1(mut)/TGFβ Trap in the combination (Group 5) based on pharmacokinetics analysis (see Example 5). It is also remarkable that the tumors in Groups 4 and 5 continued to regress after the last day of dosing, *e.g.*, average tumor size decreased from 212 mm^3 on Day 11, the last day of dosing, to 26 mm^3 on Day 24 for anti-PD-L1/TGFβ Trap treatment, demonstrating the long-lasting immunologic anti-tumor effect of targeting the two immunosuppressive mechanisms simultaneously.

EXAMPLE 10 - Anti-PD-L1/TGFβ Trap has better anti-tumor efficacy than the combination of anti-PD-L1 and TGFβ Trap in an intramuscular MC38 colorectal carcinoma model.

[0120] 8-12 week old female B6.129S2-Ighm^{tm1Cgn}/J mice (Jackson Laboratory, Bar Harbor, ME) were injected with 0.5×10^6 viable MC38 tumor cells in 0.1 ml PBS intramuscularly in the right thigh. About a week later, when average tumor size reaches about 50 mm^3, mice were sorted into groups (N=8) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated (Day 0) and repeated again two days later (Day 2). Group 1 received 400 μg of isotype antibody control; Group 2 received 400 μg of anti-PD-L1 antibody; Group 3 received 133 μg of anti-PD-L1 antibody; Group 4 received 164 μg of anti-PD-L1(mut)/TGFβ Trap; Group 5 received 492 μg of anti-PD-L1/TGFβ Trap; Group 6 received 164 μg of anti-PD-L1/TGFβ Trap; and Group 7 received a combination of 133 μg of anti-PD-L1 and 164 μg of anti-PD-L1(mut)/TGFβ Trap. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula: tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0121] All the treatments were well tolerated. The inhibition of tumor growth by the various treatments is shown in FIG. 10. Anti-PD-L1/TGFβ Trap demonstrated very potent anti-tumor efficacy, achieving T/C ratios of 0.024 ($p<0.001$) and 0.052 ($p<0.001$) for the high (492 μg, Group 5) and low (164 μg, Group 6) dose groups, respectively, on Day 15. On the other hand, equimolar doses of anti-PD-L1 were less efficacious, giving T/C ratios of 0.59 ($p<0.001$) and 0.45 ($p<0.001$) for the high (400 μg, Group 2) and low (133 μg, Group 3) dose groups, respectively. Anti-PD-L1(mut)/TGFβ Trap at164 μg (Group 4) was completely ineffective, and it

should be pointed out that although this dose is equimolar with the low dose anti-PD-L1/TGFβ Trap group (Group 6), the exposure of the TGFβ Trap should be fairly similar to that of the high dose anti-PD-L1/TGFβ Trap group (Group 5) because of the differences in pharmacokinetics (see Example 5). Therefore, the data demonstrated that anti-PD-L1/TGFβ Trap had potent synergistic anti-tumor activity in this model. It is especially noteworthy that, anti-PD-L1/TGFβ Trap was more efficacious than the combination therapy of equimolar doses of anti-PD-L1 and anti-PD-L1(mut)/TGFβ Trap, which had a T/C ratio of 0.16 ($p<0.001$ vs. Group 1 and $p>0.05$ vs. Group 6) despite a higher TGFβ Trap exposure of about threefold (see Example 5). In addition, anti-PD-L1/TGFβ Trap treatment resulted in 4 out of 10 mice with complete tumor regression, while the combination of anti-PD-L1 and the Trap control induced complete regression in only 2 out of 10 mice (data not shown). It is also remarkable that the tumors in the mice treated with anti-PD-L1/TGFβ Trap continued to regress after the last day of dosing on day 2, and stayed completely regressed thereafter (until at least Day 102), demonstrating the profound and long-lasting immunologic anti-tumor effect of this fusion protein. Without being bound by theory, the data supports a mechanism in which the anti-PD-L1/TGFβ Trap fusion protein not only exploits the synergistic effect of blocking the two major immune escape pathways, but is superior to the combination therapy due to the targeting of the tumor microenvironment by a single molecular entity. Many immunosuppressive cytokines secreted by tumor cells or subverted immune cells (*e.g.* tumor associated macrophages, myeloid-derived suppressor cells) have autocrines or paracrine functions. Therefore, anti-PD-L1/TGFβ Trap has the capability to deliver the TGFβ Trap to the tumor microenvironment via binding to PD-L1+ tumor cells, where the Trap neutralizes the locally secreted TGFβ. In addition, instead of acting just like a sink for bound TGFβ that accumulates in circulation, anti-PD-L1/TGFβ Trap bound TGFβ could be effectively destroyed through the PD-L1 receptor-mediated endocytosis (Examples 5 and 6).

EXAMPLE 11 - Treatment with anti-PDL1/TGFβ Trap or the combination of anti-PD-L1 and TGFβ Trap control at equivalent exposure in the EMT-6 orthotopic model of metastatic breast cancer.

[0122] At equimolar doses, anti-PDL1/TGFβ Trap had similar efficacy as the combination of anti-PD-L1 and TGFβ Trap control in the orthotopic EMT-6 breast cancer model (Example 9). In the following study the efficacy of anti-PDL1/TGFβ Trap or the combination of anti-PD-L1 and TGFβ Trap control administered for equivalent exposure was tested.

[0123] 8-12 week old female Jh (Igh-J^{tm1Dhu}) Balb/C mice (Taconic Farms, Hudson, NY) were inoculated with 0.25×10^6 viable EMT6 cells in 0.1 ml PBS into the right mammary pad. About a week later, when average tumor size reached about 80 mm^3, mice were sorted into groups (N=12) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated on Day 0 and repeated 7 days later. Group 1 received 133 µg of isotype antibody control; Group 2 received 164 µg of anti-PD-L1/TGFβ Trap; Group 3 received 55 µg of anti-PD-L1/TGFβ Trap; Group 4 received a combination of 133 µg of anti-

PD-L1 and 55 µg of anti-PD-L1(mut)/TGFβ Trap; and Group 5 received a combination of 44.3 µg of anti-PD-L1 and 18.3 µg of anti-PD-L1(mut)/TGFβ Trap. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula: tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy is reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0124] All the treatments were well tolerated. Anti-PD-L1/TGFβ Trap and the combination therapy demonstrated potent anti-tumor efficacy at both dose levels tested.

EXAMPLE 12 - Anti-PD-L1/TGF-β Trap has better antitumor efficacy than the combination of anti-PD-L1 and TGFβ Trap administered to give equivalent exposure in an intramuscular MC38 colorectal carcinoma model.

[0125] The results in Example 10 suggested that at equimolar doses the anti-PD-L1/TGF-β Trap has better antitumor efficacy than the combination of anti-PD-L1 and TGFβ Trap control even though the *in vivo* exposure of anti-PD-L1(mut)/TGFβ Trap control is about 3 times that of anti-PD-L1/TGFβ Trap (Example 5). In a follow-up study the anti-tumor efficacy of anti-PD-L1/TGFβ Trap and the combination of anti-PD-L1 and anti-PD-L1(mut)/TGFβ Trap based on equal exposure was compared. Lower doses than in Example 10 were administered to avoid dosing near saturating levels.

[0126] 8-12 week old female B6.129S2-Ighm^{tm1Cgn}/J mice (Jackson Laboratory, Bar Harbor, ME) were injected with 0.5×10^6 viable MC38 tumor cells in 0.1 ml PBS intramuscularly in the right thigh. A week later, when average tumor size reached about 200 mm^3, mice were sorted into groups (N=12) so that the average tumor sizes of all groups were similar. Treatment by intravenous injections was initiated (Day 0) and repeated again on Day 4. Group 1 received 133 µg of isotype antibody control; Group 2 received 164 µg of anti-PD-L1/TGFβ Trap; Group 3 received 55 µg of anti-PD-L1/TGFβ Trap; Group 4 received a combination of 133 µg of anti-PD-L1 and 55 µg of anti-PD-L1(mut)/TGFβ Trap; and Group 5 received a combination of 44.3 µg of anti-PD-L1 and 18.3 µg of anti-PD-L1(mut)/TGFβ Trap. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula: tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy is reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0127] All the treatments were well tolerated. Anti-PD-L1/TGFβ Trap demonstrated very potent

anti-tumor efficacy, achieving T/C ratios of 0.13 ($p<0.001$) and 0.19 ($p<0.001$) for the intermediate (164 µg, Group 2, called intermediate dose relative to the high dose of 492 µg that seemed to be saturating in Example 10) and low (55 µg, Group 3) dose groups, respectively, on Day 9. On the other hand, the combination of anti-PD-L1 and anti-PD-L1(mut)/TGFβ Trap were less efficacious, giving T/C ratios of 0.34 ($p<0.001$) and 0.37 ($p<0.001$) for the intermediate (Group 4) and low (Group 5) dose groups, respectively (Fig. 12A or Table). It is especially noteworthy that when administered to give equivalent *in vivo* exposure of the anti-PD-L1 antibody and the TGFβ Trap component, anti-PD-L1/TGFβ Trap was significantly more efficacious than the combination therapy of anti-PD-L1 and anti-PD-L1(mut)/TGFβ Trap at both dose levels (at the intermediate dose, T/C of 0.13 for anti-PD-L1/TGFβ Trap vs. 0.34 for the combination $p<0.0001$ (Fig. 12B); at the low dose, T/C of 0.19 for anti-PD-L1/TGFβ Trap vs. 0.37 for the combination $p<0.0001$ (Fig. 12C)).

EXAMPLE 13 - Anti-PD-L1(YW)/TGFβ Trap has superior anti-tumor effect that is synergistic of anti-PD-L1 and TGFβ Trap activities in the EMT-6 (breast carcinoma) orthotopic model.

[0128] YW243.55S70 is a human antibody that recognizes both human and murine PD-L1 (US Patent Application Publication No. US2010/0203056 A1). Its variable region sequence of the heavy chain (VH) and variable region sequence of the light chain (VL) (provided as SEQ ID NO: 14 and SEQ ID NO: 13, respectively) were used to replace the corresponding variable region sequences of the anti-PD-L1/TGFβ Trap described in Example 1 to give anti-PD-L1(YW)/TGFβ Trap by standard molecular biology techniques. After construction of the DNA coding for anti-PD-L1(YW)/TGFβ Trap, the antibody fusion protein was expressed as described in Example 1. The anti-PD-L1 antibody YW243.55S70 is similarly expressed for comparison of efficacy in murine tumor models.

[0129] 8-12 week old female Jh (Igh-Jtm1Dhu) Balb/C mice (Taconic Farms, Hudson, NY) were inoculated with 0.25×10^6 viable EMT6 cells in 0.1 ml PBS into the right mammary pad. About a week later, when average tumor size reached about 50-100 mm^3, mice were sorted into groups (N=10) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated (Day 0). Group 1 received 133 µg of isotype antibody control; Group 2 received 133 µg of anti-PD-L1(YW) antibody; Group 3 received 164 µg of anti-PD-L1(mut)/TGFβ Trap; Group 4 received 164 µg of anti-PD-L1(YW)/TGFβ Trap; and Group 5 received a combination of 133 µg of anti-PD-L1(YW) and 164 µg of anti-PD-L1(mut)/TGFβ Trap. Treatment was repeated on Days 4 and 7. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy is reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0130] All the treatments were well tolerated. The inhibition of tumor growth by the various treatments is shown in FIG. 13A, which showed the average tumor volumes of the mice on Day 17, the last day for which the average tumor volume of all the mice were available, *i.e.,* before mice with tumors over 2500 mm^3 were euthanized. Anti-PD-L1(YW)/TGFβ Trap demonstrated potent anti-tumor efficacy, achieving a T/C ratio of 0.25 ($p<0.0001$) that is slightly better than that of the combination treatment in Group 5 (T/C=0.31, $p<0.0001$), but superior to that of the anti-PD-L1(YW) antibody in Group 2 (T/C=0.57, $p<0.0001$) and the TGFβ Trap control in Group 3 (T/C=0.66, $p<0.0001$). The synergistic anti-tumor effect of the antibody fusion protein also resulted in prolonged survival of the treated mice, as shown in FIG. 13B. The anti-PD-L1/TGFβ Trap treated group had a median survival time of 65 days, which was significantly better than that of the anti-PD-L1(YW) antibody treated group (24 days) or the TGFβ Trap control treated group (21 days). It also compares favorably with the median survival time of 53.5 days for the combination treatment group. Despite dosing stopped after day 7, the continual tumor growth inhibition and the prolonged survival of the anti-PD-L1(YW)/TGFβ Trap treated mice demonstrate the long-lasting immunologic anti-tumor effect resulting from dual blockade of the two major immunosuppressive pathways.

EXAMPLE 14 - Anti-PD-L1(YW)/TGF-β Trap has superior anti-tumor effect that is synergistic of anti-PD-L1 and TGFβ Trap activities in the MC38 (colorectal carcinoma) intramuscular tumor model

[0131] 8-12 week old female B6.129S2-Ighm^{tm1Cgn}/J mice (Jackson Laboratory, Bar Harbor, ME) were injected with 0.5×10^6 viable MC38 tumor cells in 0.1 ml PBS intramuscularly in the right thigh. About a week later, when average tumor size reaches about 150-200 mm^3, mice were sorted into groups (N=10) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated (Day 0) and repeated again four days later (Day 4). Group 1 received 133 μg of isotype antibody control; Group 2 received 133 μg of anti-PD-L1(YW) antibody; Group 3 received 164 μg of anti-PD-L1(mut)/TGFβ Trap; Group 4 received 164 μg of anti-PD-L1(YW)/TGFβ Trap; and Group 5 received a combination of 133 μg of anti-PD-L1(YW) and 164 μg of anti-PD-L1(mut)/TGFβ Trap. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0132] All the treatments were well tolerated. The inhibition of tumor growth by the various treatments is shown in FIG. 14A, which showed the average tumor volumes of the mice on Day 10, the last day for which the average tumor volume of all the mice were available. Anti-PD-L1(YW)/TGFβ Trap demonstrated very potent anti-tumor efficacy, achieving a T/C ratio of 0.14

(p<0.0001) that is slightly better than that of the combination treatment in Group 5 (T/C=0.19, p<0.0001), but superior to that of the anti-PD-L1(YW) antibody in Group 2 (T/C=0.34, p<0.0001) and the TGFβ Trap control in Group 3 (T/C=0.99, p<0.0001), which had no activity in this model. The anti-tumor efficacy of anti-PD-L1(YW)/TGFβ Trap was further confirmed by tumor weight measurements taken on Day 11. By this time, the isotype control group had to be euthanized because the tumors had grown beyond 2500 mm^3. Therefore, the experiment was terminated and all the groups were euthanized and the tumor weights determined. The individual tumor weights are shown in FIG. 14B. The analysis of tumor weights confirmed that anti-PD-L1(YW)/TGFβ Trap therapy significantly inhibited MC38 tumor growth (T/C=0.13; p<0.0001). The efficacy of anti-PD-L1(YW)/TGFβ Trap was significantly better than that observed with anti-PD-L1 (T/C=0.37; p=0.003) or the TGFβ Trap control (T/C=1.0, p<0.0001). The anti-tumor efficacy of anti-PD-L1(YW)/TGFβ Trap, based on the tumor weight analysis, was not statistically better than the mice treated with the combination of anti-PD-L1 and the TGFβ Trap control (T/C=0.17; p=0.96).

EXAMPLE 15 - Combination treatment of Anti-PD-1 and TGFβ Trap do not provide any additive anti-tumor effect in an EMT-6 (breast carcinoma) orthotopic model

[0133] In this study we tested if the combination treatment of anti-PD-1 and TGFβ Trap provides any additive anti-tumor effect in the EMT-6 orthotopic model. CT-011, also known as pidiluzumab, is a humanized anti-human PD1 antibody that was tested in the clinic for treatment of hematological malignancies (Berger et al, Clin Cancer Res. 2008; 14:3044-3051). It also recognizes murine PD-1 and has shown anti-tumor activity that synergizes with cyclophosphamide and vaccine treatment in syngeneic tumor models (Mkrtichyan et al., Eur J Immunol. 2011; 41:2977-86). The VH and VL sequences of CT-011 were used to produce a recombinant antibody with human IgG1/kappa constant regions by standard molecular biology techniques.

[0134] 8-12 week old female Jh (Igh-Jtm1Dhu) Balb/C mice (Taconic Farms, Hudson, NY) were inoculated with 0.25×10^6 viable EMT6 cells in 0.1 ml PBS into the right mammary pad. About a week later, when average tumor size reached about 100 mm^3, mice were sorted into groups (N=10) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated (Day 0). Group 1 received 364 µg of isotype antibody control; Group 2 received 164 µg of anti-PD-L1(mut)/TGFβ Trap, which served as the TGFβ Trap control; Group 3 received 200 µg of anti-PD-1(CT-011); and Group 4 received a combination of 200 µg of anti-PD-1(CT-011) and 164 µg of anti-PD-L1(mut)/TGFβ Trap control. Treatment was repeated on Days 2, 4, 7, 9, and 11, *i.e.* 3 times weekly for two weeks. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with

the isotype control, respectively.

[0135] All the treatments were well tolerated. Anti-PD-1(CT-011) showed very modest antitumor efficacy (T/C = 0.87, p>0.05) in this model, while its combination with the TGFβ Trap control had the same efficacy as the TGFβ Trap control alone (FIG. 15).

EXAMPLE 16 - Combination treatment of Anti-PD-1 and TGF[3 Trap do not provide any additive anti-tumor effect in an MC38 (colorectal carcinoma) intramuscular tumor model.

[0136] In this study we tested if the combination treatment of anti-PD-1 and TGFβ Trap provides any additive anti-tumor effect in the intramuscular MC38 colorectal tumor model. 8-12 week old female B6.129S2-Ighm^{tm1Cgn}/J mice (Jackson Laboratory, Bar Harbor, ME) were injected with 0.5×10^6 viable MC38 tumor cells in 0.1 mL PBS intramuscularly in the right thigh. About a week later, when average tumor size reaches about 190 mm^3, mice are sorted into groups (N=10) so that the average tumor sizes of all groups are similar, and treatment by intravenous injections is initiated (Day 0). Group 1 received 364 µg of isotype antibody control on Days 0, 2, 4, and 7; Group 2 received 164 µg of the anti-PD-L1(mut)/TGFβ Trap control on Days 0 and 2; Group 3 received 200 µg of anti-PD-1(CT-011) on Days 0, 2, 4, and 7; and Group 4 received a combination of 200 µg of anti-PD-1(CT-011) on Days 0, 2, 4, and 7, and 164 µg of anti-PD-L1(mut)/TGFβ Trap control on Days 0 and 2. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0137] All the treatments were well tolerated. Anti-PD-1(CT-011) showed very modest antitumor efficacy (T/C = 0.87, p>0.05), while the anti-PD-L1(mut)/TGFβ Trap control had no efficacy in this model, as seen in previous examples. The combination of anti-PD-1(CT-011) with the TGFβ Trap control had no efficacy at all (FIG. 15).

EXAMPLE 17 - Combination treatment of TGF[3 Trap with either anti-LAG3 or anti-TIM-3 do not provide any additive anti-tumor effect in an EMT-6 (breast carcinoma) orthotopic model

[0138] In this study we tested if the combination treatment of TGFβ Trap with either anti-LAG3 or anti-TIM3 provides any additive anti-tumor effect in the orthotopic EMT-6 breast tumor model. The anti-LAG3 antibody used is a rat IgG1 monoclonal anti-murine LAG3 antibody

C9B7W (BioXcell, Beverly, MA), which was shown to synergize with anti-murine PD-1 treatment in syngeneic tumor models (Woo et al, Cancer Res, 2011; 72:917-27). The anti-TIM-3 antibody used is a rat IgG2a monoclonal anti-murine TIM3 antibody RMT3-23 (BioXcell, Beverly, MA), which also was shown to synergize with anti-murine PD-1 treatment in syngeneic tumor models, although its efficacy as a single agent was relatively modest (Ngiow et al, Cancer Res, 2011; 71:3540-51).

[0139] 8-12 week old female Jh (Igh-Jtm1Dhu) Balb/C mice (Taconic Farms, Hudson, NY) were inoculated with 0.25×10^6 viable EMT6 cells in 0.1 ml PBS into the right mammary pad. About a week later, when average tumor size reached about 110 mm^3, mice were sorted into groups (N=9) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections was initiated (Day 0). Group 1 received 133 µg of isotype antibody control; Group 2 received 164 µg of the anti-PD-L1(mut)/TGFβ Trap control; Group 3 received 200 µg of anti-LAG3; Group 4 received 250 µg of anti-TIM3; Group 5 received a combination of 200 µg of anti-LAG3 and 164 µg of anti-PD-L1(mut)/TGFβ Trap control; and Group 6 received a combination of 250 µg of anti-TIM3 and 164 µg of anti-PD-L1(mut)/TGFβ Trap control. Treatment was repeated on Days 2, 4, 7, 9, and 11, *i.e.* 3 times weekly for two weeks. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0140] As observed previously, the anti-PD-L1(mut)/TGFβ Trap control (Group 2) showed very modest efficacy in this EMT-6 model. Anti-TIM3 (Group 4) as a single agent showed a similarly modest efficacy as the Trap control, and in combination therapy with the Trap control (Group 6) showed no additive effect. Anti-LAG3 either as a single agent (Group 3) or in combination therapy with the Trap control (Group 5) did not show any efficacy.

EXAMPLE 18 - Combination treatment of TGFβ Trap with either anti-LAG3 or anti-TIM-3 do not provide any additive anti-tumor effect in an MC38 (colorectal carcinoma) intramuscular tumor model

[0141] In this study we tested if the combination treatment of TGFβ Trap with either anti-LAG3 (C9B7W) or anti-TIM3 (RMT3-23) provides any additive anti-tumor effect in the intramuscular MC38 colorectal tumor model.

[0142] 8-12 week old female B6.129S2-Ighm^{tm1Cgn}/J mice (Jackson Laboratory, Bar Harbor, ME) were injected with 0.5×10^6 viable MC38 tumor cells in 0.1 mL PBS intramuscularly in the right thigh. About a week later, when average tumor size reaches about 50 mm^3, mice were

sorted into groups (N=8) so that the average tumor sizes of all groups were similar, and treatment by intravenous injections is initiated (Day 0). Group 1 received 133 µg of isotype antibody control; Group 2 received 164 µg of the anti-PD-L1(mut)/TGFβ Trap control; Group 3 received 200 µg of anti-LAG3; Group 4 received 250 µg of anti-TIM3; Group 5 received a combination of 200 µg of anti-LAG3 and 164 µg of anti-PD-L1(mut)/TGFβ Trap control; and Group 6 received a combination of 250 µg of anti-TIM3 and 164 µg of anti-PD-L1(mut)/TGFβ Trap control. Treatment was repeated on Days 2, 4, 7, 9, 11, 15 and 18. Body weights were measured twice weekly to monitor toxicity. Tumor volumes were determined at different time points using the formula tumor volume (mm^3) = length × width × height × 0.5236. Any mice with tumors over 2500 mm^3 were sacrificed following the institute's animal health protocol. Anti-tumor efficacy was reported as a T/C ratio, where T and C are the average tumor volumes of the group treated with antibody or fusion protein, and the group treated with the isotype control, respectively.

[0143] As observed previously, the anti-PD-L1(mut)/TGFβ Trap control (Group 2) did not have any efficacy in this MC38 model. Anti-LAG3 as a single agent (Group 3) showed a moderate efficacy, achieving a T/C of 0.66 ($p<0.05$). However, combination with the Trap control (Group 5) did not improve its efficacy. Anti-TIM3 either as a single agent (Group 4) or in combination therapy with the Trap control (Group 6) did not show any efficacy.

SEQUENCES

[0144]

SEQ ID NO: 1

Peptide sequence of the secreted anti-PD-L1 lambda light chain

QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYDVSNRPSGVSNRF
SGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTRVFGTGTKVTVLGQPKANPTVTLFPPSSEE
LQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSH
RSYSCQVTHEGSTVEKTVAPTECS

SEQ ID NO: 2

Peptide sequence of the secreted H chain of anti-PDL1

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYIMMWVRQAPGKGLEWVSSIYPSGGITFYADTV
KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARIKLGTVTTVDYWGQGTLVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI
SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK

SEQ ID NO: 3

Peptide sequence of the secreted H chain of anti-PDL1/TGFβ Trap

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYIMMWVRQAPGKGLEWVSSIYPSGGITFYADTV
KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARIKLGTVTTVDYWGQGTLVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI

SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK

EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GAGGGGSGGGGSGGGGSGGGGSGIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQ
KSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKK
PGETFFMCSCSSDECNDNIIFSEEYNTSNPD

SEQ ID NO: 4

DNA sequence from the translation initiation codon to the translation stop codon of the anti-PD-L1 lambda light chain (the leader sequence preceding the VL is the signal peptide from urokinase plasminogen activator)

atgagggccctgctggctagactgctgctgtgcgtgctggtcgtgtccgacagcaagggcCAGT
CCGCCCTGACCCAGCCTGCCTCCGTGTCTGGCTCCCCTGGCCAGTCCATCACCATCAGCTGCAC
CGGCACCTCCAGCGACGTGGGCGGCTACAACTACGTGTCCTGGTATCAGCAGCACCCCGGCAAG
GCCCCCAAGCTGATGATCTACGACGTGTCCAACCGGCCCTCCGGCGTGTCCAACAGATTCTCCG
GCTCCAAGTCCGGCAACACCGCCTCCCTGACCATCAGCGGACTGCAGGCAGAGGACGAGGCCGA
CTACTACTGCTCCTCCTACACCTCCTCCAGCACCAGAGTGTTCGGCACCGGCACAAAAGTGACC
GTGCTGggccagcccaaggccaacccaaccgtgacactgttcccccccatcctccgaggaactgc
aggccaacaaggccaccctggtctgcctgatctcagatttctatccaggcgccgtgaccgtggc
ctggaaggctgatggctccccagtgaaggccggcgtggaaaccaccaagccctccaagcagtcc
aacaacaaatacgccgcctcctcctacctgtccctgacccccgagcagtggaagtcccaccggt
cctacagctgccaggtcacacacgagggctccaccgtggaaaagaccgtcgcccccaccgagtg
ctcaTGA

SEQ ID NO: 5

DNA sequence from the translation initiation codon to the translation stop codon (mVK SP leader: small underlined; VH: capitals; IgG1m3 with K to A mutation: small letters; (G4S)x4-G linker: bold capital letters; TGFβRII: bold underlined small letters; two stop codons: bold underlined capital letters)

atggaaacagacaccctgctgctgtgggtgctgctgctgtgggtgccccggctccacaggcGAGG
TGCAGCTGCTGGAATCCGGCGGAGGACTGGTGCAGCCTGGCGGCTCCCTGAGACTGTCTTGCGC
CGCCTCCGGCTTCACCTTCTCCAGCTACATCATGATGTGGGTGCGACAGGCCCCTGGCAAGGGC
CTGGAATGGGTGTCCTCCATCTACCCCTCCGGCGGCATCACCTTCTACGCCGACACCGTGAAGG
GCCGGTTCACCATCTCCCGGGACAACTCCAAGAACACCCTGTACCTGCAGATGAACTCCCTGCG
GGCCGAGGACACCGCCGTGTACTACTGCGCCCGGATCAAGCTGGGCACCGTGACCACCGTGGAC
TACTGGGGCCAGGGCACCCTGGTGACAGTGTCCTCCgctagcaccaagggcccatcggtcttcc
ccctggcaccctcctccaagagcacctctgggggcacagcggccctgggctgcctggtcaagga
ctacttccccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagcggcgtgcacacc
ttcccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgaccgtgccctcca
gcagcttgggcacccagacctacatctgcaacgtgaatcacaagcccagcaacaccaaggtgga
caagagagttgagcccaaatcttgtgacaaaactcacacatgcccaccgtgcccagcacctgaa
ctcctggggggaccgtcagtcttcctcttcccccaaaacccaaggacaccctcatgatctccc
ggaccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaa

ctggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaac
agcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagt
acaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagccaa
agggcagccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaac
caggtcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggaga
gcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgacggctcctt
cttcctctatagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgc
tccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtccccgggtg
ct**GGCGGCGGAGGAAGCGGAGGAGGTGGCAGCGGTGGCGGTGGCTCCGGCGGAGGTGGCTCCGG**
Aatccctccccacgtgcagaagtccgtgaacaacgacatgatcgtgaccgacaacaacggcgcc
gtgaagttccctcagctgtgcaagttctgcgacgtgaggttcagcacctgcgacaaccagaagt
cctgcatgagcaactgcagcatcacaagcatctgcgagaagccccaggaggtgtgtgtggccgt

gtggaggaagaacgacgaaaacatcaccctcgagaccgtgtgccatgaccccaagctgccctac
cacgacttcatcctggaagacgccgcctcccccaagtgcatcatgaaggagaagaagaagcccg
gcgagaccttcttcatgtgcagctgcagcagcgacgagtgcaatgacaacatcatctttagcga
ggagtacaacaccagcaaccccgacTGATAA

SEQ ID NO: 6

Polypeptide sequence of the secreted lambda light chain of anti-PD-L1(mut)/ TGFβ Trap, with mutations A31G,D52E,R99Y

QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYEVSNRPSGVSNRF
SGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTYVFGTGTKVTVLGQPKANPTVTLFPPSSEE
LQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSH
RSYSCQVTHEGSTVEKTVAPTECS

SEQ ID NO: 7

Polypeptide sequence of the secreted heavy chain of anti-PD-L1(mut)/ TGFβ Trap

EVQLLESGGGLVQPGGSLRLSCAASGFTFSMYMMMWVRQAPGKGLEWVSSIYPSGGITFYADSV
KGRFTISRDNSKNTLYLQMNSLRAEDTAIYYCARIKLGTVTTVDYWGQGTLVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI
SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GAGGGGSGGGGSGGGGSGGGGSGIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQ
KSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKK
PGETFFMCSCSSDECNDNIIFSEEYNTSNPD

SEQ ID NO: 8

Human TGFβRII Isoform A Precursor Polypeptide (NCBI RefSeq Accession No: NP_001020018)

MGRGLLRGLWPLHIVLWTRIASTIPPHVQKSDVEMEAQKDEIICPSCNRTAHPLRHINNDMIVT
DNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVCHD
PKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPDLLLVIFQV
TGISLLPPLGVAISVIIIFYCYRVNRQQKLSSTWETGKTRKLMEFSEHCAIILEDDRSDISSTC
ANNINHNTELLPIELDTLVGKGRFAEVYKAKLKQNTSEQFETVAVKIFPYEEYASWKTEKDIFS
DINLKHENILQFLTAEERKTELGKQYWLITAFHAKGNLQEYLTRHVISWEDLRKLGSSLARGIA
HLHSDHTPCGRPKMPIVHRDLKSSNILVKNDLTCCLCDFGLSLRLDPTLSVDDLANSGQVGTAR
YMAPEVLESRMNLENVESFKQTDVYSMALVLWEMTSRCNAVGEVKDYEPPFGSKVREHPCVESM
KDNVLRDRGRPEIPSFWLNHQGIQMVCETLTECWDHDPEARLTAQCVAERFSELEHLDRLSGRS
CSEEKIPEDGSLNTTK

SEQ ID NO: 9

Human TGFβRII Isoform B Precursor Polypeptide (NCBI RefSeq Accession No: NP_003233

MGRGLLRGLWPLHIVLWTRIASTIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQ
KSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKK
PGETFFMCSCSSDECNDNIIFSEEYNTSNPDLLLVIFQVTGISLLPPLGVAISVIIIFYCYRVN
RQQKLSSTWETGKTRKLMEFSEHCAIILEDDRSDISSTCANNINHNTELLPIELDTLVGKGRFA
EVYKAKLKQNTSEQFETVAVKIFPYEEYASWKTEKDIFSDINLKHENILQFLTAEERKTELGKQ
YWLITAFHAKGNLQEYLTRHVISWEDLRKLGSSLARGIAHLHSDHTPCGRPKMPIVHRDLKSSN
ILVKNDLTCCLCDFGLSLRLDPTLSVDDLANSGQVGTARYMAPEVLESRMNLENVESFKQTDVY
SMALVLWEMTSRCNAVGEVKDYEPPFGSKVREHPCVESMKDNVLRDRGRPEIPSFWLNHQGIQM
VCETLTECWDHDPEARLTAQCVAERFSELEHLDRLSGRSCSEEKIPEDGSLNTTK

SEQ ID NO: 10

A Human TGFβRII Isoform B Extracellular Domain Polypeptide

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAV
WRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSE
EYNTSNPD

SEQ ID NO: 11

$(Gly_4Ser)_4Gly$ linker

GGGGSGGGGSGGGGSGGGGSG

SEQ ID NO: 12

Polypeptide sequence of the secreted heavy chain variable region of anti-PD-L1 antibody MPDL3280A

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYY
ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSS

SEQ ID NO: 13

Polypeptide sequence of the secreted light chain variable region of anti-PD-L1 antibody MPDL3280A and the anti-PD-L1 antibody YW243.55S70

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSG
SGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGQGTKVEIKR

SEQ ID NO: 14

Polypeptide sequence of the secreted heavy chain variable region of anti-PD-L1 antibody YW243.55S70

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYYADSV
KGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSA

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Patentkrav

1. Protein, der omfatter:

a) human TGFβRII eller et fragment deraf, der er i stand til at binde TGFβ; og

b) et antistof eller antigenbindende fragment deraf, der binder humant protein Programmeret Dødsligand 1 (PD-L1) og omfatter:

(i) en variabel region med tung kæde, der omfatter en HVR-H1, HVR-H2 og HVR-H3, der har aminosyresekvenserne af henholdsvis SYIMM, SIYPSGGIT-FYADTVKG og IKLGTVTTVDY, og

(ii) en variabel region med let kæde, der omfatter en HVR-L1, HVR-L2 og HVR-L3 med aminosyresekvenserne af henholdsvis TGTSSDVGGYNYVS, DVSNRPS og SSYTSSSTRV.

2. Protein ifølge krav 1, yderligere omfattende en aminosyrelinker, der forbinder C-terminalen af antistoffets tunge kæde eller det antigenbindende fragment deraf, til N-terminalen af det humane TGFβRII eller fragment deraf.

3. Protein ifølge krav 1 eller krav 2, hvor den humane TGFβRII omfatter en aminosyresekvens med mindst 80% sekvensidentitet med SEQ ID NO: 8 eller SEQ ID NO: 9, som er mindst 20 aminosyrer lang.

4. Protein ifølge et hvilket som helst af kravene 1 til 3, hvor den humane TGFβRII omfatter en aminosyresekvens af SEQ ID NO: 10.

5. Protein ifølge et hvilket som helst af kravene 1 til 4, hvor

(a) antistoffets tunge kæde eller det antigenbindende fragment deraf omfatter en aminosyresekvens med mindst 85% sekvensidentitet med aminosyresekvensen:

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYIMMVWRQAPGKGLEWVSSIYPS
GGITFYADWKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARIKLGTVTTVD
YWGQGTLVTVSS.

og

(b) antistoffets lette kæde eller antigenbindende fragment deraf omfatter en aminosyresekvens med mindst 85% sekvensidentitet med aminosyresekvensen:

QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYDVS
NRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTRVFGTGTKVTV
L.

6. Protein ifølge et hvilket som helst af kravene 1 til 5, hvor

(a) antistoffets tunge kæde eller det antigenbindende fragment deraf omfatter aminosyresekvensen:

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYIMMVWRQAPGKGLEWVSSIYPS
GGITFYADWKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARIKLGTVTTVD
YWGQGTLVTVSS.

og

(b) antistoffets lette kæde eller det antigenbindende fragment deraf omfatter aminosyresekvensen:

QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYDVS
NRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTRVFGTGTKVTV
L.

7. Protein ifølge et hvilket som helst af kravene 1 til 6, hvor antistoffets tunge kæde omfatter en aminosyresekvens med mindst 85% sekvensidentitet med SEQ ID NO: 3.

8. Protein ifølge et hvilket som helst af kravene 1 til 7, hvor antistoffets lette kæde omfatter en aminosyresekvens med mindst 85% sekvensidentitet med SEQ ID NO: 1.

9. Nukleinsyre omfattende en første nukleotidsekvens, der koder for den humane TGFβRII, eller et fragment deraf og den variable region med tung kæde

ifølge et hvilket som helst af kravene 1-8 og en anden nukleotidsekvens, der koder for antistoffets variable region med let kæde ifølge et hvilket som helst af kravene 1- 8.

10. Celle, der på den samme eller på forskellige nukleinsyrer omfatter
en første nukleotidsekvens, der koder for et polypeptid, der omfatter (a) mindst et variabelt domæne af en tung kæde af et antistof, der binder humant PD-L1; og (b) human TGFβRII eller et opløseligt fragment deraf, der er i stand til at binde TGFβ,
hvor det variable domæne af den tunge kæde af antistoffet indbefatter en HVR-H1, HVR-H2 og HVR-H3 med aminosyresekvenserne af henholdsvis SYIMM, SIYPSGGITFYADTVKG og IKLGTVTTVDY; og
en anden nukleotidsekvens, der koder for mindst et variabelt domæne af en let kæde af et antistof, som, når det kombineres med polypeptidet, danner et antigenbindingssted, der binder PD-L1,
hvor det variable domæne af den lette kæde af antistoffet indbefatter en HVR-L1, HVR-L2 og HVR-L3 med aminosyresekvenserne af henholdsvis TGTSSDVGGYNYVS, DVSNRPS og SSYTSSSTRV.

11. Fremgangsmåde til fremstilling af et protein omfattende a) TGFβRII eller et fragment deraf, der er i stand til at binde TGFβ, og b) et antistof eller et antigenbindende fragment deraf, der binder humant protein Programmeret Dødsligand 1 (PD-L1), hvor fremgangsmåden omfatter opretholdelse af en celle ifølge krav 10 under betingelser, der tillader ekspression af proteinet, og eventuelt yderligere omfattende høst af proteinet.

12. Protein ifølge krav 1 til anvendelse i terapi.

13. Farmaceutisk sammensætning omfattende en terapeutisk effektiv mængde af et protein ifølge krav 1.

14. Protein ifølge krav 1 til anvendelse i;
(i) en fremgangsmåde til inhibering af tumorvækst hos en patient, hvilken fremgangsmåde omfatter at udsætte tumoren for proteinet; eller.
(ii) en fremgangsmåde til behandling af cancer hos en patient, hvilken fremgangsmåde omfatter indgivelse af proteinet til cancerpatienten.

15. Protein til anvendelse ifølge krav 14, hvor fremgangsmåden yderligere omfatter at udsætte cancerpatienten for stråling og/eller indgive et kemoterapeutikum.

16. Protein til anvendelse ifølge krav 14 eller krav 15, hvor tumoren eller kræften er valgt fra gruppen, der består af kolorektal-, bryst-, ovarie-, pancreas-, mave-, prostata-, nyre-, livmoderhals-, myelom-, lymfom-, leukæmi-, skjoldbruskkirtel-, endometrie-, livmoder-, blære-, neuroendokrin-, hoved- og hals-, lever-, nasopharyngeal-, testikel-, småcellet lungekræft, ikke-småcellet lungekræft, melanom, basalcellet hudkræft, pladecellekræft, dermatofibrosarcoma protuberans, Merkelcellekarcinom, glioblastom, gliom, sarkom, mesotheliom og myelodysplastiske syndromer.

17. Protein ifølge krav 1, hvor proteinet blokerer interaktionen mellem human PD-L1 og den humane PD-1 receptor.

DRAWINGS

Drawing

FIG. 1A

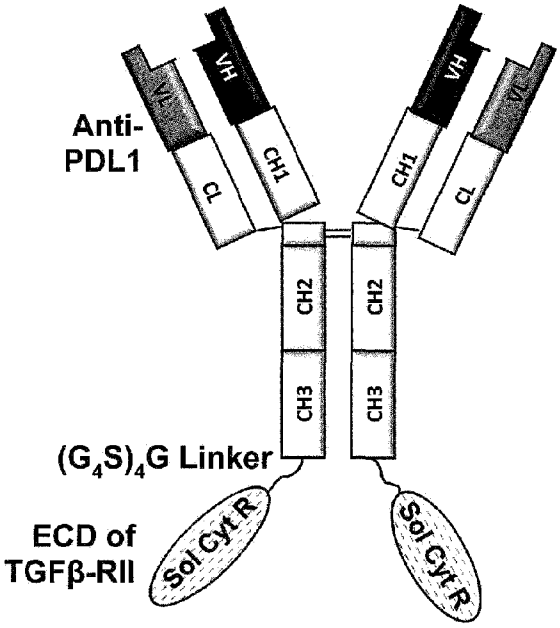


FIG. 1B

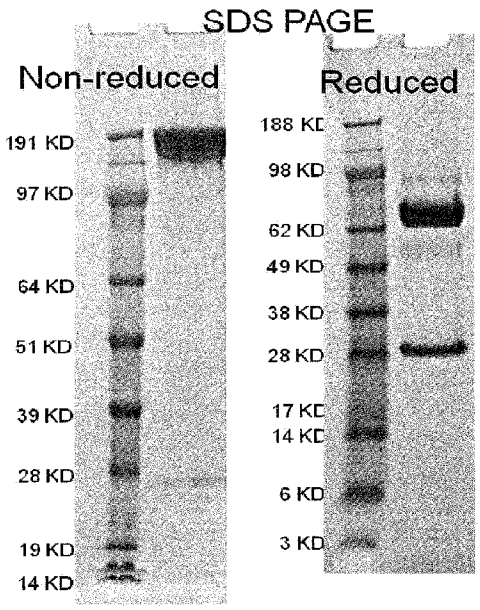
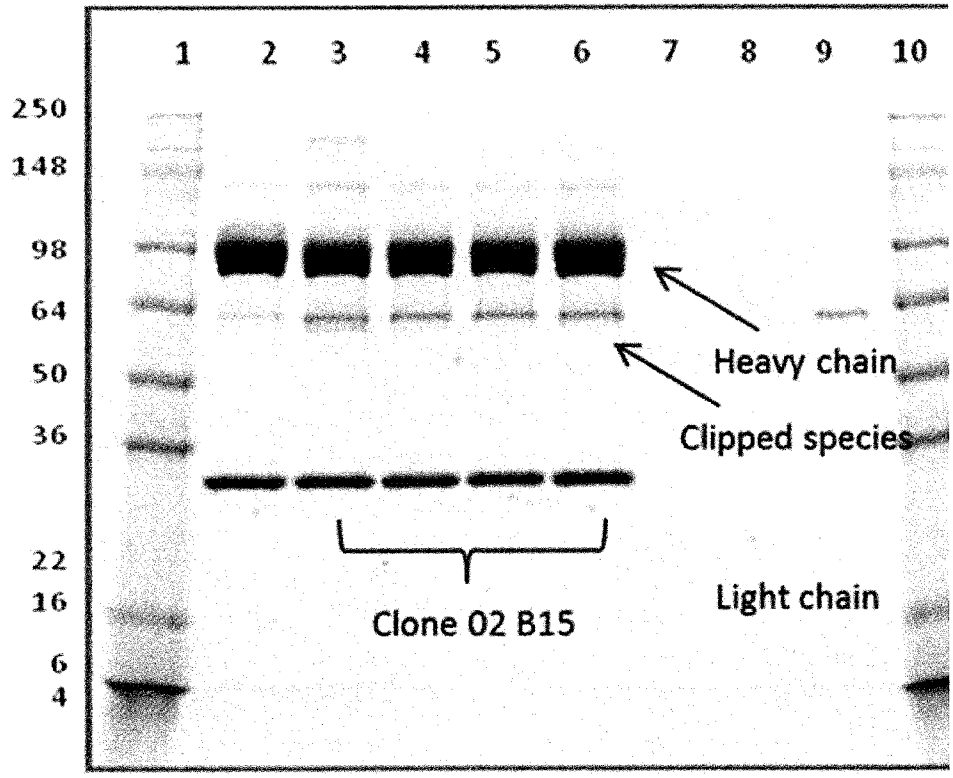

SDS PAGE
Non-reduced
Reduced
191 KD
97 KD
64 KD
51 KD
39 KD
28 KD
19 KD
14 KD
188 KD
98 KD
62 KD
49 KD
38 KD
28 KD
17 KD
14 KD
6 KD
3 KD

FIG. 2


1
2
3
4
5
6
7
8
9
10
250
148
98
64
50
36
22
16
6
4
Heavy chain
Clipped species
Light chain
Clone 02 B15

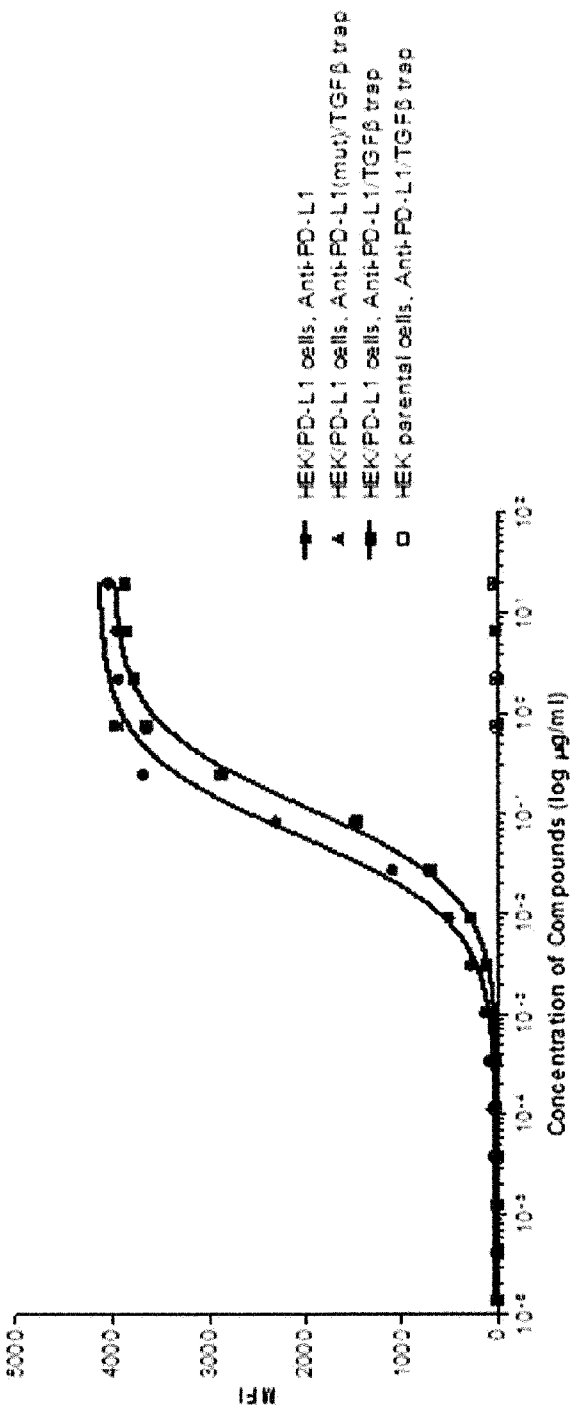

HEK/PD-L1 cells, Anti-PD-L1
HEK/PD-L1 cells, Anti-PD-L1(mut)/TGFß trap
HEK/PD-L1 cells, Anti-PD-L1/TGFß trap
HEK parental cells, Anti-PD-L1/TGFß trap
MFI
5000
4000
3000
2000
1000
0
Concentration of Compounds (log µg/ml)

FIG. 3

FIG. 4

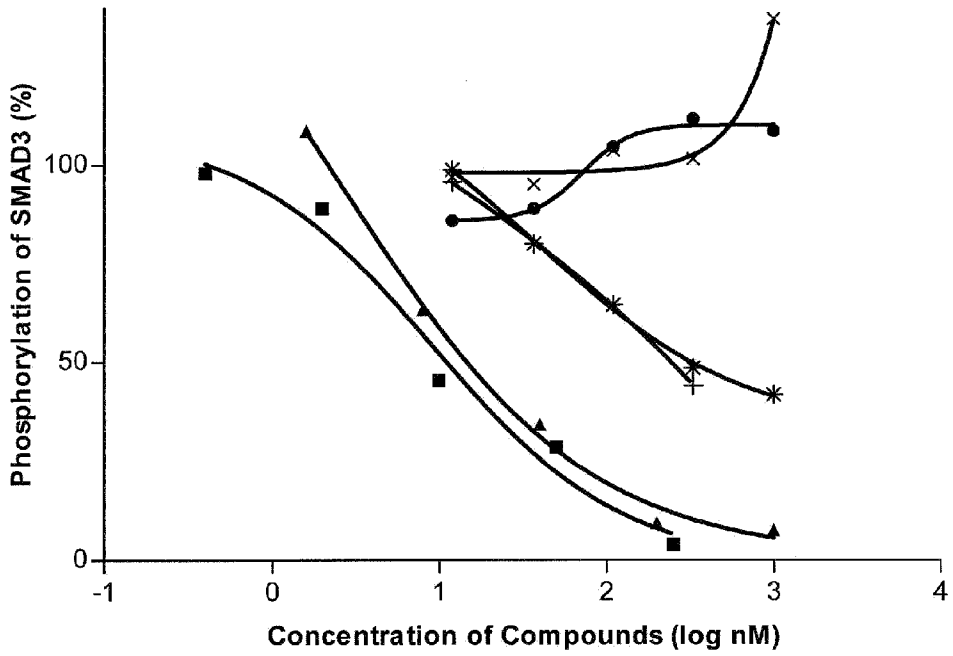

Phosphorylation of SMAD3 (%)
100
50
0
-1
0
1
2
3
4
Concentration of Compounds (log nM)

FIG. 5A

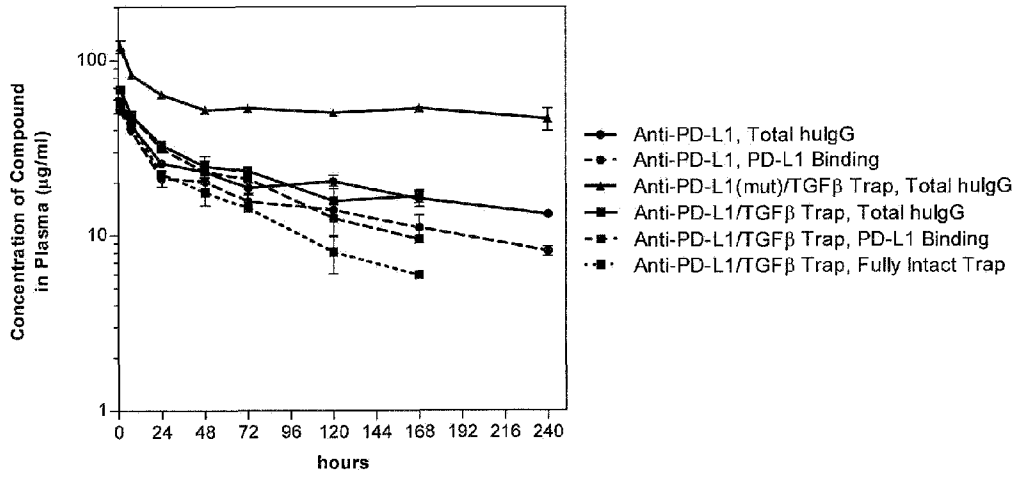

Concentration of Compound in Plasma (µg/ml)
100
10
1
0
24
48
72
96
120
144
168
192
216
240
hours
Anti-PD-L1, Total hulgG
Anti-PD-L1, PD-L1 Binding
Anti-PD-L1(mut)/TGFβ Trap, Total hulgG
Anti-PD-L1/TGFβ Trap, Total hulgG
Anti-PD-L1/TGFβ Trap, PD-L1 Binding
Anti-PD-L1/TGFβ Trap, Fully Intact Trap

FIG. 5B

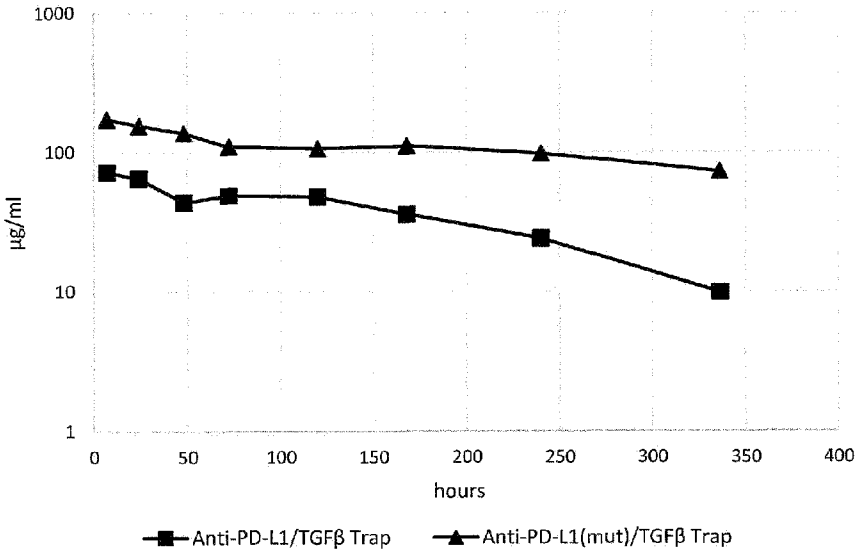

1000
100
10
1
µg/ml
0
50
100
150
200
250
300
350
400
hours
Anti-PD-L1/TGFβ Trap
Anti-PD-L1(mut)/TGFβ Trap

FIG. 6A

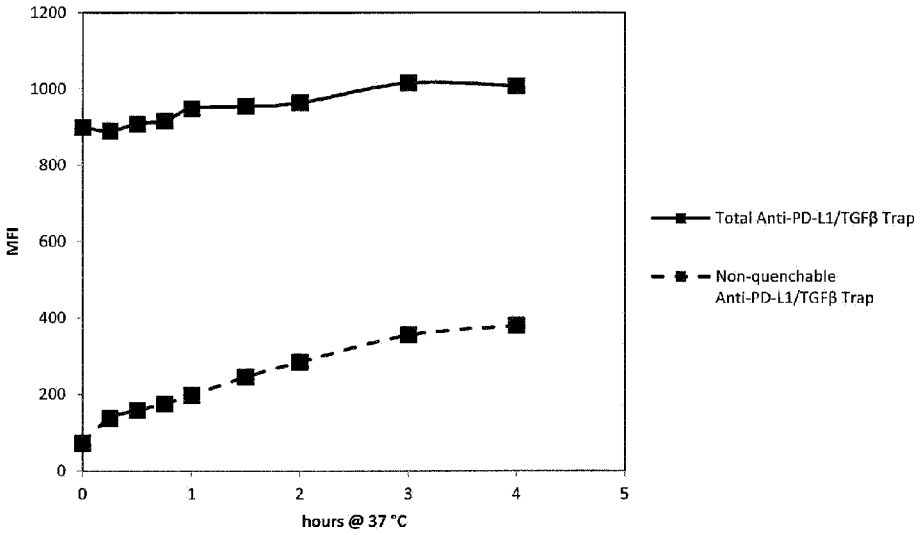

1200
1000
800
600
400
200
0
MFI
0
1
2
3
4
5
hours @ 37 °C
Total Anti-PD-L1/TGFβ Trap
Non-quenchable Anti-PD-L1/TGFβ Trap

FIG. 6B

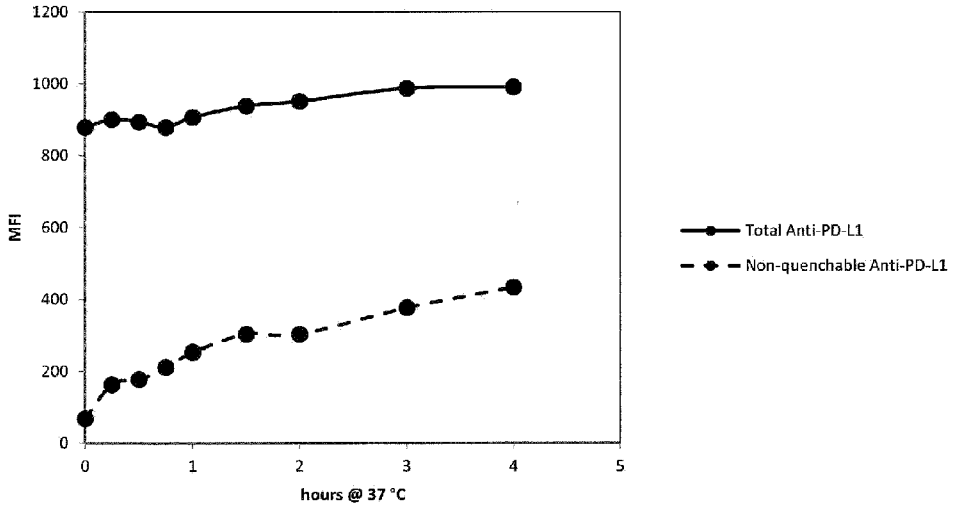

1200
1000
800
600
400
200
0
MFI
0
1
2
3
4
5
hours @ 37 °C
Total Anti-PD-L1
Non-quenchable Anti-PD-L1

FIG. 6C

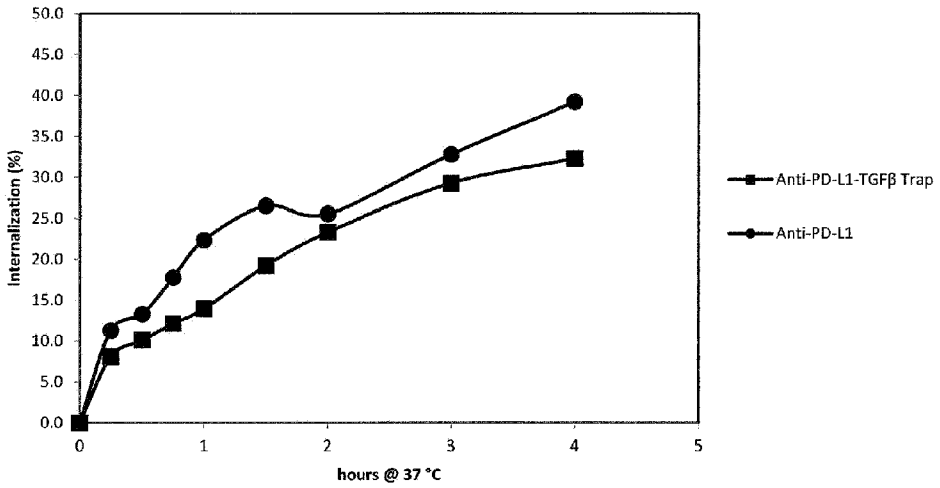
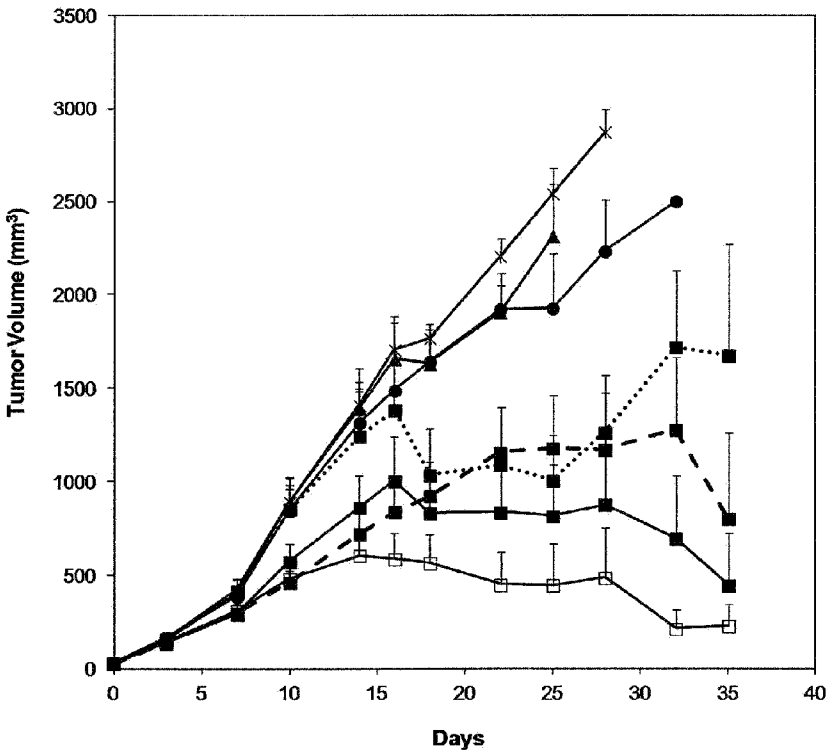

50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Internalization (%)
0
1
2
3
4
5
hours @ 37 °C
Anti-PD-L1-TGFβ Trap
Anti-PD-L1

FIG. 7A


3500
3000
2500
2000
1500
1000
500
0
Tumor Volume (mm³)
0
5
10
15
20
25
30
35
40
Days

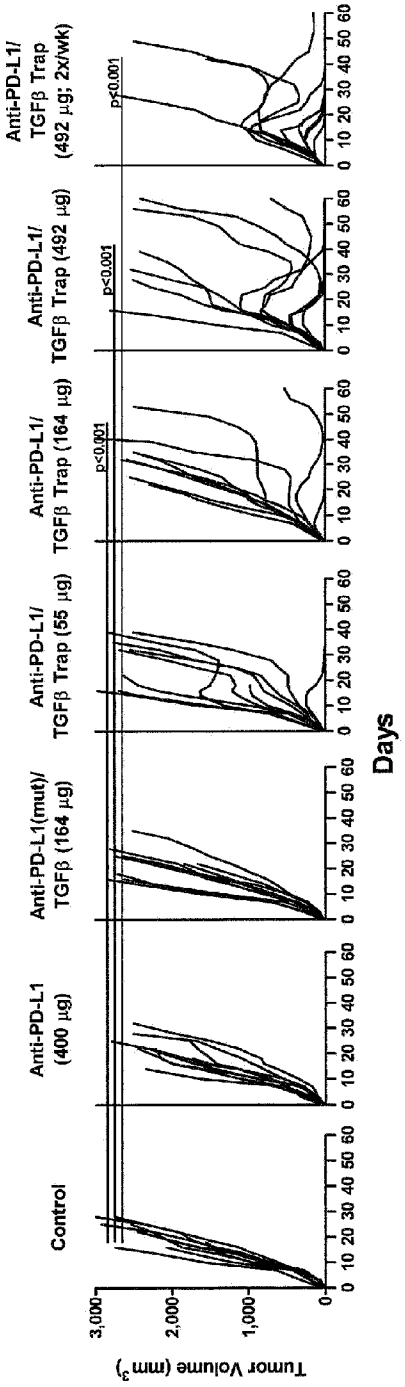

Control
Anti-PD-L1 (400 µg)
Anti-PD-L1(mut)/ TGFβ (164 µg)
Anti-PD-L1/ TGFβ Trap (55 µg)
Anti-PD-L1/ TGFβ Trap (164 µg)
Anti-PD-L1/ TGFβ Trap (492 µg)
Anti-PD-L1/ TGFβ Trap (492 µg; 2x/wk)
p<0.001
p<0.001
p<0.001
Tumor Volume (mm³)
3,000
2,000
1,000
0
0 10 20 30 40 50 60
Days

FIG. 7B

FIG. 7C

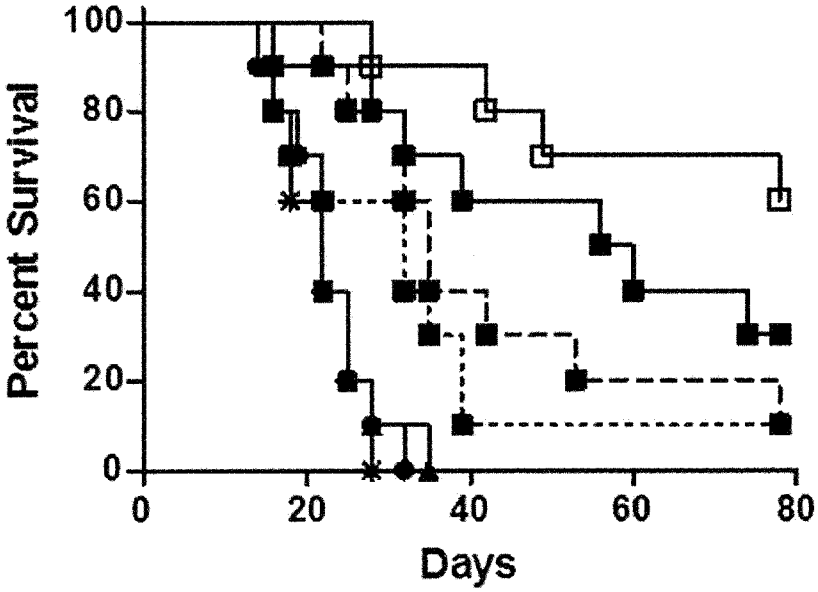


FIG. 8

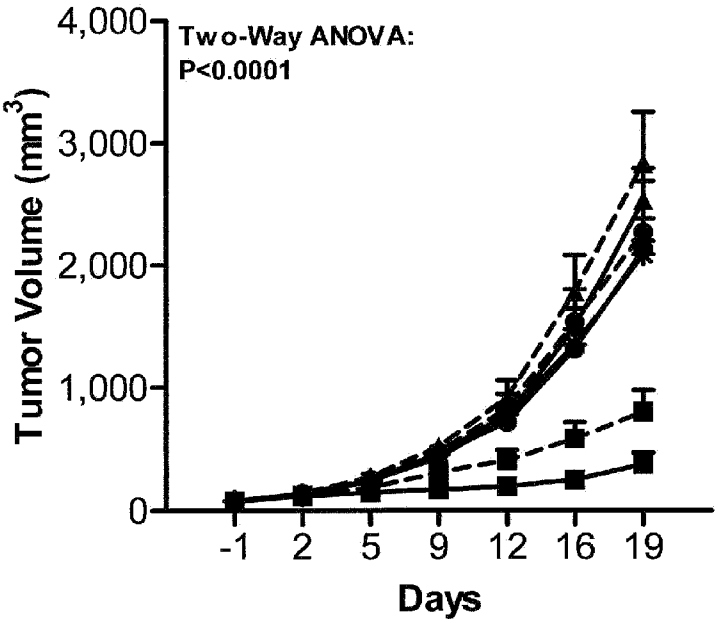

Two-Way ANOVA:
P<0.0001
Tumor Volume (mm3)
4,000
3,000
2,000
1,000
0
-1
2
5
9
12
16
19
Days

FIG. 9

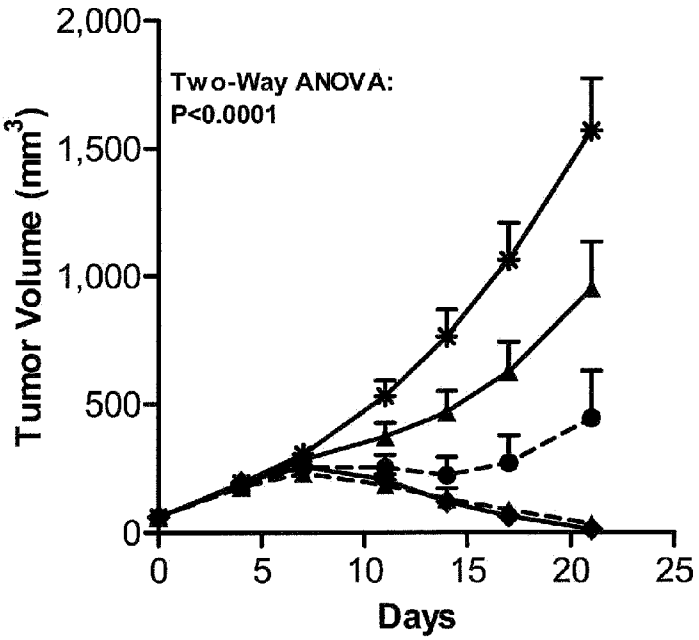

2,000
1,500
1,000
500
0
Tumor Volume (mm3)
Two-Way ANOVA:
P<0.0001
0
5
10
15
20
25
Days

FIG. 10

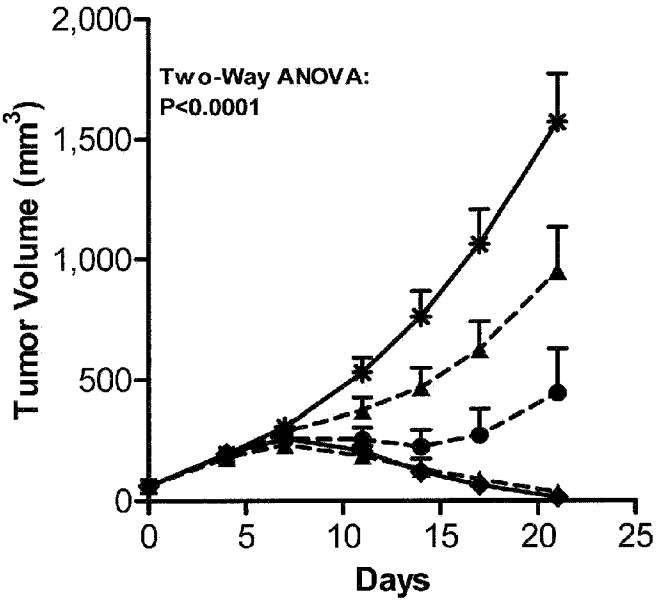

Two-Way ANOVA:
P<0.0001
Tumor Volume (mm3)
2,000
1,500
1,000
500
0
0
5
10
15
20
25
Days

FIG. 11

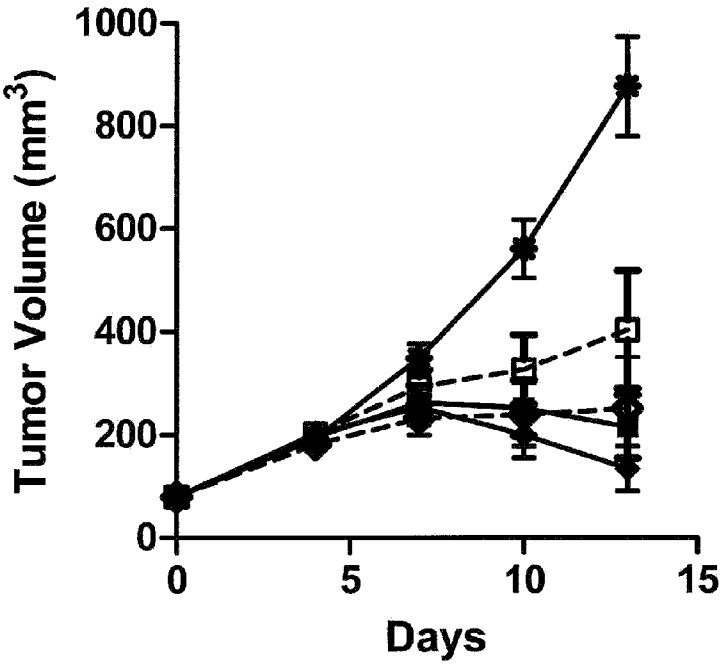


FIG. 12A

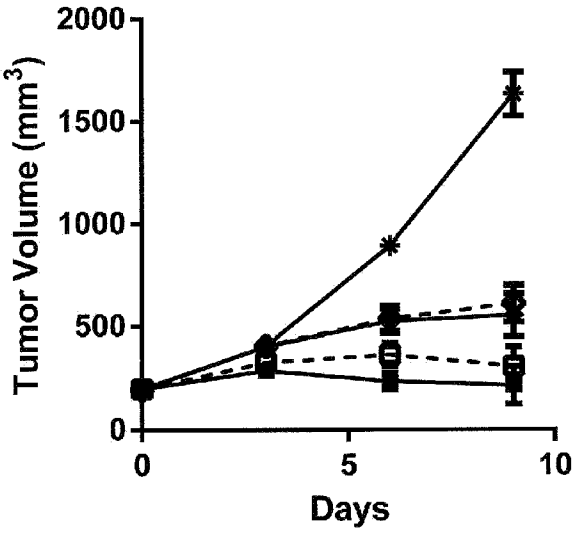


FIG. 12B

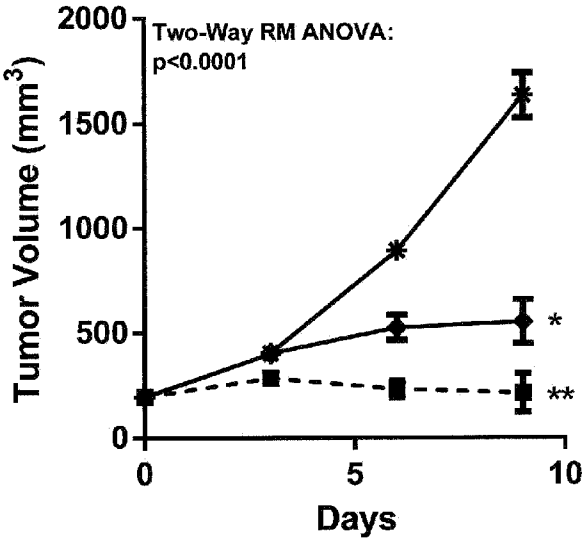


FIG. 12C

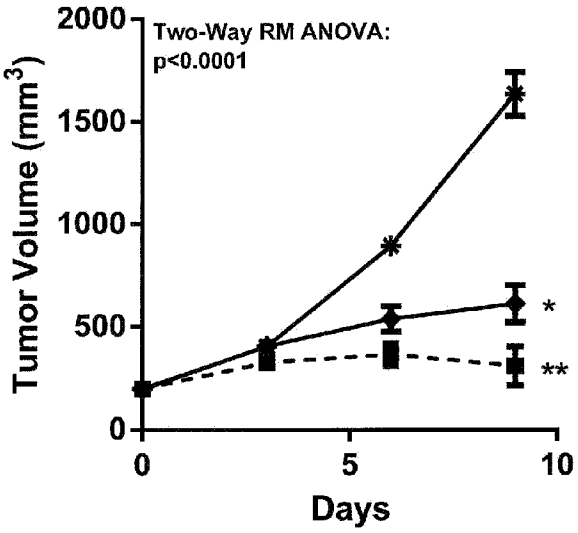

Two-Way RM ANOVA:
p<0.0001
Tumor Volume (mm3)
2000
1500
1000
500
0
0
5
10
Days
*
**

FIG. 13A

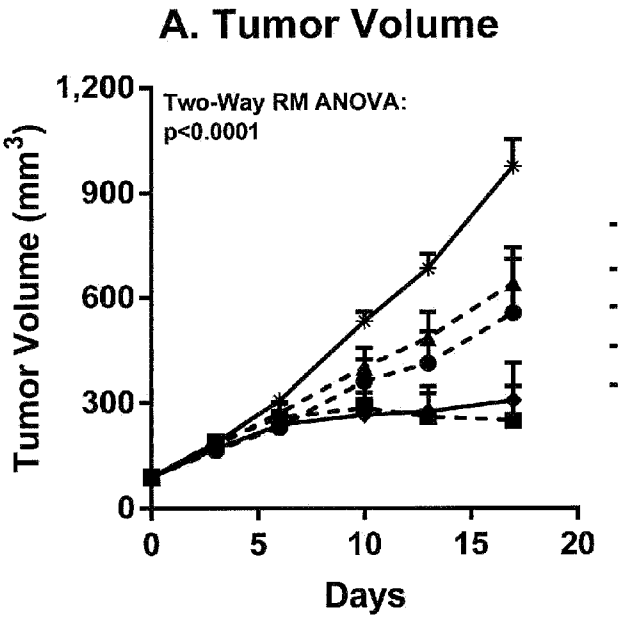

A. Tumor Volume
Two-Way RM ANOVA:
p<0.0001
Tumor Volume (mm3)
1,200
900
600
300
0
0
5
10
15
20
Days

FIG. 13B

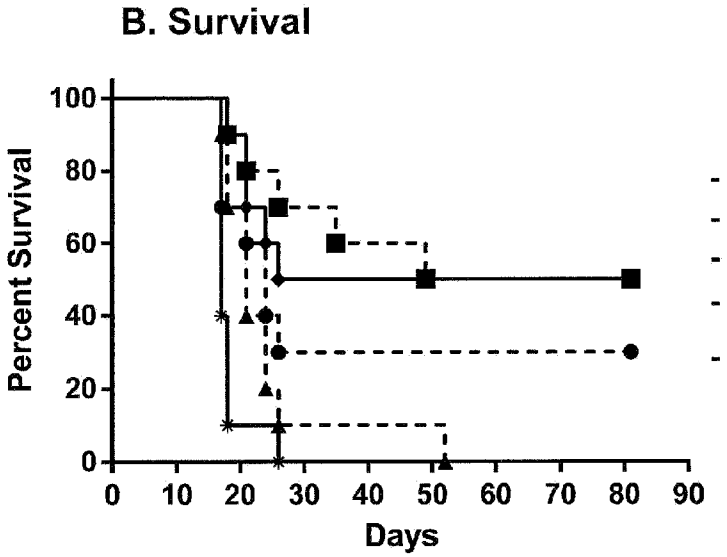

B. Survival
Percent Survival
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Days

FIG. 14A

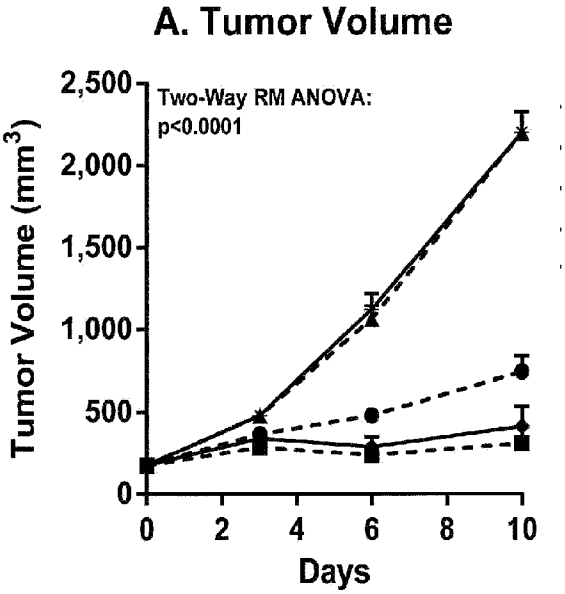

A. Tumor Volume
Two-Way RM ANOVA:
p<0.0001
Tumor Volume (mm3)
2,500
2,000
1,500
1,000
500
0
0
2
4
6
8
10
Days

FIG. 14B

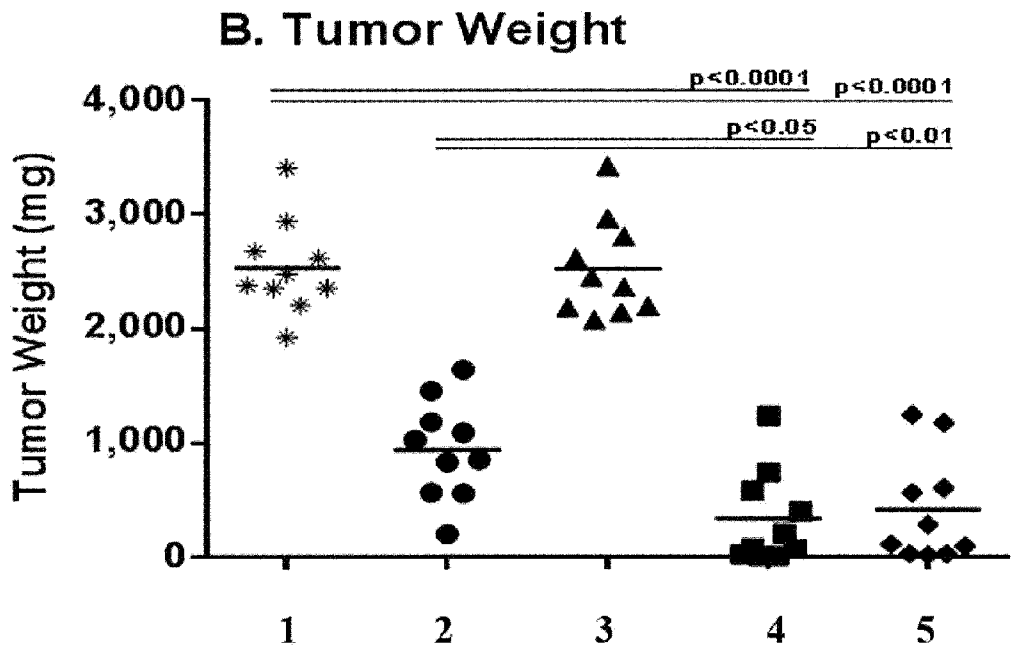

B. Tumor Weight
Tumor Weight (mg)
4,000
3,000
2,000
1,000
0
p<0.0001
p<0.0001
p<0.05
p<0.01
1
2
3
4
5

FIG. 15

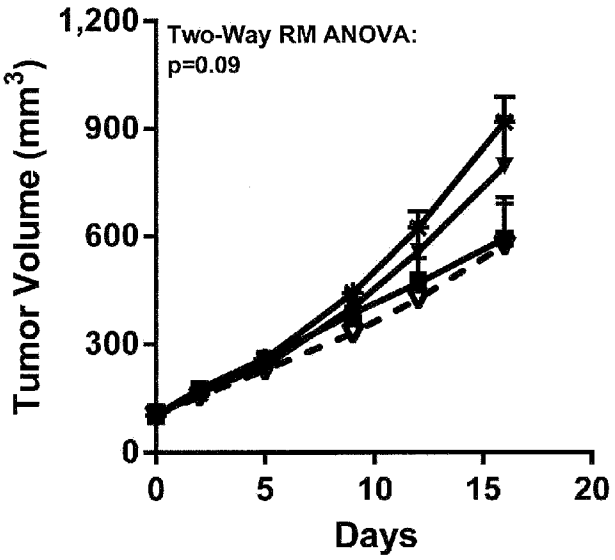

Two-Way RM ANOVA:
p=0.09
Tumor Volume (mm3)
1,200
900
600
300
0
0
5
10
15
20
Days

FIG. 16

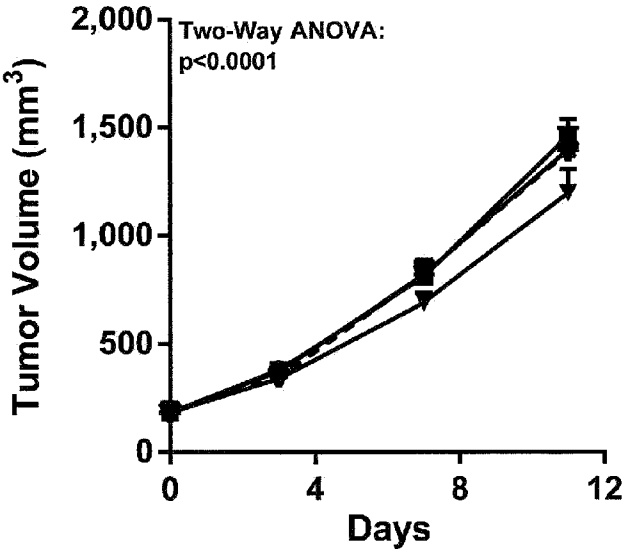

Two-Way ANOVA:
p<0.0001
Tumor Volume (mm3)
2,000
1,500
1,000
500
0
0
4
8
12
Days

FIG. 17

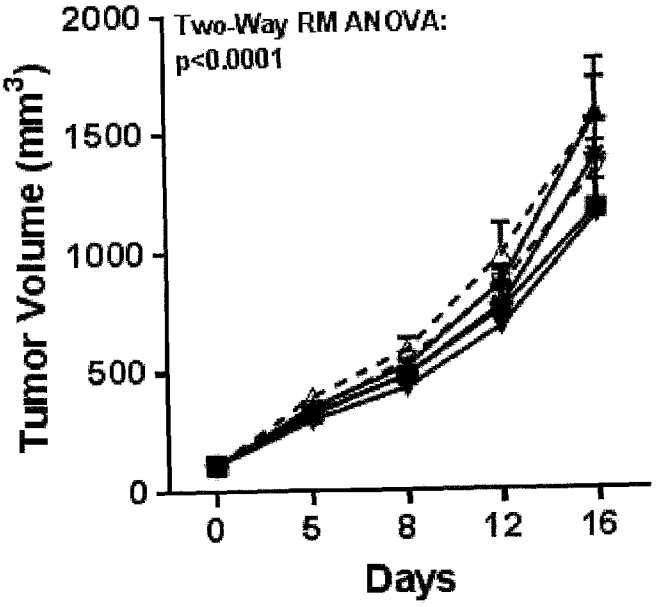
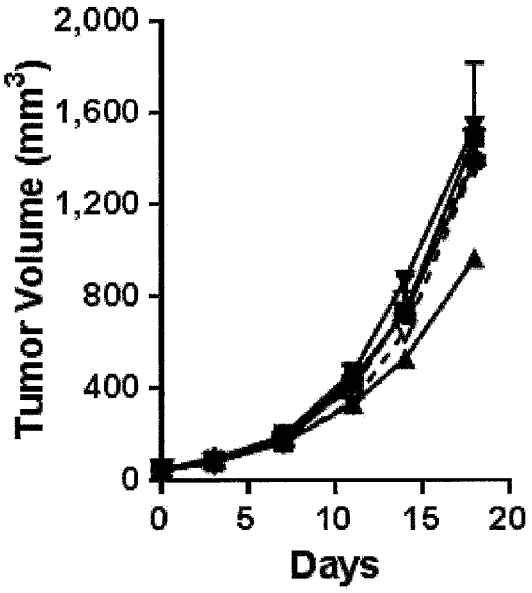

Two-Way RM ANOVA:
p<0.0001
Tumor Volume (mm3)
2000
1500
1000
500
0
0
5
8
12
16
Days

FIG. 18



SEKVENSLISTE

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