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[Continued on next page]

(54) Title: GITRL FUSION PROTEINS AND USES THEREOF

(57) Abstract: The disclosure provides GITRL fusion polypeptide subunits comprising an IgG Fc domain, a trimerization domain, and the receptor binding domain of GITR ligand, where the fusion polypeptide subunits can self-assemble into hexameric proteins. Also provided are methods of making fusion polypeptide subunits and hexameric proteins, and methods of use, e.g., treatment of cancer.

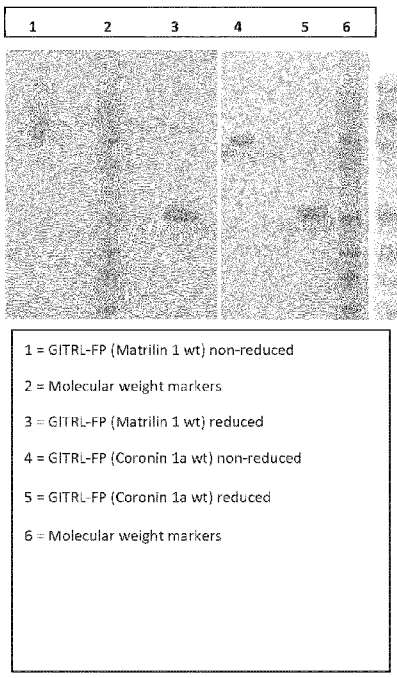

Figure 1

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GITRL FUSION PROTEINS AND USES THEREOF

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0001] The content of the electronically submitted sequence listing in ASCII text file (Name GITRLF-100P2_ST25.txt; Size: 56,159 bytes; and Date of Creation: June 15, 2016) filed with the application is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR), also known as TNFRSF18, AITR or CD357, is expressed on regulatory T cells and is up-regulated on antigen experienced $CD4^+$ helper cells and $CD8^+$ cytotoxic T cells as well as activated NK cells (Stephens et al. *J. Immunol.* (2004) 173(8): 5008-5020; Clothier and Watts, *Cytokine Growth Factor Rev.* (2014)). GITR is part of a complex system of receptors and ligands that are involved in controlling T-cell activation by antigen exposure. GITR has one known endogenous ligand, GITR ligand (GITRL), that exists in a loosely trimeric form and can cluster GITR resulting in potent cell signaling events within T cells (Chattopadhyay et al. (2007) *Proc. Natl. Acad. Sci. USA* 104(49):19452-19457). The interaction between GITR and GITRL results in delivery of positive co-stimulatory signals to T cells, which enhance their proliferation and activation by antigen exposure, help to promote memory cell generation and reprogram regulatory T cells; reducing their suppressive functions (Clothier and Watts, *Cytokine Growth Factor Rev.* (2014) Jan 4; Schaer et al. *Curr Opin Immunol.* (2012)).

SUMMARY

[0003] This disclosure relates to polypeptide subunits, each including, as a fusion polypeptide, the receptor-binding domain of GITR Ligand (GITRL), a multimerization domain, *e.g.* trimerization domain, and an IgG Fc domain, which are capable of forming stable multimeric, *e.g.*, hexameric

proteins. Compositions and methods are provided that are useful for cancer immunotherapy and treatment of viral infections.

[0004] In certain aspects, isolated single-chain polypeptide subunits that include: an IgG Fc domain; a functional multimerization domain; and a receptor binding domain of a Glucocorticoid-Induced TNF Receptor Ligand (GITRL), wherein the polypeptide subunit can self-assemble into a trimeric or a hexameric protein are provided.

[0005] In certain aspects, trimeric proteins that include three single-chain polypeptide subunits that each include: an IgG Fc domain; a functional multimerization domain; and a receptor binding domain of a Glucocorticoid-Induced TNF Receptor Ligand (GITRL), are provided.

[0006] In certain aspects, hexameric proteins that include six single-chain polypeptide subunits that each include: an IgG Fc domain; a functional multimerization domain; and a receptor binding domain of a Glucocorticoid-Induced TNF Receptor Ligand (GITRL), are provided.

[0007] In certain aspects, compositions that include the hexameric proteins and a carrier are provided.

[0008] In certain aspects, polynucleotides that include a nucleic acid that encodes the single chain polypeptide subunits or the hexameric proteins are provided.

[0009] In certain aspects, vectors that include the polynucleotides that encode the single chain polypeptide subunits or the hexameric proteins are provided.

[0010] In certain aspects, host cells that include the polynucleotides that encode the single chain polypeptide subunits or the hexameric proteins or that include the vectors that include the polynucleotides are provided.

[0011] In certain aspects, methods of producing the polypeptide subunits or of producing the hexameric proteins are provided, where the methods include culturing the host cells that include polynucleotides or vectors that encode the polypeptide subunits or hexameric proteins under conditions in which the polypeptide subunit or hexameric protein encoded by the polynucleotide or vector is expressed, and recovering the polypeptide subunit or hexameric protein.

[0012] In certain aspects, methods to promote survival or proliferation of antigen experienced T cells and/or activated NK cells are provided, where the methods include contacting antigen experienced T cells and/or activated NK cells with the hexameric protein or the composition, wherein the hexameric protein can specifically bind to GITR on the surface of the T cells and/or NK cells.

[0013] In certain aspects, methods of inducing cytokine release from activated GITR expressing immune cells are provided, where the methods include contacting these cells with the hexameric protein or the composition, wherein the hexameric protein can specifically bind to GITR on the surface of these cells.

[0014] In certain aspects, methods of promoting T cell or NK cell activation are provided, where the methods include contacting T cells or NK cells with the hexameric protein or the composition, wherein the hexameric protein can specifically bind to GITR on the surface of the T cells or NK cells.

[0015] In certain aspects, methods of treating cancer in a subject are provided, where the methods include administering to a subject in need of treatment an effective amount of the hexameric protein, or the composition, are provided.

[0016] In certain aspects, methods of enhancing an immune response in a subject, where the methods include administering to a subject in need thereof a therapeutically effective amount of the hexameric protein, or the composition, are provided.

[0017] In certain aspects, methods of treating a solid tumor in a subject, comprising administering the isolated single-chain polypeptide subunit diclosed above and an OX40 agonist to the subject, are provided.

[0018] In another aspect, methods of treating a solid tumor in a subject, comprising administering the isolated single-chain polypeptide subunit diclosed above and a T-cell priming agent to the subject, are provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1. SDS-PAGE analysis of recombinant GITRL fusion protein (FP) (Matrilin 1 wt) and GITRL FP (Coronin 1a wt) proteins purified using Protein G and size exclusion chromatography.

[0020] Figure 2A-D. Graph showing binding profile of the hexameric GITRL FP variants to GITR expressing CHO cells.

[0021] Figure 3A-D. Graph showing the inhibition profile of the hexameric GITRL FP variants competing for binding of trimeric GITRL to GITR-Fc.

[0022] Figure 4A-D. Graph showing the relative potency of the GITRL FP molecules using a human GITR transfected NF-κB luciferase gene reporter cell line.

[0023] Figure 5A-D. Unfolding transitions of hexameric GITRL FP (GCN4 pII), GITRL FP (Coronin 1a wt), GITRL FP (Langerin wt) and GITRL FP (Langerin variant).

[0024] Figure 6. The molar mass composition of eluted peaks. The graph shows that multimeric GITRL FP matrilin-1 protein (dotted line) in solution forms three species with weight-average molar mass (from left to right) of 612, 312 and 215 kDa with no easily identifiable major species. On the other hand, >90% of multimeric GITRL FP (coronin 1a wt; dashed line) and multimeric GITRL FP (GCN4 pII; solid line) protein mass elutes as a single protein species. These peaks are nevertheless not perfectly monodisperse most likely due to heterogeneity of glycans attached to the protein.

[0025] Figure 7. Schematic of a hexameric GITRL FP molecule.

[0026] Figure 8. Nucleotide and translated protein sequence of a representative GITRL IgG1 fusion polypeptide subunit. The individual domains are highlighted and annotated. ECD = extracellular domain; GITRL = glucocorticoid induced tumor necrosis factor receptor ligand; HA = hemagglutinin. The nucleic acid sequence of Figure 8 is provided as SEQ ID NO: 7 and the encoded precursor protein sequence is provided as SEQ ID NO: 8.

[0027] Figure 9. Deconvoluted LC-QTOF MS spectrum for a reduced GITRL IgG1 FP subunit. The accurate mass of GITRL IgG1 FP monomeric subunit (SEQ ID NO: 6), as determined by liquid chromatography coupled with quadrupole time of flight (QTOF) mass spectrometry (LC-QTOF MS), is consistent with the expected amino acid sequence with the addition of one biantennary glycan (predominantly G0f) per chain at the canonical glycosylation site in the Fc domain.

[0028] Figure 10. Human GITR-Fc conjugated to europium cryptate binds to hGITRL-HA in a homogeneous time resolved fluorescence assay. A titration of the IgG1 isotype control antibody does not inhibit this binding. Hexameric GITRL IgG1 FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 6 inhibits binding between hGITR and hGITRL with a half-maximal inhibitory concentration (IC50) of 0.562 nM. Experiments were conducted in duplicate wells. Error bars represent standard error of the mean. GITR(L) = glucocorticoid induced tumor necrosis factor receptor (ligand).

[0029] Figure 11. Hexameric GITRL FPs are potent agonists of the GITR receptor. Test articles were added in solution, at the concentrations indicated, to Jurkat cells transfected with hGITR and a luciferase reporter gene linked to an NFκB promoter. Luciferase activity, measured as luminescence, was determined after three hours. Hexameric GITRL IgG1 FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 6 or hexameric GITRL IgG4P FP

comprising monomeric subunits having an amino acid sequence set forth in SEQ ID NO: 40 resulted in a concentration dependent increase in luminescence. The EC_{50} of the hexameric GITRL IgG1 FP with respect to this effect was 182 pM. The EC_{50} of the hexameric GITRL IgG4P FP with respect to this effect was 289 pM. An isotype control antibody had no effect. Experiments were conducted in triplicate wells. Error bars represent standard error of the mean.

[0030] Figure 12. Hexameric GITRL FP enhances the proliferation of primary human T cells in response to anti-CD3 and anti-CD28. The proliferation of primary human T cells in response to anti-CD3 and anti-CD28 was increased by addition of plate bound hexameric GITRL IgG1 FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO:6 or hexameric GITRL IgG4P FP comprising monomeric subunits having an amino acid sequence set forth in SEQ ID NO: 40. The effect was concentration dependent, with an EC_{50} of 0.3 nM for the GITRL IgG1 FP and an EC_{50} of 0.5 nM for the GITRL IgG4P FP. The addition of an isotype control antibody had no effect. Experiments were conducted in triplicate wells. Error bars represent standard error of the mean.

[0031] Figure 13. The release of IFN-γ by primary human T cells in response to anti-CD3 and anti-CD28 was increased by addition of plate bound hexameric GITRL IgG1 FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 6 or hexameric GITRL IgG4P FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 40. The effect was concentration dependent, with an EC_{50} of 0.6 nM for the GITRL IgG1 FP and an EC_{50} of 0.8 nM for the GITRL IgG4P FP. The addition of an isotype control antibody had no effect. Experiments were conducted in triplicate wells. Error bars represent standard error of the mean.

[0032] Figure 14. Hexameric GITRL IgG1 FP mediates ADCC of primary human T cells by NK cells. Antigen experienced primary human T cells were fluorescently labelled and mixed with primary human NK cells at a ratio of 1 T cell to 32 NK cells. Test articles were added as indicated and the % lysis of T cells was calculated following 24 hours incubation. Hexameric GITRL FP IgG1 comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 6 results in an increase in the percentage of lysis. The effect was concentration dependent with an EC_{50} of 239 pM. The negative control, hexameric GITRL IgG4P FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 40, did not result in any increase in the percentage lysis of T cells.

[0033] Figure 15. ADCC mediated by hexameric GITRL IgG1 FP favors the generation of an increased CD8:CD4 T cell ratio. Antigen experienced primary human T cells were fluorescently labelled and mixed with primary human NK cells at a ratio of 1 T cell to 32 NK cells. Test articles were added as indicated and the percentage of $CD4^+$ and $CD8^+$ T cells present in the total T cell population was assessed by flow cytometry following 24 hours incubation. Hexameric GITRL IgG1 FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 6 results in a concentration dependent shift in the CD8:CD4 T cell ratio, which favors CD8 T cells.

[0034] Figure 16. Hexameric GITRL IgG1 FP overcomes regulatory T cell mediated suppression of effector T cell proliferation. The percentage of divided $CD4^+$ $CD25^-$ effector T cells was analyzed by flow cytometry following stimulation for five days with anti-CD3 and anti-CD28 antibodies. The percentage of dividing cells was reduced in the presence of increasing numbers of T-regs. Addition of plate bound isotype control further decreased the percentage of dividing cells. Addition of plate bound hexameric GITRL IgG1 FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 6 at the concentration indicated restored the percentage of dividing cells to that observed in the absence of T-regs. Experiments using effectors alone were in single wells. All other experiments were conducted in duplicate wells. Error bars represent standard error of the mean.

[0035] Figure 17. Survival of mice treated with mGITRL FP is isotype dependent. Mice were treated by intraperitoneal administration of mGITRL FP mIgG2a or mGITRL IgG1 FP, both at 5 or 10 mg/kg, daily from day 6 to day 23 following subcutaneous implantation of CT26 cells. Saline was administered as a negative control.

[0036] Figure 18. mGITRL FP results in increased proliferation of T cells. The expression of Ki67 was measured by flow cytometry in splenic T cells seven days following treatment with a single dose of either 0.2 mg/kg or 1 mg/kg mGITRL FP. Black lines with circles = CD8 T cells; Dark Grey lines with squares = $CD4^+$ $Foxp3^-$; Light Grey lines with triangles = $CD4^+$ $Foxp3^+$ cells. Significance was calculated using the Student's T test where *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

[0037] Figure 19. mGITRL FP results in increased expression of the activation marker ICOS on T cells. The expression of ICOS was measured by flow cytometry in splenic T cells seven days following treatment with a single dose of either 0.2 mg/kg or 1 mg/kg mGITRL FP. Dark grey lines with circles = CD8 T cells; black lines with squares = $CD4^+$ Foxp3. Significance was calculated using the Student's T test where *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001. Treatment with

mGITRL FP resulted in a decrease in the frequency of $CD4^+$ $FOXP3^+$ regulatory T cells and $CD4^+$ $FOXP3^-$ helper cells within the tumor, but did not alter the frequency of $CD8^+$ cytotoxic T cells. The overall result was an increased CD8:CD4 ratio within the tumor microenvironment.

[0038] Figure 20. mGITRL FP results in an increased CD8:CD4 ratio within the tumor. The frequency of CD8 T cells (black line with circles); $CD4^+$ $Foxp3^-$ cells (dark grey line with squares); $CD4^+$ $Foxp3^+$ cells (light grey lines with triangles) was measured by flow cytometry within the tumor 7 days following treatment with a single dose of either 0.2 mg/kg or 1 mg/kg mGITRL FP. Significance was calculated using the Student's T test where *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

[0039] Figure 21. ELISA data demonstrating that hexameric hGITRL IgG1 FP binds to human and cynomolgus GITR-Fc. Binding of biotinylated hexameric hGITRL IgG1 FP comprising monomeric subunits having the amino acid sequence set forth in SEQ ID NO: 6 to recombinant human and cynomolgus monkey (cyno) GITR. CD137-Fc was used as a negative control to determine the background signal in the assay. Experiments were conducted in triplicate wells. Error bars represent standard deviation. CD137 = cluster of differentiation 137 (TNFRSF9); CD137-Fc = cluster of differentiation 137 extracellular domain linked to the Fc domain of hIgG1; Cyno = cynomolgus monkey; ELISA = enzyme-linked immunosorbent assay; GITR-Fc = glucocorticoid induced tumor necrosis factor receptor extracellular domain linked to the Fc domain of hIgG1; OD450nm = optical density readings at 450 nm wavelength.

[0040] Figure 22. Measurement of %KI67 positive T cell subpopulations in hexameric hGITRL IgG1 FP treated Cynomolgus Monkeys. Cynomolgus monkeys were monitored for baseline levels of %KI67 positive T cell subpopulations for 20 days, treated with either a vehicle control (circles), 1 mg/kg hGITRL IgG1 FP (triangles), or 10 mg/kg hGITRL IgG1 FP (squares) at day 0, and then monitored for %KI67 positive T cell subpopulations days 1, 3, 5, 9, 11, 15, 18, 22, and 29.

[0041] Figure 23 A-B. Inhibition profiles for hGITRL FP proteins competing for binding of trimeric hGITRL to hGITR-Fc and IC_{50} values. hGITRL FP wt, N92D and N104D (A) and hGITRL FP, N161D (B)

[0042] Figure 24. Binding profiles for hGITRL FP proteins binding to hGITR-Fc and Kd values.

[0043] Figure 25 A-C. Graphs showing the relative potency of the GITRL FP molecules using a human GITR transfected NF-κB luciferase gene reporter cell line and EC_{50} values. hGITRL FP wt

and N92D (A); hGITRL FP wt, N161D, N129A and N129A/N161D (B); N161D and N129A/N161D (C)

[0044] Figure 26 A-C. Graphs showing the relative potency of the GITRL FP molecules using a human primary $CD3^+$ T cell re-stimulation assay with a thymidine incorporation readout. hGITRL FP wt and N92D (A); hGITRL FP wt, N92D and N104D (B); wt and N161D (C).

[0045] Figure 27. Unfolding transitions of GITRL FP wt and N92D variant.

[0046] Figure 28. Predominant oligosaccharide structures found in hGITRL FP produced in Chinese Hamster Ovary cells; Complex type (A); High mannose (B). Man = Mannose; GlcNAc = N-acetylglucosamine; Fuc = Fucose; Gal = Galactose; NANA = Nacetylneuraminic acid (Sialic acid).

[0047] Figure 29 A-C. GITRL FP peptide mapping. Extracted ion chromatograms for tryptic peptide 7 (T7), which contains the Fc N-glycosylation site, for GITRL FP wt (Ai) and GITRL FP N161D (Aii). Combined, deconvoluted mass spectra for T7, showing the predominant glycoforms, for GITRL FP wt (Aiii) and GITRL FP N161D (Aiv). Extracted ion chromatograms for tryptic peptide 40 (T40), which contains the GITRL ECD N129 N-glycosylation consensus sequence, for GITRL FP wt (Bi) and GITRL FP N161D (Bii). Combined, deconvoluted mass spectra for T40, showing a mass consistent with the absence of N-glycosylation at N129, for GITRL FP wt (Biii) and GITRL FP N161D (Biv). Extracted ion chromatograms for tryptic peptide 42-43 (T42-43) and 43 (T43) for GITRL FP wt (Ci) and GITRL FP N161D (Cii), respectively. Combined, deconvoluted mass spectra for T42-43 for GITRL FP wt (Ciii) showing the predominant glycoforms at the GITRL ECD N161 N-glycosylation site. Combined, deconvoluted mass spectra for T43 for GITRL FP N161D (Civ), showing a mass confirming the N161D substitution and the absence of N-glycosylation.

[0048] Figure 30 A-C. Structure and agonistic potential of a murine GITR ligand fusion protein. (A) Schematic of murine GITRL-FP consisting from N- to C-terminus, of a fragment crystallisable (Fc) region of an immunoglobulin G1 (IgG1) or 2a (IgG2a), a multimerisation domain (MD) and the extracellular (GITR-binding) domain (ECD) of murine GITR ligand (B) SDS-PAGE of the purified murine GITRL-FP. (C) NF-κB associated luminescence in a murine GITR receptor transduced Jurkat cell line following treatment with mGITRL-FP, DTA-1 rIgG2b isotype controls or mOX40L-FP. Data is representative of at least two independent experiments.

[0049] Figure 31 A-E. Comprehensive FcγR engagement increases antitumor activity but does not drive increased T-cell proliferation downstream of GITR (A) Tumor growth in Balb/c mice. Mice

were treated once by i.p. injection of saline control, mGITRL-FP mIgG1or mGITRL-FP mIgG2a as indicated. Number of regressions are indicated on each individual graph (B) Frequency of Ki67 expression in splenic T-cells 4 days following treatment of CT26 tumor-bearing mice. (C) Frequency of intratumoral T-cell sub-sets and (D) ratio of intratumoral CD8+ to CD4+ FoxP3+ cells 4 days following treatment of CT26 tumor-bearing mice with 10 mg/kg of mGITRL-FP or saline control as indicated. (E) Median fluorescence intensity of GITR expression on splenic and intratumoral T-cell sub-sets 4 days following treatment. Error bars indicate standard error of mean; n= 7-10 mice per group. For (B) and (C) **p<0.005 ***p<0.001 and ****p<0.0001, as calculated by two way ANOVA; Significance for C is black for changes in CD4+ Foxp3+ cells and gray for changes in CD4+ Foxp3- cells; for (D) *P<0.05, as calculated by one way ANOVA; for (E) ****p<0.0001, as calculated by Student's T-test.

[0050] Figure 32 A-B. Intratumoral T-reg depletion and CD4+ Foxp3-: T-reg ratio after treatment with mGITRL-FP mIgG2a or mIgG1. CT26 tumor bearing mice were injected with either saline control, mGITRL-FP mIgG1 (10 mg/kg) or mGITRL-FP mIgG2a (10 mg/kg) once i.p. at 6 days post CT26 implantation. (A) Flow cytometric plots showing the proportion of CD4+ Foxp3+ T-regs in the tumor 4 days after treatment. CD4+ Foxp3+ flow cytometry analysis gate is positioned based on Foxp3 fluorescence minus one (FMO) control. (B) Intratumoral CD4+ Foxp3-: T-reg ratio measured at 4 days after treatment as indicated. Statistical analysis carried out using one way ANOVA where **** indicates a P-value <0.0001.

[0051] Figure 33 A-C. Murine GITRL-FP mIgG2a mediates antitumor activity in a dose and schedule dependent manner. Tumor growth in Balb/c mice. Mice were treated by i.p. injection of (A) a single dose of mGITRL-FP mIgG2a, at the dose level indicated or (B) multiple doses of 0.2 mg/kg mGITRL-FP mIgG2a given daily [Q1D] or weekly [Q1W]. (C) Predicted serum concentration of mGITRL-FP following administration using the dose and schedule indicated. Dotted line indicates the blood concentration threshold of mGITRL-FP mIgG2a required to achieve maximum antitumor activity.

[0052] Figure 34 A-D. Murine GITRL-FP mIgG2a mediates PD changes in T-cell proliferation and activation in a dose and schedule dependent manner. Frequency of (A) Ki67, (B) ICOS, (C) PD-1 and (D) OX40 positive CD4+ T-cells in the tumor draining lymph node of CT26 tumor bearing mice 7 days following treatment with 0.2 or 1 mg/kg mIgG2a mGITRL-FP once, every three days [Q3D]

or every day [Q1D]. Error bars represent the standard error of the mean from 7-8 mice per group. *p<0.05, **p< 0.01 ***p<0.001, ****0<0.0001, as calculated by one way ANOVA.

[0053] Figure 35 A-B. Binding and potency profile of a mouse OX40 ligand fusion protein. (A) Binding ELISA showing that mGITRL-FP mIgG1 and mIgG2a each binds specifically to recombinant mouse GITR-Fc (black bars) and not to recombinant mouse OX40-Fc and that mOX40L-FP mIgG1 and mIgG2a each binds specifically to recombinant mouse OX40 (grey bars) and not recombinant mouse GITR. mOX40L-FP Y182A isotype control binds minimally to recombinant mouse OX40-Fc. (B) Binding of mOX40L-FP mIgG1 (black circles) or Y182A isotype control (open circles) to human OX40 on Jurkat human OX40 NF-κB reporter cell line. Mouse OX40L FP mIgG1 induced NF κB signalling in the reporter assay but this was not evident for the mOX40L-FP Y182A isotype control.

[0054] Figure 36 A-B. The antitumor activity of mGITRL-FP is superior to that of mOX40L-FP in the CT26 model. (A) Tumor growth in Balb/c mice. Mice were treated twice weekly with an i.p. injection of 5 mg/kg mIgG2a or mIgG1 mGITRL-FP or mOX40L-FP, 5 mg/kg mIgG1 fusion protein isotype control or saline, Number of total regressions are indicated on each individual graph. (B) Frequency of intratumoral CD4+, FoxP3+ T-regs in CT26 tumor-bearing Balb/c mice at 10 days following treatment as indicated.

[0055] Figure 37 A-E. The pharmacodynamics (PD) changes mediated by mGITRL-FP mIgG2a and mOX40L-FP mIgG1 are differential and can be enhanced through combination. Frequency of (A) CD4+ FoxP3- or CD8+, Ki67+ (B) CD4+ or CD8+, CD44+ CD62L Lo/- effector memory, (C) CD4+ CD44+ CD62L+ central memory, (D) CD4+ or CD8+, T-bet+ and (E) CD4+ EOMES+ T-cells in the spleens of CT26 tumor bearing mice 14 days following twice weekly treatment with either 25 mg/kg mGITRL-FP mIgG2a, 15 mg/kg mOX40L-FP mIgG1 or a combination of both molecules. *p<0.05, **p< 0.01 ***p<0.001, ****0<0.0001, as calculated by one way ANOVA.

[0056] Figure 38 A-B. Combination of mGITRL-FP mIgG2a and mOX40L-FP mIgG1 synergise to induce increased antitumor activity in B16F10-Luc2 and CT26 tumor bearing mice. Tumor growth in B16F10-Luc2 and CT26 tumor bearing mice. (A) B16F10-Luc2 tumor bearing mice were dosed i.p. with saline, 25 mg/kg mGITRL-FP mIgG2a biweekly for two weeks, 15 mg/kg mOX40L-FP mIgG1 bi weekly for three weeks or a combination of both molecules and tumor growth measured. (B) CT26 tumor bearing mice were untreated or treated by i.p. injection of isotype control, 7.5 mg/kg of mOX40L-FP mIgG1 twice weekly for two doses, a single suboptimal dose of mGITRL-FP

mIgG2a at 0.1 mg/kg or the combination of both molecules. Number of total regressions are indicated next to each individual graph.

[0057] Figure 39. Combination of mGITRL-FP mIgG2a and mOX40L-FP mIgG1 induces increased survival of mice bearing B16F10-Luc2 tumors compared to monotherapy treatment. B16F10-Luc2 tumor bearing mice were dosed i.p. with saline, 25 mg/kg mGITRL-FP mIgG2a biweekly for two weeks, 15 mg/kg mOX40L-FP mIgG1 bi weekly for three weeks or a combination of both molecules and survival measured. Log Rank test, where *** indicates a P-value <0.001, ** indicates a P-value <0.01 and * indicates a P-value of <0.05.

[0058] Figure 40 (A)-(G). (A) CT26 cells were implanted subcutaneously into Balb/C mice, $5x10^5$ cells/mouse. The mice were randomized by tumor volume on day 6 and dosing was initiated (Group n=10 mice). The mice were dosed IP with mGITRL FP IgG2a either with (B) a single dose or (C) every other day for 9 doses, Q2Dx9. They were dosed at 5, 1, 0.5, 0.2, 0.1 and 0.04 mg/kg. Data shown is a representative of two repeat experiments. (D) On day 11 they were randomized based on tumor size and were treated with nothing, DTA-1 (anti-GITR mAb), or mGITRL-FP (Group n=9). (E) Mice were depleted of CD8 T-cells on day 8, 10, 12, 14, and 16. On day 11 they were randomized based on tumor size and treated with nothing, DTA-1, or mGITRL-FP IgG2a. (F) Median survival. (G) On day 18, untreated mice with CT26 tumors were sacrificed to examine GITR expression on CD8 T-cells and Tregs in the spleen and tumor.

[0059] Figure 41 (A) – (H). (A) CT26 cells were implanted subcutaneously into Balb/C mice, $5x10^5$ cells/mouse. The mice were randomized by tumor volume on day 10 and dosing was initiated. The mice were dosed IP with mGITRL-FP IgG2a biweekly for 4 total doses doses. On day 18 mice were sacrificed to examine (B) Tregs, (C) CD8 T-cells. (D) Mouse spleens a tumors were re-stimulated with 10μg/mL AH1 peptide/Protein Transport inhibitor for 5 hours and stained for IFNgamma and TNFalpha. (E) GITR Expression on CD8 cells. (F) GITR Expression on Tregs in the spleen, lymph node, and tumor. (G) KI-67 on CD4 T-cells (H) KI-67 on the CD8 T-cells.

[0060] Figure 42 (A)-(C). mGITRL FP expands antigen specific T-cells in a dose dependent manner. (A) CT26 tumor bearing mice treated with a single dose of mGITRL FP IgG2a clear tumors and are protected from to rechallenge with 5E5 CT26 cells/mouse on day 85[R Arrow]. (B) After re-challenge with CT26, on day 120, mouse spleen was harvested [PD Arrow], processed to single cell and restimulated with 10μg/mL AH1 peptide/Protein Transport inhibitor for 5 hours. Mice had

a dose dependent increase in AH1 specific T cells. (C) Representative plot of 5 mice from each group. For comparison, naïve mice and untreated mice with CT26 tumors at day 10 are included.

[0061] Figure 43 (A)-(D). (A)-(B) TC-1 cells were implanted into the footpad of C57BL/6 mice, $2x10^4$ cells/mouse. The mice were randomized by tumor volume on day 14 and dosing was initiated. The mice were dosed IP with mGITRL-FP IgG2a biweekly for 4 total doses. On day 24, untreated mice were sacrificed to examine (C) GITR expression on Tregs and GITR expression on CD8 T-cells. Mice were evaluated for E7 specific T-cells, and none were detected by E7 restim or by dextramer.

[0062] Figure 44 (A)-(K). (A) To generate E7 specific T-cells, naïve C57BL/6 mice were injected with 10ug of E7 SLP in CpG (Addavax) at the base of tail. Mice were then treated with mGITRL-FP IgG2a at 1mg/kg for 3 doses. Mice were evaluated for splenic (B) CD4 T-cells (C) CD8 T-cells (D) E7 Dextramer+ T-cells (E) Tregs, (F) GITR levels on the antigen specific cells. (G) TC-1 cells were implanted into the footpad of C57BL/6 mice, $2x10^4$ cells/mouse. The mice were randomized by tumor volume on day 14 and dosing was initiated. C57BL/6 mice were injected with 10ug of E7 SLP in CpG (Addavax) at the base of tail. At day 28, mice were sacrificed. Spleen and tumor were evaluated for (H)-(I) E7 and specific CD8 T-cells (J)-(K).

[0063] Figure 45. (A) TC-1 cells were implanted into the footpad of C57BL/6 mice, $2x10^4$ cells/mouse. The mice were randomized by tumor volume on day 14 and dosing was initiated. (B) Vaccinated C57BL/6 mice were injected with 3.3ug of E7 SLP in CpG (Addavax) at the base of tail. Treated mice were dosed IP with GITRL-FP IgG2a biweekly for 4 total doses. (C) Kaplan-Meier survival of mice after TC-1 implant with a $P < 0.05$. (D) Median survival of the groups. (E) To examine pharmacodynamic effects, groups of mice treated the same in (A) were sacrificed and the spleens and tumor harvested. Tumors were pooled and spleens were left as individuals. (F) CD45+ cells were evaluated in the tumor. (G) Mouse spleens and tumors were restimulated with 1μg/mL E7 peptide/Protein Transport inhibitor for 5 hours and stained for IFNgamma and TNFalpha, and spleen and tumor Tregs were measured.

DETAILED DESCRIPTION

[0064] Engagement of the GITR receptor on T cells, *e.g.*, $CD4^+$ T cells or $CD8^+$ T cells during, or shortly after, priming by an antigen results in an increased response of the T cells, *e.g.*, $CD4^+$ T cells or $CD8^+$ T cells to the antigen. Engagement of the GITR receptor on NK cells or B cells, *e.g.*, during, or shortly after, priming by an activating signal (*e.g.*, antigen exposure) results in an increased response of the NK cells or B cells. In the context of the present disclosure, the term "engagement" refers to binding to and stimulation of at least one activity mediated by the GITR receptor. For example, engagement of the GITR receptor on antigen specifics, *e.g.*, $CD4^+$ T cells or $CD8^+$ T cells results in increased T-cell proliferation and increased cytokine production, as compared to the response to antigen alone. The elevated response to the antigen can be maintained for a period of time substantially longer than in the absence of GITR receptor engagement. Thus, stimulation via the GITR receptor enhances the antigen specific immune response by boosting T-cell, NK-cell, or B-cell recognition of non-self, *e.g.*, tumor antigens or viral antigens. GITR has been implicated in T-cell mediated control of certain chronic viral infections (Pascutti, *et al.*, PLoS Pathog. 2015 Mar 4;11(3); Clouthier, *et al.*, PLoS Pathog. 2015 Jan 15;11(1)).

[0065] GITR agonists can enhance antigen specific immune responses in a subject, such as a human subject, when administered to the subject during or shortly after priming of T cells by an antigen. GITR agonists include GITR ligand ("GITRL"), such as soluble GITRL fusion proteins and anti-GITR antibodies or fragments thereof. A specific example is a fusion polypeptide subunit comprising the receptor binding domain of GITRL, a multimerization domain, *e.g.*, trimerization domain, *e.g.*, an alpha helical coiled coil domain derived from Coronin 1a, and a IgG Fc domain, where the polypeptide subunit self-assembles into a multimeric (*e.g.*, trimeric or hexameric) fusion protein. Also described herein are nucleic acids including polynucleotide sequences that encode such fusion polypeptides. This disclosure also provides methods for enhancing an antigen specific immune response in a subject using the multimeric GITRL fusion proteins. The compositions and methods disclosed herein with respect to GITRL fusion proteins can be more generally applied to the production and use of multimeric (*e.g.*, trimeric and hexameric) receptor-binding fusion proteins, for example, in a method of treating cancer, a method of treating a viral infection, or a method of enhancing an immune response in a subject.

Definitions

[0066] The term "a" or "an" entity refers to one or more of that entity; for example, "polypeptide subunit" is understood to represent one or more polypeptide subunits. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0067] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[0068] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects or aspects of the disclosure, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0069] As used herein, the phrase “antigen experienced’ is used to describe a cell that has been exposed to an antigen where the exposure to that antigen has elicited a response in the cell.

[0070] As used herein, the term “polypeptide” is intended to encompass a singular “polypeptide” as well as plural “polypeptides,” and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, “protein,” “amino acid chain,” or any other term used to refer to a chain or chains of two or more amino acids are included within the definition of "polypeptide,” and the term “polypeptide” can be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-standard amino acids. A polypeptide can be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a

designated nucleic acid sequence. It can be generated in any manner, including by chemical synthesis.

[0071] A "protein" as used herein can refer to a single polypeptide, *i.e.*, a single amino acid chain as defined above, but can also refer to two or more polypeptides that are associated, *e.g.*, by disulfide bonds, hydrogen bonds, or hydrophobic interactions, to produce a multimeric protein. As used herein, the term "polypeptide subunit" refers to a polypeptide chain of amino acids which can interact with other polypeptide subunits, either identical or different, to form a multimeric protein, *e.g.*, a hexameric protein as described herein.

[0072] A polypeptide as disclosed herein can be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides can have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides that do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, and are referred to as unfolded.

[0073] An "isolated" substance, composition, entity, and/or any combination of substances, compositions, or entities, or any grammatical variants thereof, e.g., isolated biological material, is a substance that is not in its natural milieu. No particular level of purification is required. For example, an isolated antibody is an antibody that is not produced or situated in its native or natural environment. Recombinantly produced biological materials are considered isolated as disclosed herein, as are materials that are produced in a non-native cell, such as a hybridoma. A substance, e.g., biological material, is also considered "isolated" if it has been separated, fractionated, or partially or substantially purified by any suitable technique. In certain aspects, an isolated substance, e.g., isolated biological material, can be "non-naturally occurring."

[0074] As used herein, the term "non-naturally occurring" substance, composition, entity, and/or any combination of substances, compositions, or entities, or any grammatical variants thereof, is a conditional term that explicitly excludes, but only excludes, those forms of the substance, composition, entity, and/or any combination of substances, compositions, or entities that are well-understood by persons of ordinary skill in the art as being "naturally-occurring," or that are, or might be at any time, determined or interpreted by a judge or an administrative agency such as the United States Patent and Trademark Office, or judicial body to be, "naturally-occurring." For example, the

term "a non-naturally occurring antibody explicitly excludes those antibodies that exist in nature, *e.g.*, an antibody that would naturally be present in the immune system of a mouse exposed to a normal milieu of antigenic stimulus, or an antibody finally determined by an administrative body, *e.g.*, the United States Patent and Trademark Office, or a judicial body, *e.g.*, a federal court, to be "naturally-occurring."

[0075] Other polypeptides disclosed herein are fragments, derivatives, analogs, or variants of the foregoing polypeptides, and any combination thereof. The terms "fragment," "variant," "derivative" and "analog" when referring to polypeptide subunit or multimeric protein as disclosed herein can include any polypeptide or protein that retain at least some of the activities of the complete polypeptide or protein, but which is structurally different. Fragments of polypeptides include, for example, proteolytic fragments, as well as deletion fragments. Variants include fragments as described above, and also polypeptides with altered amino acid sequences due to amino acid substitutions, deletions, or insertions. Variants can occur spontaneously or be intentionally constructed. Intentionally constructed variants can be produced using art-known mutagenesis techniques. Variant polypeptides can comprise conservative or non-conservative amino acid substitutions, deletions or additions. Derivatives are polypeptides that have been altered so as to exhibit additional features not found on the native polypeptide. Examples include fusion proteins. Variant polypeptides can also be referred to herein as "polypeptide analogs." As used herein a "derivative" refers to a subject polypeptide having one or more amino acids chemically derivatized by reaction of a functional side group. Also included as "derivatives" are those peptides that contain one or more standard or synthetic amino acid derivatives of the twenty standard amino acids. For example, 4-hydroxyproline can be substituted for proline; 5-hydroxylysine can be substituted for lysine; 3-methylhistidine can be substituted for histidine; homoserine can be substituted for serine; and ornithine can be substituted for lysine.

[0076] A "conservative amino acid substitution" is one in which one amino acid is replaced with another amino acid having a similar side chain. Families of amino acids having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan,

histidine). For example, substitution of a phenylalanine for a tyrosine is a conservative substitution. Methods of identifying nucleotide and amino acid conservative substitutions which do not eliminate protein activity are well-known in the art (see, *e.g.*, Brummell *et al.*, *Biochem.* 32: 1180-1 187 (1993); Kobayashi *et al.*, *Protein Eng.* 12(10):879-884 (1999); and Burks *et al.*, *Proc. Natl. Acad. Sci. USA* 94:.412-417 (1997)).

[0077] As used herein, the term "antibody" (or a fragment, variant, or derivative thereof) refers to at least the minimal portion of an antibody which is capable of binding to antigen, *e.g.*, at least the variable domain of a heavy chain (VH) and the variable domain of a light chain (VL) in the context of a typical antibody produced by a B cell. Basic antibody structures in vertebrate systems are relatively well understood. See, *e.g.*, Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988).

[0078] Antibodies or antigen-binding fragments, variants, or derivatives thereof include, but are not limited to, polyclonal, monoclonal, human, humanized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, *e.g.*, Fab, Fab' and F(ab')2, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VL or VH domain, and fragments produced by a Fab expression library. ScFv molecules are known in the art and are described, *e.g.*, in US patent 5,892,019. Immunoglobulin or antibody molecules encompassed by this disclosure can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

[0079] The term "polynucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, *e.g.*, messenger RNA (mRNA) or plasmid DNA (pDNA). A polynucleotide can comprise a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond, such as found in peptide nucleic acids (PNA)). The term "nucleic acid" refers to any one or more nucleic acid segments, *e.g.*, DNA or RNA fragments, present in a polynucleotide. By "isolated" nucleic acid or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant polynucleotide encoding a polypeptide subunit contained in a vector is considered isolated as disclosed herein. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of polynucleotides. Isolated polynucleotides or nucleic acids further include such

molecules produced synthetically. In addition, polynucleotide or a nucleic acid can be or can include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

[0080] As used herein, a "coding region" is a portion of nucleic acid comprising codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it can be considered to be part of a coding region, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, and the like, are not part of a coding region. Two or more coding regions can be present in a single polynucleotide construct, *e.g.*, on a single vector, or in separate polynucleotide constructs, *e.g.*, on separate (different) vectors. Furthermore, any vector can contain a single coding region, or can comprise two or more coding regions, *e.g.*, a single vector can separately encode an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region. In addition, a vector, polynucleotide, or nucleic acid can encode heterologous coding regions, either fused or unfused to a nucleic acid encoding a polypeptide subunit or fusion protein as provided herein. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain.

[0081] In certain embodiments, the polynucleotide or nucleic acid is DNA. In the case of DNA, a polynucleotide comprising a nucleic acid that encodes a polypeptide normally can include a promoter and/or other transcription or translation control elements operably associated with one or more coding regions. An operable association or linkage is when a coding region for a gene product, *e.g.*, a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" or “operably linked” if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter can be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated

with the polynucleotide to direct cell-specific transcription. Suitable promoters and other transcription control regions are disclosed herein.

[0082] A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions that function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (the immediate early promoter, in conjunction with intron-A), simian virus 40 (the early promoter), and retroviruses (such as Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit β-globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (*e.g.*, promoters inducible by interferons or interleukins).

[0083] Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from picornaviruses (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence).

[0084] In other embodiments, a polynucleotide can be RNA, for example, in the form of messenger RNA (mRNA).

[0085] Polynucleotide and nucleic acid coding regions can be associated with additional coding regions that encode secretory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide as disclosed herein, *e.g.*, a polynucleotide encoding a polypeptide subunit provided herein. According to the signal hypothesis, proteins secreted by mammalian cells have a signal peptide or secretory leader sequence that is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells generally have a signal peptide fused to the N-terminus of the polypeptide, which is cleaved from the complete or "full length" polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, *e.g.*, an immunoglobulin heavy chain or light chain signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, can be used. For example, the wild-type leader sequence can be

substituted with the leader sequence of influenza A virus haemaglutinin, human tissue plasminogen activator (TPA) or mouse β-glucuronidase.

[0086] A "vector" is a nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector can also include one or more selectable marker gene and other genetic elements known in the art.

[0087] A "transformed" cell, or a "host" cell, is a cell into which a nucleic acid molecule has been introduced by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule can be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration. A transformed cell or a host cell can be a bacterial cell or a eukaryotic cell.

[0088] By "specifically binds," it is generally meant that a molecule, *e.g.*, a GITRL or receptor-binding fragment thereof, binds to another molecule, *e.g.*, GITR, via its receptor-binding domain, and that the binding entails some complementarity between the ligand and its receptor . According to this definition, a ligand is said to "specifically bind" to a receptor when it binds to that receptor, via its receptor-binding domain more readily than it would bind to a random, unrelated molecule. The term "specificity" is used herein to qualify the relative affinity by which a certain ligand binds to a certain receptor. For example, ligand "A" may be deemed to have a higher specificity for a given receptor than ligand "B," or ligand "A" may be said to bind to receptor "C" with a higher specificity than it has for related receptor "D."

[0089] By "a receptor-binding domain," it is intended a binding domain comprised in a ligand, *e.g.*, a GITRL as disclosed herein.

[0090] A ligand, *e.g.*, a GITRL fusion polypeptide subunit or multimeric GITRL fusion protein as disclosed herein can bind to a receptor, *e.g.*, GITR, with an off rate (k(off)) of less than or equal to 5 X 10^{-2} sec^{-1}, 10^{-2} sec^{-1}, 5 X 10^{-3} sec^{-1} or 10^{-3} sec^{-1}. A ligand, *e.g.*, a GITRL fusion polypeptide subunit or multimeric GITRL fusion protein as disclosed herein can bind to a receptor, *e.g.*, GITR, with an off rate (k(off)) less than or equal to 5 X 10^{-4} sec^{-1}, 10^{-4} sec^{-1}, 5 X 10^{-5} sec^{-1}, or 10^{-5} sec^{-1} 5 X 10^{-6} sec^{-1}, 10^{-6} sec^{-1}, 5 X 10^{-7} sec^{-1} or 10^{-7} sec^{-1}.

[0091] The terms "inhibit," "block," and "suppress" are used interchangeably herein and refer to any statistically significant decrease in biological activity, including full blocking of the activity. For

example, "inhibition" can refer to a decrease of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% in biological activity.

[0092] As used herein, the term "affinity" refers to a measure of the strength of the binding of a ligand to its cognate receptor. As used herein, the term "avidity" refers to the overall stability of the complex between a population of ligands and receptors, that is, the functional combining strength of a combination of ligands and receptors, *e.g.*, interaction of a hexameric GITRL IgG Fusion Protein (GITRL FP) to cell surface GITR. Avidity is related to both the affinity of individual receptor binding domains in the population with specific receptors, and also the valencies of the ligands and the receptors.

[0093] A ligand, *e.g.*, a GITRL fusion polypeptide subunit or multimeric GITRL fusion protein as disclosed herein can also be described or specified in terms of its binding affinity to a ligand. For example, a ligand can bind to a receptor with a dissociation constant or K_D no greater than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

[0094] A ligand, *e.g.*, a GITRL fusion polypeptide subunit or multimeric GITRL fusion protein as disclosed herein can bind to a receptor, *e.g.*, GITR, with an on rate (k(on)) of greater than or equal to 10^3 M^{-1} sec^{-1}, 5×10^3 M^{-1} sec^{-1}, 10^4 M^{-1} sec^{-1} or 5×10^4 M^{-1} sec^{-1}. A ligand, *e.g.*, a GITRL fusion polypeptide subunit or multimeric GITRL fusion protein as disclosed herein can bind to a receptor, *e.g.*, GITR, with an on rate (k(on)) greater than or equal to 10^5 M^{-1} sec^{-1}, 5×10^5 M^{-1} sec^{-1}, 10^6 M^{-1} sec^{-1}, or 5×10^6 M^{-1} sec^{-1} or 10^7 M^{-1} sec^{-1}.

[0095] GITR, or "GITR receptor" is a protein, also variously termed glucocorticoid-induced TNF-related protein, tumor necrosis factor ligand superfamily member 18, TNSF18, activation-inducible TNF-related receptor, AITR, CD357, and RP5-902P8.2, is expressed on the surface of activated NK cells and antigen experienced T-cells, *e.g.*, $CD4^+$ and $CD8^+$ T cells, as well as on $CD4^+CD25^+FOXP3^+$ regulatory T-cells (Tregs; Stephens et al. *J. Immunol.* (2004) 173(8): 5008-5020). GITR is, *e.g.*, the protein of SEQ ID NO: 47. "GITR ligand" ("GITRL"), also variously termed glucocorticoid-induced TNF-related ligand, tumor necrosis factor ligand superfamily member 18 ligand, TNFSF18 ligand, TL6, activation-inducible TNF-related ligand, AITR ligand, AITRL, and RP1-15D23, is found largely on antigen presenting cells (APCs; Stephens et al. *J.*

Immunol. (2004) 173(8): 5008-5020). GITRL is expressed on the surface of cells and includes an intracellular, a transmembrane and an extracellular receptor-binding domain.

[0096] As used herein, the term "GITRL" refers to the entire GITR ligand, soluble GITR ligand, and functionally active portions of the GITR ligand. Also included within the definition of GITRL are both naturally occurring allelic variants of GITRL, GITR ligand variants which vary in amino acid sequence from naturally occurring GITR ligand molecules, and combinations of such variants, where the variants retain the ability to specifically bind to a GITR receptor. Certain variants of GITRL comprising amino acid residue substitutions are identified herein by residue number in the mature GITRL protein of SEQ ID NO: 1. For example, N161D refers to a substitution of an asparagyl residue at position 161 of a mature human GITRL of SEQ ID NO: 1 with an aspartyl residue and also to that same substitution in the equivalent position in the extracellular domain of a human GITRL of SEQ ID NO: 6 and SEQ ID NO: 8. In referring to various substitutions in the GITRL sequence of SEQ ID NO: 1, equivalent substitutions of the corresponding residues in GITRL polypeptides other than the GITRL polypeptide of SEQ ID NO: 1 are also provided herein. Such corresponding residues can be readily identified by aligning the SEQ ID NO: 1 sequence with the GITRL sequence to be substituted. For example, a GITRL peptide having a single amino acid N-terminal addition to SEQ ID NO: 1 could have a substitution of an asparagyl residue at position 162 that would be equivalent to a substitution of an asparagyl residue at position 161 of SEQ ID NO: 1.

[0097] As used herein, the term “GITRL fusion polypeptide subunit” or “GITRL FP subunit” refers to a single-chain polypeptide subunit comprising: a human IgG Fc domain; a functional trimerization domain; and a receptor binding domain of a Glucocorticoid-Induced TNF Receptor Ligand (GITRL), wherein the polypeptide subunit can self-assemble into a multimeric e.g. a trimeric or a hexameric protein. The terms “multimeric GITRL fusion protein” or “multimeric GITRL FP” refer to self-assembled multimers of a GITRL fusion polypeptide subunit including, e.g., trimers and hexamers. When an IgG Fc domain of a certain isotype is used in a GITRL FP subunit, the GITRL FP having that isotype is described as a “GITRL IgGX FP”, where X can be 1, 2, 2a, 3, 4, or 4P, *e.g.*, GITRL IgG1 FP, GITRL IgG2 FP, GITRL IgG2a FP, GITRL IgG3 FP, GITRL IgG4 FP, and GITRL IgG4P FP.

[0098] As used herein, "OX40 polypeptide" means a polypeptide or fragment thereof having at least about 85% amino acid identity to NCBI Accession No. NP_003318. OX40 is a member of the TNFR-superfamily of receptors that is expressed on the surface of antigen-activated mammalian

CD4+ and CD8+ T lymphocytes. See, for example, Paterson et al., Mol Immunol 24, 1281-1290 (1987); Mallett et al., EMBO J 9, 1063-1068 (1990); and Calderhead et al., J Immunol 151, 5261-5271 (1993)). OX40 is also referred to as CD134, ACT-4, and ACT35. OX40 receptor sequences are known in the art and are provided, for example, at GenBank Accession Numbers: AAB33944 or CAE11757.

[0099] An exemplary human OX40 amino acid sequence is provided below:

```
1   mcvgarrlgr gpcaallllg lglstvtglh cvgdtypsnd rcchecrpgn gmvsrcsrsq
61  ntvcrpcgpg fyndvvsskp ckpctwcnlr sgserkqlct atqdtvcrcr agtqpldsyk
121 pgvdcapcpp ghfspgdnqa ckpwtnctla gkhtlqpasn ssdaicedrd ppatqpqetq
181 gpparpitvq pteawprtsq gpstrpvevp ggravaailg lglvlgllgp laillalyll
241 rrdqrlppda hkppgggsfr tpiqeeqada hstlaki (SEQ ID NO: 52)
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[0100] By "OX40 ligand" is meant a polypeptide or fragment thereof having at least about 85% amino acid identity to NCBI Accession No. NP_003317 and that specifically binds the OX40 receptor. See, for example, Baum P.R., *et al. EMBO J. 13*:3992-4001(1994)). The term OX40L includes the entire OX40 ligand, soluble OX40 ligand, and fusion proteins comprising a functionally active portion of OX40 ligand covalently linked to a second moiety, *e.g.*, a protein domain. Also included within the definition of OX40L are variants which vary in amino acid sequence from naturally occurring OX4L but which retain the ability to specifically bind to the OX40 receptor. Further included within the definition of OX40L are variants which enhance the biological activity of OX40. OX40 ligand sequences are known in the art and are provided, for example, at GenBank Accession Numbers: NP_003318.

[0101] An exemplary human OX40 ligand amino acid sequence is provided below:

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MERVQPLEENVGNAARPRFERNKLLLVASVIQGLGLLLCFTYICLHFSALQVSHRYPRIQSIKVQFTE
YKKEKGFILTSQKEDEIMKVQNNSVIINCDGFYLISLKGYFSQEVNISLHYQKDEEPLFQLKKVRSVN
SLMVASLTYKDKVYLNVTTDNTSLDDFHVNGGELILIHQNPGEFCVL (SEQ ID NO: 53)
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[0102] As used herein, "OX40 agonist" means an OX40 ligand that specifically interacts with and increases the biological activity of the OX40 receptor. Desirably, the biological activity is increased by at least about 10%, 20%, 30%, 50%, 70%, 80%, 90%, 95%, or even 100%. In certain aspects, OX40 agonists as disclosed herein include OX40 binding polypeptides, such as anti-OX40

antibodies (e.g., OX40 agonist antibodies), OX40 ligands, or fragments or derivatives of these molecules.

[0103] As used herein, "OX40 antibody" means an antibody that specifically binds OX40. OX40 antibodies include monoclonal and polyclonal antibodies that are specific for OX40 and antigen-binding fragments thereof. In certain aspects, anti-OX40 antibodies as described herein are monoclonal antibodies (or antigen-binding fragments thereof), e.g., murine, humanized, or fully human monoclonal antibodies. In one particular embodiment, the OX40 antibody is an OX40 receptor agonist, such as the mouse anti-human OX40 monoclonal antibody (9B12) described by Weinberg et al., J Immunother 29, 575-585 (2006). In other embodiments, the antibody which specifically binds to OX40, or an antigen-binding fragment thereof binds to the same OX40 epitope as mAb 9B12. In another aspect, the antibody is MEDI0562. See, for example, US Pub. No. 2016/0137740.

[0104] As used herein, "OX40 ligand fusion protein (OX40L FP)" means a protein that specifically binds the OX40 receptor and increases an immune response. In one embodiment, binding of an OX40 ligand fusion protein to the OX40 receptor enhances a tumor antigen specific immune response by boosting T-cell recognition. Exemplary OX40 ligand fusion proteins are described in U.S. Patent 7,959,925, entitled, "Trimeric OX40 Immunoglobulin Fusion Protein and Methods of Use." Other OX40 ligand fusion proteins are described, for example, in US Patent No. U.S. Pat. No. 6,312,700. In one embodiment, an OX40 ligand fusion protein enhances tumor-specific T-cell immunity. In one embodiment, the OX40 ligand fusion protein is MEDI6383 (SEQ ID NO: 50). See, for example, US Pub. No. 2016/0024176.

[0105] A "trimerization domain" is an amino acid sequence within a polypeptide that promotes assembly of the polypeptide into trimers. For example, a trimerization domain can promote assembly into trimers via associations with other trimerization domains (of additional polypeptides with the same or a different amino acid sequence). The term is also used to refer to a polynucleotide that encodes such a peptide or polypeptide.

[0106] The term "Fc" domain refers to a portion of an antibody constant region. Traditionally, the term Fc domain refers to a protease (*e.g.*, papain) cleavage product encompassing the paired CH2, CH3 and hinge regions of an antibody. In the context of this disclosure, the term Fc domain or Fc refers to any polypeptide (or nucleic acid encoding such a polypeptide), regardless of the means of

production, that includes all or a portion of the CH2, CH3 and hinge regions of an immunoglobulin polypeptide.

[0107] As used herein, the term "IgG Fc domain" refers to an Fc domain of an IgG1, IgG2, IgG3, or IgG4 immunoglobulin, and variants of such Fc domains. Variants of an IgG4 Fc domain include, but are not limited to, an IgG4P Fc domain.

[0108] As used herein the term "CH2 domain" includes the portion of the Fc domain of a heavy chain molecule that extends, *e.g.*, from about amino acid 244 to amino acid 360 of an antibody using conventional numbering schemes (amino acids 244 to 360, Kabat numbering system; and amino acids 231-340, EU numbering system). It is also well documented that the CH3 domain extends from the CH2 domain to the C-terminal of the IgG molecule and comprises approximately 108 amino acids.

[0109] As used herein, the term "linker region" includes any peptide that is used to fuse or join two protein domains. Such linkers include, but are not limited to, peptides that comprise $(Gly_4)n$ motif(s), a $(Gly_4Ser)n$ motif(s) (SEQ ID NO: 19), and $Ser(Gly_4Ser)n$ motif(s) (SEQ ID NO: 22).

[0110] As used herein, the term "IgG hinge region" includes the portion of the Fc domain of a heavy chain IgG molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 amino acids and is flexible, thus allowing the two N-terminal antigen binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux *et al.*, *J. Immunol. 161*:4083 (1998)).

[0111] As used herein the term "disulfide bond" includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In certain aspects provided herein, a human IgG4 Fc domain can be mutated in the hinge region to insure disulfide bond formation between two hinge regions, specifically, a serine to proline mutation at position 228 (according to EU numbering). Human IgG4 Fc domains comprising the S228P mutation are referred to herein as "IgG4P Fc domains."

[0112] As used herein, the terms "linked," "fused" or "fusion" can be used interchangeably. These terms refer to the joining together of two more elements or components, by whatever means including chemical conjugation or recombinant means. An "in-frame fusion" refers to the joining of two or more polynucleotide open reading frames (ORFs) to form a continuous longer ORF, in a manner that maintains the correct translational reading frame of the original ORFs. Thus, a recombinant fusion protein is a single protein containing two or more segments that correspond to

polypeptides encoded by the original ORFs (which segments are not normally so joined in nature), *e.g.*, a GITRL fusion polypeptide subunit as provided herein. Although the reading frame is thus made continuous throughout the fused segments, the segments can be physically or spatially separated by, for example, in-frame linker sequence.

[0113] In the context of polypeptides, a "linear sequence" or a "sequence" is an order of amino acids in a polypeptide in an amino to carboxyl terminal direction in which amino acids that neighbor each other in the sequence are contiguous in the primary activated structure of the polypeptide.

[0114] The term “expression” as used herein refers to a process by which a gene produces a biochemical, for example, a polypeptide. The process includes any manifestation of the functional presence of the gene within the cell including, without limitation, gene knockdown as well as both transient expression and stable expression. It includes without limitation transcription of the gene into messenger RNA (mRNA), and the translation of such mRNA into polypeptide(s). If the final desired product is a biochemical, expression includes the creation of that biochemical and any precursors. Expression of a gene produces a "gene product." As used herein, a gene product can be either a nucleic acid, *e.g.*, a messenger RNA produced by transcription of a gene, or a polypeptide which is translated from a transcript. Gene products described herein further include nucleic acids with post transcriptional modifications, *e.g.*, polyadenylation, or polypeptides with post translational modifications, *e.g.*, methylation, glycosylation, the addition of lipids, association with other protein subunits, proteolytic cleavage, and the like.

[0115] As used herein the terms "treat," "treatment," or "treatment of" when used in the context of treating cancer (*e.g.*, in the phrase "treating a cancer patient ") refers to reducing the potential for disease pathology, reducing the occurrence of disease symptoms, *e.g.*, to an extent that the subject has a longer survival rate or reduced discomfort. For example, treating can refer to the ability of a therapy when administered to a subject, to reduce disease symptoms, signs, or causes. Treating also refers to mitigating or decreasing at least one clinical symptom and/or inhibition or delay in the progression of the condition and/or prevention or delay of the onset of a disease or illness.

[0116] As used herein the terms "treat," "treatment," or "treatment of" when used in the context of treating a viral infection (e.g., in the phrase "treating a viral infection") refers to reducing the pathological conditions and/or symptoms associated with the viral infection.

[0117] By "subject" or "individual" or "animal" or "patient" or “mammal,” is meant any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian

subjects include humans, domestic animals, farm animals, sports animals, and zoo animals, including, *e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, bears, and so on.

[0118] The term "pharmaceutical composition" refers to a preparation that is in such form as to permit the biological activity of the active ingredient to be effective, and that contains no additional components that are unacceptably toxic to a subject to which the composition would be administered. Such composition can be sterile.

[0119] An "effective amount" of an antibody as disclosed herein is an amount sufficient to carry out a specifically stated purpose. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose.

GITRL Fusion Polypeptide Subunits

[0120] The present disclosure relates to a GITRL fusion polypeptide subunit that can assemble into a multimeric, *e.g.,* trimeric or hexameric protein with improved properties that include, but are not limited to, improved yield when expressed in transfected mammalian cell cultures; improved binding affinity for GITR; improved activity in various biological assays and/or improved homogeneity when purified or partially purified in comparison to previously disclosed polypeptides containing GITRL (*See, e.g.,* Wyzgol *et al.*, *J Immunol* 2009; 183:1851-1861). GITRL fusion polypeptide subunits provided herein can comprise an IgG Fc domain, *e.g.*, a human IgG Fc domain, a trimerization domain, and the receptor binding domain of human GITRL. An exemplary embodiment is illustrated schematically in FIG. 7. Typically, the IgG Fc domain, the trimerization domain and the GITRL receptor binding domain are arranged in an N-terminal to C-terminal direction. An exemplary GITRL IgG1 fusion polypeptide subunit is represented by SEQ ID NO: 6.

[0121] In certain embodiments, the GITRL receptor binding domain is an extracellular domain of a human GITRL. The sequence of one such domain is represented by SEQ ID NO: 37.

[0122] In certain aspects, the GITRL receptor binding domain is a GITRL variant extracellular domain. GITRL variant extracellular domains that can be used include, but are not limited to, polypeptides having at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity across the entire length of SEQ ID NO: 37. In certain aspects, the GITRL variant extracellular domain is a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity across the entire length of SEQ ID NO: 34. In certain aspects, the

GITRL variant extracellular domain residue corresponding to asparagyl 161 of SEQ ID NO: 1 is substituted with any amino acid or with an aspartyl residue. GITRL variant extracellular domains where the residue corresponding to asparagyl 161 of SEQ ID NO: 1 is substituted with any amino acid or with an aspartyl residue include, but are not limited to, polypeptides having at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99%, sequence identity across the entire length of SEQ ID NO: 35, SEQ ID NO: 36, and SEQ ID NO: 37. Such substitutions can reduce or eliminate N-linked glycosylation of this site in the GITRL variant extracellular domain. In certain aspects, the GITRL variant extracellular domain residue corresponding to asparagyl 106 of SEQ ID NO: 1 is substituted with an alanyl residue. In certain contexts, N106A substitutions of hGITRL can result in increased binding to GITR and an improved T-cell proliferation response (Chattopadhyay et al. (2007) *Proc. Natl. Acad. Sci. USA* 104(49):19452-19457). In certain aspects, GITRL variant extracellular domain residues corresponding to Glu 52, Phe 62, Pro 66, Pro 67, Met 71, Pro 77, Val 79, Asn 92, Ser 83, Gly 99, Asn 104, Pro 112, Arg 116, Met 123, Asn 153, Val 158, Asn 161, Iso 167, Iso 168, and combinations thereof are independently substituted with a non-naturally occurring amino acid residue or an amino acid residue of an allelic variant. A non-limiting example of a GITRL variant extracellular domain is provided in SEQ ID NO: 35, where X_1= Glu or Ala, X_2= Ser or Phe, X_3=Thr or Pro, X_4=Leu or Ser, X_5=Thr or Met, X_6= Leu or Pro, X_7= Met or Val, X_8= Thr, Phe, or Ser, X_9= Ser or Gly, X_{10}= Arg or Pro, X_{11}= Trp or Arg, X_{12}= Leu or Met, X_{13}= Ser or Asn, X_{14}=Phe or Val, X_{15}=any amino acid other than Asn or Asp, X_{16}= Val or Ile, X_{17}=Leu or Ile, and where X_1-X_{17} are independently selected and can be present in any combination.

[0123] Any GITRL polypeptide sequence that retains the desired property of binding to the GITR receptor is suitable in the fusion polypeptides and methods described herein.

[0124] Adjacent to the GITRL receptor binding domain is a trimerization domain. The term "adjacent" includes, for example, contiguous with, or associated via a linker region or heterologous agent. Such domains, when contiguous to one another, are domains that are fused directly to one another. The trimerization domain serves to promote self-assembly of individual GITRL fusion polypeptide subunits into a trimeric protein or into a hexameric protein. In certain embodiments, a GITRL fusion polypeptide subunit with a trimerization domain self-assembles into a hexameric GITRL fusion protein. In one embodiment, the trimerization domain is a coiled coil domain, e.g., a leucine zipper domain. An exemplary trimeric leucine zipper domain is the engineered yeast GCN4 pII variant described by Harbury *et al.* (1993) Science 262:1401-1407, the disclosure of which is

incorporated herein for all purposes. Exemplary trimerization domains include: TNF receptor-associated factor-2 (TRAF2) (GENBANK® Accession No. Q12933 [gi:23503103]; amino acids 310-349); Thrombospondin 1 (Accession No. PO7996 [gi:135717]; amino acids 291-314); Matrilin-4 (Accession No. O95460 [gi:14548117]; amino acids 594-618; cartilage matrix protein (matrilin-1) (Accession No. NP002370 [gi:4505111]; amino acids 463-496; Heat shock transcription factor (HSF) (Accession No. AAX42211 [gi:61362386]; amino acids 165-191; and Cubilin (Accession No. NP001072 [gi:4557503]; amino acids 104-138.

[0125] In certain aspects, the trimerization domain comprises an alpha-helical coiled coil domain. Useful alpha-helical coiled coil domains include, but are not limited to those derived from Matrilin 1, Coronin 1a, dystrophia myotonica kinase (DMPK), Langerin, and combinations thereof. Such derivatives include, but are not limited to, coiled coil domains with wild type sequences as well as variants comprising one or more amino acid substitutions in the coiled coil domain wild type sequence. Coronin 1a proteins containing Coronin 1a trimerization domains are also sometimes synonymously referred to as any of Coronin-like protein A, Clipin-A, Coronin-like protein p57, Tryptophan aspartate-containing coat protein and the HUGO name CORO1A. Non-limiting examples of wild-type coiled coil domains derived from various proteins that can be used include Matrilin 1 (SEQ ID NO: 28), DMPK (SEQ ID NO: 30), Langerin (SEQ ID NO: 32), and Coronin 1a (SEQ ID NO: 11). Variants of the alpha-helical coiled coil domains can include allelic variants, engineered variants, and combinations thereof. Alpha-helical coiled coil domains are typically organized in heptad sequence repeats "hpphcpc" (or abcdefg) that can be independently substituted at one or more of the "h" positions ("a" and/or "d" positions) in one or more of the repeats with Alanine, Leucine, Isoleucine, or Valine residues. In such "hpphcpc" heptad repeats, h represents hydrophobic residues, c represents, typically, charged residues, and p represents polar (and, therefore, hydrophilic) residues. Alpha-helical coiled coil domain variants of Matrilin 1, Coronin 1a, dystrophia myotonica kinase (DMPK), and Langerin where one or more of the "a" and/or "d" positions in one or more of the heptad repeats of those trimerization domains are substituted with Alanine, Leucine, Isoleucine, or Valine residues are provided. Non-limiting examples of alpha-helical coiled coil domain variants that can be used include Matrilin 1 (SEQ ID NO: 29), DMPK (SEQ ID NO: 31), Langerin (SEQ ID NO: 32), and Coronin 1a (SEQ ID NO: 12-18). In certain aspects, a variant Matrilin 1 trimerization domain used in the GITRL fusion polypeptide subunit can include, but is not limited to, a polypeptide having at least 70%, 75%, 80%,

85%, 90%, 95%, 97%, 98%, or 99% sequence identity across the entire length of SEQ ID NO: 29. In certain aspects, a variant DMPK trimerization domain used in the GITRL fusion polypeptide subunit can include, but is not limited to, a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% sequence identity across the entire length of SEQ ID NO: 31. In certain aspects, a variant Langerin trimerization domain used in the GITRL fusion polypeptide subunit can include, but is not limited to, a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% sequence identity across the entire length of SEQ ID NO: 33. In certain aspects, a Coronin 1a trimerization domain used in the GITRL fusion polypeptide subunit can include, but is not limited to, a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% sequence identity across the entire length of SEQ ID NO: 11. In certain aspects, a Coronin 1a trimerization domain variant consensus sequence where one or more of the "A" and/or "D" positions in one or more of the heptad repeats of those trimerization domains are substituted with Alanine, Leucine, Isoleucine, or Valine residues that can be used in the GITRL fusion polypeptide subunits is provided as SEQ ID NO: 10. Exemplary coronin 1a wt and variant sequences that can be used in a GITRL fusion polypeptide subunit are presented in Table A.

Table A Coronin 1a Coiled Coil domains and variants thereof

Trimerization Domain	**Sequence**	**SEQ ID NO:**	**ProCoil prediction score**	**Predicted oligomeric state**
heptad sequence repeat	hpphcpc-hpphcpc-hpphcpc-hpphcpc-hpph; where h represents hydrophobic residues, c represents, typically, charged residues, and p represents polar (and, therefore, hydrophilic) residues. The positions of the heptad repeat can also be denoted by the lowercase letters *abcdefg*.	NA		
hCor1a Consensus (X= Ala, Leu, Ile, or Val)	XSRXEEEXRKXQATXQELQKRXDRLEETVQAK	10		
hCor1a wt	VSRLEEEMRKLQATVQELQKRLDRLEETVQAK	11	0.101544216	TRIMER
hCor1a variant 1	VSRLEEEIRKLQATVQELQKRLDRLEETVQAK	12	0.205919088	TRIMER
hCor1a variant 2	VSRIEEEIRKLQATVQELQKRLDRLEETVQAK	13	0.386363142	TRIMER
hCor1a variant 3	ISRIEEEIRKLQATVQELQKRLDRLEETVQAK	14	0.431631235	TRIMER
hCor1a variant 4	ISRIEEEIRKIQATVQELQKRLDRLEETVQAK	15	0.509021117	TRIMER
hCor1a variant 5	ISRIEEEIRKIQATVQELQKRIDRLEETVQAK	16	0.668080151	TRIMER

Trimerization Domain	Sequence	SEQ ID NO:	ProCoil prediction score	Predicted oligomeric state
hCor1a variant 6	ISRIEEEIRKINATVQELQKRIDRLEETVQAK	17	0.785463418	TRIMER
hCor1a variant 7	ISRIEEEIRKINATIQELQKRIDRLEETVQAK	18	0.729421966	TRIMER

[0126] A GITRL fusion polypeptide subunit having certain trimerization domains provided herein (*e.g.* a coronin 1a trimerization domain or variant thereof) can exhibit improved properties when compared to other GITRL fusion polypeptide subunits having distinct trimerization domains. More specifically, a GITRL fusion polypeptide subunit having a coronin 1a trimerization domain or variant thereof can exhibit improved properties that include, but are not limited to, improved yield when expressed in transformed mammalian cell cultures; e.g. CHO cells; improved binding affinity for GITR; improved activity in various biological assays (*e.g.* activation of the NF-κB signaling pathway); and/or improved homogeneity when purified or partially purified. Without seeking to be limited by theory, it is believed that such improved properties of a GITRL fusion polypeptide subunit having a coronin 1a trimerization domain or variant thereof can facilitate the manufacture of GITRL fusion polypeptide subunits and/or improve the efficacy of multimeric GITRL fusion proteins in various therapeutic applications.

[0127] In addition to the GITRL receptor binding domain and the trimerization domain, a GITRL fusion polypeptide subunit as provided herein includes an immunoglobulin domain, such as a constant region or "Fc" domain. In certain aspects the present disclosure provides both a human IgG1 and IgG4 Fc domain including at least the hinge region. In certain aspects the human IgG1 or IgG4 Fc domain further includes the CH2 domain. In certain aspects the human IgG1 or IgG4 Fc domain further includes the CH3 domain. In certain aspects, the Fc domain is a human IgG1 Fc domain or variant thereof. A human IgG Fc domain can comprise a peptide having an amino acid sequence having at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 21. Variants of an IgG Fc domain (*e.g.* an IgG1 Fc domain) that can be used include, but are not limited to, the IgG Fc domain containing one or more amino acid residues independently selected from the group consisting of 252Y, 254T, 256E, and combinations thereof, wherein the residues are numbered according to EU numbering. In certain aspects the hinge region of an IgG4 Fc region can comprise a serine to proline mutation at position 228 (according to EU

numbering) which confers complete inter-heavy chain disulfide bond formation. In certain aspects the IgG4 hinge region comprises amino acids 1 to 12 of SEQ ID NO: 40. In certain aspects the human IgG4 Fc domain is an IgG4P-Fc domain with the S228P mutation of SEQ ID NO: 38.

[0128] In combination with the trimerization domain which brings together three GITRL receptor binding domains, the disulfide bond formation between two IgG Fc domains results in the formation of a hexameric protein (Fig. 7). Thus, the immunoglobulin domain serves as a dimerization domain that promotes assembly between two trimeric GITRL fusion proteins into a stable hexamer (that is a multimer that contains six GITRL fusion polypeptide subunits) via interactions between unpaired immunoglobulin domains. In certain aspects, a human IgG4 Fc domain provides stability to the hexameric protein without promoting effector functions such as antibody dependent cellular cytotoxicity (ADCC) or complement-dependent cellular cytotoxicity. In other aspects, a human IgG1 Fc domain provides stability to the hexameric protein while promoting effector functions such as antibody dependent cellular cytotoxicity (ADCC) or complement-dependent cellular cytotoxicity.

[0129] In certain aspects, this disclosure provides a single-chain polypeptide subunit that self-assembles to form a hexameric protein that can specifically bind to GITR. An exemplary polypeptide subunit comprises a human IgG Fc domain, a functional trimerization domain, and a receptor binding domain of GITRL. In specific aspects the polypeptide subunit can self-assemble into a hexameric protein. In certain aspects, the polypeptide subunit is arranged, from the amino terminus to the carboxy terminus, as follows: the human IgG Fc domain, followed by the trimerization domain, followed by the GITRL receptor binding domain. The three domains can be immediately adjacent. For example, in certain aspects the carboxy terminus of the human IgG Fc domain is fused directly to the amino terminus of the trimerization domain, and the carboxy terminus of the trimerization domain is fused directly to the amino terminus of the GITRL receptor binding domain. Alternatively, two or three domains can be separated by one or more linkers, spacers, or other heterologous polypeptides. Useful linkers include, but are not limited to, a $(Gly_4)n$ motif, a $(Gly_4Ser)n$ motif (SEQ ID NO: 19), $Ser(Gly_4Ser)n$ motif (SEQ ID NO: 22), GGGGSGGGGSGGGGSAL (SEQ ID NO: 23), or GGGGSGGGGSGGGGSA (SEQ ID NO: 24), and combinations thereof, where n is a positive integer selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10.

[0130] In certain aspects, a GITRL fusion polypeptide subunit as provided herein can specifically bind to human GITR. In certain aspects, a GITRL fusion polypeptide subunit as provided herein can

specifically bind to a non-human primate GITR, *e.g.*, cynomolgus monkey GITR or rhesus monkey GITR. In certain aspects, a GITRL fusion polypeptide subunit as provided herein does not bind to mouse GITR or to rat GITR.

[0131] A GITRL fusion polypeptide subunit as provided herein can contain one or more conservative amino acid changes, *e.g.*, up to ten conservative changes (*e.g.*, two substituted amino acids, three substituted amino acids, four substituted amino acids, or five substituted amino acids, etc.), provided that the changes can be made in the polypeptide without changing a biochemical function of the GITRL fusion polypeptide subunit or multimeric GITRL FP.

[0132] For example, one or more conservative changes can be made in a GITRL receptor binding domain without changing its ability to bind to GITR. Similarly, one or more conservative changes can be made in trimerization domain without altering its ability to trimerize.

[0133] GITRL fusion polypeptide subunits provided herein can also contain one or more amino acid substitutions, insertions, or deletions that block or reduce N-linked glycosylation of asparagyl residue 161 of GITRL. In certain aspects, asparagyl residue 161 of GITRL is substituted with any amino acid other than an asparagyl residue to block or reduce glycosylation. In certain aspects, asparagyl residue 161 of GITRL is substituted with an aspartyl residue, *e.g.*, is a N161D variant of GITRL as shown in SEQ ID NO: 4. In certain aspects, N-linked glycosylation of asparagyl residue 161 of GITRL is blocked or reduced by amino acid substitutions, insertions, or deletions that disrupt the N-linked glycosylation site sequence NNT of GITRL residues 161-163 such that this sequence no longer conforms to the canonical N-linked glycosylation site sequence NX(T, S, or C). In certain embodiments, substitution of threonyl residue 163 with an amino acid residue other than serinyl or cysteinyl can be used to block or reduce N-linked glycosylation of asparagyl residue 161 of GITRL, provided that the changes can be made in the polypeptide without changing a biochemical function of the GITRL fusion polypeptide subunit or multimeric GITRL fusion protein.

[0134] Additionally, part of a polypeptide domain can be deleted without impairing or eliminating all of its functions. Similarly, insertions or additions can be made in the polypeptide chain, for example, adding epitope tags, without impairing or eliminating its functions, as described below. Other modifications that can be made without materially impairing one or more functions of a polypeptide include, for example, *in vivo* or *in vitro* chemical and biochemical modifications that incorporate unusual amino acids. Such modifications include, for example, acetylation, carboxylation, phosphorylation, glycosylation, labeling, *e.g.*, with radionuclides, and various

enzymatic modifications, as will be readily appreciated by those of ordinary skill in the art. A variety of methods for labeling polypeptides, and labels useful for such purposes, are well known in the art, and include radioactive isotopes such as ^{32}P, fluorophores, chemiluminescent agents, enzymes, and antiligands.

[0135] The fusion polypeptide subunit can further include a heterologous agent, *e.g.*, a stabilizing agent, an immune response modifier, or a detectable agent. In certain aspects the heterologous agent comprises one or more additional polypeptide sequences fused to the polypeptide subunit via a peptide bond, such as a signal sequence (*e.g.*, a secretory signal sequence), a linker sequence, an amino acid tag or label, or a peptide or polypeptide sequence that facilitates purification. In certain aspects, the heterologous polypeptide can be fused to the N-terminus of the IgG-Fc domain, the heterologous polypeptide can be fused to the C-terminus of the receptor binding domain of GITRL, the heterologous polypeptide can be fused to the C-terminus of the IgG-Fc domain and to the N-terminus of the trimerization domain, or the heterologous polypeptide can be fused to the C-terminus of the trimerization domain and to the N-terminus of the receptor binding domain of GITRL. Alternatively the heterologous polypeptide can be fused internally within any of the IgG Fc domain, the trimerization domain, or the GITRL receptor binding domain, as long as the functional characteristics of the domains are maintained.

[0136] In certain aspects, the heterologous agent can be chemically conjugated to the polypeptide subunit. Exemplary heterologous agents that can be chemically conjugated to the polypeptide subunit include, without limitation, linkers, drugs, toxins, imaging agents, radioactive compounds, organic and inorganic polymers, and any other compositions which might provide a desired activity that is not provided by the polypeptide subunit itself. Specific agents include, without limitation, polyethylene glycol (PEG), a cytotoxic agent, a radionuclide, an imaging agent, biotin.

[0137] In certain aspects, the GITRL fusion polypeptide subunits as well as any trimers or hexameric proteins comprising those subunits can be used as controls, reference standards for developing or executing diagnostic assays (*e.g.* for dosing determinations), or research tools. For example, the disclosure provides a GITRL fusion polypeptide subunit as described above, where the GITRL receptor binding domain comprises any of SEQ ID NOs: 34, 35, 36, or 37. In certain aspects, the control, reference standard, or tool can comprise a GITRL fusion polypeptide subunit having at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% sequence identity to SEQ ID NO: 6. In another example, the disclosure provides GITRL fusion polypeptide subunits that can

form a multimeric protein as described above, but where the GITRL receptor binding domain is a mouse or rat GITRL receptor binding domain, and the Fc domain is an Fc domain of human or murine origin, and the multimerisation domain is, *e.g.,* a trimerization domain, *e.g.,* coronin 1a trimerization domain. This fusion protein can be used to conduct *in vivo* experiments in rodents. Without seeking to be limited by theory, IgG Fc domain effector functions conferred by distinct IgG isotypes in mice generally differ from those conferred by the same IgG isotype in humans. However, it has been previously shown that specific mouse IgG isotypes are considered analogous or comparable to alternative IgG isotypes in human, for example mouse IgG2a is considered analogous to human IgG1 and mouse IgG1 is considered comparable to human IgG4. As such, results obtained in mice with a given mouse IgG Fc domain isotype can be used to predict results obtained in humans with analogous or comparable human IgG isotypes.

Multimeric GITRL Fusion Proteins

[0138] A GITRL fusion polypeptide subunit as described above can self-assemble into a hexameric GITRL FP. Accordingly, the disclosure provides a hexameric protein comprising six polypeptide subunits as described above. An exemplary polypeptide subunit described in the Examples self-assembles into a hexameric protein designated herein as a "hexameric GITRL FP." A non-limiting example of an amino acid sequence of a GITRL fusion polypeptide subunit that self-assembles into a hexameric GITRL FP is provided in SEQ ID NO: 6. Nonetheless, one of ordinary skill in the art will recognize in light of the instant disclosure that numerous other sequences also fulfill the criteria set forth herein for hexameric GITRL FPs. Hexameric GITRL FPs comprising six GITRL fusion polypeptide subunits with at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% sequence identity to SEQ ID NO: 6 are also provided herein.

[0139] In certain aspects, certain GITRL fusion polypeptide subunits provided herein can also self-assemble into a trimeric GITRL FP comprising three GITRL fusion polypeptide subunits. This could occur, for example, where an Fc domain that cannot dimerize is used in a GITRL FP to generate a trimeric protein. Examples of Fc domains that cannot dimerize and are thus suitable for production of trimeric GITRL FP include, but are not limited to, monomeric IgG1 Fc molecules (Ying et al. J Biol Chem. Jun 1, 2012; 287(23): 19399–19408) and monovalent IgG4 molecules (Wilkinson, et al. MAbs. May 1, 2013; 5(3): 406–417).

[0140] A multimeric GITRL fusion protein as provided herein, *e.g.*, a hexameric GITRL FP, can specifically bind to GITR as expressed on primary antigen experienced T-cells, *e.g.*, primary antigen experienced T-cells, from human, cynomolgus monkey, rhesus monkey, or any combination thereof.

[0141] A hexameric protein as provided herein, *e.g.*, a hexameric GITRL FP, can specifically bind to recombinant GITR. In certain aspects, a hexameric protein as provided herein, *e.g.*, a hexameric GITRL FP, can bind to recombinant human GITR with a binding affinity of about 1 nM to about 120 nM, e.g., about 10 nM to about 100 nM, e.g., about 20 nM to about 100 nM, e.g., about 60 nM to about 100 nM, all as measured by kinetic exclusion assay. For example, a hexameric protein as provided herein, *e.g.*, a hexameric GITRL FP, can bind to recombinant human GITR with a binding affinity of about 0.1 nM, about 0.5 nM, about 1 nM, about 2 nM, about 3 nM, about 4 nM, about 5 nM, about 6 nM, about 7 nM, about 8 nM, about 9 nM, about 10 nM, about 20 nM, about 30 nM, about 40 nM, about 50 nM, about 60 nM, about 70 nM, about 80 nM, about 90 nM, about 100 nM, about 120 nM, about 250 nM or about 500 nM, all as measured by a kinetic exclusion assay. In certain aspects, a hexameric protein as provided herein, *e.g.*, a hexameric GITRL FP, can bind to recombinant human GITR with a binding affinity of any of about 0.1 nM, about 0.5 nM, about 1 nM, about 2 nM, about 3 nM, about 4 nM, about 5 nM, about 6 nM, about 7 nM, about 8 nM, about 9 nM, about 10 nM, about 20 nM, about 30 nM, about 40 nM, about 50 nM, about 60 nM, or about 70 nM to any of about 90 nM, about 100 nM, about 120 nM, about 250 nM, or about 500 nM, all as measured by kinetic exclusion assay. In certain aspects, a hexameric protein as provided herein, *e.g.*, a hexameric GITRL FP, can bind to recombinant human GITR with a binding affinity of about 82 nM as measured by kinetic exclusion assay. Binding affinity can be measured by a number of different methods and/or instruments, and the relative binding affinities can vary depending on the method or instrument, as is well understood by persons or ordinary skill in the art.

[0142] In another example, a hexameric protein as provided herein, *e.g.*, GITRL FP, can bind to cynomolgus monkey GITR expressed on primary antigen experienced cynomolgus monkey T cells, for example $CD4^+$ or $CD8^+$ T cells.

[0143] In certain aspects, a hexameric protein as provided herein, *e.g.*, GITRL FP, can induce dose-dependent proliferation of antigen experienced $CD3^+$ T cells in a plate-based assay. For example, in an *in vitro* assay using a hexameric protein as provided herein, *e.g.*, GITRL, a 20% maximal proliferation response (EC_{20}) can be achieved in primary antigen experienced human $CD3^+$ T cells at a hexameric protein concentration of about 0.03 nM to about 0.2 nM, *e.g.*, about 0.16 nM, a 50%

maximal proliferation response (EC_{50}) can be achieved in primary antigen experienced human $CD3^+$ T cells at a hexameric protein concentration of about 0.2 nM to about 1 nM, *e.g.*, about 0.4 nM, and a 90% maximal proliferation response (EC_{90}) can be achieved in primary antigen experienced human $CD3^+$ T cells at a hexameric protein concentration of about 0.7 nM to about 5 nM, *e.g.*, about 1.8 nM, all as measured by thymidine incorporation.

[0144] In certain aspects, a hexameric protein as provided herein, *e.g.*, GITRL IgG Fusion Protein, can induce dose-dependent cytokine release from antigen experienced T cells, *e.g.*, human primary antigen experienced $CD3^+$ T cells. In certain aspects, the released cytokine is IFNγ, TNFα, IL-5, IL-10, IL-2, IL-4, IL-13, IL-8, IL-12 p70, IL-1β, or any combination thereof. In certain aspects, the cytokine is IFNγ, TNFα, IL-5, IL-10, or any combination thereof. Similarly, a hexameric protein as provided herein, *e.g.*, GITRL FP, can enhance Tcell proliferation and cytokine release in primary antigen experienced cynomolgus monkey T cells and in primary antigen experienced rhesus monkey T cells.

[0145] In additional aspects, a hexameric protein as provided herein, e.g., GITRL FP can activate the NFκB pathway in GITR expressing T cells. For example, a hexameric protein as provided herein, e.g., GITRL FP can activate the NFκB pathway in GITR-expressing Jurkat NFκB-luciferase reporter cells that produce luciferase in response to stimulation of the NFκB signaling pathway, with an EC_{50} of about 20 pM to about 300 pM, e.g., about 182 pM for hexameric GITRL IgG1FP, and 289 pM for hexameric GITRL IgG4FP. Alternatively, a hexameric protein as provided herein, e.g., GITRL FP can activate the NFκB pathway in cells expressing human GITR, cynomolgus monkey GITR or rhesus monkey GITR.

[0146] In yet another aspect a hexameric protein as provided herein, *e.g.*, GITRL FP can facilitate cancer treatment, *e.g.*, by slowing tumor growth, stalling tumor growth, or reducing the size of existing tumors, when administrated as an effective dose to a subject in need of cancer treatment. In certain aspects the facilitation of cancer treatment can be achieved in the presence of T cells. In certain aspects, a hexameric protein as provided herein, *e.g.*, GITRL FP, when administered as an effective dose to a subject in need of treatment, can reduce tumor growth by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or at least 100% compared to administration of an isotype-matched control molecule.

[0147] In yet another aspect a hexameric protein as provided herein, e.g., GITRL FP can facilitate treatment of viral infections, e.g., by slowing viral multiplication, stalling viral multiplication, or reducing infection reoccurrance or infection reoccurance frequency, when administrated as an effective dose to a subject in need of the treatment, e.g. a subject infected with the virus. Such subjects can have either a chronic or a latent viral infection. In certain aspects, the treatments are of subjects having latent viral infections and reduce infection reoccurrence or infection reoccurrence frequency in comparison to subjects treated with a placebo. In certain aspects the facilitation of treatment of the viral infection can be achieved in the presence of T cells. In certain aspects, a hexameric protein as provided herein, e.g., GITRL FP, when administered as an effective dose to a subject in need of treatment, can reduce viral load by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 100% compared to administration of an isotype-matched control molecule. In certain aspects, a hexameric protein as provided herein, e.g., GITRL FP, when administered as an effective dose to a subject in need of treatment, can reduce the reoccurrence of the viral infection by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, or at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 100% compared to administration of an isotype-matched control molecule.

[0148] In yet further aspects, a hexameric protein as provided herein, *e.g.*, a GITRL IgG4 Fusion Protein, can induce proliferation of antigen experienced, GITR-expressing T cells through binding to GITR, but does not substantially trigger complement-dependent or antibody-dependent cytotoxicity against the antigen experienced T cells. Moreover in certain aspects, a hexameric protein as provided herein, *e.g.*, a multimeric GITRL IgG1 Fusion Protein, can induce proliferation of antigen experienced, GITR-expressing T cells through binding to GITR, but does bind to C1q and trigger Fc receptor-mediated antibody-dependent cellular cytotoxicity or phagocytosis of antigen experienced $CD4^+$ T cells, in particular $FOXP3^+$ $CD4^+$ regulatory T cells.

Polynucleotides Encoding GITRL IgG Fusion Polypeptide Subunits

[0149] The disclosure further provides a polynucleotide comprising a nucleic acid that encodes a GITRL fusion polypeptide subunit or a hexameric protein as provided herein, *e.g.*, GITRL FP. An exemplary polynucleotide sequence that encodes a GITRL fusion polypeptide subunit is represented by SEQ ID NO: 5. In certain aspects, nucleic acid sequences encoding the IgG Fc domain, the

trimerization domain and the GITRL receptor binding domains are joined in a 5' to 3' orientation, *e.g.*, contiguously linked in a 5' to 3' orientation. In other aspects, the provided polynucleotide can further comprise a signal sequence encoding, *e.g.*, a secretory signal peptide or membrane localization sequence. Polynucleotides encoding any and all GITRL fusion polypeptide subunits or multimeric, *e.g.*, hexameric proteins comprising the subunits, are provided by this disclosure.

[0150] In certain aspects, the disclosure provides a polynucleotide comprising a nucleic acid that encodes GITRL fusion polypeptide subunit. In certain aspects the nucleic acid sequence comprises SEQ ID NO: 5.

[0151] Polynucleotides encoding a GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP include deoxyribonucleotide (DNA, cDNA) or ribodeoxynucleotide (RNA) sequences, or modified forms of either nucleotide, which encode the fusion polypeptides described herein. The term includes single and double stranded forms of DNA and/or RNA.

[0152] Also provided are polynucleotides comprising nucleic acid sequences comprising one or a small number of deletions, additions and/or substitutions. Such changes can be contiguous or can be distributed at different positions in the nucleic acid. A substantially identical nucleic acid sequence can, for example, have 1, or 2, or 3, or 4, or even more nucleotide deletions, additions and/or substitutions. In certain aspects, the one or more deletions, additions and/or substitutions do not alter the reading frame encoded by the polynucleotide sequence, such that a modified ("mutant") but substantially identical polypeptide is produced upon expression of the nucleic acid.

[0153] The similarity between amino acid (and/or nucleic acid) sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity); the higher the percentage, the more similar are the primary activated structures of the two sequences. “Percent (%) identity” is defined herein as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a selected sequence, after aligning the sequences and introducing gaps in the candidate and/or selected sequence, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative amino acid substitutions as part of the sequence identity.

[0154] Thus, a polynucleotide comprising a nucleic acid that encodes a GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP, can be at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at

least 96%, frequently at least 97%, 98%, or 99% identical to SEQ ID NO: 5 or to at least one subsequence thereto. Alignment for purposes of determining percent homology (*i.e.*, sequence similarity) or percent identity can be achieved in various ways that are within the skill in the art, for instance, using publicly or proprietary algorithms. For instance, sequence similarity can be determined using pairwise alignment methods, *e.g.*, BLAST, BLAST-2, ALIGN, or ALIGN-2 or multiple sequence alignment methods such as Megalign (DNASTAR), ClustalW or T-Coffee software. Those skilled in the art can determine appropriate scoring functions, *e.g.*, gap penalties or scoring matrices for measuring alignment, including any algorithms needed to achieve optimal alignment quality over the full-length of the sequences being compared. In addition, sequence alignment can be achieved using structural alignment methods (*e.g.*, methods using secondary or tertiary structure information to align two or more sequences), or hybrid methods combining sequence, structural, and phylogenetic information to identify and optimally align candidate protein sequences.

[0155] The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, J. Mol. Biol. (1990) 215:403) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD.) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. A description of how to determine sequence identity using this program is available on the NCBI website on the internet.

[0156] Thus, a nucleic acid sequence that is substantially identical, or substantially similar to SEQ ID NO: 5 is encompassed within the present disclosure. A sequence is substantially identical to SEQ ID NO: 5 if the sequence is identical, on a nucleotide by nucleotide basis, with at least a subsequence of the reference sequence (*e.g.*, SEQ ID NO: 4). Such nucleic acids can include, *e.g.*, insertions, deletions, and substitutions relative to SEQ ID NO: 4. For example, such nucleic acids can be at least about 70%, 80%, 90%, 95%, 96%, 97%, 98% or even 99% identical to a reference nucleic acid, or encode a polypeptide at least about 70%, 80%, 90%, 95%, 96%, 97%, 98% or even 99% identical to the reference polypeptide sequence, *e.g.*, SEQ ID NO: 4.

[0157] Additionally, a polynucleotide comprising a nucleic acid encoding a GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP, can also include polynucleotide sequences, such as expression regulatory sequences and/or vector sequences that facilitate the expression or replication of the nucleic acids. Similarly, a polynucleotide comprising a nucleic acid encoding a GITRL fusion polypeptide subunit, or a hexameric protein as provided

herein, *e.g.*, GITRL FP, can include additional coding sequences that confer functional attributes on the encoded polypeptide. Such sequences include, but are not limited to, secretory signal sequences and membrane localization signals. A non-limiting example of a nucleic acid encoding a signal peptide that is operably linked to a nucleic acid encoding a GITRL fusion polypeptide subunit is provided in SEQ ID NO: 7.

[0158] A polynucleotide comprising a nucleic acid encoding a GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP, can be introduced into a vector, such as a eukaryotic expression vector, by conventional techniques. Accordingly, the disclosure provides a vector comprising a polynucleotide as provided herein. An expression vector is designed to permit the transcription of the polynucleotide sequence encoding a GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP in cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. Numerous expression vectors are known to those of skill in the art, and are available commercially, or can be assembled from individual components according to conventional molecular biology procedures.

[0159] The choice of expression control sequence and expression vector will depend upon the choice of host cell. A wide variety of expression host/vector combinations can be employed. Useful expression vectors for eukaryotic hosts include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pCR 1, pBR322, pMB9 and their derivatives, wider host range plasmids, such as M13 and filamentous single-stranded DNA phages.

[0160] Suitable host cells for expression of a GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP include prokaryotes, yeast, insect or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed. Additional information regarding methods of protein production, including antibody production, can be found, *e.g.*, in U.S. Patent Publication No. 2008/0187954, U.S. Patent Nos. 6,413,746 and 6,660,501, and International Patent Publication No. WO 2004/009823, each of which is hereby incorporated by reference herein in its entirety.

[0161] Also provided is a host cell comprising a polynucleotide or vector as provided herein. Various mammalian or insect cell culture systems can be advantageously employed to express polypeptide subunits or hexameric proteins provided herein. Expression of recombinant proteins in mammalian cells can be performed because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include HEK-293 and HEK-293T, the COS-7 lines of monkey kidney cells, described by Gluzman (Cell 23:175, 1981), and other cell lines including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors can comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, BioTechnology 6:47 (1988).

[0162] The expression and purification of proteins, such as a GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP, can be performed using standard laboratory techniques. Examples of such methods are discussed or referenced herein. After expression, purified proteins have many uses, including for instance functional analyses, antibody production, and diagnostics, as well as the prophylactic and therapeutic uses described below. For example, polypeptide subunits or hexameric proteins provided herein can be used to produce pharmaceutical compositions, including vaccine compositions suitable for prophylactic and/or therapeutic administration.

[0163] A GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP produced by a transformed host, can be purified according to any suitable method. Such standard methods include chromatography (*e.g.*, ion exchange, affinity and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexahistidine, maltose binding domain, influenza coat sequence and glutathione-S-transferase can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be physically characterized using such techniques as proteolysis, nuclear magnetic resonance and x-ray crystallography.

[0164] For example, supernatants from systems that secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, can be employed to further purify an influenza B/Yamagata virus-binding molecule. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

[0165] A GITRL fusion polypeptide subunit, or a hexameric protein as provided herein, *e.g.*, GITRL FP produced in bacterial culture, can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. High performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Pharmaceutical Compositions and Administration Methods

[0166] Methods of preparing and administering a hexameric protein as provided herein, *e.g.*, GITRL FP as provided herein, to a subject in need thereof, *e.g.*, to enhance an immune response in a cancer patient, *e.g.*, to inhibit or reduce tumor growth, or patient having a viral infection, *e.g.*, to reduce viral load or to reduce reoccurance of viral infection, are well known to or can be readily determined by those skilled in the art. The route of administration of a hexameric protein as provided herein, *e.g.*, GITRL FP can be, for example, oral, parenteral, by inhalation or topical. The term parenteral as used herein includes, *e.g.*, intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal, or vaginal administration. While all these forms of administration are clearly contemplated as suitable forms, another example of a form for administration would be a solution for injection, in

particular for intravenous or intraarterial injection or drip. Usually, a suitable pharmaceutical composition can comprise, without limitation, a buffer (*e.g.* acetate, phosphate or citrate buffer), a surfactant (*e.g.* polysorbate), a stabilizer agent (*e.g.* human albumin), etc. In other methods compatible with the teachings herein, a hexameric protein as provided herein, *e.g.*, GITRL FP as provided herein can be delivered directly to the site of the adverse cellular population thereby increasing the exposure of the diseased tissue to the therapeutic agent.

[0167] Certain pharmaceutical compositions provided herein can be orally administered in an acceptable dosage form including, *e.g.*, capsules, tablets, aqueous suspensions or solutions. Certain pharmaceutical compositions also can be administered by nasal aerosol or inhalation. Such compositions can be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, and/or other conventional solubilizing or dispersing agents.

[0168] The amount of a hexameric protein as provided herein, *e.g.*, GITRL FP that can be combined with carrier materials to produce a single dosage form will vary depending upon the subject treated and the particular mode of administration. The composition can be administered as a single dose, multiple doses or over an established period of time in an infusion. Dosage regimens also can be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response).

[0169] By "therapeutically effective dose or amount" or "effective amount" is intended an amount of a hexameric protein as provided herein, *e.g.*, GITRL FP, that when administered brings about a positive therapeutic response with respect to treatment of a patient with a disease or condition to be treated.

Kits

[0170] This disclosure further provides kits that comprise a hexameric protein as provided herein, *e.g.*, GITRL FP described herein and that can be used to perform the methods described herein. In certain embodiments, a kit comprises at least one purified a hexameric protein as provided herein, *e.g.*, GITRL FP, in one or more containers. One skilled in the art will readily recognize that the disclosed hexameric protein as provided herein, *e.g.*, GITRL FP, can be readily incorporated into one of the established kit formats that are well known in the art.

Immunoassays

[0171] A hexameric protein as provided herein, *e.g.*, GITRL FP can be assayed for specific and/or selective binding by any method known in the art. The immunoassays that can be used include but are not limited to competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), fluorescent focus assay (FFA), microneutralization assay, hemagglutination inhibition assay (HAI), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, to name but a few. Such assays are routine and well known in the art (see, *e.g.*, Ausubel *et al.*, eds, (1994) Current Protocols in Molecular Biology (John Wiley & Sons, Inc., NY) Vol. 1, which is incorporated by reference herein in its entirety). FFA, microneutralization assay, and HAI will be discussed in details in the Examples below.

[0172] Methods and reagents suitable for determination of binding characteristics of a hexameric protein as provided herein are known in the art and/or are commercially available. Equipment and software designed for such kinetic analyses are commercially available (*e.g.,* BIAcore®, BIAevaluation® software, GE Healthcare; KINEXA® Software, Sapidyne Instruments).

Methods of Immune Enhancement and Treatment

[0173] The enhancement of an antigen-specific immune response in a subject (*e.g.*, a mammalian subject, such as a human subject) by engaging GITR on antigen experienced T cells, *e.g.*, antigen experienced $CD4^+$ or $CD8^+$ T cells during or after antigen activation can be accomplished using a wide variety of methods. The method of choice will primarily depend upon the type of antigen against which it is desired to enhance the immune response, and various methods available are discussed below. Whatever method is selected, a hexameric protein as provided herein, *e.g.*, GITRL FP, can be administered to a subject, *e.g.*, a human patient such that it is presented to T cells of the subject during or shortly after priming of the T cells by antigen. Exemplary methods of activating an immune response in a subject, *e.g.*, a human subject using an OX40 hexameric protein, are presented in US Pub. No. 2016/0024176, which is incorporated by reference herein in its entirety, and can be adapted for use with the GITRL FPs provided herein.

[0174] In certain aspects, the disclosure provides a method to promote survival or proliferation of antigen experienced T cells, *e.g.*, antigen experienced $CD4^+$ or $CD8^+$ T cells, comprising contacting

the antigen experienced T cells, *e.g.*, antigen experienced $CD4^+$ or $CD8^+$ T cells, with a hexameric protein as provided herein, *e.g.*, GITRL FP, under conditions where the hexameric protein can specifically bind to GITR on the surface of the T cells, *e.g.*, antigen experienced $CD4^+$ or $CD8^+$ T cells. In certain aspects the contacting is *in vitro*. In certain aspects the contacting is *in vivo*, *e.g.* via administration of an effective dose of the hexameric protein to a subject in need of treatment. In certain aspects the contacting can occur at the same time as T-cell activation, *e.g.*, antigen activation, in certain aspects the contacting can occur after T-cell activation.

[0175] In further aspects, the disclosure provides a method of enhancing cytokine release from antigen experienced T cells, *e.g.*, antigen experienced $CD4^+$ or $CD8^+$ T cells, comprising contacting the antigen experienced T cells, *e.g.*, antigen experienced $CD4^+$ or $CD8^+$ T cells, with a hexameric protein as provided herein, *e.g.*, GITRL FP, wherein the hexameric protein can specifically bind to GITR on the surface of the antigen experienced T cells, *e.g.*, antigen experienced $CD4^+$ or $CD8^+$ T cells. In certain aspects the contacting is *in vitro*. In certain aspects the contacting is *in vivo*, *e.g.* via administration of an effective dose of the hexameric protein to a subject in need of treatment. In certain aspects the contacting can occur at the same time as T-cell-activation, *e.g.*, antigen activation, in certain aspects the contacting can occur after T-cell activation. In certain aspects the cytokine can be IFNγ, TNFα, IL-5, IL-10, IL-2, IL-4, IL-13, IL-8, IL-12 p70, IL-1β, GM-CSF, or any combination thereof. In certain aspects the cytokine is IFNγ, TNFα, IL-5, IL-10, IL-4, IL-13, GM-CSF, or any combination thereof.

[0176] In certain aspects, the antigen experienced T cells, *e.g.*, antigen experienced $CD4^+$ or $CD8^+$ T cells are human $CD4^+$ or $CD8^+$ T cells, cynomolgus monkey $CD4^+$ or $CD8^+$ T cells, rhesus monkey $CD4^+$ or $CD8^+$ T cells, or a combination thereof.

[0177] The disclosure further provides a method of promoting T-cell activation, comprising contacting T cells with a hexameric protein as provided herein, *e.g.*, GITRL Fusion Protein, wherein the hexameric protein can specifically bind to GITR on the surface of the T cells. In certain aspects the contacting occurs in the presence of antigen, *e.g.*, a tumor antigen. In certain aspects the method further comprises cross-linking of the hexameric GITRL Fusion Protein through interaction of the IgG-Fc domain of the GITRL FP with a cell expressing FcγR, *e.g.*, a B cell, monocyte, macrophage, myeloid or plasmacytoid dendritic cell, follicular dendritic cell, Langerhans cell, endothelial cell, NK cell, neutrophil, eosinophil, platelet, mast cell, a $CD45^+$ cell from a primary human tumor or tumor-draining or non-draining lymph node, a $CD45^+$ cell from other secondary or tertiary lymphoid

structures, or a combination thereof. In certain aspects, the T-cell activation can be measured as stimulation of the NFκB signal transduction pathway. In certain aspects, the GITRL FP that promotes T-cell activation is a GITRL IgG1 FP, a GITRL IgG4 FP, or variant thereof. In certain aspects the contacting is *in vitro*. In certain aspects the contacting is *in vivo*, *e.g.* via administration of an effective dose of the hexameric protein to a subject in need of treatment.

[0178] Also provided herein are methods for treating cancer, comprising administration of GITRL FP, and an OX40 agonist (*e.g.*, an OX40 ligand fusion protein or OX40 agonist antibody). Administration of GITRL FP and OX40 ligand fusion protein resulted in a reduction in tumor volume and increased survival in a mouse tumor model. In certain aspects, a patient presenting with a solid tumor is administered GITRL FP and an OX40 ligand fusion protein (e.g., MEDI6383).

[0179] Effective treatment with a cancer therapy including GITRL FP and OX40 agonist includes, for example, reducing the rate of progression of the cancer, retardation or stabilization of tumor or metastatic growth, tumor shrinkage, and/or tumor regression, either at the site of a primary tumor, or in one or more metastases. In some aspects the reduction or retardation of tumor growth can be statistically significant. A reduction in tumor growth can be measured by comparison to the growth of patient's tumor at baseline, against an expected tumor growth, against an expected tumor growth based on a large patient population, or against the tumor growth of a control population. In other embodiments, the methods of the invention increase survival.

[0180] Clinical response to administration of a cancer therapy including GITRL FP and OX40 agonist can be assessed using diagnostic techniques known to clinicians, including but not limited to magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, flow cytometry or fluorescence-activated cell sorter (FACS) analysis, histology, gross pathology, and blood chemistry, including but not limited to changes detectable by ELISA, RIA, and chromatography.

[0181] The disclosure further provides methods of treating cancer or viral infections in a subject, comprising administering to a subject in need of treatment an effective amount of a hexameric protein as provided herein, *e.g.*, GITRL FP, or a composition or formulation comprising the hexameric protein. In certain aspects, the cancer is a solid tumor. According to this method, administration of the hexameric protein or composition can inhibit tumor growth; can promote tumor reduction, or both. In certain aspects, the tumor growth inhibition is achieved in the presence of T cells.

[0182] The terms "cancer", "tumor", "cancerous", and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancers include but are not limited to, carcinoma including adenocarcinomas, lymphomas, blastomas, melanomas, sarcomas, and leukemias. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, glioma, cervical cancer, ovarian cancer, liver cancer such as hepatic carcinoma and hepatoma, bladder cancer, breast cancer (including hormonally mediated breast cancer, see, *e.g.*, Innes *et al.* (2006) Br. J. Cancer 94:1057-1065), colon cancer, colorectal cancer, endometrial carcinoma, myeloma (such as multiple myeloma), salivary gland carcinoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, haematological cancers including, but not limited to, acute myeloid leukemia (AML) and multiple myeloma (MM), various types of head and neck cancer including, but not limited to, squamous cell cancers, and cancers of mucinous origins, such as, mucinous ovarian cancer, cholangiocarcinoma (liver) and renal papillary carcinoma. In certain embodiments, the haematological cancer is selected from the group consisting of Hodgkins lymphoma, non-Hodgkins lymphoma, multiple myeloma, acute lymphoblastic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, and chronic myelogenous leukemia.

[0183] Some embodiments are directed to a method of preventing or treating a cancer or a viral infection in a subject in need thereof, comprising administering to the subject an effective amount of a hexameric protein as provided herein, *e.g.*, GITRL FP, a composition or formulation comprising the hexameric protein, or a polynucleotide, a vector, or a host cell as described herein.

[0184] Methods of treating viral infections in subjects in need thereof comprising administering to the subject an effective amount of the hexameric protein or a composition comprising the hexameric protein are also provided. In certain embodiments, the viral infection can be a chronic or a latent viral infection. Such chronic viral infections are viral infections characterized by weeks, months, or years of viral infection where virion multiplication occurs. Such latent viral infections are viral infections characterized by a period where virions are not multiplying. Subjects in need thereof can in certain embodiments be identified by performing an assay diagnostic for the presence of the virus in the subject or in a sample obtained from the subject. In certain embodiments, the treatment can provide for a decrease in viral load, decrease in viral reactivation, amelioration of symptoms

associated with said infection, or the combination thereof. In certain embodiments, the decrease in viral load, reactivation, or symptoms is in comparison to control subject treated with a placebo. In certain embodiments of any of the aforementioned methods, the viral infection is by a virus selected from the group consisting of Human Immunodeficiency Virus (HIV), Hepatitis B virus , Hepatitis C virus, measles, Epstein-Barr virus (EBV), Cytomegalovirus (CMV), adenovirus (AdV), Human Papillomavirus (HPV), Herpes Simplex Virus (HSV), Varicella-Zoster virus (VZV), and combinations thereof.

[0185] The compositions of the disclosure can be administered by any suitable method, *e.g.*, parenterally, intraventricularly, orally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The term "parenteral" as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques.

[0186] The disclosure further provides a method of enhancing an immune response in a subject comprising administering to a subject in need thereof a therapeutically effective amount of a hexameric protein as provided herein, *e.g.*, GITRL FP, or a composition or formulation comprising the hexameric protein.

[0187] The subject to be treated can be any animal, *e.g.*, mammal, in need of treatment, in certain aspects, subject is a human subject.

[0188] In its simplest form, a preparation to be administered to a subject is a hexameric protein as provided herein, *e.g.*, GITRL FP, administered in conventional dosage form, and preferably combined with a pharmaceutical excipient, carrier or diluent as described elsewhere herein.

[0189] A hexameric protein as provided herein, *e.g.*, GITRL FP, can be administered by any suitable method as described elsewhere herein, *e.g.*, via IV infusion. In certain aspects, a hexameric protein as provided herein, *e.g.*, GITRL FP, can be introduced into a tumor, or in the vicinity of a tumor cell.

[0190] All types of tumors are potentially amenable to treatment by this approach including, without limitation, carcinoma of the breast, lung, pancreas, ovary, kidney, colon and bladder, as well as melanomas, sarcomas and lymphomas.

T Cell Priming agents

[0191] Methods of treating cancer in subjects in need thereof comprising administering to the subject an effective amount of the hexameric protein or a composition comprising the hexameric protein in combination with a T cell priming agent are also provided. In certain aspects, the T-cell

priming agent is a DNA vaccine plus an adjvant. In specific aspects, this combination is, e.g., E7 Synthetic Loing Peptide (SLP) and CpG Oligodeoxynucleotide. In further aspects, the T cell priming agent is an epigenetic modifier, e.g, 5-aza-2'-deoxycytidine or histone modifiers such as HDAC inhibitors. In additional aspects, the T cell priming agent is a virus, e.g., Vaccinia, Listeria, or Newcastle Disease Virus.

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[0192] This disclosure employs, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook *et al.*, ed. (1989) Molecular Cloning A Laboratory Manual (2nd ed.; Cold Spring Harbor Laboratory Press); Sambrook *et al.*, ed. (1992) Molecular Cloning: A Laboratory Manual, (Cold Springs Harbor Laboratory, NY); D. N. Glover ed., (1985) DNA Cloning, Volumes I and II; Gait, ed. (1984) Oligonucleotide Synthesis; Mullis *et al.* U.S. Pat. No. 4,683,195; Hames and Higgins, eds. (1984) Nucleic Acid Hybridization; Hames and Higgins, eds. (1984) Transcription And Translation; Freshney (1987) Culture Of Animal Cells (Alan R. Liss, Inc.); Immobilized Cells And Enzymes (IRL Press) (1986); Perbal (1984) A Practical Guide To Molecular Cloning; the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Miller and Calos eds. (1987) Gene Transfer Vectors For Mammalian Cells, (Cold Spring Harbor Laboratory); Wu *et al.*, eds., Methods In Enzymology, Vols. 154 and 155; Mayer and Walker, eds. (1987) Immunochemical Methods In Cell And Molecular Biology (Academic Press, London); Weir and Blackwell, eds., (1986) Handbook Of Experimental Immunology, Volumes I-IV; Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); and in Ausubel *et al.* (1989) Current Protocols in Molecular Biology (John Wiley and Sons, Baltimore, MD.).

[0193] General principles of antibody engineering are set forth in Borrebaeck, ed. (1995) Antibody Engineering (2nd ed.; Oxford Univ. Press). General principles of protein engineering are set forth in Rickwood *et al.*, eds. (1995) Protein Engineering, A Practical Approach (IRL Press at Oxford Univ. Press, Oxford, Eng.). General principles of antibodies and antibody-hapten binding are set forth in: Nisonoff (1984) Molecular Immunology (2nd ed.; Sinauer Associates, Sunderland, Mass.); and Steward (1984) Antibodies, Their Structure and Function (Chapman and Hall, New York, N.Y.). Additionally, standard methods in immunology known in the art and not specifically described are generally followed as in Current Protocols in Immunology, John Wiley & Sons, New York; Stites *et*

al., eds. (1994) Basic and Clinical Immunology (8th ed; Appleton & Lange, Norwalk, Conn.) and Mishell and Shiigi (eds) (1980) Selected Methods in Cellular Immunology (W.H. Freeman and Co., NY).

[0194] Standard reference works setting forth general principles of immunology include Current Protocols in Immunology, John Wiley & Sons, New York; Klein (1982) J., Immunology: The Science of Self-Nonself Discrimination (John Wiley & Sons, NY); Kennett *et al.*, eds. (1980) Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses (Plenum Press, NY); Campbell (1984) "Monoclonal Antibody Technology" in Laboratory Techniques in Biochemistry and Molecular Biology, ed. Burden *et al.*, (Elsevier, Amsterdam); Goldsby *et al.*, eds. (2000) Kuby Immunology (4th ed.; H. Freemand & Co.); Roitt *et al.* (2001) Immunology (6th ed.; London: Mosby); Abbas *et al.* (2005) Cellular and Molecular Immunology (5th ed.; Elsevier Health Sciences Division); Kontermann and Dubel (2001) Antibody Engineering (Springer Verlag); Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press); Lewin (2003) Genes VIII (Prentice Hall 2003); Harlow and Lane (1988) Antibodies: A Laboratory Manual (Cold Spring Harbor Press); Dieffenbach and Dveksler (2003) PCR Primer (Cold Spring Harbor Press).

[0195] All of the references cited above, as well as all references cited herein, are incorporated herein by reference in their entireties.

[0196] The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Table B: List of Abbreviations and Definitions of Terms

Abbreviation or Term	Definition
A	Alanine
ADCC	Antibody-dependent cellular cytotoxicity
°C	Degrees Celsius
CDC	Complement-dependent cytotoxicity
CR	complete response
Cyno	cynomolgus
F	Phenylalanine
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
LC-QTOF MS	liquid chromatography coupled with quadruple time of flight mass spectrometry
M	Molarity

mAb	monoclonal antibody
hGITRL	human GITRL
µg	micrograms
MFI	Mean fluorescence intensity
min	Minutes
mL	Milliliter
Multimeric mGITRL FP	A multimeric mouse GITR ligand mouse IgG Fusion Protein
NIP228	A human monoclonal antibody against 4-hydroxy-3-iodo-5-nitrophenylacetic acid
NK	Natural killer
PBS	Phosphate buffered saline
pM	Picomolar
RBD	receptor binding domain
Rh	Recombinant human
ROA	route of administration
rpm	Revolutions per minute
RT	Room temperature
SC	subcutaneous
SD	Standard Deviation
TCR	T cell receptor
TGI	tumor growth inhibition
TNFR	Tumor necrosis factor receptor
Treg	T regulatory
V	volume

EXAMPLE 1: Engineering of GITRL IgG Fc Fusion Protein

Identification of a suitable human trimerization motif that could be utilized for generating a multimeric human GITRL-FP molecule

[0197] Efforts to generate a human hexameric GITRL FP had originally focused on the use of the GCN4 pII trimerization motif to stabilize the GITRL trimer and form a hexameric protein. This motif, however, is derived from a yeast protein and the administration of GITRL FP containing a non-human motif to humans may result in immunogenicity. Therefore, it was considered desirable to generate an equivalent GITRL FP containing a trimerization motif derived from a human protein. Amino acid sequences from 51 proteins that form trimeric coiled-coil motifs, as demonstrated by their three-dimensional crystal structure or those of orthologs, were identified via the Protein Data

Bank (PDB; accessible through the internet via the world wide web site "wwpdb.org") and a subset of 4 coiled-coil motifs were selected for incorporation into GITRL FP molecules for empirical experimental testing.

Methods

[0198] The three-dimensional crystal structures of coronin-1A (PDB code: 2akf), matrilin 1 (1aq5), langerin (3kqg) and dystrophia myotonica kinase DMPK (PDB code: 1wt6) coiled-coil motifs were analyzed using PyMol Visualisation Graphics software.

Results

[0199] Following a search of the Protein Data Bank (PDB) for trimeric coiled coil protein sequences that identified 51 candidate sequences, four sequences were selected for use in generating multimeric GITRL FPs (Table 1-1). These four sequences were coiled-coil sequences from human proteins coronin 1a, matrilin 1, langerin and DMPK. The structure of the coiled-coil motif from mouse coronin 1a (PDB code: 2akf) is reported to be stable trimer in solution (Kammerer, R.A. et al. PNAS, vol: 102, p13891-13896 (2005)). The human coronin 1a coiled-coil sequence shows high sequence identity to the mouse protein (78.1% sequence identity) so this sequence was predicted to form a trimeric coiled-coil structure. Similarly, the structure of the coiled-coil motif from chicken matrilin 1 (PDB code: 1aq5) is reported to be a trimer (Dames, S. A. et al. NAT. STRUCT. BIOL. 5:687-691 (1998); PDB code: 1aq5). The human matrilin 1 coiled-coil sequence was predicted to form a trimeric coiled-coil structure based on its high sequence identity (60.0% sequence identity) to the chicken ortholog. The human langerin (PDB code: 3kqg) and human DMPK (PDB code: 1wt6) coiled-coil sequences also are reported to be trimeric (Feinberg, H. et al., J. BIOL. CHEM. 285:13285-13293 (2010)) and were selected for use in generating multimeric human GITRL fusion proteins.

[0200] To provide additional coiled-coil sequences for use in the GITRL fusion proteins, variants of the wild-type coiled-coil sequences from human coronin-1A, matrilin-1, langerin and DMPK were produced. To generate these variant sequences the online implementation of the ProCoil algorithm (accessible through the internet via the world wide web site “bioinf.jku.at/software/procoil/”) was used. This algorithm predicts the probabilities of given sequences to form dimeric and trimeric coiled-coil structures. Several variants of the wild-type coiled-coil sequences mentioned above were

predicted to have higher probability scores for forming trimers compared to the wild-type sequences. The variant sequence with the highest probability for being a trimer was selected for use in generating hexameric GITRL FP.

[0201] The variant coiled-coil sequences chosen for all the above four human proteins are shown in **Table 1-1**.

Table 1-1: Sequences for use as trimeric coiled-coil motifs in GITRL FP constructs

Trimerization Motif	Sequence and SEQ ID NO
GCN4 pII	IKQIEDKIEEILSKIYHIENEIARIKKL (SEQ ID NO: 27)
Matrilin 1 wt	CACESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVV (SEQ ID NO: 28)
Matrilin 1 variant	CACESLVKFQAKVEGLIQALTRKLEAVSKRIAILENTVV (SEQ ID NO: 29)
Coronin 1a wt	VSRLEEEMRKLQATVQELQKRLDRLEETVQAK (SEQ ID NO: 11)
Coronin 1a variant	ISRIEEEIRKINATVQELQKRIDRLEETVQAK (SEQ ID NO: 17)
DMPK wt	EAEAEVTLRELQEALEEEVLTRQSLSREMEAIRTDNQNFASQLREAEARNRDLEAHVRQLQERMELLQAE (SEQ ID NO: 30)
DMPK variant	IAEIEVTIRELQEAIEEEVLTRQSLSREIEAIRTDIQNIASQLREIEARIRDLEAHVRQLQERMELLQAE (SEQ ID NO: 31)
Langerin wt	ASALNTKIRALQGSLENMSKLLKRQNDILQVVS (SEQ ID NO: 32)

In silico analysis to rank the trimerization motifs

[0202] *In silico* predictions of motifs that would generate a more stable GITRL trimers, and therefore a more stable multimeric GITRL FP, were made by analyzing the sequences in Table 1-1 with the algorithm (LOGICOIL) to predict their oligomeric state. In addition, the scores obtained for the formation of a parallel trimer were compared to those from coiled coils identified from the entire human proteome via the combination of two algorithms (MARCOIL and LOGICOIL).

Methods

[0203] The genome-wide prediction was performed on the human proteome downloaded from the ftp site "ftp.ncbi.nlm.nih.gov/refseq/H_sapiens/mRNA_Prot/human.protein.faa.gz." This version includes a total number of 71861 protein sequences. These sequences were scanned and the coiled-coil motifs were identified by executing the software MARCOIL, downloaded from the worldwide

web site "bcf.isb-sib.ch/Delorenzi/Marcoil/Marcoilcode.tar.gz". All of the motifs that passed certain specific thresholds (i.e. 0.01, 0.10, 0.50, 0.90 and 0.99) were further analyzed using the LOGICOIL algorithm, downloaded from the internet via the world wide web site "coiledcoils.chm.bris.ac.uk/LOGICOIL/LOGICOIL_Source.zip", to predict the oligomerization state (parallel or anti-parallel dimer, trimer or tetramer) of coiled-coil sequences. This package was implemented in the R language (version 3.0.2 - 2013-09-25). An ad-hoc perl script was developed to convert the outputs from MARCOIL to the file format expected by LOGICOIL. The histograms were plotted in R using the lattice package (version 0.20-23).

Results

[0204] Coiled-coil motifs in all human sequences from Refseq were predicted (using MARCOIL). The number of coiled-coil motifs predicted by the MARCOIL algorithm for different thresholds is shown in **Table 1-2**. The oligomeric states of all the motifs from the 0.99 threshold were then predicted, using LOGICOIL, alongside the eight selected trimeric coiled coil domains in Table 1-2.

The distribution of these scores and the ranking of the motifs used in this study are shown in **Table 1-3**.

Table 1-2: The number of coiled-coil motifs predicted by the MARCOIL algorithm for different thresholds

Threshold	Number of Motifs
0.01	168583
0.10	68793
0.50	41762
0.90	34714
0.99	27240

Table 1-3: Ranking of the eight coiled-coil motifs in the context of the whole proteome, including LOGICOIL TRIM scores

PROTEOME RANKING	SUBSET RANKING	SEQUENCE	LOGICOIL TRIM
20	1	GCN4 pII	2.43
201	2	hDMPK-mutant	1.74
219	3	hLangerin-wt	1.71
241	4	hLangerin-variant	1.68
427	5	hCor1a-wt	1.58

PROTEOME RANKING	SUBSET RANKING	SEQUENCE	LOGICOIL TRIM
1065	6	hCor1a-mutant	1.44
3620	7	hDMPK-wt	1.18
12473	8	hMatrilin-variant	0.96
18993	9	hMatrilin-wt	0.81

Generation of GITRL FP variants that contain different human trimerization motifs

[0205] The sequences for each of the selected trimerization motifs, in addition to the GCN4 pII sequence, were cloned in an appropriate configuration, into DNA vectors that encoded the other elements of the GITRL FPs *i.e.*, between a human Fc domain and the extracellular domain of human GITRL, separated by short flexible amino acid linker sequences. These vectors were used to transiently transfect mammalian cells, enabling the secretion and subsequent purification of the recombinant hexameric GITRL FP proteins.

Method

[0206] Suspension CHO cells were transiently transfected, using PEI, with DNA vectors encoding the different hexameric GITRL FPs and grown for eight days at 37 °C, shaking at 140 rpm with 80% humidity. Forty milliliters of the conditioned media containing the secreted proteins was separated from cells and cell debris by centrifugation at 1,600 x g and filtration. The proteins were purified using Mab SelectSure™ resin and their size and integrity was analyzed by reducing SDS-PAGE.

Larger scale expression and a two-step purification protocol were adopted subsequently for hexameric GITRL FP (coronin 1a wt) and hexameric GITRL FP (matrilin 1 wt). Here, a larger volume of the CHO conditioned (400 ml) was subjected to Protein G chromatography followed by a size-exclusion chromatography step (S200 16/60). These purified proteins were used for SEC-MALLS analysis.

Results

[0207] All recombinant hexameric GITRL FP proteins expressed at similar levels, but the hexameric GITRL-FP containing the coronin 1a wt motif did appear to provide a higher yield than the other proteins (**Table 1-4:**, Figure 1).

Table 1-4: Protein yields from transient expression in CHO cells for the eight GITRL FP proteins

GITRL FP	Concentration (mg/ml)
Matrilin 1 wt	0.51
Matrilin 1 variant	0.57
Coronin 1a wt	0.9
Coronin 1a variant	0.63
DMPK wt	0.66
DMPK variant	0.34
Langerin wt	0.37
Langerin variant	0.48

Characterization of GITRL FP variants that contain different human trimerization motifs

Binding of GITRL FP variants to cells expressing GITR

[0208] In order to determine whether the hexameric GITRL FP molecules could bind to cell surface-expressed GITR, they were incubated with cells overexpressing GITR and their binding was detected via their Fc domain.

Method

[0209] A fixed concentration of a DyLight-649 conjugated anti-human IgG antibody buffer, followed by a 2-fold dilution of the purified hexameric GITRL FP proteins described above in section 1.3 and an isotype control antibody (NIP228) were added in duplicate wells to a 384 well black walled clear bottom plate. CHO cells stably expressing GITR were added to all wells and the plate incubated at room temperature for four hours. Binding to the CHO-GITR cells was determined using the Mirrorball™ plate cytometer with a 640 nm laser and the fluorescence measured.

Results

[0210] Results are presented in Figures 2A-D. The binding profile obtained demonstrates a "hook effect" which is observed when the concentration of the hexameric GITRL FP exceeds the concentration of the DyLight-649 conjugated detection antibody, resulting in less binding being

detected at the higher concentrations of hexameric GITRL FP; thus a bell-shaped binding profile is produced. Despite this phenomenon, the different hexameric GITRL FP molecules produce a very similar binding profile.

Competition of GITRL Competition of GITRL FP variants with recombinant trimeric ligand for binding to GITR

[0211] The effect of the hexameric GITRL FP molecules on human GITRL binding to human GITR was determined using a Homogeneous Time Resolved Fluorescence (HTRF) assay.

Method

[0212] The hexameric GITRL FP molecules were titrated into an HTRF assay in which binding of GITRL-HA (hemagglutinin tag) to GITR-Fc was measured. The human GITR Fc was conjugated with europium cryptate and an anti-HA antibody conjugated with XL665 was used to detect the GITRL-HA protein. IC_{50} values were determined by curve fitting the analyzed data to a four parameter logistic equation with Prism 5.01 software (GraphPad).

Results

[0213] Results are presented in Figures 3A-D and **Table 1-5**. The hexameric GITRL FP molecules with the different trimerization motifs are all potent inhibitors of trimeric GITRL-HA binding to GITR-Fc. The majority of GITRL FP variants produced similar inhibition profiles and IC_{50} values with the exception of GITRL FP (Langerin wt) that demonstrated an 8-fold lower potency than GITRL FP (GCN4). GITRL FP (Coronin 1a) had the lowest IC_{50} value at 0.61 nM.

Table 1-5: IC_{50} (nM) values for GITRL FP proteins competing for binding of trimeric GITRL to GITR-Fc

Test sample	IC50 (nM)
GITRL FP (GCN4 pII)	1.06
GITRL FP (Matrilin 1 wt)	1.36
GITRL FP (Matrilin 1 variant)	0.83
GITRL FP (Coronin 1a wt)	0.61
GITRL FP (Coronin 1a variant)	1.44

Test sample	IC50 (nM)
GITRL FP (DMPK wt)	2.73
GITRL FP (DMPK variant)	2.43
GITRL FP (Langerin wt)	8.05
GITRL FP (Langerin variant)	2.0
NIP228 IgG-TM	No Inhibition

Activity of GITRL FP variants in a reporter assay

[0214] The functional activity of the different GITRL FP molecules (hexameric GITRL IgG1 FP (SEQ ID NO: 6) and hexameric GITRL IgG4 FP (SEQ ID NO: 40) was determined in an assay using NFκB-luciferase reporter cells stably expressing GITR. Luminescence, driven by agonism of GITR and subsequent activation of the NFκB pathway, was measured.

Method

[0215] GITRL FP proteins were serially diluted 4-fold for a six-point data curve and added in triplicate to 96 well plates. Then Jurkat NF-κB luciferase reporter cells transfected with human GITR were added to all wells of the assay plates and incubated at 37 °C for three hours. Luciferase expression was detected by adding Steady-Glo reagent to all wells of the assay plates. The plates were incubated for five minutes at room temperature and then luminescence was measured and EC_{50} values were generated using log (agonist) vs. response variable slope nonlinear curve fit in GraphPad Prism 5.01 (GraphPad).

Results

[0216] Results are presented in Figures 4A-D and **Table 1-6**. All the hexameric GITRL FP proteins trigger NF-κB signaling. The proteins generated similar potency profiles and EC_{50} values to GITRL FP (GCN4).

Table 1-6: EC_{50} (nM) values for GITRL FP variants using a human GITR transfected NF-κB luciferase gene reporter cell line

	Test sample	EC_{50} (nM)
	GITRL FP (GCN4 pII)	1.02

	Test sample	EC_{50} (nM)
	GITRL FP (Matrilin 1 wt)	1.12
	GITRL FP (Matrilin 1 variant)	0.90
	GITRL FP (Coronin 1a wt)	0.95
	GITRL FP (Coronin 1a variant)	0.74
	GITRL FP (DMPK wt)	1.37
	GITRL FP (DMPK variant)	1.39
	GITRL FP (Langerin wt)	1.54
	GITRL FP (Langerin variant)	0.75

Melting temperature of GITRL FP variants

[0217] The melting temperature of the GITRL FP proteins was determined using a fluorescent dye (Sypro Orange) whose emission properties change in the presence of an unfolded protein.

Method

[0218] Thermostability of multimeric GITRL FP variants was assessed using a Sypro Orange-based assay to calculate melting temperatures (Tm). The different proteins were first diluted to 0.5 mg/mL in 2×PBS before dispensing into a 96 well PCR plate. Sypro Orange™ was added to each well on the plate, which was then sealed. Plates were read on a Real-Time™ PCR machine using a Chromo4™ continuous fluorescence detector. The temperature was set to increase from 20 °C to 90 °C with a read every 1 °C and a hold time of 1s. Unfolding transitions were determined by plotting the fluorescence intensity and fluorescence derivative as a function of temperature. Each multimeric GITRL-FP protein was analyzed in duplicate.

Results

[0219] Results are presented in Figures 5A-D. Melting temperatures for each of the nine multimeric GITRL- FP proteins are summarized in **Table 1-7** below. All variants have a similar profile with the exception of GITRL FP (DMPK wt) which displays a single transition peak at 59 °C compared to 62-64 °C for the majority of the others, GITRL FP (Langerin wt) and GITRL FP (Langerin variant)

which both display an additional transition peak at a low temperature (45-50 °C) suggesting some structural instability.

Table 1-7: Transition temperatures for the nine GITRL FP proteins

	Test sample	Tm1 (°C)	Tm2 (°C)
	GITRL FP (GCN4 pII)	/	63
	GITRL FP (Matrilin 1 wt)	/	62.5
	GITRL FP (Matrilin 1 variant)	/	63
	GITRL FP (Coronin 1a wt)	/	63
	GITRL FP (Coronin 1a variant)	/	63
	GITRL FP (DMPK wt)	/	59
	GITRL FP (DMPK variant)	/	63.5
	GITRL FP (Langerin wt)	47.5	63
	GITRL FP (Langerin variant)	45-50	62

In silico immunogenicity analysis of GITRL FP variants

[0220] The predicted immunogenicity of the 8 coiled coil domains and their surrounding sequences was determined using the ProPred algorithm.

Method

[0221] Using ProPred [Singh & Raghava (2001) Bioinformatics 17(12)], the T-score of the eight selected human trimerization motifs, and yeast GCN4 pII were evaluated. The T-score of an arbitrary amino acid sequence quantifies the number of strongly binding MHC class II epitopes relative to the total length of the amino acid sequence. A nine-mer subsequence is considered a strongly binding epitope, if ProPred returns a binding score that lies above the 95 percentile of scores obtained by evaluating a set of 10,000 randomly generated nine-mer sequences. The T-score was calculated for each motif, including the 2x[G4S] sequence preceding the motif and the [G4] sequence and first 4 amino acids of the GITRL domain following the motif, for the eight most common human alleles. The overall T-score was then formed as the sum of the individual allele T-scores.

Results

[0222] The overall T score for each trimerization motif and its surrounding sequence is shown in **Table 1-8**. Note that the maximal score is equal to eight, which corresponds to a case where every nine-mer in the sequence is a strongly binding epitope for all of the eight alleles that are tested. Interestingly this global analysis predicts that almost all candidates, except for DMPK wt have slightly stronger immunogenic profiles than GCN4. Matrilin wt and variant both have the highest overall T score.

Table 1-8: Overall T scores for the eight human trimerization motifs and GCN4 pII

#	Coiled coil motif	T score
1	GCN4 pII	0.131579
2	Matrilin 1	0.270833
3	Matrilin 1 variant	0.270833
4	Coronin 1a wt	0.142857
5	Coronin 1a variant	0.166667
6	DMPK wt	0.0875
7	DMPK variant	0.175
8	Langerin wt	0.209302
9	Langerin variant	0.186047

SEC-MALLS analysis of GITRL FP variants

Method

[0223] Proteins were analyzed on a BioSep-Sec-S™ 4000 (void volume, V0= 5.7 ml) and all runs were performed at the flow rate of 0.5 ml/min for 30 min. Elution was monitored with a UV280, refractive index and multi-angle laser light scattering detectors. Samples (at 1-2 mg/ml) were loaded on the column and the molar mass and particle diameter were then calculated.

Results

Multimeric GITRL FP (Matrilin 1 wt)

[0224] GITRL FP with the matrilin-1 trimerization motif yielded three distinct peaks on the gel-filtration column: peak 2 has the weight-average molar mass of 312 kDa in agreement with the expected molar mass of the glycosylated hexamer and constitutes 40.9% of the total protein mass, while peak 1 has weight-average molar mass corresponding to the dimer of hexamers (~610 kDa)

and constitutes 41.3 % of the total mass injected. Peak 3 has a lower than expected mass (~215 kDa) which could be either a result of fragmentation or a loss of a dimer from the hexamer. It constitutes 17.7% of the total protein mass. The composition of this protein preparation is therefore heterogeneous, with no single dominant oligomeric species.

Multimeric GITRL FP (Coronin 1a wt)

[0225] Analysis of the overlay of UV280, refractive index and 900-laser scattering traces shows that 93.9 % of this protein elutes at 16.4 min as a monodisperse species of 300 kDa. This observed mass is consistent with the predicted mass of the hexameric GITRL FP polypeptide (274.95 kDa). The difference between calculated and observed mass of 25 kDa is most likely caused by glycosylation. The minor peak at 14.7 min has the weight-average molar mass of 690 kDa which could be attributed to the dimer of hexamers.

Multimeric GITRL FP (GCN4 pII)

[0226] Similarly, analysis of the laser scattering and refractive index chromatograms of GITRL FP with the GCN4 pII trimerization motif demonstrates that 93.75% of this protein elutes as a monodisperse species of 330 kDa, consistent with the glycosylated hexamer. The peak eluting at 15 minutes contains only 6.25% of total protein and has the weight-average molar mass of approximately 700 kDa probably reflecting the presence of a dimer of hexamers.

Molar mass versus time profiles

[0227] Figure 6 shows the molar mass composition of eluted peaks for each of the three Multimeric GITRL FP proteins. For all three proteins, the molar mass across early peaks with ~15 min retention time is highly variable, particularly in the case of GITRL FP (Matrilin 1 wt) where the mass of molecules eluted at the beginning and at the end of the same peak differs by more than 100 kDa. Peaks eluting at ~17 minutes are less heterogeneous in their molecular composition, with the maximal molar mass variation in GITRL FP (coronin 1a wt) of less than 20 kDa. The variation in the weight-average molar mass is most likely caused by differences in the glycan content of the glycoproteins being eluted within the same peak.

Summary of characterization data

[0228] In analyzing the compiled data as shown in

[0229] **Table 1-9** that compares the performance of various coiled coil trimerization domains in multimeric GITRL FP, the coronin 1a coiled coil domain provides improved expression yield. Comparison of the prediction scores of the coronin-1a coiled-coil motif and the motifs from human sequences classified as trimeric coiled-coil, however, shows that the coronin-1a wt had a LOGICOIL ranking of 5.

Table 1-9: Summary of characterization data

Trimerization Motif	LOGICOIL ranking	Expression yield (mg/ml)	Tm1 (Sypro Orange)	Receptor/ Ligand inhibition IC50 (HTRF)	Cell binding (Mirrorball)	Agonism EC_{50} (NFkB Reporter)	*In silico* Immunogenicity
GCN4	1		63	1.06	Good	0.89	0.132
Matrilin 1 wt	9	0.51	62.5	1.36	Good	0.92	0.271
Matrilin 1 variant	8	0.57	63	0.83	Good	0.89	0.271
Coronin 1a wt	5	0.9	63	0.61	Good	0.75	0.143
Coronin 1a variant	6	0.63	63	1.44	Good	0.71	0.167
DMPK wt	7	0.66	59	2.73	Good	0.96	0.088
DMPK variant	2	0.34	63.5	2.43	Good	1.38	0.175
Langerin wt	3	0.37	47.5	8.05	Good	1.17	0.209
Langerin variant	4	0.48	45-50	2	Good	0.57	0.186

EXAMPLE 2: Generation and Characterization of a GITRL Fusion Protein, Characterization of the Same, and Characterization of Mouse GITRL Fusion Proteins

Derivation and Composition

[0230] A hexameric GITRL IgG1 Fusion Protein comprising monomeric subunits having the amino acid sequence of SEQ ID NO: 6 was constructed. Each GITRL IgG1 FP monomeric subunit of SEQ ID NO: 6 comprises 3 distinct domains: 1) a human IgG1 Fc domain; 2) an alpha helical coiled coil trimerization domain derived from the human coronin 1A protein and 3) the human GITRL ECD, with a single point mutation (N161D) in the human GITRL ECD that eliminates the sole occupied glycosylation site. Each domain is separated by flexible glycine and serine-rich or glycine-rich, *e.g.*, $(Gly)_4$ linkers (Figure 8). The human GITRL ECD domain in each monomer forms weak non-covalent trimers with two further monomers in solution, and this association is strengthened through interactions between the human coronin 1A trimerization domains resulting in a stabilized trimeric structure. The interaction of the IgG1 Fc domains present in each trimer leads to subsequent formation of a dimer of GITRL trimers resulting in the final hexameric conformation.

[0231] The DNA molecule set forth in SEQ ID NO: 7, which encodes the precursor GITRL IgG1 FP polypeptide subunit having the amino acid sequence of SEQ ID NO: 8, was synthesized and cloned, using standard molecular biology techniques, into an expression vector that enabled efficient transient recombinant protein expression.

[0232] The nucleotide and deduced amino acid sequences of the subunit monomer of the GITRL IgG1 FP polypeptide subunit set forth in SEQ ID NO: 6, are shown in Figure 8.

Expression and purification of recombinant protein

[0233] CHO cells growing in suspension in a chemically defined media similar to CD-CHO™ (Life Technologies Ltd, Paisley, UK) in wavebag bioreactors, were transfected with DNA vectors encoding the different hexameric GITRL FPs. The cultures were maintained as a fed batch, using a nutrient feed similar to CHO CD- Efficient Feed A ™ (Life Technologies Ltd, Paisley, UK), for 10-12 days at which point they were harvested using filtration for cell removal. The conditioned medium was then refrigerated prior to purification.

[0234] Protein was purified from conditioned media using MabSelectSure™ resin. The eluted peak was neutralized and filtered prior to further purification on Hydroxyapatite Type 1 resin. Elution was carried out with a salt gradient. Purity was monitored by SEC HPLC throughout the process.

Glycosylation Analysis of GITRL IgG1 FP polypeptide subunit

[0235] The glycosylation status of the GITRL IgG1 FP polypeptide subunit of SEQ ID NO: 6 was determined using liquid chromatography coupled with quadrupole time of flight (QTOF) mass spectrometry (LC-QTOF MS), and peptide mapping with mass spectrometry. The results are shown in Figure 9.

[0236] The accurate mass of the GITRL IgG1 FP polypeptide subunit of SEQ ID NO: 6, obtained by LC-QTOF MS analysis of the reduced polypeptide subunit, is consistent with the expected amino acid sequence with the addition of one biantennary glycan (predominantly G0f) per chain. The mass profile is consistent with Fc domain glycosylation with no N-glycan occupancy at N129 in the GITRL ECD (N369 in fusion protein) of the GITRL IgG1 FP polypeptide subunit of SEQ ID NO: 6 as confirmed by peptide mapping. These data also confirm that the N161D mutation in the GITRL ECD (N401 in the fusion protein) results in an aglycosylated GITRL ECD, with N-glycosylation present only at the canonical glycosylation site in the Fc domain.

In Vitro Characterization of a hexameric GITRL IgG1 FP

Effect of a hexameric GITRL IgG1 FP on Ligand-receptor Binding

[0237] The effect of a hexameric GITRL IgG1 FP having the monomeric GITRL IgG1 FP subunit sequence of SEQ ID NO: 6 on human GITRL binding to human GITR was determined using a Homogeneous Time Resolved Fluorescence (HTRF) assay. The GITRL IgG1 FP was titrated into an HTRF assay in which binding of GITRL-HA (hemagglutinin tag) to GITR-Fc was measured. The human GITR-Fc was conjugated with europium cryptate and an anti-HA antibody conjugated with XL665 was used to detect the GITRL-HA protein. IC_{50} values were determined by curve fitting the analyzed data to a four parameter logistic equation with Prism 6.0x software (GraphPad). Representative results shown in Figure 10 demonstrate that the GITRL IgG1 FP inhibits the binding of GITRL-HA to GITR with an IC_{50} of 0.562 nM. No inhibition was observed with isotype control antibody NIP228.

GITR Agonism

[0238] The purpose of this experiment was to determine the ability of a GITRL fusion protein (FP) to deliver signals via the GITR receptor.

Method

[0239] Hexameric GITRL FP was added in solution to Jurkat cells transfected with hGITR and a luciferase reporter gene linked to a nuclear factor kappa B (NFκB) promoter. In this assay activation of the GITR receptor results in signaling via the NFκB pathway, which in turn results in an increase in luciferase activity that can be measured via luminescence.

Results

[0240] The addition of hexameric GITRL FP to the assay system resulted in an increase in luminescence. The observed effect was concentration dependent with an EC_{50} of approximately 180 pM. In contrast the addition of an isotype control antibody had no effect. Results are shown in Figure 11. These data demonstrate that hexameric GITRL FP is a potent agonist of the GITR receptor.

Primary T Cell Activation

[0241] This experiment was conducted to assess the impact of GITR agonism, mediated by hexameric GITRL FP, on the proliferation and function of human T cells.

Method

[0242] Total human T cells were isolated from healthy human blood and antigen experienced, by culture in the presence of plate bound anti-CD3 for 4 days, in order to up-regulate the expression of GITR. Antigen experienced cells were rested for 2 days by culture in media alone before being re-stimulated with a sub-optimal concentration of anti-CD3 and anti-CD28 in the presence of hexameric GITRL FP. Proliferation of cells was assessed by quantitating the incorporation of thymidine into cells over an 18 hour period. The release of interferon gamma (IFN-γ) was quantitated using meso scale discovery.

Results

[0243] The addition of anti-CD3 together with anti-CD28 resulted in a minimal level of proliferation and a minimal release of IFN-γ. The addition of plate bound hexameric GITRL FP together with plate bound anti-CD3 and anti-CD28 resulted in a concentration dependent increase in the level of proliferation as well as the release of IFN-γ. Representative data is shown in Figure 12 and Figure 13. An isotype control antibody had no effect on proliferation or the release of cytokines.

ADCC

[0244] A mouse hexameric GITRL IgG1 FP has been shown *in vivo* to deplete intratumoral CD4 positive T cells, including FOXP3 positive regulatory T cells, resulting in an increased ratio of $CD8^+$ to $CD4^+$ T cells within the tumor. This experiment was conducted to determine the ability of hexameric GITRL FP to mediate depletion of human T cells via ADCC and to assess the resulting change in CD8:CD4 ratio in the surviving cell population.

Method

[0245] Primary human T cells, isolated from healthy human blood, were antigen experienced with phytohemagglutinin (PHA) and IL-2 in order to up-regulate GITR expression. They were then labelled with a fluorescent dye and incubated for 24 hours with primary NK cells, at the indicated

ratio together with hexameric GITRL FP, at the indicated concentrations. Flow cytometry was used to quantitate the percentage of live T cells present at the end of the assay, and this was used to calculate a percentage lysis for treated relative to untreated wells. The proportion of CD4 and CD8 cells was also quantitated by flow cytometry.

Results

[0246] NK cells alone mediated a small amount of lysis of the primary T cells, which was significantly enhanced by the addition of hexameric GITRL FP comprising a GITRL fusion polypeptide subunit set forth in SEQ ID NO: 6 containing an IgG1 Fc domain in a concentration dependent manner. Hexameric GITRL FP comprising a GITRL fusion polypeptide subunit of SEQ ID NO: 40 containing an IgG4 Fc domain that cannot bind the Fc gamma receptors on NK cells, was used as a negative control and had no effect on the level of lysis measured (Figure 14).

Flow cytometric analysis of the percentage of $CD4^+$ and $CD8^+$ T cells present at the end of the assay indicated that ADCC mediated by hexameric GITRL FP favors the generation of an increased CD8:CD4 T cell ratio (Figure 15).

Regulatory T-cell Assays

[0247] GITR is expressed at increased levels on regulatory T cells (T-regs) and signaling through GITR has been suggested to impact the ability of T-regs to suppress other T cells. This experiment was conducted in order to assess the impact of hexameric GITRL FP on T-reg function.

Method

[0248] $CD4^+$ $CD25^-$ effector T cells and $CD4^+$ $CD25^+$ T-regs were isolated from the peripheral blood of healthy human donors. Effector T cells were labelled with CFSE prior to culture in the presence of anti-CD3 antibody, anti-CD28 antibody, T-regs at the ratio indicated and test articles. The percentage of proliferating effector T cells was analyzed by flow cytometry.

Results

[0249] Effector T cell proliferation was observed in response to the addition of anti-CD3 and anti-CD28. The addition of increasing numbers of T-regs resulted in a reduction in the percentage of effector T cells that had divided during the assay. The addition of plate bound isotype control further decreased the percentage of divided cells, while addition of plate bound hexameric GITRL FP

restored the percentage of divided to that observed in the absence of T-regs. Results are shown in Figure 16.

[0250] This study demonstrates the ability of hexameric GITRL FP to overcome the effects of regulatory T-cell mediated suppression on other T cells.

Tests for biological/functional activity *in vivo*

Isotype Dependent Antitumor Activity

[0251] Because human GITRL does not cross-react to mouse GITR the human GITRL FP cannot be tested in immunocompetent mouse models of cancer. To enable this testing surrogate mouse mGITRL IgG1 FP and mGITRL IgG2a FP were generated. This study was conducted in order to evaluate the antitumor activity of mGITRL FP in the CT26 model of cancer, and to determine the impact of Fc isotype on the magnitude of this activity.

Method

[0252] Balb/c mice were implanted with the CT26 mouse colorectal cancer cell line. On day 6 following implantation animals were administered mGITRL FP by intraperitoneal (i.p.) injection either once or daily for 17 days. Two different versions of the mGITRL FP were tested; one containing a mIgG2a Fc domain and the other containing a mIgG1 Fc domain Two different dose levels of each mGITRL FP were tested; 5 mg/kg and 10 mg/kg. Saline treatment was employed as a negative control.

Results

[0253] The median survival in the saline treated group was 22 days and none of the mice in this group survived until the end of the study. Treatment with the mGITRL FP at 10 mg/kg extended median survival to 32 days and resulted in a 50% survival rate at the end of the study. In the group treated with the mGITRL FP at 5 mg/kg 9 out of 10 mice were alive at the end of the study and no median survival time could be defined. Treatment with the mGITRL FP mIgG1 at 5 or 10 mg/kg extended median survival to 28 and 22.5 days respectively and resulted in 2 out of 10 mice surviving until the end of the study. Results are presented in Figure 17.

This study demonstrates the potential of the mGITRL FP to mediate antitumor activity and indicates that the isotype of the FP can impact the level of antitumor activity observed.

Pharmacodynamic Effects

[0254] This study was conducted to determine the impact of mGITRL FP on the activation state and proportion of T-cells in the spleen and the tumor of tumor bearing mice.

Method

[0255] CT26 tumor bearing Balb/c female mice aged 7-9 weeks were treated by intraperitoneal injection with mGITRL FP at either 0.2 or 1 mg/kg as indicated. Saline treatment was employed as a negative control. Mice were sacrificed 7 days following initiation of treatment and flow cytometry was used to assess the frequency and phenotype of cells in the spleen and the tumor.

Results

[0256] Treatment with mGITRL FP resulted in an increase in the expression of the proliferation marker Ki67 in all T-cell sub-sets in the spleen suggesting an increase in the proliferation of these cells. See Figure 18.

[0257] Treatment with mGITRL FP resulted in an increase in the expression of the activation marker ICOS in all T-cell sub-sets in the spleen suggesting an increase in the activation of these cells. See Figure 19.

[0258] Treatment with mGITRL FP resulted in a decrease in the frequency of CD4+ FOXP3+ regulatory T-cells and CD4+ FOXP3- helper cells within the tumor, but did not alter the frequency of CD8+ cytotoxic T-cells. The overall result was an increased CD8:CD4 ratio within the tumor microenvironment. See Figure 20.

Method

Cell lines and Reagents

[0259] The TC-1 tumor line was obtained from ATCC (Cat # CRL 6475, Manassas, VA) and maintained in DMEM + 10% FBS + 1% penicillin/streptomycin. The CT26 tumor line was obtained from ATCC (Manassas, VA) and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. DTA-1 and isotype antibodies were purchased from Bio X Cell (West Lebanon, NH).

Tumor models

[0260] TC-1 experiments used female C57BL/6 mice (Cat# 000664) mice obtained from Jackson Labs (Bar Harbor, ME). CT26 experiments used female Balb/C mice obtained from Envigo (Frederick, MD). Mice were between 6 and 8 weeks of age at the time of tumor implantation. All animal experiments were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee. For TC-1 tumor implantation, 2 x 10^4 viable TC-1 cells was implanted subcutaneously into the left hind footpad. For CT26 tumor implantation, 5 × 10^5 cells were implanted in the right flank. Tumor growth was evaluated by direct measurement with calipers1. Bi-directional measurements were collected every 2–4 days, and tumor volume calculated using volume = (length · width2)/2. Tumors were allowed to develop for 6-14 days and then tumor bearing mice were randomized to treatment groups by tumor volume. Mice were euthanized when the primary tumor exceeded 1000 mm^3 for TC-1/footpad and 2000mm^3 for CT26/flank in accordance with IACUC protocol. For PD studies, mice were euthanized and tumors and spleens were harvested, crushed through a 70 uM filter (Corning, Corning, NY), and processed to a single cell suspension.

Functional T-cell responses and Flow Cytometry

[0261] For antigen specific stimulations, 1-2x10^6 live cells were plated per well with 1 ug AH1 peptide sequence SPSYVYHQF (SEQ ID NO: 56) (Anaspec, Fremont, CA) For all stains the order of stain was live/dead blue (Life Tech), extracellular proteins, FOXP3 Fix/Perm Kit (Ebioscience), and followed by intracellular cytokines. Antibodies included GITR (Clone DTA-1). All samples were run on either an LSR-II or Fortessa (BD San Jose, CA). All data was analyzed using FlowJo (Treestar, Ashland, OR).

Results

mGITRL-FP is a highly effective GITR agonist and this leads to the rejection of CT26 tumors

[0262] It has previously been shown that GITR agonism is effective at causing the regression and elimination of the CT26 tumor in Bablc mice. Balb/c mice were implanted with CT26 tumor cells and after 6 days were randomized by tumor volume (group n=10) and treated with a single administration or repeat administration of mGITRL-FP IgG2a (Q2Dx9). The dose range was from 5 mg/kg body weight down to 0.04 mg/kg body weight. For the single administration, doses down to

1mg/kg of mGITRL-FP eliminated all but one CT26 mouse tumor to day 30 (Figure 40B). The lowest doses yielded 3 eliminations in 0.2mg/kg and 2 in the 0.1 mg/kg groups. Adding additional administrations of mGITRL-FP increased efficacy and all but the lowest dose group of 0.04 mg/kg saw a 100% elimination rate (Figure 40C). The lowest repeat administration group saw 4 eliminations.

[0263] For evaluation of mGITRL-FP function, it was compared to DTA-1, a known monoclonal GITR agonist. Use of a dose ranging from 100 ug to 500 ug per mouse has previously been described. Each mouse was estimated to be roughly 20 grams, so doses ranged from 5 mg/kg to 25 mg/kg. Balb/c mice were implanted with CT26 tumor cells and after 10 days were randomized by tumor volume (group n=10) and then treated with a single administration of either mGITRL-FP IgG2a at 1mg/kg or DTA-1 at 5mg/kg or 25mg/kg (Figure 40D).

[0264] Increasing the dose of DTA-1 increased efficacy, however 1mg/kg of mGITRL-FP showed similar efficacy and tumor growth kinetics as those of DTA-1 at 5mg/kg. To determine if CD8 T-cells were necessary for our drug effect, the same groups were evaluated but selectively depleted CD8 T-cells using a monoclonal depletion antibody (Figure 40E). Without CD8 T-cells, the ability for either DTA-1 or mGITRL-FP to completely eliminate CT26 tumor was significantly diminished. Only 1-2 mice from each group survived tumor free.

[0265] Additionally, without CD8 T-cells, mGITRL-FP was less effective than DTA-1 at increasing median overall survival (Figure 40F). To understand how how mGITRL-FP interacts with CD8s, GITR expression on individual lymphoid populations was evaluated. CD4+ Tregs express higher levels of GITR than CD8 T-cells; however, both Tregs and CD8 T-cells in the tumor express higher levels of GITR than their respective populations in the spleen (Figure 40G).

mGITRL-FP depletes Tregs and increases tumor antigen specific T-cells

[0266] It has previously been shown that GITR agonists decrease Tregs as well as increase high avidity T-cell responses. To understand what pharmacodynamic effects GITRL-FP was capable of mediating, the CT26 model was evaluated during tumor regression. Balb/c mice with CT26 were treated with mGITRL-FP IgG2a and 8 days after dosing began, spleens and tumor were harvested. Two arms of the study were included, a TGI (tumor growth inhibition) group (n=5) and a PD (pharmacodynamic) group (n=5). All but a single mouse treated with mGITRL-FP was cured of CT26 (Figure 41A). On day 18, the PD group was sacrificed and evaluated both the phenotypes of

the immune cells present in the spleen and tumor. CD8 T-cell function was then evaluated by restimulation with AH1, the CT26 immunodominant epiptope. mGITRL-FP significantly increased decreased the number of Tregs in the spleen and tumor (Figure 41B). While mGITRL-FP did not change the percentage of CD8s, it significantly increased the number of CD8s that are antigen specific for AH1 (Figure 41C-D). In the spleen 5-10 % of the total CD8s were antigen specific and capable of producing IFNgamma and TNF alpha. In the tumor, the number was 5-15%. This is significant expansion of both tumor infiltrating lymphocytes as well as the peripheral reservoir. To evaluate whether mGITRL-FP had bound to both CD4 Tregs and CD8 T-cells, GITR was stained for using the DTA-1 antibody (Figure 41 E-F). Tregs showed more than a 75% reduction in GITR MFI (mean fluorescence intensity). This same effect was observed on the CD8 T-cells, showing that mGITRL-FP was altering DTA-1 binding on the CD8 T-cells. Based on this data, it was hypothesized T-cells would show increased KI-67 proliferation. After evaluation on day 8 after treatments, both CD4 and CD8 T-cells show significantly more KI-67 positive cells in both the tumor and spleen (Figure 41G-H).

mGITRL-FP expands antigen specific CD8 T-cells in a dose dependent manner

[0267] It was next investigated if mice cured of CT26 were protected from rechallenge with the same tumor. To evaluate this, cured mice were rechallenged from groups treated with a single dose of 1.0, 0.5, or 0.2 mg/kg of mGITRL-FP IgG2a (Figure 42A). All mice from the initial dose down are show, but only mice cured at D85 were rechallenged. All mice originally cured of CT26 were protected from rechallenge of the tumor, although in the lowest dosed groups, a small mass was measured but was quickly eliminated. To further interrogate this result, at day 120 the mice were sacrificed, splenocytes pulled, and restimulated against AH1 peptide. There was a dose dependent increase in the numbers of AH1 specific T-cells (Figure 42 B-C). In the 1 mg/kg mGITRL-FP group, 25% of the splenic CD8 T-cells are specific for a single epitope of CT26. These mice showed no increased in tumor size during re-challenge. The lowest dose, 0.2 mg/kg, had 6% antigen specific CD8s and showed a small growing mass upon rechallenge that was cleared by day 120.

Binding Affinity of a hexameric GITRL IgG1 FP to human GITR

[0268] The solution KD (dissociation constant) of a hexameric GITRL IgG1 FP comprising GITRL IgG1 FP monomeric subunits of SEQ ID NO: 6 for recombinant human GITR was determined using

a kinetic exclusion assay (KinExA) using a KinExA 3200 instrument (Sapidyne Instruments, Boise Idaho).

[0269] 2 nM solutions of the hexameric GITRL IgG1 FP in D-PBS, 0.02% sodium and 1 mg mL^{-1} bovine serum albumin buffer were titrated with recombinant human GITR and equilibrated overnight at 25 °C. Samples were transferred to the KinExA 3200 instrument which was temperature controlled at 25 °C. Sampling of the equilibrated mixtures was achieved using azlactone bead-bound streptavidin that had been titrated with minimally amine-biotinylated human GITR. The secondary detection reagent used was the Fc specific reagent DyLight 650 labelled Protein G' (a fragment of Protein G available from Sigma). Data was processed and interpreted using KinExAPro software (version 3.6.8.). A K_D of 82 nM was obtained for the binding of the hexameric GITRL IgG1 FP to human GITR.

Binding of a hexameric GITRL IgG1 FP to Recombinant Cynomolgus Monkey GITR by KinExA

[0270] The solution K_D of a hexameric GITRL IgG1 FP comprising GITRL IgG1 FP monomeric subunits of SEQ ID NO: 6 for recombinant cynomolgous GITR was determined using a KinExA in the same manner as described in Section 6.1 for human GITR. A K_D for the binding of the hexameric GITRL IgG1 FP to cynomolgus GITR of 107 nM was obtained.

Binding of a Hexameric GITRL IgG1 FP to Recombinant Cynomolgus Monkey GITR by ELISA

[0271] The cross-reactivity of a hexameric GITRL IgG1 FP comprising GITRL IgG1 FP monomeric subunits of SEQ ID NO: 6 to cynomolgus GITR was determined using an ELISA. The hexameric GITRL IgG1 FP was biotinylated and binding to immobilized cynomolgus GITR-Fc was detected using streptavidin-HRP and TMB substrate. Figure 21 shows that the binding of the hexameric GITRL IgG1 FP to cynomolgus GITR is very similar to human GITR, and that no binding is observed to the negative control CD137-Fc protein.

EXAMPLE 3: *In vivo* pharmacodynamic effect of intravenous GITRL IgG1 FP treatment in the Cynomolgus Monkey

[0272] To determine the pharmacodynamic parameters of a hexameric GITRL IgG1 fusion protein (FP) in the cynomolgus monkey, hexameric GITRL IgG1 FP comprising GITRL IgG1 FP

monomeric subunits of SEQ ID NO: 6 was administered by intravenous (IV) bolus injection to groups (5/group) of male Cynomologus monkeys.

Method

[0273] Separate groups of animals were injected on day 1, day 3 and day 5 with 1 or 10 mg/kg hexameric GITRL IgG1 FP. Control animals received vehicle (20mM Sodium Phosphate, 230 mM Sucrose, 0.02% P80, pH 7.5). Blood samples were taken during the predose phase and on Days 1, 3, 5, 9, 11, 15, 18, 22, and 29 for the measurement of pharmacodynamic endpoints.

Using standard flow cytometry methods, Ki67 positive T-cell populations were measured from the whole blood.

Results

[0274] An increase in the % Ki67 positive T-cell subpopulations was observed e.g. (CD3+CD4+CD95high CD28+/dim/- total memory CD4+ and CD3+CD8+CD95high CD28+/dim/- total memory CD8+ T-cells) indicative of cell proliferation (Figure 22). This observation reached a maximum at Day 11 or 15 of the dosing phase and then declined towards the end of the study.

EXAMPLE 4: Mutation of amino acids within GITRL FP and their impact on receptor binding, agonist activity and thermal stability

[0275] Generation of hGITRL FP variants with mutations in the GITRL Receptor Binding Domain

hGITRL FP variants were generated and tested for their ability to bind to and agonise GITR. In some cases their thermal stability was also assessed.

Method

[0276] Using gene synthesis and standard DNA cloning techniques, DNA vectors encoding GITRL FP variants were generated. Suspension CHO cells were transiently transfected, using PEI, with DNA vectors encoding the different hexameric hGITRL fusion proteins and grown for eight days at 37°C, shaking at 140rpm with 80% humidity. Forty millilitres of the conditioned media containing the secreted proteins was separated from cells and cell debris by centrifugation at 1,600 x g and filtration. The proteins were purified using Mab SelectSure™ resin and their size and integrity was analysed by reducing SDS-PAGE.

Competition of GITRL FP variants with recombinant trimeric ligand for binding to GITR

[0277] The effect of the GITRL FP molecules on human GITRL binding to human GITR was determined using a Homogeneous Time Resolved Fluorescence (HTRF) assay.

Method

[0278] The GITRL FP molecules were titrated into an HTRF assay in which binding of GITRL-HA (hemagglutinin tag) to GITR-Fc was measured. The human GITR Fc was conjugated with europium cryptate and an anti-HA antibody conjugated with XL665 was used to detect the GITRL-HA protein. IC_{50} values were determined by curve fitting the analyzed data to a four parameter logistic equation with Prism 5.01 software (GraphPad).

Results

[0279] The mutated hexameric GITRL FP molecules (hGITRL-FP wt, N92D, N104D and N161D) are all potent inhibitors of trimeric GITRL-HA binding to GITR-Fc and produced similar inhibition profiles and IC_{50} values (Figure 23 A, B).

Binding of GITRL FP variants to recombinant GITR-Fc

[0280] The functional activity of the different hGITRL FP molecules was determined in an assay using NFκB-luciferase reporter cells stably expressing hGITR. Luminesence, driven by agonism of hGITR and subsequent activation of the NFκB pathway, was measured.

Method

[0281] The hGITRL FP molecules were titrated into an HTRF assay in which binding of the hGITRL FP to hGITR-Fc was measured. The human hGITR Fc was conjugated with europium cryptate and an anti-FLAG antibody conjugated with XL665 was used to detect the hGITRL FP protein. KD values were determined by curve fitting the analyzed data to a one site saturation binding equation with Prism 5.01 software (GraphPad).

Results

[0282] The binding profiles for hGITRL FP wt and hGITRL FP N161D were very similar (KDs of 1.225nM and 1.079nM, respectively), whereas the N129A mutated hGITRL FP showed reduced binding to hGITRFc (KD = 3.774nM). When the N129A mutation was combined with the N161D mutation, the binding capacity of the hGITRL FP molecules was further negatively impacted (KD = 11.85nM) (*see* Fig. 24).

Activity of GITRL FP variants in a reporter assay

[0283] The functional activity of the different hGITRL-FP molecules was determined in an assay using NFκB-luciferase reporter cells stably expressing hGITR. Luminescence, driven by agonism of hGITR and subsequent activation of the NFκB pathway, was measured.

Method

[0284] hGITRL FP proteins were serially diluted 4-fold for a 6-point data curve and added in triplicate to 96 well plates. Then Jurkat NF-κB luciferase reporter cells transfected with human GITR were added to all wells of the assay plates and incubated at 37 °C for three hours. Luciferase expression was detected by adding Steady-Glo reagent to all wells of the assay plates. The plates were incubated for five minutes at room temperature and then luminescence was measured and EC_{50} values were generated using log(agonist) vs. response variable slope nonlinear curve fit in GraphPad™ Prism 5.01 (GraphPad Software, Inc. La Jolla, CA USA).

Results

[0285] All GITRL FP proteins trigger NF-κB signalling. The N92D and N161D mutant hGITRL FP proteins generated similar potency profiles and EC_{50} values to GITRL FP (wt). The N129A and N129A/N161D mutations negatively impacted the ability of the hGITRL FP to agonise GITR in this assay (Figure 25 A-C).

Activity of GITRL FP variants in a T cell re-stimulation assay

[0286] The functional activity of the different hGITRL FP molecules was determined in a co-stimulation assay using primary human T cells and a thymidine incorporation readout.

Methods

[0287] Human peripheral blood mononuclear cells (PBMCs)-derived $CD3^+$ T cells were stimulated via incubation in TC treated plates coated with mouse anti-human CD3 antibody for four days at 37°C. They were then pelleted by centrifugation, resuspended in assay media, added to a TC treated plate and incubated at 37°C for two days (rest phase). hGITRL FP molecules were serially diluted in assay media 2-fold over 10 points and added in triplicate to TC treated plates pre-coated with mouse anti-human CD3. After two hours the assay plates were washed and the $CD3^+$ T cells prepared previously were added to each well and incubated at 37°C for four days. After four days, tritiated thymidine in assay media was added into each well and plates were incubated at 37°C for a further 18 hours. After the incubation, the incorporation of thymidine was measured using a Topcount™.

Results

[0288] All hGITRL FP mutants tested demonstrated equivalent activity to hGITRL FP wt, with the exception of N92D which had reduced activity in two independent experiments (Figure 26 A-C).

Melting temperature of GITRL FP variants

[0289] The melting temperature of the GITRL FP wt and N92D proteins was determined using a fluorescent dye (Sypro™ Orange), the emission properties of which change in the presence of an unfolded protein.

Method

[0290] Thermostability of wt and N92D GITRL FP variants was assessed using a Sypro Orange-based assay to calculate melting temperatures (Tm). The proteins were first diluted to 0.5mg/mL in 2×PBS before dispensing into a 96 well PCR plate. Sypro™ Orange was added to each well on the plate, which was then sealed. Plates were read on a Real-Time PCR machine using a Chromo4™ continuous fluorescence detector. The temperature was set to increase from 20°C to 90°C with a read every 1°C and a hold time of 1s. Unfolding transitions were determined by plotting the fluorescence intensity and fluorescence derivative as a function of temperature. Each GITRL FP protein was analysed in duplicate.

Results

[0291] Melting temperatures for the two GITRL FP proteins are summarised in Table 4-1 below. Both variants have a transition peak at 67°C, however, hGITRL FP N92D displays an additional broad transition peak at a low temperature (54°C) suggesting structural instability (Figure 27).

Table 4-1: Transition temperatures for the 2 GITRL FP proteins

	Test sample	Tm1 (°C)	Tm2 (°C)
	GITRL FP wt	/	67
	GITRL FP N92D	54	67

Data summary and conclusions

[0292] The N104D GITRL FP protein demonstrated equivalent activity to the wild-type hGITRL FP counterpart in the GITR/GITRL competition binding assay and the primary T cell re-stimulation assay suggesting that N104 does not play a key role in the activity of hGITRL FP. The mutation of Asn161 to Asp represents the removal of an N-linked glycosylation site (see data in example 4) and this mutant (hGITRL FP N161D) retained activity in all assays (GITR/GITRL competition binding, direct binding, reporter and primary T cell re-stimulation assays), suggesting that Asn161, and the glycans at this site are not involved in important functions such as binding and agonism of GITR.

[0293] The N129A mutant demonstrated reduced binding to hGITR and reduced activity in the reporter assay suggesting it plays a role in binding to hGITR and subsequent agonism of hGITR. Interestingly, when this mutation was combined with the N161D mutation (which alone did not impact activity) the binding and agonism of GITR was reduced even further.

[0294] The N92D mutation did not appear to impact activity of hGITRL FP when it was tested in the reporter assay, however, reduced activity of hGITRL FP N92D was observed in the primary T cell re-stimulation assay. When the thermal stability of the N92D mutant was investigated, an additional very broad lower melting temperature transition peak was observed compared to hGITRL FP, suggesting some structural instability at lower temperatures (including 37°C). Due to the longer timecourse of the primary T cell assay (4 days), it could be envisaged that the hGITRL FP N92D molecule becomes unstable and unfolds, leading to reduced activity in this assay compared to the

reporter assay (3 hours). Thus, the mutation of Asn92 to Asp appears to be unfavourable in terms of structural stability.

EXAMPLE 5: Identification of amino acid residues within hGITRL FP that are glycosylated.

[0295] There are two potential N-glycosylation consensus sites within the GITR binding domain (N161 and N129). The presence and structure of the glycans at these sites, as well as at the canonical N-glycosylation site within the Fc domain (N297 in the context of an IgG; N78 in the mature hGITRL FP sequence), were determined by mass spectrometry.

Methods

Recombinant protein expression and purification

[0296] Recombinant hGITRL FP wt and N161D proteins were purified from the conditioned media of CHO cells transiently transfected with vectors encoding the relevant proteins using affinity chromatography and subsequent size exclusion chromatography.

Tryptic peptide mapping

[0297] Samples of hGITRL FP wt and hGITRL FP N161D were denatured, reduced and the reduced cysteines were alkylated. The samples were then digested with trypsin. After 4 hours at 37 C, the digestion was quenched by addition of acid. The peptides were separated by reverse phase on a UPLC and measured using a UV detector and a mass spectrometer. The resultant tryptic peptide sequences are provided in Table 5-1.

Results:

Table 5-1

ID	Sequence	Sample
T7	EEQY**N**STYR (SEQ ID NO:43)	hGITRL FP wt & hGITRL FP N161D
T40	DMIQTLT**N**K (SEQ ID NO:44)	hGITRL FP wt & hGITRL FP N161D

ID	Sequence	Sample
T42-43	IQNVGGTYELHVGDTIDLIFNSEHQVLK**N**N TYWGIILLANPQFIS (SEQ ID NO:45)	hGITRL FP wt
T43'	DNTYWGIILLANPQFIS (SEQ ID NO:46)	hGITRL FP N161D

N = Asparagine part N-glycosylation consensus sequence.

D = N161D substitution in hGITRL FP N161D

[0298] A schematic diagram of the types of oligosaccharide structures found in the various hGITRL FP is provided in Figure 28. Mass spectrometry data is provided in Figures 29 A, B, and C.

Conclusion

[0299] Peptide mapping analysis was used to determine N-glycosylation site occupancy in the GITRL and Fc domains, as well as the structure of the predominant oligosaccharides at each site. In both hGITRL FP wt and hGITRL FP N161D proteins the canonical Fc N-glycosylation site (N78) was glycosylated and the predominant carbohydrate structures were neutral, biantennary complex type oligosaccharides; typical for IgG Fc regions expressed in CHO cells. The N-glycosylation consensus site at N129 within the GITRL RBD was not occupied with any oligosaccharide structures in either hGITRL FP wt or hGITRL FP N161D. The N-glycosylation consensus site at N161 within the GITRL RBD was occupied in hGITRL FP wt and the predominant carbohydrate structures were found to be neutral and charged, biantennary complex type oligosaccharides. In the hGITRL FP N161D protein, peptide mapping confirmed the N161D amino acid substitution, which removes the N161 N-glycosylation consensus site from the GITRL RBD. As expected no N-glycosylation was detected at D161.

EXAMPLE 6: Murine models and OX40 combination studies

[0300] In order to better understand targeting of GITR, the activity and pharmacodynamic effects of murine GITRL-FP were compared to those of an agonistic murine OX40L FP targeting OX40.

Materials and Methods

NF-κB reporter assay

[0301] Jurkat mGITR or human OX40 NFκB cells were cultured at 37°C, 5% CO_2 and 85% humidity in 96 well plates at 50,000 cells (Jukat mGITR) or 200,000 cells (Jurkat human OX40) / well together with anti-GITR antibody DTA-1 (Biolegend), NIP rIgG2b isotype control, mGITRL-

FP mIgG2a or plate immobilized mOX40L-FP mIgG1 or isotype control (comprised a single amino acid mutation at a position (Y182A) that rendered the protein unable to signal via OX40) as indicated. Steady Glo® Reagent (Promega) containing luciferin substrate, was added to the plates after 5 hours or after 16 hours for assays containing mOX40L-FP mIgG1 and the plates were incubated for 30 min in the dark on a plate shaker. Assay signal was detected using an Envision plate reader (Perkin Elmer).

SDS PAGE

[0302] Five micrograms of protein were mixed with loading buffer and reducing agent, denatured at 80°C for 10 mins and loaded onto a 4-20% Tris-Glycine SDS-PAGE gel (Thermo Fisher), alongside a protein molecular weight marker (Rainbow Marker, GE Healthcare). The proteins were electrophoresed for 45 mins at200V and stained using Instant Blue protein stain (Sigma).

Mice and tumor models

[0303] 8-10-week-old BALB/c or C57BL/6 female mice were obtained from Charles River UK Ltd. or Harlan Laboratories Inc. A 100 µL suspension of CT26 or B16F10-Luc2 cells in PBS at a cell density of 5×10^6 cells/mL or 5×10^4 cells/mL 100 µl was subcutaneously injected into the right flank of each animal. The B16F10-Luc2 cell line incorporated a luciferase reporter under the control of the CAG promoter and cells were implanted in 50 % PBS and 50 % growth factor reduced and phenol red free matrigel (Corning). Measurable tumors were randomized based on tumor volume. The length (mm) and width (mm) of each tumor was measured with an electronic caliper 3 times per week. Volumes of tumors (mm^3) were calculated based on the formula (length [mm] × width $[mm]^2$)/ 2. Tumor growth responses were categorized as a response if there was no measureable tumor or a sustained tumor growth inhibition such that volume was less than $200mm^3$ at the end of the study. Number of regressions indicated on each spider plot is the proportion relative to the total number of tumors implanted. Mice were dosed i.p. with either mGITRL-FP or mOX40L-FP at day 6 post implantation of syngeneic tumors, or when they reached a volume of $200mm^3$. Dosing of 25 mg/kg mGITRL-FP in B16F10-Luc2 bearing mice was only tolerated for four doses.

Flow Cytometry

[0304] Tumour, spleen or tumor draining lymph node were dissected and placed into RPMI-1640 media on ice. Tissues were disaggregated by passing each through a 40 or 100 μm nylon cell strainer (Falcon), and cells were pelleted by centrifugation and resuspended in red blood cell lysis buffer (Sigma). Following incubation for 2 minutes at room temperature, cells were washed and resuspended in flow cytometry buffer (Ebioscience). Tumor tissue samples were processed using the gentle MACS dissociator and tumor dissociation kit (Miltenyi Biotec) following manufacturer's instructions.

[0305] Samples were stained for viability using live dead fixable blue (Life Technologies) following manufacturer's instructions and then blocked with anti-CD16/32 (ebioscience) before staining with fluorochrome conjugated antibodies. CD4 (Rm4.5), Foxp3 (FJK-16S), Ki67 (Sol A15), ICOS (7E.17G9), Eomes (Dan11mag), T-bet (Apr-46) and GITR (DTA-1) were purchased from ebioscience. CD45 (30-F11), CD44 (IM7), CD62L (MEL-14), PD-1 (29F.1A12), and OX40 (OX86) were purchased from Biolegend. CD8 (53-6.7) was purchased from BD Pharmingen. For staining of intracellular antigens, the Foxp3 staining kit (ebioscience) was used according to manufacturer's instructions. Samples were fixed in 3.7% formalin before acquisition of samples using a Fortessa (Becton Dickinson). Data were analyzed with FlowJo software (Ashland, OR).

Pharmacokinetic modelling

[0306] Balb/c mice were administered 5 or 15 mg/kg mGITRL-FP mIgG2a once. Three mice per group were sacrificed 5 minutes 0.5, 1, 2, 6, 24, 72, 144, and 240 hours following treatment. Serum samples were collected and the levels of circulating mGITRL-FP mIgG2a present were assessed in a sandwich ELISA using anti-murine GITRL mAb (R&D Systems) as both capture and detection.

[0307] Pharmacokinetic (PK) data obtained from the 2 dosing groups were pooled and simultaneously modeled using a population approach. Population analysis was conducted using a pharmacostatistical software package NONMEM (Version 7.2, ICON Development Solutions, Ellicott City, Maryland). The FOCE method with Interaction option was employed. Murine GITRL-FP mIgG2a PK was adequately descripted by a two-compartment model.

Cell Lines

[0308] HEK293T-17 cells (ATCC, CRL-11268) were maintained in DMEM (Invitrogen) plus 10% v/v heat inactivated fetal bovine serum (HI FBS, HyClone) and 1 X non-essential amino acids (NEAA, Invitrogen). Jurkat cells (ATCC, TIB-152) were maintained in RPMI 1640 (Invitrogen) plus 10% v/v HI FBS. Jurkat mGITR NFκB cells were maintained in RPMI 1640 supplemented with 10% HI FBS, 5 μg/mL blasticidin (Invitrogen) and 5 μg/mL puromycin (Invitrogen).

Cell Line Generation

[0309] Jurkat mGITR NFκB cell line was generated by lentiviral transduction using a third generation lentiviral system (Systems Biosciences). The murine GITR gene (NM_009400.2) was cloned into the expression plasmid under the control of a CAG promoter and containing a puromycin resistance gene. The NFκB reporter expression plasmid was designed to express five copies of the NFκB response element under a minimal promoter and upstream of a firefly luciferase reporter (luc2, Promega), together with a blasticidin resistance gene. Each expression plasmid alongside the packaging plasmids (Systems Biosciences) were co-transfected into HEK293T-17 using lipofectamine 2000 (Invitrogen). Supernatant containing viral particles was collected 48 h post transfection and used to transduce Jurkat cells. Virally transduced Jurkat cells were cultured in culture media plus selection antibiotics, 5 μg/mL blasticidin and 5 μg/mL puromycin from 48 h post transduction. Jurkat human OX40 NFκB cell line was generated using similar procedures, except a lentiviral vector designed to constitutively express human OX40 was transduced. Mouse OX40L binds to human OX40; therefore, a human OX40-expressing NFκB luciferase reporter Jurkat cell line can be used to assess the activity of murine OX40L fusion proteins.

ELISA

[0310] Recombinant mouse GITR-Fc and OX40-Fc (R&D Systems) glycoproteins were coated overnight at 1 μg/mL in PBS onto 96-well plates (Greiner). Plates were washed with PBST (PBS + 0.01 % Tween-20), blocked for 1 hour at room temperature with PBS containing 1 % (w/v) BSA, and washed in PBST again. Twenty-five microlitres of 1 μg/mL mouse GITRL or OX40L FPs diluted in assay buffer [PBS + 1 % bovine serum albumin (BSA)] was added to wells, and plates were incubated for 2 hours at room temperature. After 3 washes, 25 μL of 1 μg/mL horseradish peroxidase–conjugated anti-mouse Fc antibody (Sigma) diluted in assay buffer was added to each

well, and plates were incubated for 1 hour. After incubation, plates were washed 3 times in PBST, 25 µL of tetramethylbenzidine substrate solution (KPL) was added to each well, and plates incubated for 5 minutes. After incubation 15 µL of 0.5 M sulphuric acid stop solution was added to all wells. The optical density at 450 nm was measured using an EnVision plate reader (PerkinElmer).

Statistics

[0311] All statistical analysis was carried out using the Prism Statistical Software Version 6.

Results

mGITRL-FP *in vitro* potency

[0312] In order to investigate the effects of GITR receptor signalling on immune cell activation and antitumor activity in vivo, a tetrameric mGITRL-FP was generated. The mGITRL-FP was designed to elicit avid binding to the GITR receptor and FcγRs on effector cells. The molecule consisted, from N- to C-terminus, of a fragment crystallisable (Fc) region of an immunoglobulin G (IgG), an isoleucine zipper domain (ILZ) and the extracellular (GITR-binding) domain (ECD) of murine GITR ligand (Figure 30A). When the purified denatured mGITRL-FP was visualised by SDS-PAGE (Figure 30B), it demonstrated a high degree of homogeneity and a molecular weight that was slightly higher than the expected molecular weight of 48kDa presumably due to glycosylation of the Fc and mGITRL domains. Both mGITRL-FP mIgG2a and an anti-GITR antibody (DTA-1) were able to induce NF-κB signalling in a GITR-dependent NFκB reporter gene cell assay, whereas a murine OX40L FP mIgG1 (mOX40L-FP mIgG1), and isotype control lacked any detectable signal (Figure 30C). Importantly, the tetrameric mGITRL-FP demonstrated an EC_{50} of 0.05 nM with respect to GITR agonism in this assay, which was nearly 50 times more potent than DTA-1, which demonstrated an EC_{50} of 2.31 nM.

Antitumor activity of mGITRL-FP engineered with a mIgG2a Fc

[0313] Previous studie have shown that activating FcγRs are required for the antitumor activity of the DTA-1 antibody, and that the antitumor activity of this antibody is increased when it carries a mIgG2a Fc, compared to a rIgG2b Fc or a N297A mutant Fc which lacks FcγR binding. To determine the impact of the Fc isotype on the antitumor activity of mGITRL-FP, CT26 tumor bearing mice were treated with mGITRL-FP with a mIgG2a or mIgG1 Fc isotype. Treatment of mice

with 5 or 10 mg/kg of either isotype resulted in notable antitumor activity compared to saline treated controls, as evidenced by reduced tumor volume (Figure 31A). The antitumor activity overall, however, was greater following treatment with mGITRL-FP mIgG2a (11/20 total regressions), as opposed to mGITRL-FP mIgG1 (7/20 total regressions, Figure 31A).

[0314] To determine if this difference in activity of the Fc variants was due to a difference in their ability to mediate T-cell activation and subsequent events downstream of GITR agonism, the proliferation of splenic T-cell populations was assessed using flow cytometric analysis of Ki67 expression on $CD4^+$ $FoxP3^-$ (effector T-cells), $CD8^+$ (effector T-cells) and $CD4^+$ $FoxP3^+$ (T-regs) cells. Both isotypes of the mGITRL-FP caused a comparable significant increase in the proliferation of all three splenic T-cell subsets when compared to saline controls (Figure 31B). The splenic T-reg cell population exhibited the highest expression of Ki67 after treatment (53.95 % ± 0.75 after mGITRL-FP mIgG2a and 58.43 % ± 1.26 after mGITRL-FP mIgG1 treatment), followed by $CD4^+$ $FoxP3^-$ (9.52 % ± 0.58 with mGITRL-FP mIgG1 and 10.89 % ± 0.56 with mGITRL-FP mIgG1) and $CD8^+$ T-cells (7.60 % ± 0.27 with mGITRL-FP mIgG2a and 7.79 ± 0.43 with mGITRL-FP mIgG1). This data suggested that splenic T-cell activation alone could not account for the differences observed in antitumor immunity between mIgG1 and mIgG2a Fc isotypes of the mGITRL-FP.

[0315] It was next investigated whether intratumoral changes in T-cell populations could explain the increased antitumor activity observed with the mIgG2a versus mIgG1 variants of mGITRL-FP. Treatment of mice with mGITRL-FP mIgG2a induced a significant decrease in the frequency of intratumoral T-regs from 13.31 % ± 1.06 in saline treated animals to 3.60 % ± 0.79 with a significance value of $p<0.0001$ (Figure 31C and Figure. 32) and a subsequent increase in the CD8:T-reg (Figure. 31D) and CD4:T-reg (Figure 32B) ratios compared to control treated animals. In contrast, the decrease in intratumoural T-regs was not evident for the mIgG1 Fc variant of mGITRL-FP (12.95 % ± 1.34). There was also evidence for a significant decrease in the proportion of intratumoral $CD4^+$ $Foxp3^-$ T-cells after treatment with mGITRL-FP mIgG2a (8.64 % ± 1.21) compared to control animals (13.01 % ± 1.12), but this was not to the extent of that observed for T-regs. The preferential depletion of T-regs by mGITRL-FP mIgG2a is likely attributable to the high expression of GITR on intratumoral T-regs (Figure 31E) and the high expression of activating FcγRs in the tumor microenvironment of CT26 tumors, and is suggestive of clearance by antibody dependent cellular cytotoxicity (ADCC) or antibody dependent cellular phagocytosis (ADCP). Collectively these data suggest that for optimal antitumor activity following mGITRL-FP treatment,

proliferation of peripheral $CD4^+$ and $CD8^+$ T-cells coincident with a decrease in intratumoral T-regs is required.

mGITRL-FP mIgG2a mediated antitumor activity

[0316] Because mGITRL-FP mIgG2a elicited increased antitumor activity compared to the mGITRL-FP mIgG1, how exposure levels and time of exposure of the mGITRL-FP mIgG2a in the blood related to antitumor responses in CT26 tumor-bearing mice was next characterised. First, the effect of increasing dose level on tumor growth by treating mice with one single dose of saline control or 0.2, 1, or 5 mg/kg mGITRL-FP mIgG2a was measured. Treatment with 0.2 mg/kg mGITRL-FP mIgG2a resulted in only 1/10 complete regressions, however there was evidence for a dose dependent increase in antitumor immunity when the dose was raised from 0.2 to 1 mg/kg or 5 mg/kg, which resulted in 6/10 and 9/10 regressions respectively (Figure 33A). Furthermore, the antitumor activity could also be improved by increasing the frequency of 0.2 mg/kg dosing to once every day (Q1D) or once every week (Q1W) (Figure 33B). These results indicated that there was a required exposure level of mGITRL-FP mIgG2a needed to induce optimal antitumor activity, as defined by complete tumor regression and minimal residual tumor volume, which was reached only when using the Q1D schedule. To determine this threshold the concentration of mGITRL-FP mIgG2a was measured in the blood of mice treated with 2 alternative dose levels of mGITRL-FP and then these results were incorporated into a PK model. Based on this model, the blood concentration of mGITRL-FP mIgG2a sustained by the Q1D schedule, and considered as required to maintain optimal antitumor activity was calculated to be equal to or greater than 1 μg/mL (Figure 33C).

[0317] PD biomarkers of anti-tumor activity was next investigated following mGITRL-FP mIgG2a treatment. The expression of Ki67 was used to assess proliferation, while ICOS, PD-1 and OX40, which are all known to be expressed on T-cells following activation, were also analysed. Increasing the exposure of mGITRL-FP mIgG2a, either by increasing the dose level or by increasing the frequency of administration, resulted in progressively greater increases in the frequency of Ki67, ICOS, PD-1 and OX40 expressing peripheral $CD4^+$ T-cells (Figure 34A-D).

Comparative analysis of mGITRL-FP and mOX40L-FP antitumor activity

[0318] In order to understand the relative mechanism of GITR, as opposed to OX40 targeting, the antitumor activities of the mGITRL-FP were directly compared with mOX40L-FP, which comprises a similar protein structure, does not cross react with the GITR receptor and is able to induce NFκB expression in an OX40 reporter cell assay (Figure 35A and B).

[0319] CT26 tumor bearing mice were treated twice a week with 5 mg/kg mGITRL-FP or mOX40L-FP of either mIgG1 or mIgG2a Fc isotypes and tumor growth was measured. As seen with mGITRL-FP, the antitumor activity observed with mOX40L-FP was greater following treatment with the mIgG2a isotype (9/10 regressions) than with the mIgG1 (1/10 regressions; Figure. 36A). However, treatment with the mGITRL-FP mIgG1 (6/10 regressions) elicited increased antitumor activity relative to that seen following treatment with mOX40L-FP mIgG1 and was marginally better with a mIgG2a isotype (10/10) compared to mOX40L-FP mIgG2a (Figure 36A). A similar depletion in intratumoral T-regs was observed following treatment with both mGITRL-FP and mOX40L-FPs with mIgG2a Fc isotypes, but this was not evident for mIgG1 Fc isotypes of either molecule (Fig. 5B). This data indicates that the mIgG2a isotype is superior to the mIgG1 for inducing antitumor activity of both GITR and OX40 agonists in CT26 tumor bearing mice.

mGITRL-FP mIgG2a and mOX40L-FP mIgG1 combination studies

[0320] Given the upregulation of OX40 receptor on $CD4^+$ T-cells following treatment with mGITRL-FP mIgG2a (Figure 34D), the potential to maximise T-cell activation was further investigated. mGITRL-FP mIgG2a and mOX40L-FP mIgG1 were used to assess the potential benefits of combining agents targeting the GITR and OX40 pathways.

[0321] Biweekly treatment with mGITRL-FP mIgG2a and mOX40L-FP mIgG1 combination resulted in significantly increased expression of Ki67 in splenic $CD4^+$ (29.16 % ± 1.51) and $CD8^+$ (24.4 % ± 0.87) T-cells compared to saline controls (7.82 % ± 0.30 and 9.22 % ± 0.31) or treatment with either monotherapy (14.2 % ± 0.45 and 15.52 % ± 0.66 for mGITRL-FP treated CD4 and CD8 T-cells and 17.02 % ± 0.49 and 16.22 % ± 1.22 for mOX40L-FP treated CD4 and CD8 T-cells; Figure 37A), showing an additive effect of combining both molecules. Additional analysis of the splenic $CD4^+$ and $CD8^+$ T-cell populations showed that combination treatment increased the frequency of both the $CD4^+$ and $CD8^+$ effector memory compartment, defined as $CD44^+$ and $CD62^{lo}$ (Fig. 37B) and the $CD4^+$ central memory population, as defined as $CD44^+$ and $CD62^+$ (Figure 37C) above that of monotherapy treatment. The expression of the transcription factors T-bet (Figure 37D)

and Eomes (Fig. 37E) which have shown redundancy in IFNy production and cytotoxicity in $CD8^+$ T-cell and also have roles in Th1 differentiation, were also increased to a greater degree on $CD4^+$ and $CD8^+$ T-cells during combination than with either monotherapy. A mechanistic difference between the GITR and OX40 pathways was the significantly higher expression of T-bet and Eomes on $CD4^+$ T-cells in mOX40L-FP mIgG1 treated animals as compared to animals treated with mGITRL-FP mIgG2a.

[0322] Based on these findings, the effect of combining mGITRL-FP mIgG2a with mOX40L-FP mIgG1 on tumor growth was determined. It was next investigated whether treatment of mice with mOX40L-FP mIgG1 could be further improved by combination with a single sub-optimal dose of mGITRL-FP mIgG2a. Treatment with mOX40L-FP mIgG1 monotherapy induced 5/10 regressions, mGITRL-FP mIgG2a monotherapy treatment induced 3/10 regressions, but the combination of both mGITRL-FP mIgG2a and mOX40L-FP mIgG1 resulted in enhanced antitumor activity showing 8/10 regressions (Figure 38A).

[0323] Given that monotherapy treatment with mGITRL-FP mIgG2a at high doses induced complete tumor regression in the majority of mice in the CT26 model, the combination of high doses of mOX40L-FP mIgG1 and mGITRL-FP mIgG2a was investigated for increased benefit in a B16F10-Luc 2 model. Dosing with either monotherapy did not induce any tumor regression in this model, however the combination of both molecules resulted in improved antitumor activity; a delay in tumour growth and the survival increased compared to monotherapy treatment (Figure 38B and Figure 39).

EXAMPLE 7: T cell priming agent in combination with mGITRL-FP

Materials and Methods

Cell lines and Reagents

[0324] The TC-1 tumor line was obtained from ATCC (Cat # CRL 6475, Manassas, VA) and maintained in DMEM + 10% FBS + 1% penicillin/streptomycin. The CT26 tumor line was obtained from ATCC (Manassas, VA) and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. DTA-1 and isotype antibodies were purchased from Bio X Cell (West Lebanon, NH).

E7 SLP and Vaccination

[0325] E7 Synthetic Long Peptide (SLP) consisting of the 45-mer HPV16-E7 sequence SSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVD (SEQ ID NO: 57) was synthesized from New England Peptide (Gardner, MA). E7 SLP was dosed at 10 or 3.3 μg and was formulated with 20 μg CpG ODN 2395 (TriLink, San Diego, CA) in Addavax (Life Technologies, Carlsbad, CA) and PBS in a total volume of 50 μL. Vaccinations were administered subcutaneously into the dorsal surface of the base of tail.

Tumor models

[0326] TC-1 experiments used female C57BL/6 mice (Cat# 000664) mice obtained from Jackson Labs (Bar Harbor, ME). CT26 experiments used femail Balb/C mice obtained from Envigo (Frederick, MD). Mice were between 6 and 8 weeks of age at the time of tumor implantation. All animal experiments were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee. For TC-1 tumor implantation, 2 x 10^4 viable TC-1 cells was implanted subcutaneously into the left hind footpad. For CT26 tumor implantation, 5 × 10^5 cells were implanted in the right flank. Tumor growth was evaluated by direct measurement with calipers1. Bi-directional measurements were collected every 2–4 days, and tumor volume calculated using volume = (length · width2)/2. Tumors were allowed to develop for 6-14 days and then tumor bearing mice were randomized to treatment groups by tumor volume. Mice were euthanized when the primary tumor exceeded 1000 mm^3 for TC-1/footpad and 2000mm^3 for CT26/flank in accordance with IACUC protocol. For PD studies, mice were euthanized and tumors and spleens were harvested, crushed through a 70 uM filter (Corning™, Corning, NY), and processed to a single cell suspension.

Functional T-cell responses and Flow Cytometry

[0327] For antigen specific stimulations, 1-2x10^6 live cells were plated per well with 1 ug AH1 peptide sequence SPSYVYHQF (SEQ ID NO: 56) (Anaspec, Fremont, CA) or 10 ug E7 peptide sequence RAHYNIVTF (SEQ ID NO: 58) (Anaspec) and Protein Transport Inhibitor (Ebioscience, Santa Clara, CA). After 5 hours, cells were stained. For all stains the order of stain was live/dead blue (Life Tech), extracellular proteins, FOXP3 Fix/Perm Kit (Ebioscience), and followed by intracellular cytokines. Antibodies included CD45 (Clone 30-F11), CD4 (Clone RM4-5) CD8

(Clone 53-6.7), GITR (Clone DTA-1), IFNgamma (Clone XMG 1.2), TNFalpha (Clone MP6-XT22), FOXP3 (Clone FJK-16s), KI-67 (Clone SolA15). H2-Db E7 Dextramer loaded with RAHYNIVTF was purchased from Immudex (Fairfax, VA) and we followed their protocol for staining. All samples were run on either an LSR-II or Fortessa (BD San Jose, CA). All data was analyzed using FlowJo (Treestar, Ashland, OR).

mGITRL-FP requires antigen specific cells

[0328] CT26 is a very immunogenic tumor model with high Intratumoral CD45 and basal CTL levels. CT26 has been shown to self prime low levels of AH1 specific T-cells. To assess whether mGITRL-FP could lead to the de novo generation of antigen specific responses the E6/E7 transformed TC-1 tumor model was used. This model has known CD8 T-cell epitopes and that E7 specific immune response can lead to protection from tumors that carry E7. TC-1 tumor cells were implanted in the footpad of C57/b6 mice. Mice were measured at day 14, randomized based on tumor size and treated with mGITRL-FP IgG2a at 1mg/kg, 5 mg/kg, and 20mg/kg (Figure 43A). There was delay or inhibition of tumor growth at any dose tested (Figure 43B). On day 24, subsets of untreated and the 1mg/kg treated mice were sacrificed and spleens and tumors harvested. T-cell populations and E7 antigen specific responses were assessed. GITR levels were present on the CD4 and CD8 T-cells, and were significantly higher in the tumor (Figure 43C). Without E7 priming, mGITRL-FP could not delay TC-1 tumor growth or generate an anti-tumor immune response.

mGITRL-FP expands primed antigen specific CD8 T-cells

[0329] Vaccination with DNA vaccines has been shown to be effective in generation of E7 specific responses that lead to the prevention and inhibition of tumor growth of the TC-1 tumor model. This study hypothesized that mGITRL-FP may need primed antigen specific T-cells to drive an anti-tumor response. To generate this response, naïve C57/b6 mice were vaccinated with 10 ug of an E7 synthetic long peptide (SLP) with CpG (addavax) at the base of tail. During this time the mice were treated with 3 doses of mGITRL-FP IgG2a. After 7 days, splenocytes were pulled and evaluated T-cells for antigen specificity with an E7 dextramer (Figure 44A). No differences in CD4 and CD8 percentages were seen, but a large increase in antigen specific cells was observed when treated with mGITRL-FP (Figure 44 B-D). Additionally, GITR levels on vaccinated mice alone were evaluated, the antigen specific Dex+ cells had a higher GITR MFI than Dex- CD8 T-cells (Figure 44E-F). It

was hypothesized that this difference would be even higher in mice with tumor, as GITR levels seemed to be highest in the tumor microenvironment. C57/b6 mice were implanted with TC-1 tumors and vaccinated with E7 SLP/CpG (Addavax) (Figure 44G). Priming of antigen specific E7 cells was observed as measured by dextamer in both spleen and in the tumor (Figure 44H-I). The cells that stained positive with the dextramer were also higher for GITR in both the spleen and the tumor (Figure 44J-K). E7 SLP was able to successfully prime an E7 specific response with or without the presence of tumor and the antigen specific cells that were primed expressed higher levels of GITR than other CD8 T-cells.

mGITRL-FP can expand antigen specific cells primed by E7 SLP and generate protective immune response to TC-1 tumors

[0330] To evaluate whether mGITRL-FP could expand an E7 sepcific response to TC-1 tumor cells, TC-1 tumor cells were implanted into the footpad and randomized mice by tumor volume on day 14. A single dose of E7 SLP given with CpG (Addavax) at the base of tail with mGITRL-FP IgG2a for four doses starting the day of vaccination (Figure 45A). Two arms of the study were included, a TGI (tumor growth inhibition) group (n=10) and a PD (pharmacodynamic) group (n=5). Control mice tumors quickly grew and all mice died with a median survival of 27 days. E7 SLP alone provided survival advantage to mice with TC-1 tumors with 1 of 10 mice alive and tumor free at day 85 and a median survival of 46.5 days (Figure 45B). E7 SLP + mGITRL-FP significantly delayed tumor growth with 3/10 mice tumor free and 5/10 alive at day 85 and a media survival of 80.5 days (Figure 45C-D). Vaccination alone provided a delay in tumor progression and adding mGITRL-FP on top further delay progression. It was hypothesized that this occurred because of selection expansion of tumor specific CD8s. At day 21, the group of PD mice was sacrificed and harvested spleen and tumor for pharmacodynamics analysis. Tumors from the footpad were pooled due to amount of tissue harvested (Figure 45E). Vaccination alone or in combination with mGITRL-FP resulted in a significant increase in CD45+ cells into the tumor (Figure 45F). To evaluate antigen specific function, single cell suspensions of tumor or spleen were restimulated with 1ug/mL of E7 peptide. Vaccination provided a basal increase in antigen specific cells and addition of mGITRL-FP further boosted this to higher levels in both the spleen and tumor (Figure 45G). mGITRL-FP appeared to not deplete Tregs in the spleen and was only able to deplete Tregs in the tumor when E7 SLP vaccine was present (Figure 45G).

EXAMPLE 8: Nucleotide and protein sequences.

[0331] Annotated versions of nucleotide and protein sequences disclosed here are provide in Table 6-1.

Table 6-1.

SEQ ID NO.	Description	Sequence
1	Amino Acid Sequence of full-length WT GITRL	MCLSHLENMPLSHSRTQGAQRSSWKLWLFCSIVMLLFLCSFS WLIFIFLQLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSD WKLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQ TLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLKNNTYWGII LLANPQFIS
2	Amino Acid Sequence of full-length GITRL Variant	MCLSHLENMPLSHSRTQGAQRSSWKLWLFCSI**X1X2**LLFLCSFS WLIFIFLQL**X3**TAKEPCMAK**X4**GPL**X5X6**KWQ**X7**ASSEP**X8**C**X9**N KV**X10**DWKLEILQNGLYLIY**X11**QVAPNANYNDVA**X12**FEV**X13**L YKNKD**X14**IQTLTNKSKIQNVGGTYELHVGDTIDLIF**X15**SEHQ**X 16**LK**X17**NTYWG**X18X19**LLANPQFIS X_1= Gly or Val, X_2= Thr, Met, or Val; X_3= Glu or Ala, X_4= Ser or Phe, X_5=Thr or Pro , X_6=Leu or Ser , X_7=Thr or Met, X_8= Leu or Pro, X_9= Met or Val, X_{10}= Thr, Phe, or Ser , X_{11}= Ser or Gly, X_{12}= Arg or Pro, X_{13}= Trp or Arg, X_{14}= Leu or Met, X_{15}= Ser or Asn, X_{16}=Phe or Val, X_{17}=Any amino acid other than Asn , X_{18}= Val or Ile , and X_{19}=Leu or Ile
3	Amino Acid Sequence of full-length GITRL N161X Variant	MCLSHLENMPLSHSRTQGAQRSSWKLWLFCSIVMLLFLCSFS WLIFIFLQLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSD WKLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQ TLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLK**X**NTYWGII LLANPQFIS X is any amino acid except Asn.
4	Amino Acid Sequence of full-length GITRL N161D Variant	MCLSHLENMPLSHSRTQGAQRSSWKLWLFCSIVMLLFLCSFS WLIFIFLQLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSD WKLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQ TLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLK**D**NTYWGII LLANPQFIS
5	Nucleic Acid sequence encoding a mature GITRL	CTGGACAAGACCCATACCTGTCCTCCATGCCCTGCCCCCGA ACTGCTGGGAGGCCCTTCTGTGTTCCTGTTCCCCCCAAAGC CCAAGGACACCCTGATGATCTCCCGGACCCCCGAAGTGAC CTGCGTGGTGGTGGATGTGTCCCACGAGGACCCTGAAGTG

	IgG1 FP subunit (w/o signal peptide encoding region)	AAGTTCAATTGGTACGTGGACGGCGTGGAAGTGCACAACG CCAAGACCAAGCCCAGAGAGGAACAGTACAACTCCACCTA CCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGATTGGC TGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAGGC CCTGCCTGCCCCCATCGAAAAGACCATCTCCAAGGCCAAG GGCCAGCCCCGGGAACCCCAGGTGTACACACTGCCCCCTA GCCGGGAAGAGATGACCAAGAACCAGGTGTCCCTGACCTG TCTCGTGAAGGGCTTCTACCCCTCCGATATCGCCGTGGAAT GGGAGTCCAACGGCCAGCCTGAGAACAACTACAAGACCAC CCCCCCTGTGCTGGACTCCGACGGCTCATTCTTCCTGTACTC CAAGCTGACAGTGGACAAGTCCCGGTGGCAGCAGGGCAAC GTGTTCTCCTGCTCCGTGATGCACGAGGCCCTGCACAACCA CTACACCCAGAAGTCCCTGTCCCTGAGCCCTGGAAAAGGC GGCGGAGGATCTGGCGGAGGCGGTTCTGGTGGTGGCGGAT CTGGGGGCGGAGGTAGCGGAGGTGGTGGCTCTGTGTCTCG GCTGGAAGAGGAAATGCGGAAGCTGCAGGCCACCGTGCAG GAACTGCAGAAGCGGCTGGACAGACTGGAAGAGACAGTGC AGGCTAAGGGCGGTGGCGGACAGCTCGAGACAGCCAAAG AACCCTGCATGGCCAAGTTCGGCCCCCTGCCTTCCAAGTGG CAGATGGCCTCTTCCGAGCCCCCCTGCGTGAACAAAGTGTC CGACTGGAAGCTGGAAATCCTGCAGAACGGCCTGTACCTG ATCTACGGCCAGGTGGCCCCCAACGCCAACTACAACGATG TGGCCCCCTTCGAAGTGCGGCTGTACAAGAACAAGGACAT GATCCAGACCCTGACCAACAAGAGCAAGATCCAGAACGTG GGCGGCACCTACGAGCTGCACGTGGGCGATACCATCGACC TGATCTTCAACTCCGAGCACCAGGTGCTGAAGGACAACAC CTACTGGGGCATCATCCTGCTGGCCAACCCCCAGTTCATCT CC
6	Amino Acid Sequence of a mature GITRL IgG1 FP subunit (Linker 1 and Linker 2 are underlined)	LDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLSPGKGGGGSGGGGSGGGGSGGGGSGGGG SVSRLEEEMRKLQATVQELQKRLDRLEETVQAKGGGGQLET AKEPCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQNGL YLIYGQVAPNANYNDVAPFEVRLYKNKDMIQTLTNKSKIQNV GGTYELHVGDTIDLIFNSEHQVLKDNTYWGIILLANPQFIS
7	Nucleic Acid encoding precursor GITRL IgG1 FP subunit (including signal peptide	ATGGCCATCATCTACCTGATCCTGCTGTTCACCGCCGTGCG GGGCCTGGACAAGACCCATACCTGTCCTCCATGCCCTGCCC CCGAACTGCTGGGAGGCCCTTCTGTGTTCCTGTTCCCCCCA AAGCCCAAGGACACCCTGATGATCTCCCGGACCCCCGAAG TGACCTGCGTGGTGGTGGATGTGTCCCACGAGGACCCTGA AGTGAAGTTCAATTGGTACGTGGACGGCGTGGAAGTGCAC AACGCCAAGACCAAGCCCAGAGAGGAACAGTACAACTCCA

	encoding region)	CCTACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGAT TGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACA AGGCCCTGCCTGCCCCCATCGAAAAGACCATCTCCAAGGC CAAGGGCCAGCCCCGGGAACCCCAGGTGTACACACTGCCC CCTAGCCGGGAAGAGATGACCAAGAACCAGGTGTCCCTGA CCTGTCTCGTGAAGGGCTTCTACCCCTCCGATATCGCCGTG GAATGGGAGTCCAACGGCCAGCCTGAGAACAACTACAAGA CCACCCCCCCTGTGCTGGACTCCGACGGCTCATTCTTCCTG TACTCCAAGCTGACAGTGGACAAGTCCCGGTGGCAGCAGG GCAACGTGTTCTCCTGCTCCGTGATGCACGAGGCCCTGCAC AACCACTACACCCAGAAGTCCCTGTCCCTGAGCCCTGGAA AAGGCGGCGGAGGATCTGGCGGAGGCGGTTCTGGTGGTGG CGGATCTGGGGGCGGAGGTAGCGGAGGTGGTGGCTCTGTG TCTCGGCTGGAAGAGGAAATGCGGAAGCTGCAGGCCACCG TGCAGGAACTGCAGAAGCGGCTGGACAGACTGGAAGAGAC AGTGCAGGCTAAGGGCGGTGGCGGACAGCTCGAGACAGCC AAAGAACCCTGCATGGCCAAGTTCGGCCCCCTGCCTTCCAA GTGGCAGATGGCCTCTTCCGAGCCCCCCTGCGTGAACAAA GTGTCCGACTGGAAGCTGGAAATCCTGCAGAACGGCCTGT ACCTGATCTACGGCCAGGTGGCCCCCAACGCCAACTACAA CGATGTGGCCCCCTTCGAAGTGCGGCTGTACAAGAACAAG GACATGATCCAGACCCTGACCAACAAGAGCAAGATCCAGA ACGTGGGCGGCACCTACGAGCTGCACGTGGGCGATACCAT CGACCTGATCTTCAACTCCGAGCACCAGGTGCTGAAGGAC AACACCTACTGGGGCATCATCCTGCTGGCCAACCCCCAGTT CATCTCC
8	Amino Acid Sequence of a precursor GITRL IgG1 FP subunit (including signal peptide encoding region; Linker 1 and Linker 2 are underlined)	MAIIYLILLFTAVRGLDKTHTCPPCPAPELLGGPSVFLFPPKPKD TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGGGSGGGGSG GGGSGGGGSGGGGSVSRLEEEMRKLQATVQELQKRLDRLEE TVQAKGGGGQLETAKEPCMAKFGPLPSKWQMASSEPPCVNK VSDWKLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNKD MIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLKDNTY WGIILLANPQFIS
9	Amino Acid Sequence of Human Coronin 1a (hCor1a)	MSRQVVRSSKFRHVFGQPAKADQCYEDVRVSQTTWDSGFC AVNPKFVALICEASGGGAFLVLPLGKTGRVDKNAPTVCGHTA PVLDIAWCPHNDNVIASGSEDCTVMVWEIPDGGLMLPLREPV VTLEGHTKRVGIVAWHTTAQNVLLSAGCDNVIMVWDVGTG AAMLTLGPEVHPDTIYSVDWSRDGGLICTSCRDKRVRIIEPRK GTVVAEKDRPHEGTRPVRAVFVSEGKILTTGFSRMSERQVAL WDTKHLEEPLSLQELDTSSGVLLPFFDPDTNIVYLCGKGDSSIR YFEITSEAPFLHYLSMFSSKESQRGMGYMPKRGLEVNKCEIAR FYKLHERRCEPIAMTVPRKSDLFQEDLYPPTAGPDPALTAEEW

		LGGRDAGPLLISLKDGYVPPKSRELRVNRGLDTGRRRAAPEA SGTPSSDAVSRLEEEMRKLQATVQELQKRLDRLEETVQAK
10	Amino Acid Sequence of hCor1a Trimerization Domain Consensus	XSRXEEEXRKXQATXQELQKRXDRLEETVQAK X= Ala, Leu, Ile, or Val
11	Amino Acid Sequence of hCor1a wt Trimerization Domain	VSRLEEEMRKLQATVQELQKRLDRLEETVQAK
12	Amino Acid Sequence of hCor1a variant 1 Trimerization Domain	VSRLEEEIRKLQATVQELQKRLDRLEETVQAK
13	Amino Acid Sequence of hCor1a variant 2 Trimerization Domain	VSRIEEEIRKLQATVQELQKRLDRLEETVQAK
14	Amino Acid Sequence of hCor1a variant 3 Trimerization Domain	ISRIEEEIRKLQATVQELQKRLDRLEETVQAK
15	Amino Acid Sequence of hCor1a variant 4 Trimerization Domain	ISRIEEEIRKIQATVQELQKRLDRLEETVQAK
16	Amino Acid Sequence of hCor1a variant 5 Trimerization Domain	ISRIEEEIRKIQATVQELQKRIDRLEETVQAK
17	Amino Acid Sequence of hCor1a variant 6 Trimerization Domain	ISRIEEEIRKINATVQELQKRIDRLEETVQAK
18	Amino Acid Sequence of	ISRIEEEIRKINATIQELQKRIDRLEETVQAK

	hCor1a variant 7 Trimerization Domain	
19	Amino Acid Sequence of Gly(4)Ser Motif where n=1	GGGGS
20	Amino Acid Sequence of Linker 1	GGGGSGGGGSGGGGSGGGGSGGGGS
21	Amino Acid Sequence of IgG1 Fc region used in the GITRL IgG1 FP of SEQ ID NO:6	LDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLSPGK
22	Amino Acid Sequence of Ser(Gly_4Ser)n motif where n=1	SGGGGS
23	Amino Acid Sequence of Linker region	GGGGSGGGGSGGGGSAL
24	Amino Acid Sequence of Linker region	GGGGSGGGGSGGGGSA
25	Amino Acid Sequence of Gly(4)Ser Motif where n=3	GGGGSGGGGSGGGGS
26	Amino Acid Sequence of Linker region	GGGGSGGGGSGGGG
27	Amino Acid Sequence of yeast GCN4 pII	IKQIEDKIEEILSKIYHIENEIARIKKL
28	Amino Acid Sequence of Matrilin 1 wt trimerisation domain	CACESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVV
29	Amino Acid Sequence of Matrilin 1	CACESLVKFQAKVEGLIQALTRKLEAVSKRIAILENTVV

	variant trimerisation domain	
30	Amino Acid Sequence of DMPK wt trimerisation domain	EAEAEVTLRELQEALEEEVLTRQSLSREMEAIRTDNQNFASQL REAEARNRDLEAHVRQLQERMELLQAE
31	Amino Acid Sequence of DMPK variant trimerisation domain	IAEIEVTIRELQEAIEEEVLTRQSLSREIEAIRTDIQNIASQLREIE ARIRDLEAHVRQLQERMELLQAE
32	Amino Acid Sequence of Langerin wt trimerisation domain	ASALNTKIRALQGSLENMSKLLKRQNDILQVVS
33	Amino Acid Sequence of Langerin variant trimerisation domain	ISALNTKIRAIQGSIENMSKLIKRQNDIIQVVS
34	Amino Acid Sequence of Mature WT GITRL extracellular domain	QLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQ NGLYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQTLTNKSKI QNVGGTYELHVGDTIDLIFNSEHQVLKNNTYWGIILLANPQFI S
35	Amino Acid Sequence of Mature GITRL Variant extracellular domain	QL**X**$_1$TAKEPCMAK**X**$_2$GPL**X**$_3$**X**$_4$KWQ**X**$_5$ASSEP**X**$_6$C**X**$_7$NKV**X**$_8$DW KLEILQNGLYLIY**X**$_9$QVAPNANYNDVA**X**$_{10}$FEV**X**$_{11}$LYKNKD**X**$_{12}$ IQTLTNKSKIQNVGGTYELHVGDTIDLIF**X**$_{13}$SEHQ**X**$_{14}$LK**X**$_{15}$NT YWG**X**$_{16}$**X**$_{17}$LLANPQFIS X_1= Glu or Ala, X_2= Ser or Phe, X_3=Thr or Pro , X_4=Leu or Ser , X_5=Thr or Met, X_6= Leu or Pro, X_7= Met or Val, X_8= Thr, Phe, or Ser , X_9= Ser or Gly, X_{10}= Arg or Pro, X_{11}= Trp or Arg, X_{12}= Leu or Met, X_{13}= Ser or Asn, X_{14}=Phe or Val, X_{15}=Any amino acid other than Asn , X_{16}= Val or Ile , and X_{17}=Leu or Ile
36	Amino Acid Sequence of Mature GITRL N161X Variant extracellular domain	QLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQ NGLYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQTLTNKSKI QNVGGTYELHVGDTIDLIFNSEHQVLK**X**NTYWGIILLANPQFI S X IS ANY AMINO ACID EXCEPT Asn.

37	Amino Acid Sequence of Mature GITRL N161D Variant extracellular domain	QLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQ NGLYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQTLTNKSKI QNVGGTYELHVGDTIDLIFNSEHQVLK**D**NTYWGIILLANPQFI S
38	Amino Acid Sequence of IgG1 Fc region used in the GITRL IgG4P FP (S228P mutation underlined)	ESKYGPPCP**P**CPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPRE PQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH EALHNHYTQKSLSLSLGK
39	Nucleic Acid encoding a mature GITRL IgG4P FP subunit (w/o signal peptide encoding region)	GAGTCTAAGTACGGCCCTCCTTGTCCTCCTTGCCCTGCCCCT GAGTTTCTGGGCGGACCTTCCGTGTTCCTGTTCCCCCCAAA GCCCAAGGACACCCTGATGATCTCCCGGACCCCCGAAGTG ACCTGCGTGGTGGTGGATGTGTCCCAGGAAGATCCCGAGG TGCAGTTCAATTGGTACGTGGACGGCGTGGAAGTGCACAA CGCCAAGACCAAGCCCAGAGAGGAACAGTTCAACTCCACC TACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGATTG GCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAG GGCCTGCCCTCCAGCATCGAAAAGACCATCTCCAAGGCCA AGGGCCAGCCCCGGGAACCCCAGGTGTACACACTGCCTCC AAGCCAGGAAGAGATGACCAAGAACCAGGTGTCCCTGACC TGTCTCGTGAAGGGCTTCTACCCCTCCGATATCGCCGTGGA ATGGGAGTCCAACGGCCAGCCTGAGAACAACTACAAGACC ACCCCCCCTGTGCTGGACTCCGACGGCTCCTTCTTCCTGTA CTCCCGCCTGACCGTGGACAAGTCCAGATGGCAGGAAGGC AACGTGTTCTCCTGCTCCGTGATGCACGAGGCCCTGCACAA CCACTACACCCAGAAGTCCCTGTCCCTGTCTCTGGGCAAGG GCGGCGGAGGATCTGGCGGAGGCGGTTCTGGTGGTGGTGG ATCTGGTGGCGGAGGAAGTGGGGGAGGGGGATCTGTGTCT CGGCTGGAAGAGGAAATGCGGAAGCTGCAGGCCACCGTGC AGGAACTGCAGAAGCGGCTGGACAGACTGGAAGAGACAG TGCAGGCTAAGGGCGGTGGCGGACAGCTCGAGACAGCCAA AGAACCCTGCATGGCCAAGTTCGGCCCCCTGCCTTCCAAGT GGCAGATGGCCTCTTCCGAGCCCCCCTGCGTGAACAAAGT GTCCGACTGGAAGCTGGAAATCCTGCAGAACGGCCTGTAC CTGATCTACGGCCAGGTGGCCCCCAACGCCAACTACAACG ATGTGGCCCCCTTCGAAGTGCGGCTGTACAAGAACAAGGA CATGATCCAGACCCTGACCAACAAGAGCAAGATCCAGAAC GTGGGCGGCACCTACGAGCTGCACGTGGGCGATACCATCG ACCTGATCTTCAACTCCGAGCACCAGGTGCTGAAGGACAA CACCTACTGGGGCATCATCCTGCTGGCCAACCCCCAGTTCA TCTCC

40	Amino Acid Sequence of a mature GITRL IgG4P FP subunit	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPRE PQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH EALHNHYTQKSLSLSLGKGGGGSGGGGSGGGGSGGGGSGGG GSVSRLEEEMRKLQATVQELQKRLDRLEETVQAKGGGGQLE TAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQNG LYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQTLTNKSKIQN VGGTYELHVGDTIDLIFNSEHQVLKDNTYWGIILLANPQFIS
41	Nucleic Acid encoding a precursor GITRL IgG4P FP subunit (including signal peptide encoding region)	ATGGCCATCATCTACCTGATCCTGCTGTTCACCGCCGTGCG GGGCGAGTCTAAGTACGGCCCTCCTTGTCCTCCTTGCCCTG CCCCTGAGTTTCTGGGCGGACCTTCCGTGTTCCTGTTCCCCC CAAAGCCCAAGGACACCCTGATGATCTCCCGGACCCCCGA AGTGACCTGCGTGGTGGTGGATGTGTCCCAGGAAGATCCC GAGGTGCAGTTCAATTGGTACGTGGACGGCGTGGAAGTGC ACAACGCCAAGACCAAGCCCAGAGAGGAACAGTTCAACTC CACCTACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGG ATTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAA CAAGGGCCTGCCCTCCAGCATCGAAAAGACCATCTCCAAG GCCAAGGGCCAGCCCCGGGAACCCCAGGTGTACACACTGC CTCCAAGCCAGGAAGAGATGACCAAGAACCAGGTGTCCCT GACCTGTCTCGTGAAGGGCTTCTACCCCTCCGATATCGCCG TGGAATGGGAGTCCAACGGCCAGCCTGAGAACAACTACAA GACCACCCCCCCTGTGCTGGACTCCGACGGCTCCTTCTTCC TGTACTCCCGCCTGACCGTGGACAAGTCCAGATGGCAGGA AGGCAACGTGTTCTCCTGCTCCGTGATGCACGAGGCCCTGC ACAACCACTACACCCAGAAGTCCCTGTCCCTGTCTCTGGGC AAGGGCGGCGGAGGATCTGGCGGAGGCGGTTCTGGTGGTG GTGGATCTGGTGGCGGAGGAAGTGGGGGAGGGGGATCTGT GTCTCGGCTGGAAGAGGAAATGCGGAAGCTGCAGGCCACC GTGCAGGAACTGCAGAAGCGGCTGGACAGACTGGAAGAG ACAGTGCAGGCTAAGGGCGGTGGCGGACAGCTCGAGACAG CCAAAGAACCCTGCATGGCCAAGTTCGGCCCCCTGCCTTCC AAGTGGCAGATGGCCTCTTCCGAGCCCCCCTGCGTGAACA AAGTGTCCGACTGGAAGCTGGAAATCCTGCAGAACGGCCT GTACCTGATCTACGGCCAGGTGGCCCCCAACGCCAACTAC AACGATGTGGCCCCCTTCGAAGTGCGGCTGTACAAGAACA AGGACATGATCCAGACCCTGACCAACAAGAGCAAGATCCA GAACGTGGGCGGCACCTACGAGCTGCACGTGGGCGATACC ATCGACCTGATCTTCAACTCCGAGCACCAGGTGCTGAAGG ACAACACCTACTGGGGCATCATCCTGCTGGCCAACCCCCAG TTCATCTCC
42	Amino Acid Sequence of a precursor	MAIIYLILLFTAVRGESKYGPPCPPCPAPEFLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKT KPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPS

	GITRL IgG4P FP subunit (with signal peptide encoding region)	SIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSLSLGKGGGGSGGGGS GGGGSGGGGSGGGGSVSRLEEEMRKLQATVQELQKRLDRLE ETVQAKGGGGQLETAKEPCMAKFGPLPSKWQMASSEPPCVN KVSDWKLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNK DMIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLKDNTY WGIILLANPQFIS
43	hGITRL-FP wt & hGITRL-FP N161D polypeptide subunit tryptic fragment	EEQY**N**STYR
44	hGITRL-FP wt & hGITRL-FP N161D polypeptide subunit tryptic fragment	DMIQTLT**N**K
45	hGITRL-FP wt polypeptide subunit tryptic fragment	IQNVGGTYELHVGDTIDLIFNSEHQVLK**N**NTYWGIILLANPQFI S
46	hGITRL-FP N161D polypeptide subunit tryptic fragment	DNTYWGIILLANPQFIS
47	hGITR (human GITR)	MAQHGAMGAFRALCGLALLCALSLGQRPTGGPGCGPGRLLL GTGTDARCCRVHTTRCCRDYPGEECCSEWDCMCVQPEFHCG DPCCTTCRHHPCPPGQGVQSQGKFSFGFQCIDCASGTFSGGHE GHCKPWTDCTQFGFLTVFPGNKTHNAVCVPGSPPAEPLGWLT VVLLAVAACVLLLTSAQLGLHIWQLRSQCMWPRETQLLLEVP PSTEDARSCQFPEEERGERSAEEKGRLGDLWV
48	mGITRL-FP	MAIIYLILLFTAVRGIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVL MISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHR EDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERT ISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDI YVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNW VERNSYSCSVVHEGLHNHHTTKSFSRTPGKGGGGSGGGGSGG GGSGGGGSGGGGSIKQIEDKIEEILSKIYHIENEIARIKKLGGGG IESCMVKFELSSSKWHMTSPKPHCVNTTSDGKLKILQSGTYLI YGQVIPVDKKYIKDNAPFVVQIYKKNDVLQTLMNDFQILPIGG VYELHAGDNIYLKFNSKDHIQKTNTYWGIILMPDLPFIS
49	mOX40L-FP	VPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVV

		DISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELP IMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVY TIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQWNGQPAENY KNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGL HNHHTEKSLSHSPGKRLDQDKIEALSNKVQQLERSIGLKDLA MADLEQKVSELEVSTSSPAKDPPIQRLRGAVTRCEDGQLFISS YKNEYQTMEVQNNSVVIKCDGLYIIYLKGSFFQEVKIDLHFRE DHNPISIPMLNDGRRIVFTVVASLAFKDKVYLTVNAPDTLCEH LQINDGELIVVQLTPGYCAPEGSYHSTVNQVPL
50	MEDI6383	LATDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGKELLGGGSIKQIEDKIEEILSKIYHI ENEIARIKKLIGERGHGGGSNSQVSHRYPRFQSIKVQFTEYKK EKGFILTSQKEDEIMKVQNNSVIINCDGFYLISLKGYFSQEVNI SLHYQKDEEPLFQLKKVRSVNSLMVASLTYKDKVYLNVTTD NTSLDDFHVNGGELILIHQNPGEFCVL
51	MEDI0562 VH	QVQLQESGPGLVKPSQTLSLTCAVYGGSFSSGYWNWIRKHPG KGLEYIGYISYNGITYHNPSLKSRITINRDTSKNQYSLQLNSVT PEDTAVYYCARYKYDYDGGHAMDYWGQGTLVTVSSASTKG PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSD IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK
52	MEDI0562 VL	DIQMTQSPSSLSASVGDRVTITCRASQDISNYLNWYQQKPGKA PKLLIYYTSKLHSGVPSRFSGSGSGTDYTLTISSLQPEDFATYY CQQGSALPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C
53	Human OX40	MCVGARRLGR GPCAALLLLG LGLSTVTGLH CVGDTYPSND RCCHECRPGN GMVSRCSRSQNTVCRPCGPG FYNDVVSSKP CKPCTWCNLR SGSERKQLCTATQDTVCRCR AGTQPLDSYK PGVDCAPCPP GHFSPGDNQA CKPWTNCTLA GKHTLQPASN SSDAICEDRD PPATQPQETQGPPARPITVQ PTEAWPRTSQ GPSTRPVEVP GGRAVAAILG LGLVLGLLGP LAILLALYLL RRDQRLPPDA HKPPGGGSFR TPIQEEQADA HSTLAKI
54	Human OX40 ligand	MERVQPLEENVGNAARPRFERNKLLLVASVIQGLGLLLCFTYI CLHFSALQVSHRYPRIQSIKVQFTEYKKEKGFILTSQKEDEIMK VQNNSVIINCDGFYLISLKGYFSQEVNISLHYQKDEEPLFQLKK

		VRSVNSLMVASLTYKDKVYLNVTTDNTSLDDFHVNGGELILI HQNPGEFCVL
55	mOX40L-FP Y182A	VPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVV DISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELP IMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVY TIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQWNGQPAENY KNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGL HNHHTEKSLSHSPGKRLDQDKIEALSNKVQQLERSIGLKDLA MADLEQKVSELEVSTSSPAKDPPIQRLRGAVTRCEDGQLFISS YKNEYQTMEVQNNSVVIKCDGLYIIYLKGSFFQEVKIDLHFRE DHNPISIPMLNDGRRIVFTVVASLAFKDKVYLTVNAPDTLCEH LQINDGELIVVQLTPGACAPEGSYHSTVNQVPL
56	AH1 peptide	SPSYVYHQF
57	E7 Synthetic Long Peptide (SLP)	SSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRLCVQSTH VD
58	E7 peptide	RAHYNIVTF

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[0332] The breadth and scope of the present disclosure should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents.

WHAT IS CLAIMED IS:

1. An isolated single-chain polypeptide subunit comprising: an IgG Fc domain; a functional multimerization domain; and a receptor binding domain of a Glucocorticoid-Induced TNF Receptor Ligand (GITRL), wherein the polypeptide subunit can self-assemble into a trimeric or a hexameric protein.

2. The polypeptide subunit of claim 1, wherein the multimerization domain is a trimerization domain.

3. The polypeptide subunit of claim 1 or 2 comprising, from the amino terminus to the carboxy terminus, the IgG Fc domain, followed by the multimerization or trimerization domain, followed by the GITRL receptor binding domain.

4. The polypeptide subunit of any one of claims 1 to 3, wherein the carboxy terminus of the IgG Fc domain is fused to the amino terminus of the multimerization or trimerization domain via a first linker region.

5. The polypeptide subunit of any one of claims 1 to 4, wherein the carboxy terminus of the multimerization or trimerization domain is fused to the amino terminus of the GITRL receptor binding domain via a second linker region.

6. The polypeptide subunit of any one of claims 1 to 4, wherein the carboxy terminus of the IgG Fc domain is fused directly to the amino terminus of the multimerization or trimerization domain.

7. The polypeptide subunit of claim 5 or 6 wherein the IgG Fc domain includes an IgG hinge region at its amino terminus.

8. The polypeptide subunit of claim 5 or 6, wherein the IgG hinge region comprises a mutation that confers complete inter heavy chain disulfide bond formation.

9. The polypeptide subunit of claim 7 or 8, wherein the IgG hinge region comprises an IgG1 hinge region, an IgG4 hinge region, or variants thereof.

10. The polypeptide subunit of claim 9, wherein the IgG4 hinge region has a serine to proline mutation at position 228 (S228P) according to EU numbering (IgG4P).

11. The polypeptide subunit of any one of claims 1 to 10, wherein the IgG Fc domain is a human IgG Fc domain.

12. The polypeptide subunit of any one of claims 1 to 4 or to 6 to 10, wherein the carboxy terminus of the multimerization or trimerization domain is fused directly to the amino terminus of the GITRL receptor binding domain.

13. The polypeptide subunit of any one of claims 5 to 12, wherein the first linker region, the second linker region, or the first and second linker regions, when present, are independently selected from a group consisting of a linker region containing a (Gly_4)n motif, a (Gly_4Ser)n motif (SEQ ID NO: 19), a Ser(Gly_4Ser)n motif (SEQ ID NO: 22), GGGGSGGGGSGGGGSAL (SEQ ID NO: 23), GGGGSGGGGSGGGGSA (SEQ ID NO: 24), and combinations thereof, wherein n is a positive integer selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10.

14. The polypeptide subunit of claim 13, wherein the first and second linker regions are independently selected from a group consisting of GGGGSGGGGSGGGGS (SEQ ID NO: 25), and GGGGSGGGGSGGGG (SEQ ID NO: 26).

15. The polypeptide subunit of claim 13 or claim 14, wherein the first linker region is GGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 20) and the second linker region is a (Gly_4) motif.

16. The polypeptide subunit of any one of claims 1 to 15, wherein the IgG Fc domain comprises an IgG1, IgG2, IgG3, IgG4, IG4P Fc domain, or variants thereof.

17. The polypeptide subunit of any one of claims 1 to 16, wherein the IgG Fc domain contains one or more amino acid residue substitutions selected from the group consisting of 252Y, 254T, 256E, and combinations thereof, wherein the residues are numbered according to EU numbering.

18. The polypeptide subunit of any one of claims 1 to 17, wherein the IgG Fc domain comprises a CH2 region.

19. The polypeptide subunit of claim 18, wherein the IgG Fc domain further comprises a CH3 region.

20. The polypeptide subunit of claim 19, wherein the IgG Fc domain comprises an amino acid sequence having at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 21.

21. The polypeptide subunit of claim 19, wherein the IgG Fc domain comprises the amino acid sequence of SEQ ID NO: 21.

22. The polypeptide subunit of any one of claims 1 to 21, wherein the trimerization domain comprises an alpha-helical coiled coil domain, a leucine zipper domain, or a combination thereof.

23. The polypeptide subunit of claim 22, wherein the trimerization domain is derived from Matrilin 1, Coronin 1a, dystrophia myotonica kinase (DMPK), Langerin, or a combination thereof.

24. The polypeptide subunit of claim 23, wherein the trimerization domain is derived from Matrilin 1 or Coronin 1a.

25. The polypeptide subunit of claim 24, wherein the trimerization domain is derived from Coronin 1a.

26. The polypeptide subunit of claim 25, wherein the trimerization domain comprises a Coronin 1a trimerization domain having at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 11.

27. The polypeptide subunit of claim 26, wherein the Coronin 1a trimerization domain comprises the amino acid sequence of SEQ ID NO: 11.

28. The polypeptide subunit of claim 26, wherein the trimerization domain comprises a Coronin 1a trimerization domain of SEQ ID NO: 10.

29. The polypeptide subunit of claim 26, wherein the Coronin 1a trimerization domain comprises the amino acid sequence of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, or any combination or variant thereof.

30. The polypeptide subunit of claim 24, wherein the trimerization domain comprises a Matrilin 1 trimerization domain having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 28.

31. The polypeptide subunit of claim 23, wherein the trimerization domain comprises a DMPK trimerization domain having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 30.

32. The polypeptide subunit of claim 23, wherein the trimerization domain comprises a langerin trimerization domain having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 32.

33. The polypeptide subunit of claim 23, wherein the trimerization domain comprises the amino acid sequence of SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, or SEQ ID NO: 33.

34. The polypeptide subunit of any one of claims 1 to 33, wherein the GITRL receptor binding domain comprises an amino acid sequence with at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 34.

35. The polypeptide subunit of any one of claims 1 to 33, wherein the GITRL receptor binding domain comprises an amino acid sequence with at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 37 where residue 161 is not an asparagyl residue.

36. The polypeptide subunit of claim 35, wherein the GITRL receptor binding domain comprises the amino acid sequence of SEQ ID NO: 35.

37. The polypeptide subunit of any one of claims 1 to 33, wherein the GITRL receptor binding domain comprises the amino acid sequence of SEQ ID NO: 35 wherein residue 161 is an aspartyl residue.

38. The polypeptide subunit of claim 37 wherein the GITRL receptor binding domain comprises the amino acid sequence of SEQ ID NO: 36.

39. The polypeptide subunit of claim 38 wherein the GITRL receptor binding domain comprises the amino acid sequence of SEQ ID NO: 37.

40. The polypeptide subunit of any one of claims 1 to 39, wherein a hexameric protein assembled from six of the polypeptide subunits can specifically bind to human GITR.

41. The polypeptide subunit of any one of claims 1 to 40, wherein the protein is non-glycosylated.

42. The polypeptide subunit of any one of claims 1 to 41, comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 6.

43. The polypeptide subunit of claim 42, comprising the amino acid sequence of SEQ ID NO: 6.

44. The polypeptide subunit of any one of claims 1 to 43, wherein the subunit has agonist activity.

45. The polypeptide subunit of any one of claims 1 to 44, further comprising an associated heterologous agent.

46. The polypeptide subunit of claim 45, wherein the heterologous agent is a heterologous polypeptide and is fused to the polypeptide subunit via a peptide bond.

47. The polypeptide subunit of claim 46, wherein the heterologous polypeptide is fused to the N-terminus of the IgG-Fc domain, is fused to the C-terminus of the receptor binding domain of GITRL, is fused to the C-terminus of the IgG-Fc domain and to the N-terminus of the trimerization domain, or is fused to the C-terminus of the trimerization domain and to the N-terminus of the receptor binding domain of GITRL.

48. The polypeptide subunit of claim 45, wherein the heterologous agent is chemically conjugated to the polypeptide subunit.

49. The polypeptide subunit of any one of claims 45 to 48, wherein the heterologous agent comprises a cytotoxic molecule, a stabilizing agent, an immune response modifier, or a detectable agent.

50. A trimeric protein comprising three polypeptide subunits of any one of claims 1 to 49.

51. A hexameric protein comprising six polypeptide subunits of any one of claims 1 to 49.

52. The hexameric protein of claim 51 which can specifically bind to Glucocorticoid-Induced TNF Receptor (GITR) as expressed on $CD4^+$ or $CD8^+$ T cells, B cells, or NK cells, wherein the $CD4^+$ or $CD8^+$ T cells or B cells are optionally antigen experienced, or the NK cells are optionally activated, from human, or a non-human primate, optionally a cynomolgus monkey, a rhesus monkey, or any combination thereof.

53. The hexameric protein of claim 52, which can specifically bind to GITR as expressed on primary $CD4^+$ or $CD8^+$ T cells from human, or a non-human primate, optionally, a cynomolgus monkey, a rhesus monkey, or any combination thereof.

54. The hexameric protein of any one of claims 52 or 53, wherein the $CD4^+$ or $CD8^+$ T cells are antigen experienced.

55. The hexameric protein of claim 51 or claim 54, wherein the binding affinity for human GITR is about 54 nM to about 111 nM as measured by kinetic exclusion assay.

56. The hexameric protein of claim 55, wherein the binding affinity is about 82 nM as measured in a kinetic exclusion assay (KinExA).

57. The hexameric protein of any one of claims 51 to 56, which can induce dose-dependent proliferation and dose-dependent cytokine release from GITR positive immune cells.

58. The hexameric protein of claim 57, wherein the GITR positive immune cells are T cells in a plate-based assay.

59. The hexameric protein of any one of claims 52 to 58, wherein the cells are $CD4^+$, $CD8^+$, NK, or B cells.

60. The hexameric protein of any one of claims 57 to 59, wherein the GITR positive immune cells, T cells, $CD4^+$, $CD8^+$, or B cells are antigen experienced and/or NK cells are activated.

61. The hexameric protein of claim 60, which can stimulate proliferation of primary antigen experienced human T cells with an EC_{50} of about 0.1 to about 2.7 nM, as measured by thymidine incorporation.

62. The hexameric protein of claim 61, wherein the EC_{50} is about 0.5 nM.

63. The hexameric protein of claim 57, wherein the cytokine is IFNγ, TNFα, IL-5, IL-10, IL-2, IL-4, IL-13, IL-8, IL-12 p70, IL-1β, or any combination thereof.

64. The hexameric protein of any one of claims 51 to 63, which can activate downstream signalling pathways in GITR expressing cells, optionally wherein the downstream signaling pathway is the NFκB pathway or the MAPK pathway.

65. The hexameric protein of claim 64, wherein the GITR-expressing cells are T cells.

66. The hexameric protein of claim 64, wherein the GITR-expressing T cells are GITR-expressing Jurkat NFκB-luciferase reporter cells that produce luciferase in response to stimulation of the NFκB signaling pathway.

67. The hexameric protein of any one of claims 51 to 66, wherein administration of an effective dose to a subject in need of cancer treatment can inhibit tumor growth in the subject.

68. The hexameric protein of claim 67, wherein the tumor growth inhibition is achieved in the presence of GITR positive immune cells, optionally T-cells or NK cells.

69. The hexameric protein of claim 67 or 68, wherein tumor growth is inhibited by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 100% compared to administration of an isotype-matched control.

70. The hexameric protein of any one of claims 51 to 69, wherein the polypeptide subunit comprises an IgG1, IgG2, IgG3, IgG4 or IgG4P Fc domain and can induce cytokine activation and proliferation of GITR-expressing $CD4^+$ or $CD8^+$ T cells through binding to GITR, but does not substantially trigger complement-dependent or antibody-dependent cytotoxicity against the $CD4^+$ or $CD8^+$ T cells.

71. The hexameric protein of any one of claims 51 to 69, wherein the polypeptide subunit comprises an IgG1 Fc domain and triggers Fc receptor dependent cytotoxicity against cells expressing high levels of GITR.

72. The hexameric protein of any one of claims 51 to 69, wherein the polypeptide subunit comprises an IgG2, IgG3, IgG4, or IG4P Fc domain and can induce proliferation of GITR-expressing cells through binding to GITR.

73. The hexameric protein of claim 71, wherein the Fc receptor dependent cytotoxicity is antibody-dependent cellular cytotoxicity or antibody dependent phagocytosis.

74. The hexameric protein of claim 71, wherein the cells expressing high levels of GITR are tumor cells.

75. The hexameric protein of claim 71, wherein the cells expressing high levels of GITR are $CD4^+$ $FOXP3^+$ T cells or antigen experienced $CD4^+$ $FOXP3^-$ T cells.

76. The hexameric protein of claim 71, wherein the hexameric protein provides a decrease in the frequency of $CD4^+$ $FOXP3^+$ regulatory T cells in comparison to a control.

77. The hexameric protein of claim 71, wherein the hexameric protein provides a decrease in the frequency of $CD4^+$ $FOXP3^+$ regulatory T cells within a tumor in comparison to a control.

78. A composition comprising the hexameric protein of any one of claims 51 to 77, and a carrier.

79. A polynucleotide comprising a nucleic acid that encodes the polypeptide subunit of any one of claims 1 to 49, or the hexameric protein of any one of claims 51 to 78.

80. The polynucleotide of claim 79, wherein the polynucleotide further comprises a nucleic acid sequence encoding a signal peptide that is operably linked to the polypeptide subunit.

81. The polynucleotide of claim 80, wherein the nucleic acid encoding the signal peptide is operably linked to a nucleic acid encoding the amino-terminus of the IgG Fc domain of the polypeptide subunit.

82. The polynucleotide of claim 79, comprising SEQ ID NO: 5.

83. A vector comprising the polynucleotide of any one of claims 79 to 82.

84. A host cell comprising the polynucleotide of any one of claims 79 to claim 82 or the vector of claim 83.

85. A method of producing the polypeptide subunit of any one of claims 1 to 49, or the hexameric protein of any one of claims 51 to 77, comprising culturing the host cell of claim 84 under

conditions in which the polypeptide subunit or hexameric protein encoded by the polynucleotide or vector is expressed, and recovering the polypeptide subunit or hexameric protein.

86. A method to promote survival or proliferation of antigen experienced T cells and/or NK cells, comprising contacting antigen experienced T cells and/or NK cells with the hexameric protein of any one of claims 51 to 77 or the composition of claim 78, wherein the hexameric protein can specifically bind to GITR on the surface of the T cells and/or NK cells.

87. A method of inducing cytokine release from activated GITR expressing immune cells, comprising contacting these cells with the hexameric protein of any one of claims 51 to 77 or the composition of claim 78 wherein the hexameric protein can specifically bind to GITR on the surface of these cells.

88. The method of claim 87, wherein the cytokine is IFNγ, TNFα, IL-10, GM-CSF, or any combination thereof.

89. The method of any one of claims 87 or 88, wherein the GITR expressing immune cells are antigen experienced $CD4^+$ T cells, antigen experienced $CD8^+$ T cells, activated NK cells or a combination thereof.

90. The method of any one of claims 86 to 89, wherein the activated NK cells, antigen experienced $CD4^+$ or antigen experienced $CD8^+$ T cells are human NK cells, $CD4^+$ or $CD8^+$ T cells, cynomolgus monkey NK cells, $CD4^+$ or $CD8^+$ T cells, rhesus monkey NK cells, $CD4^+$ or $CD8^+$ T cells, or a combination thereof.

91. A method of promoting T cell or NK cell activation, comprising contacting T cells or NK cells with the hexameric protein of any one of claims 51 to 77 or the composition of claim 78 wherein the hexameric protein can specifically bind to GITR on the surface of the T cells or NK cells.

92. The method of claim 91, further comprising cross-linking of the hexameric protein through interaction of the Fc domain with a cell expressing FcγR.

93. The method of claim 92, wherein the cell expressing FcγR is a B cell, a monocyte, a macrophage, a myeloid or plasmacytoid dendritic cell, a follicular dendritic cell, a Langerhans cell, an endothelial cell, an NK cell, a neutrophil, a eosinophil, a platelet, a mast cell, a $CD45^+$ cell from a primary human tumor or tumor-draining or non-draining lymph node, a $CD45^+$ cell from other secondary or tertiary lymphoid structures, or a combination thereof.

94. The method of any one of claims 91 to 93, wherein NK cell or T-cell activation can be measured through stimulation of the NFκB or MAPK signal transduction pathway.

95. The method of any one of claims 91 to 94, wherein the NK or T cells are activated NK cells, antigen experienced $CD4^+$ T cells, antigen experienced $CD8^+$ T cells, or a combination thereof.

96. The method of claim 95, wherein the NK cells, $CD4^+$ or CD8+ T cells are primary human NK cells, $CD4^+$ or CD8+ T cells, cynomolgus monkey NK cells, $CD4^+$ or CD8+ T cells, rhesus monkey NK cells, $CD4^+$ or CD8+ T cells, or a combination thereof.

97. The method of any one of claims 86 to 96, wherein the contacting comprises administering an effective amount of the hexameric protein or a composition comprising the hexameric protein to a subject.

98. A method of treating cancer in a subject, comprising administering to a subject in need of treatment an effective amount of the hexameric protein of any one of claims 51 to 77, or the composition of claim 78.

99. The method of claim 98, wherein the subject is a human subject or a canine subject.

100. The method of claim 98, wherein the cancer is a solid tumor.

101. The method of claim 100, wherein the solid tumor is associated with a cancer selected from the group consisting of colorectal cancer, breast cancer, hepatocellular cancer, non-small cell lung cancer, small cell lung cancer, mesothelioma, head and neck cancer, gastric cancer, pancreatic cancer, melanoma, uveal melanoma, renal cancer, ovarian cancer, cervical cancer, glioblastoma, testicular cancer, thyroid cancer, prostate cancer, and oesophageal cancer.

102. The method of claim 98, wherein the cancer is a haematological cancer.

103. The method of claim 102, wherein the haematological cancer is selected from the group consisting of Hodgkins lymphoma, non-Hodgkins lymphoma, multiple myeloma, acute lymphoblastic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, and chronic myelogenous leukemia.

104. The method of any one of claims 98 to 103, wherein administration of the hexameric protein or composition can inhibit tumor growth, can promote tumor reduction, or both.

105. The method of any one of claims 98 to 104, wherein tumor growth inhibition is achieved in the presence of NK cells or T cells.

106. The method of claim 104 or claim 105, wherein tumor growth is inhibited by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70, at least 80%, at

least 90%, at least 95%, at least 98% or 100% compared to administration of an isotype-matched control that lacks a GITRL receptor-binding domain.

107. The method of any one of claims 98 to 106 wherein the hexameric protien comprises an IgG1, IgG2, IgG3, IgG4 or IgG4P Fc domain and can induce proliferation of antigen experienced, GITR-expressing immune cells through binding to GITR, but does not substantially trigger complement-dependent or antibody-dependent cytotoxicity against the activated immune cells.

108. The method of claim 107, wherein the GITR-expressing immune cells are $CD4^+$ T cells, NK cells, or $CD8^+$ T cells

109. The method of any one of claims 98 to 106, wherein the hexameric protein comprises an IgG1 Fc domain and triggers NK cell- and/or macrophage- mediated antibody-dependent cellular cytotoxicity (ADCC) or antibody dependent cell phagocytosis of cells expressing high levels of GITR.

110. The method of claim 109, wherein the cells expressing high levels of GITR are tumor cells.

111. The method of claim 109, wherein the cells expressing high levels of GITR are $CD4^+$ $FOXP3^+$ T cells.

112. The method of claim 111, wherein the hexameric protein provides a decrease in the frequency of $CD4^+$ $FOXP3^+$ regulatory T cells in comparison to a control.

113. The method of claim 111, wherein the hexameric protein provides a decrease in the frequency of $CD4^+$ $FOXP3^+$ regulatory T cells within a tumor in comparison to a control.

114. A method of enhancing an immune response in a subject comprising administering to a subject in need thereof a therapeutically effective amount of the hexameric protein of any one of claims 51 to 77, or the composition of claim 78.

115. The method of claim 114, wherein the subject is a human subject or a canine subject.

116. The method of claim 115, wherein the subject in need thereof has cancer.

117. The method of claim 116, wherein the cancer is a solid tumor.

118. The method of any one of claims 114 to 117, wherein the subject has colorectal cancer, breast cancer, hepatocellular cancer, non-small cell lung cancer (NSCLC), small cell lung cancer, mesothelioma, head and neck cancer, gastric cancer, pancreatic cancer, melanoma, uveal melanoma, renal cancer, ovarian cancer, cervical cancer, glioblastoma, testicular cancer, thyroid cancer, prostate

cancer, oesophageal cancer, bladder cancer, squamous cell carcinoma of the head and neck (SCCHN), colorectal cancer (CRC), haematological cancer, or a combination thereof.

119. The method of claim 118, wherein the cancer is a haematological cancer.

120. The method of claim 119, wherein the haematological cancer is selected from the group consisting of Hodgkins lymphoma, non-Hodgkins lymphoma, multiple myeloma, acute lymphoblastic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, and chronic myelogenous leukemia.

121. The method of any one of claims 114 to 120, wherein administration of the hexameric protein or composition can inhibit tumor growth, can promote tumor reduction, or both.

122. The method of any one of claims 114 to 121, wherein tumor growth inhibition is achieved in the presence of NK cells or T cells.

123. The method of any one of claims 121 or 122, wherein tumor growth is inhibited by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70, at least 80%, at least 90%, at least 95%, at least 98% or 100% compared to administration of an isotype-matched control that lacks a GITRL receptor-binding domain.

124. The method of any one of claims 114 to 123, wherein the hexameric protein comprises an IgG1, IgG2, IgG3, IgG4 or IgG4P Fc domain and can induce proliferation of activated, GITR-expressing immune cells through binding to GITR, but does not substantially trigger complement-dependent or antibody-dependent cytotoxicity against the activated immune cells.

125. The method of claim 124, wherein the GITR-expressing immune cells are $CD4^+$ T cells, NK cells, or $CD8^+$ T cells

126. The method of any one of claims 114 to 123, wherein the hexameric protein comprises an IgG1 Fc domain and triggers NK cell- and/or macrophage- mediated antibody-dependent cellular cytotoxicity (ADCC) or antibody dependent cell phagocytosis of cells expressing high levels of GITR.

127. The method of claim 126, wherein the cells expressing high levels of GITR are tumor cells.

128. The method of claim 126, wherein the cells expressing high levels of GITR are $CD4^+$ $FOXP3^+$ T cells.

129. The method of claim 126, wherein the hexameric protein provides a decrease in the frequency of $CD4^+$ $FOXP3^+$ regulatory T cells in comparison to a control.

130. The method of claim 126, wherein the hexameric protein provides a decrease in the frequency of $CD4^+$ $FOXP3^+$ regulatory T cells within a tumor in comparison to a control.

131. A method of treating a solid tumor in a subject, comprising administering the isolated single-chain polypeptide subunit of claim 1 and an OX40 agonist to the subject.

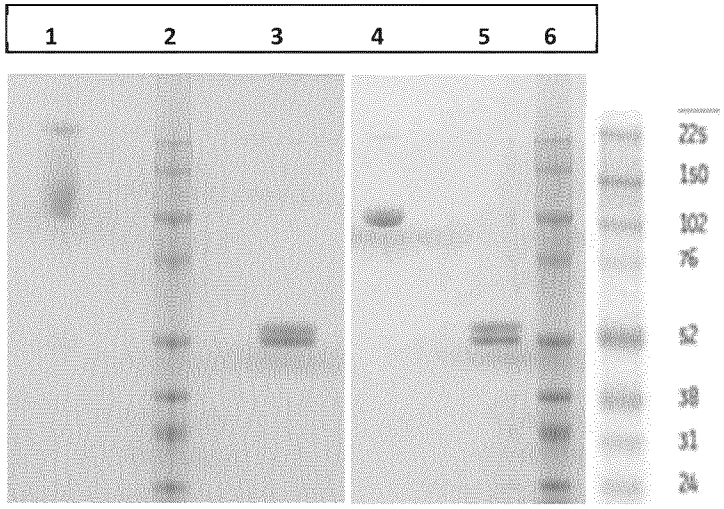
132. The method of claim 131, wherein the OX40 agonist is one or more of an OX40 ligand fusion protein or anti-OX40 antibody.

133. The method of claim 132, wherein the OX40 ligand fusion protein is MEDI6383.

134. The method of claim 132, wherein the anti-OX40 antibody is MEDI0562.

135. A method of treating cancer in a subject, comprising administering to a subject in need of treatment an effective amount of the hexameric protein of any one of claims 51 to 77, or the composition of claim 78 in combination with a T-cell priming agent.

136. The method of claim 136 wherein the T-cell priming agent is E7 Synthetic Loing Peptide (SLP) and plus CpG Oligodeoxynucleotide.



1 = GITRL-FP (Matrilin 1 wt) non-reduced

2 = Molecular weight markers

3 = GITRL-FP (Matrilin 1 wt) reduced

4 = GITRL-FP (Coronin 1a wt) non-reduced

5 = GITRL-FP (Coronin 1a wt) reduced

6 = Molecular weight markers

Figure 1

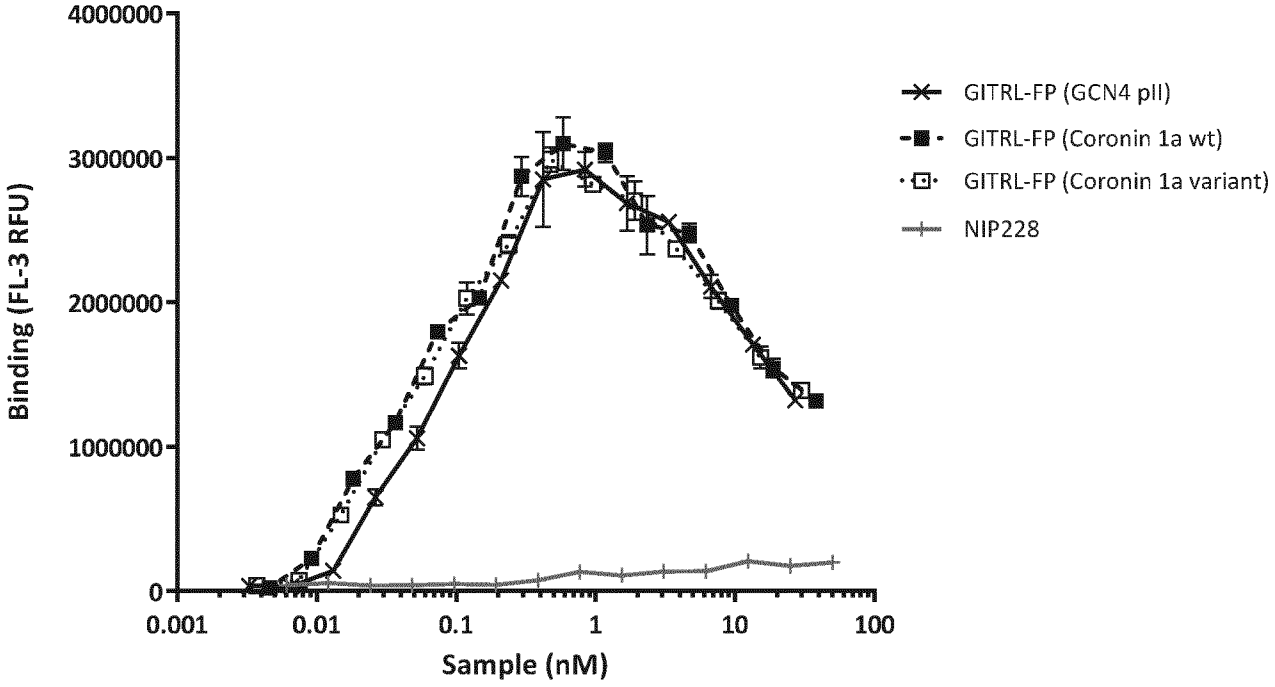


Figure 2A

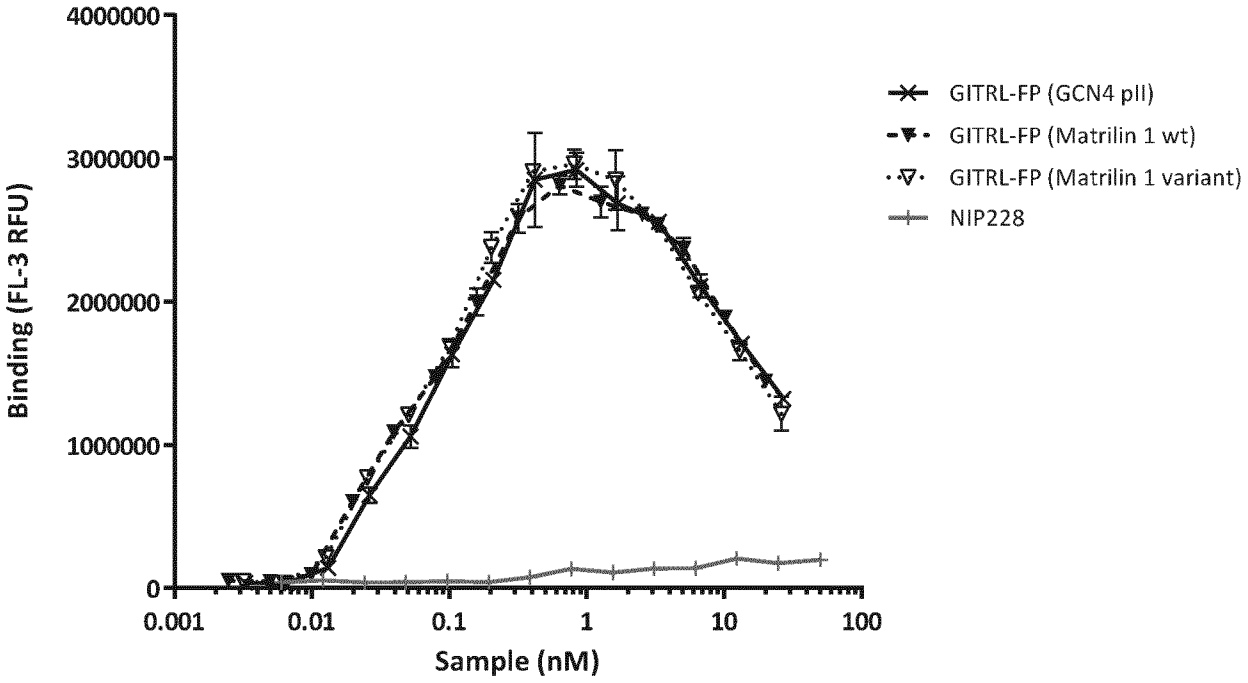


Figure 2B

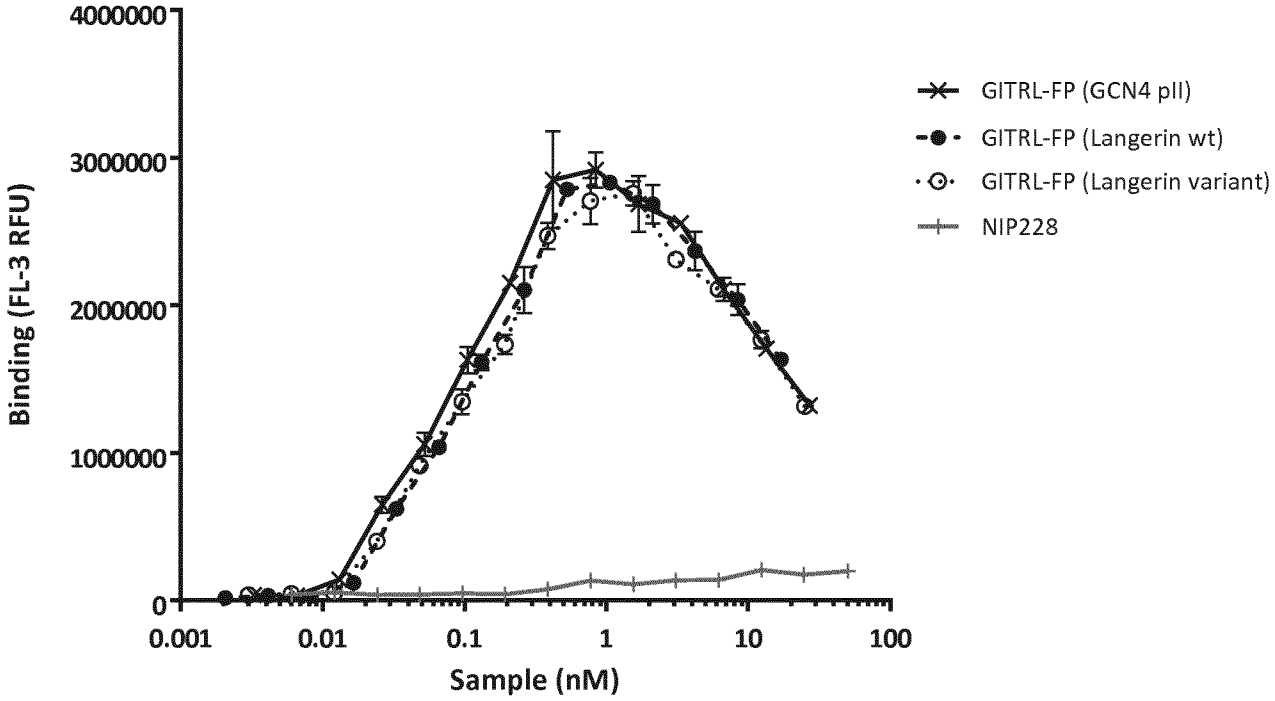


Figure 2C

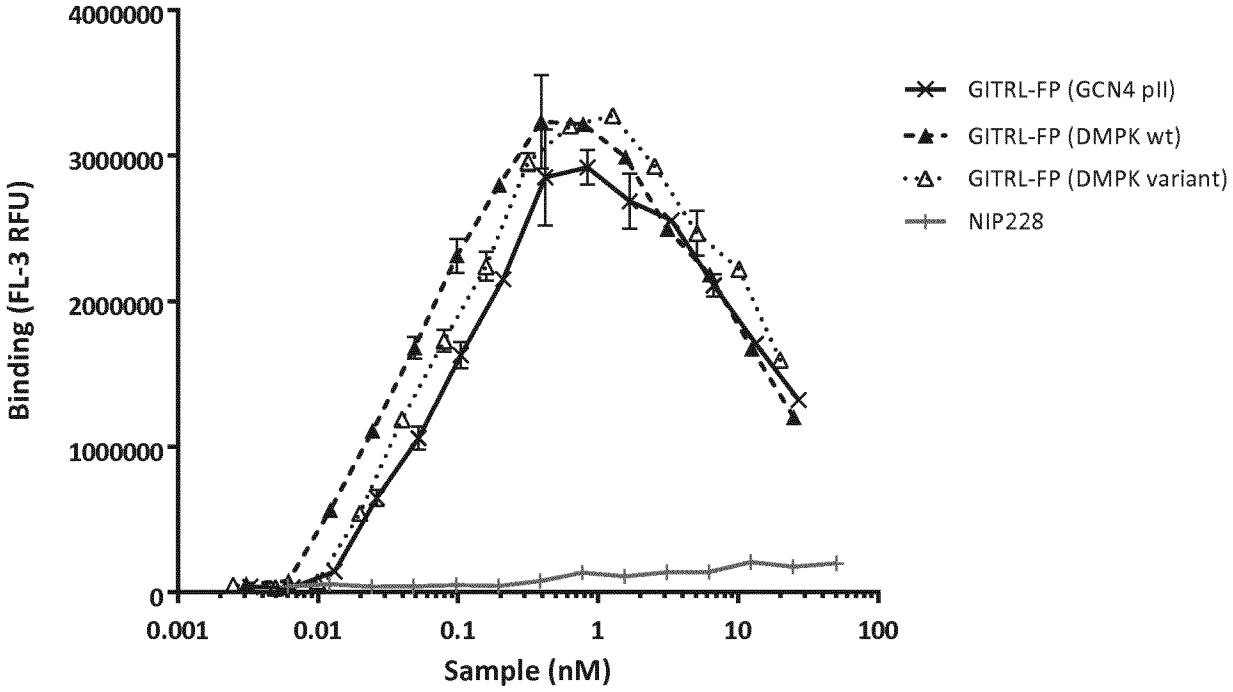


Figure 2D

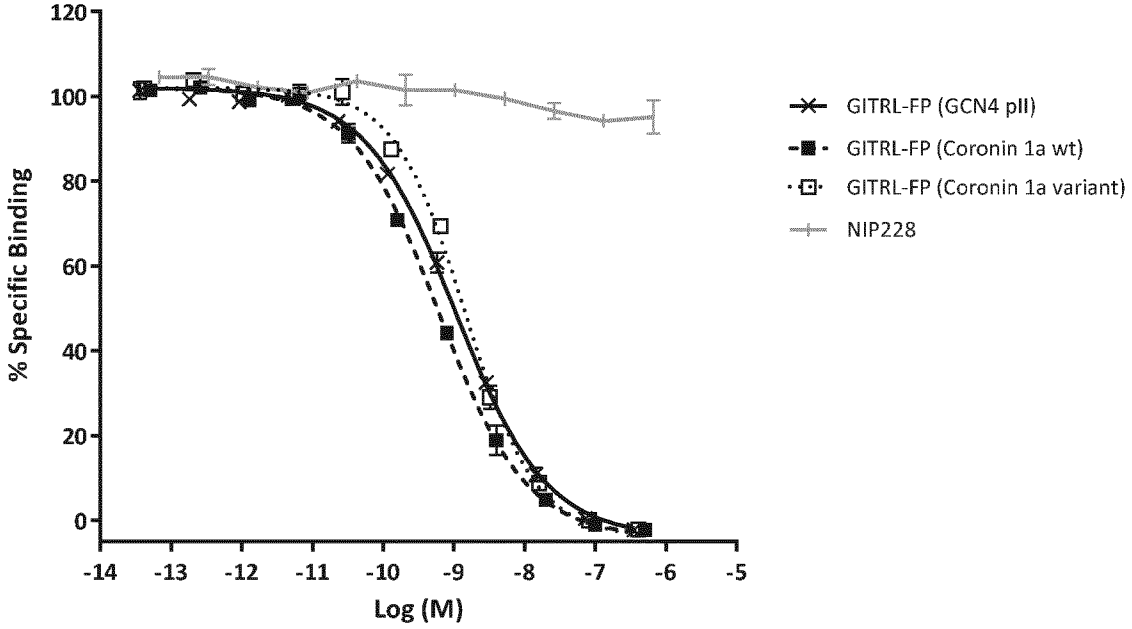


Figure 3A

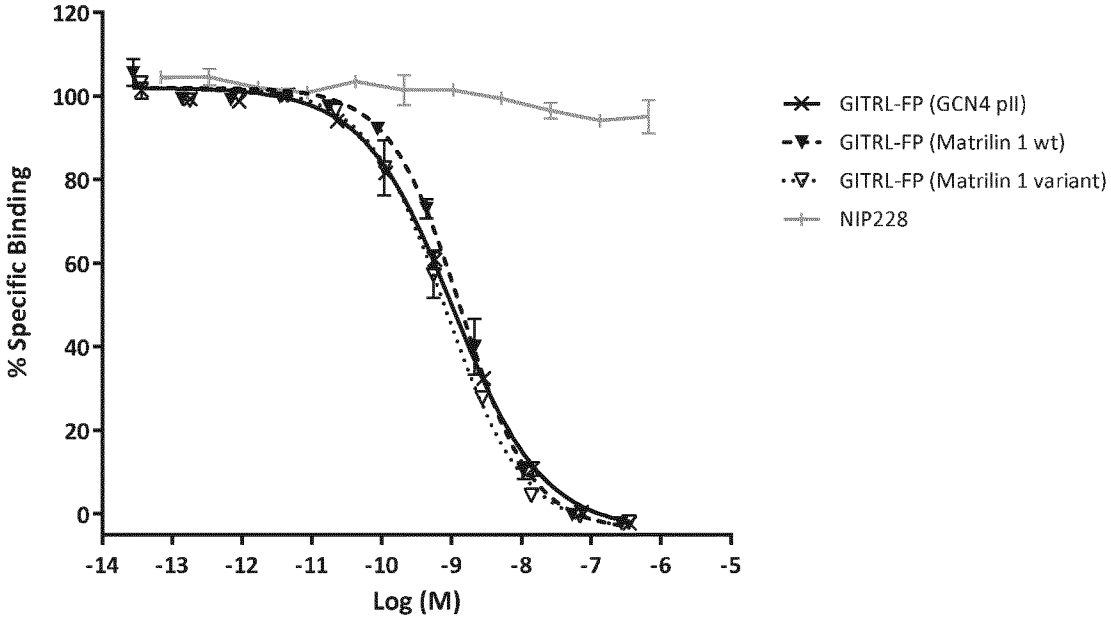


Figure 3B

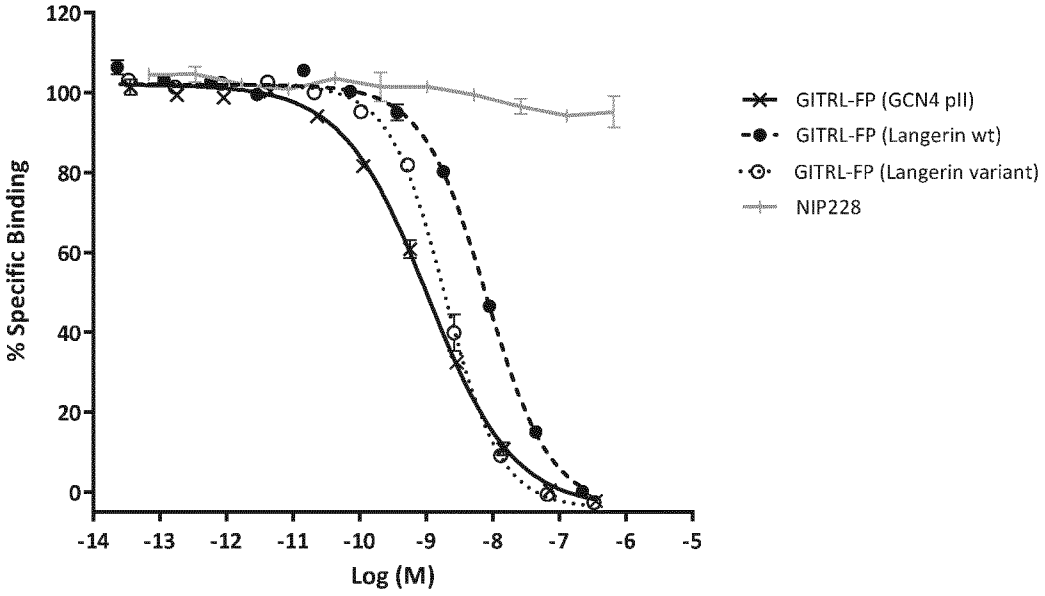


Figure 3C

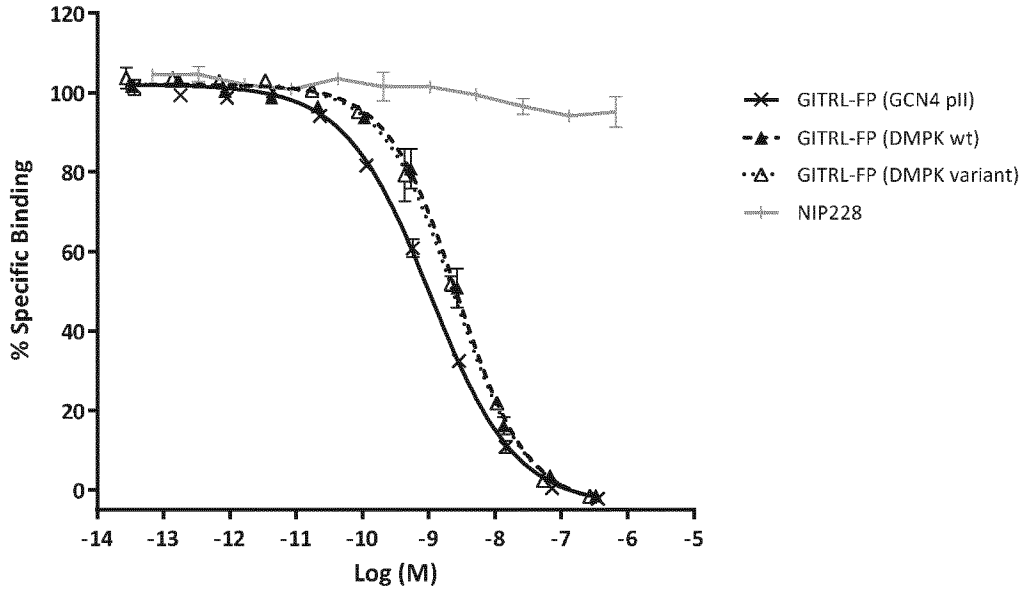


Figure 3D

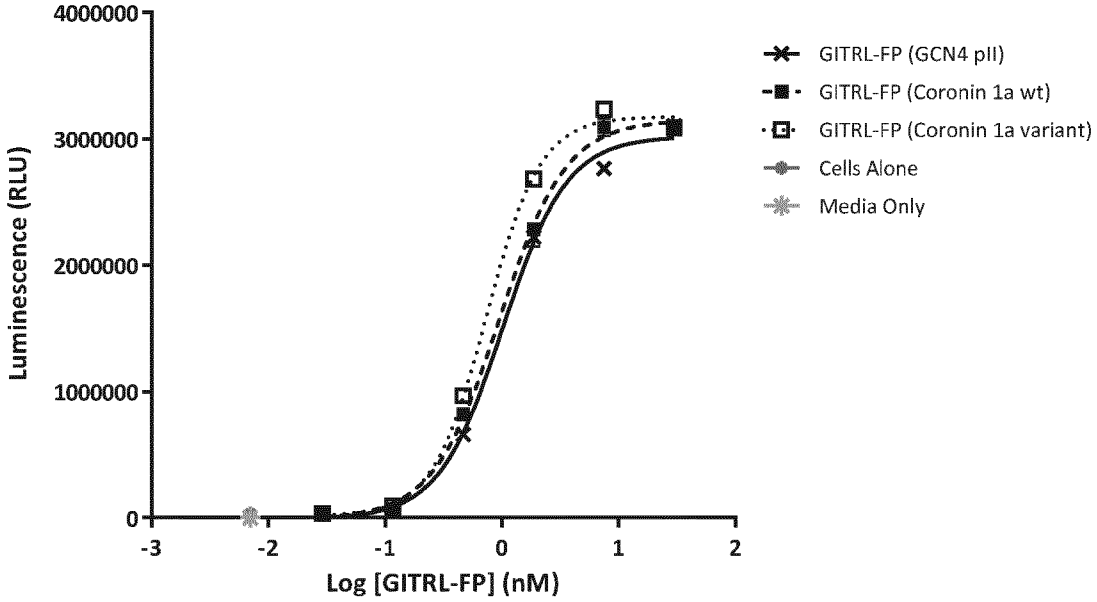


Figure 4A

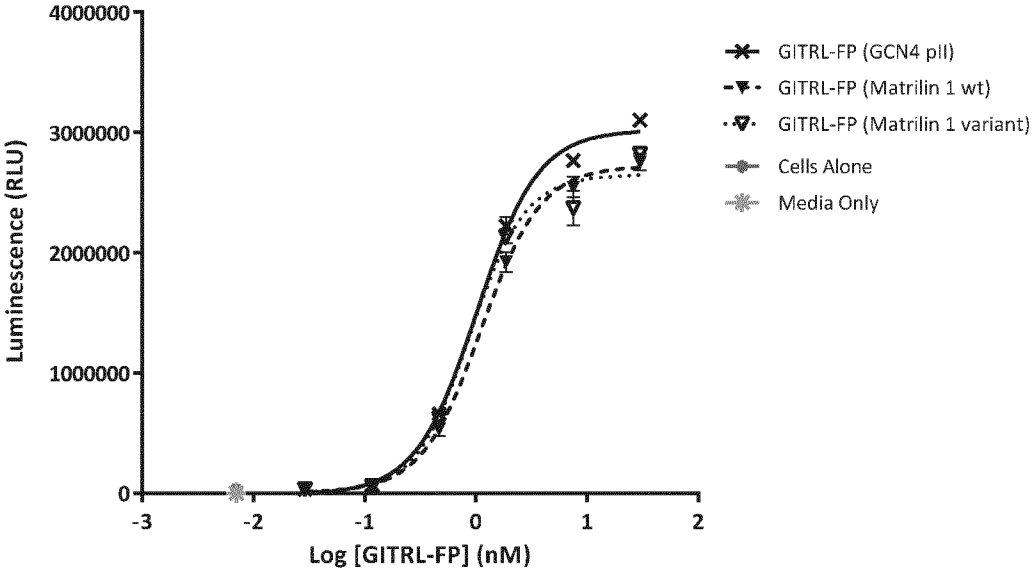


Figure 4B

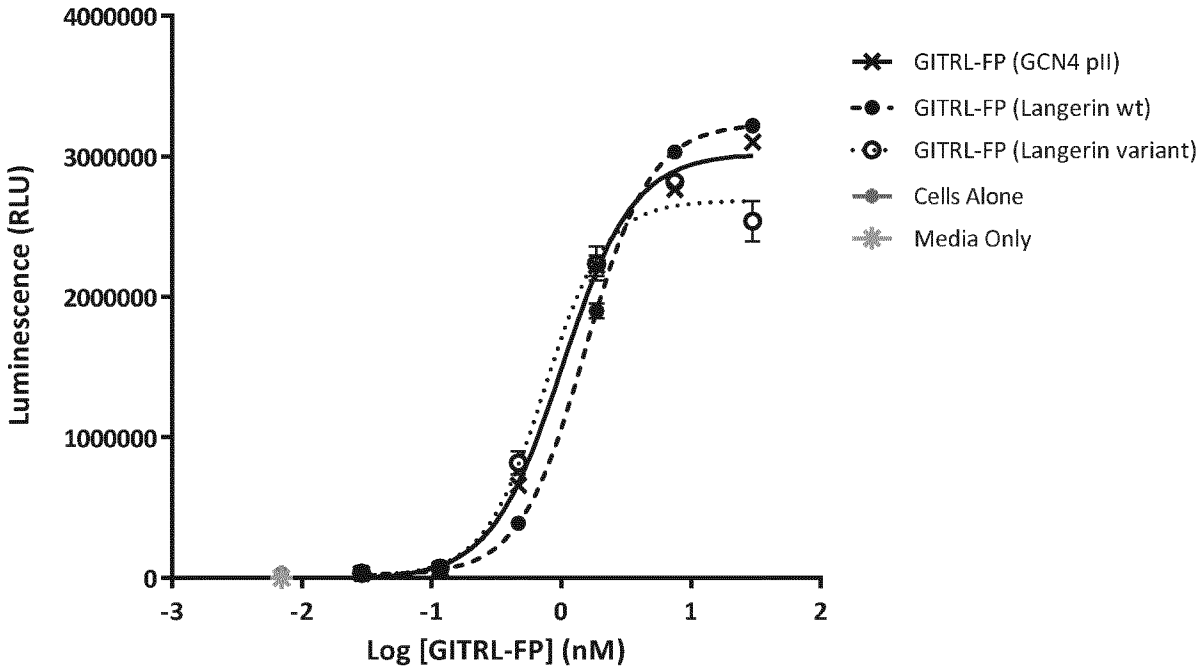


Figure 4C

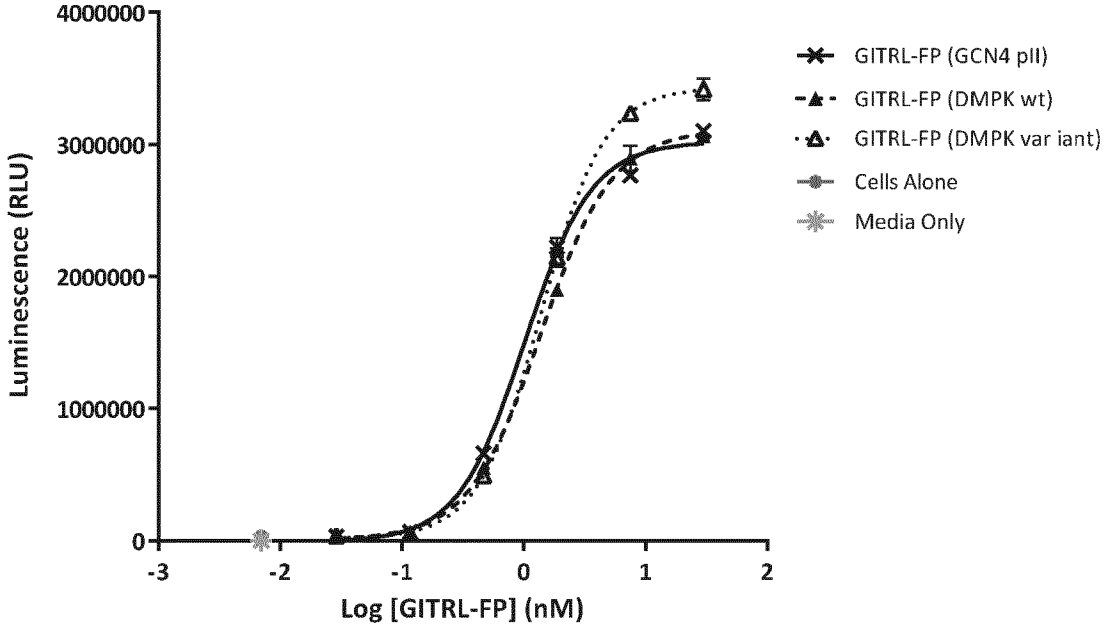
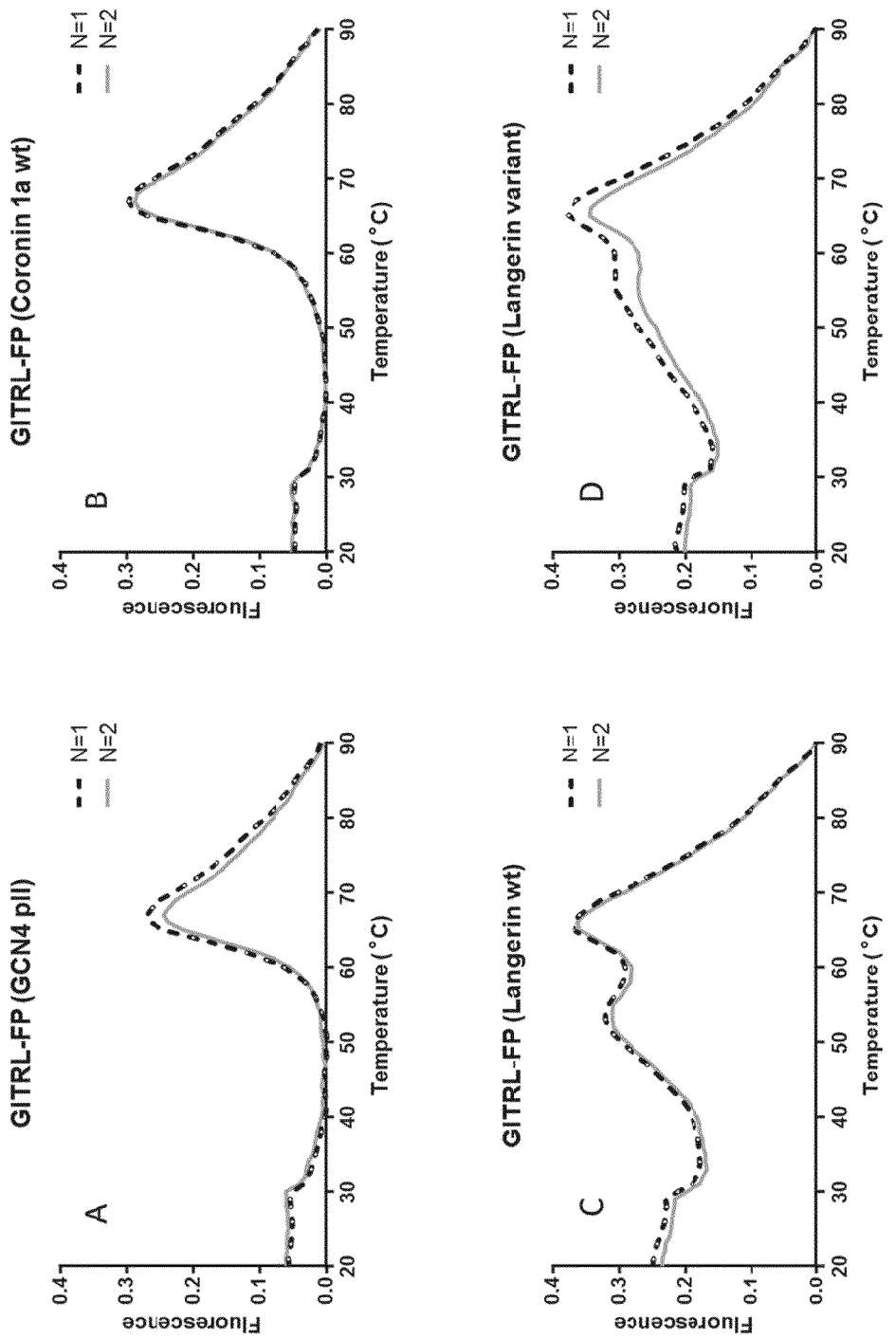


Figure 4D


GITRL-FP (GCN4 pII)
A
GITRL-FP (Coronin 1a wt)
B
GITRL-FP (Langerin wt)
C
GITRL-FP (Langerin variant)
D
N=1
N=2
Fluorescence
Temperature (°C)
0.0
0.1
0.2
0.3
0.4
20
30
40
50
60
70
80
90

Figures 5A-D

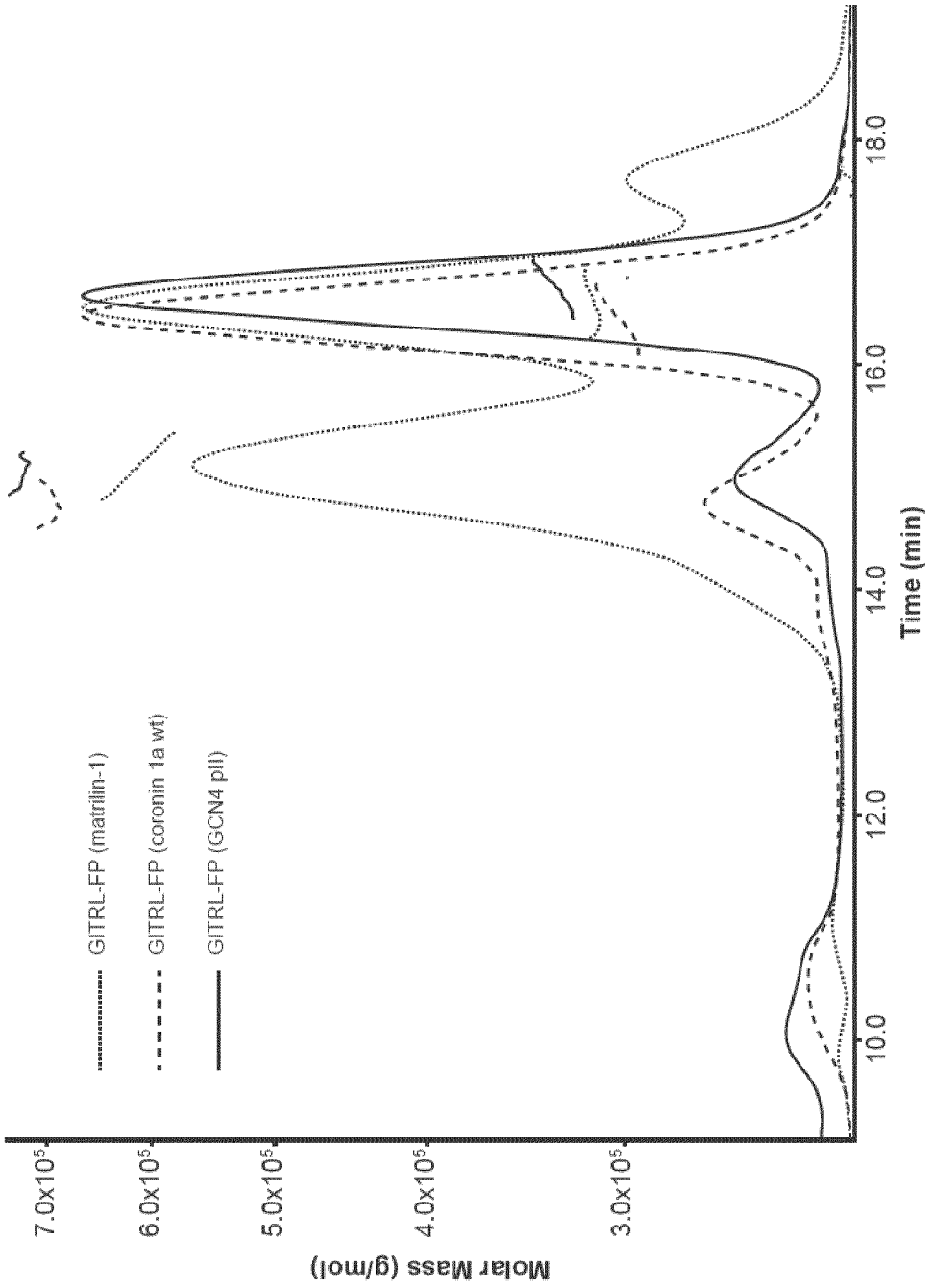

GITRL-FP (matrilin-1)
GITRL-FP (coronin 1a wt)
GITRL-FP (GCN4 pII)
Molar Mass (g/mol)
7.0x10^5
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
10.0
12.0
14.0
16.0
18.0
Time (min)

Figure 6

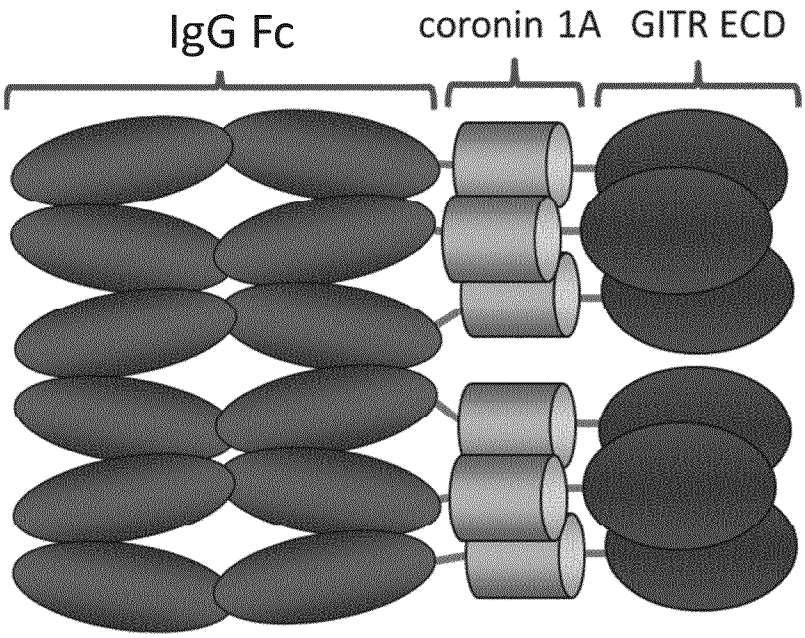


Figure 7

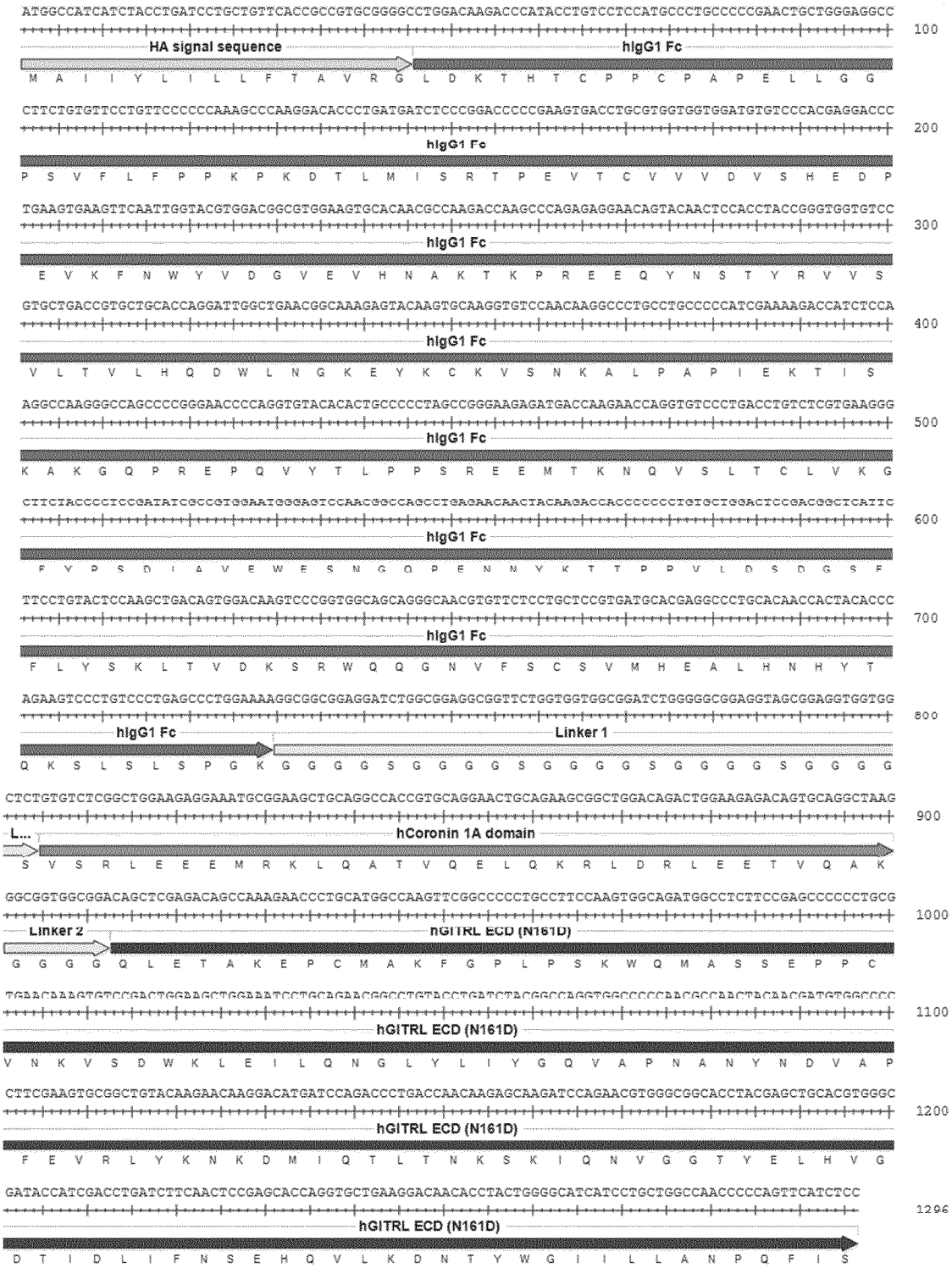

HA signal sequence
hIgG1 Fc
Linker 1
Linker 2
hCoronin 1A domain
hGITRL ECD (N161D)

Figure 8

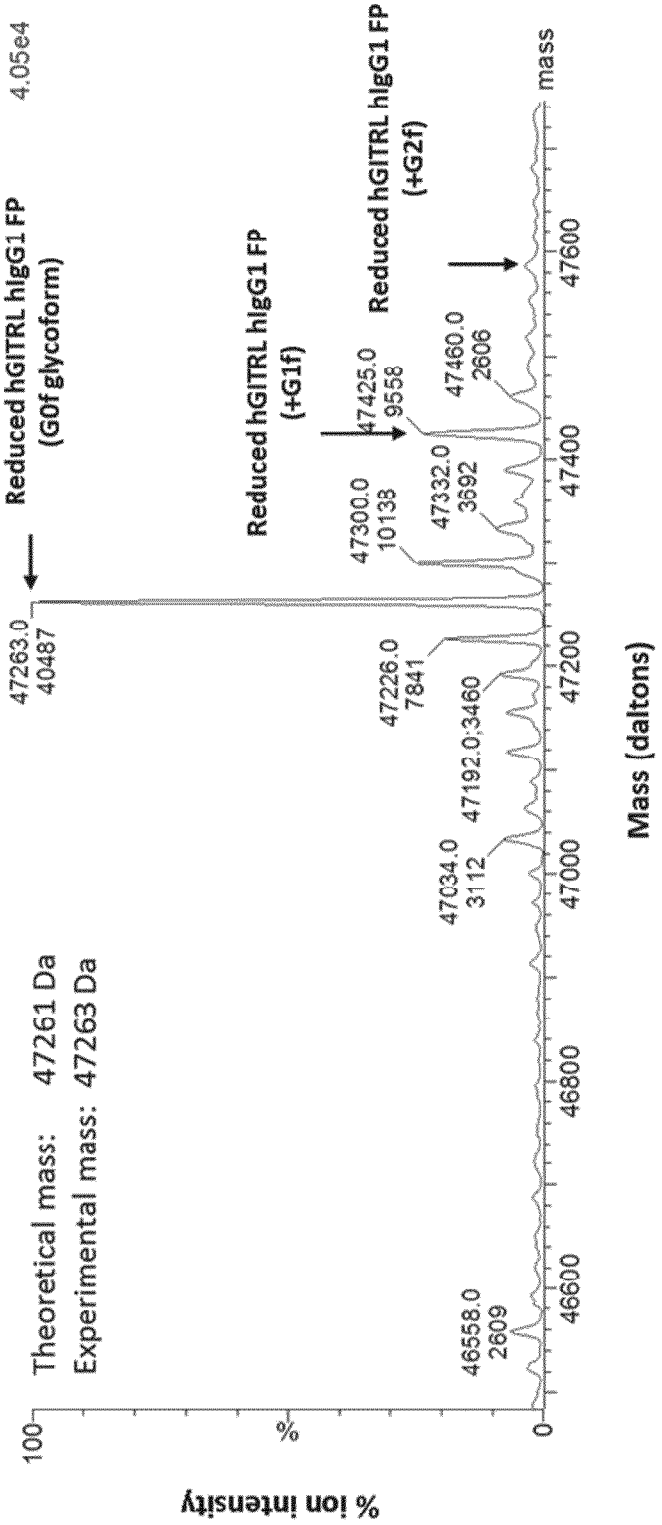

Reduced hGITRL hIgG1 FP
(G0f glycoform)
4.05e4
47263.0
40487
Reduced hGITRL hIgG1 FP
(+G1f)
47300.0
10138
47425.0
9558
Reduced hGITRL hIgG1 FP
(+G2f)
47332.0
3692
47460.0
2606
47226.0
7841
47192.0;3460
47034.0
3112
46558.0
2609
Theoretical mass: 47261 Da
Experimental mass: 47263 Da
100
%
0
46600
46800
47000
47200
47400
47600
mass
Mass (daltons)
% ion intensity

Figure 9

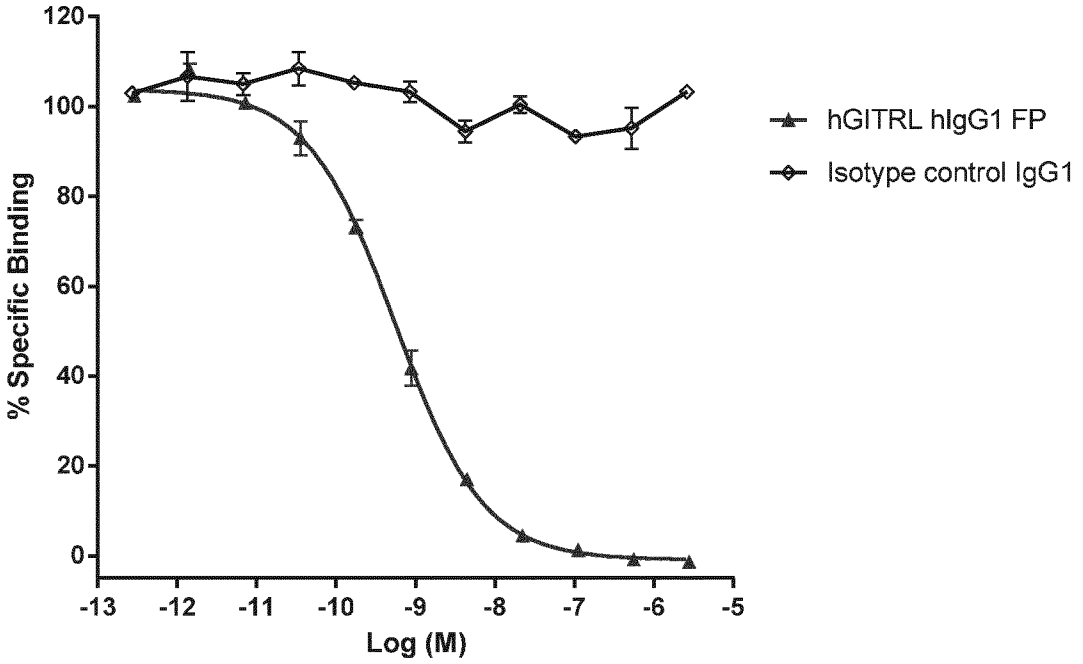


Figure 10

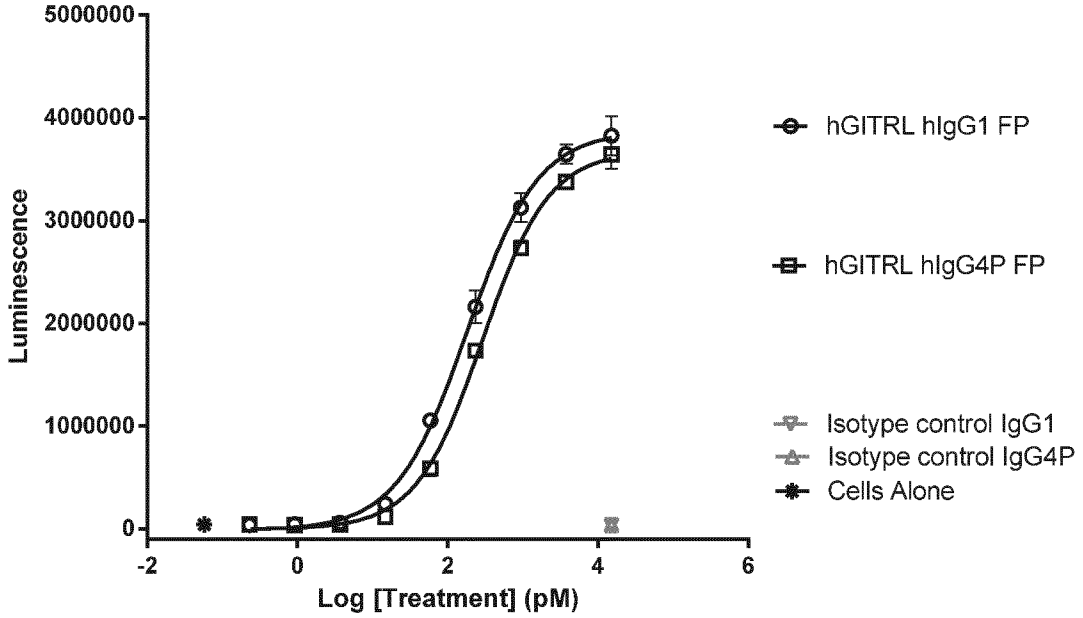


Figure 11

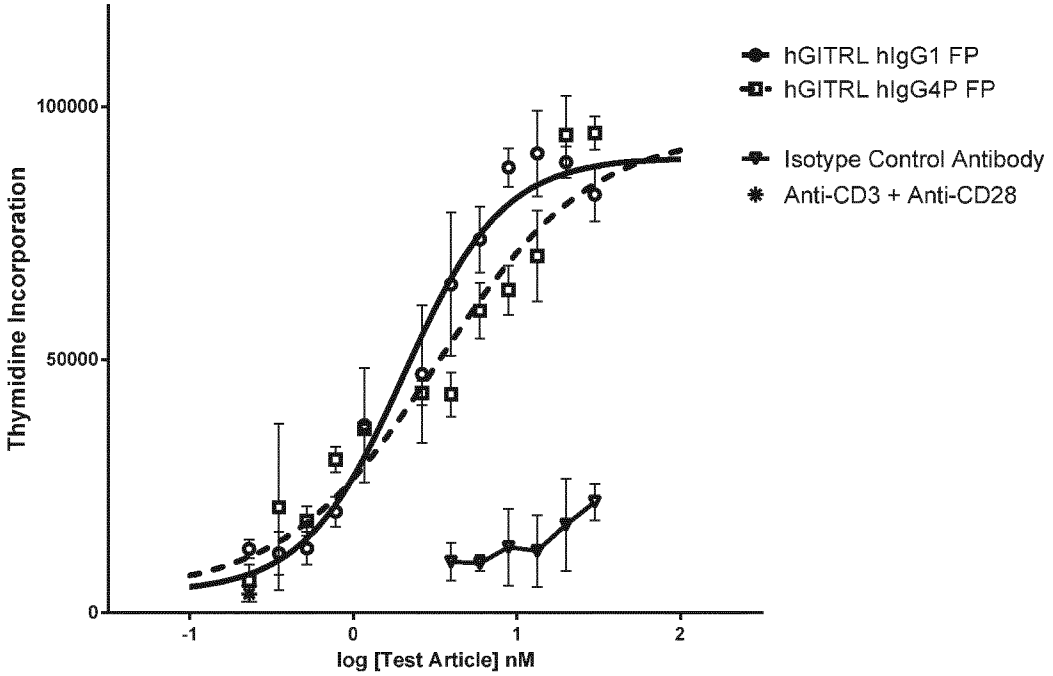


Figure 12

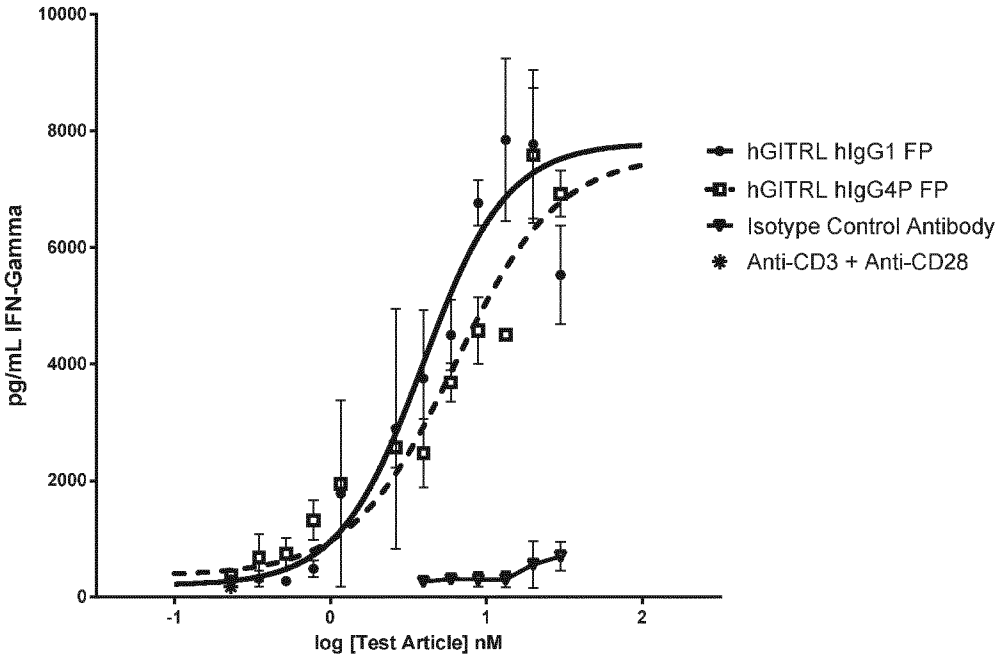


Figure 13

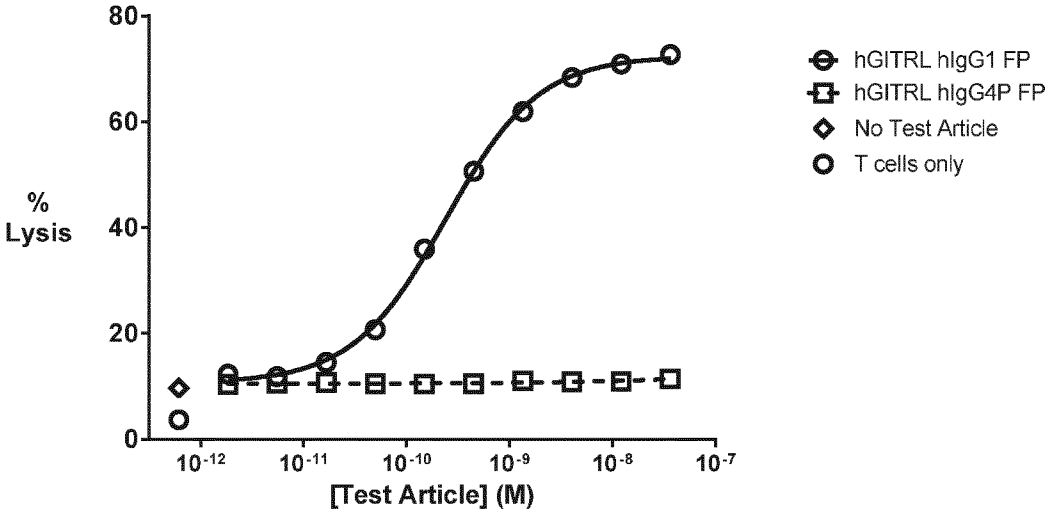


Figure 14

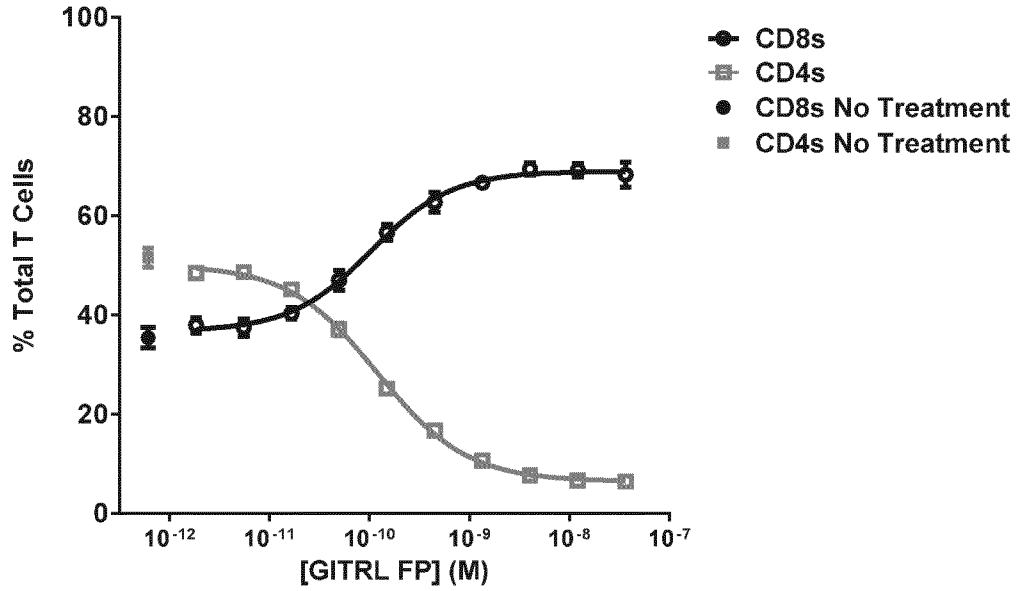


Figure 15

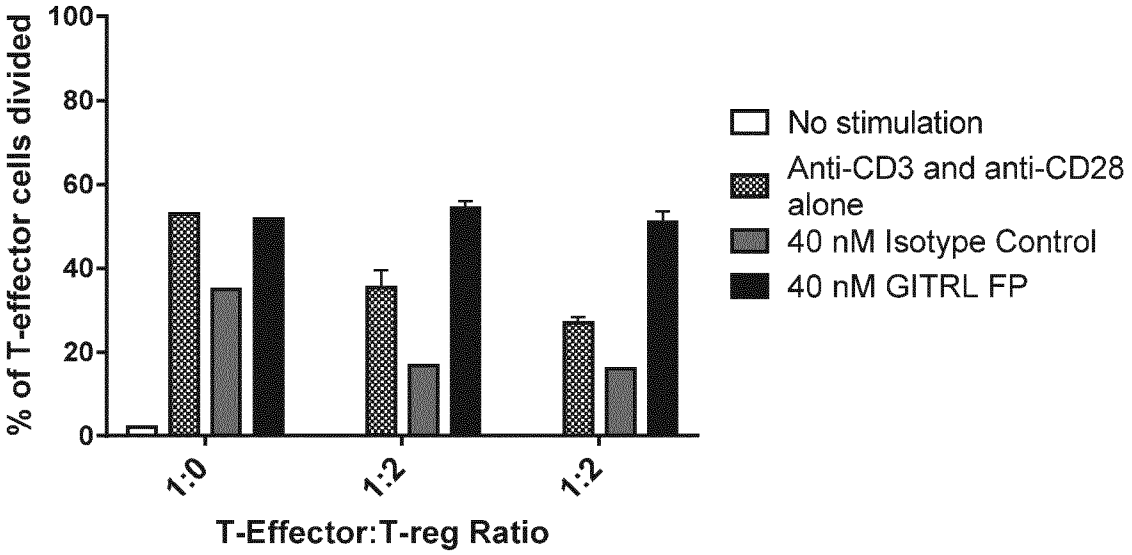


Figure 16

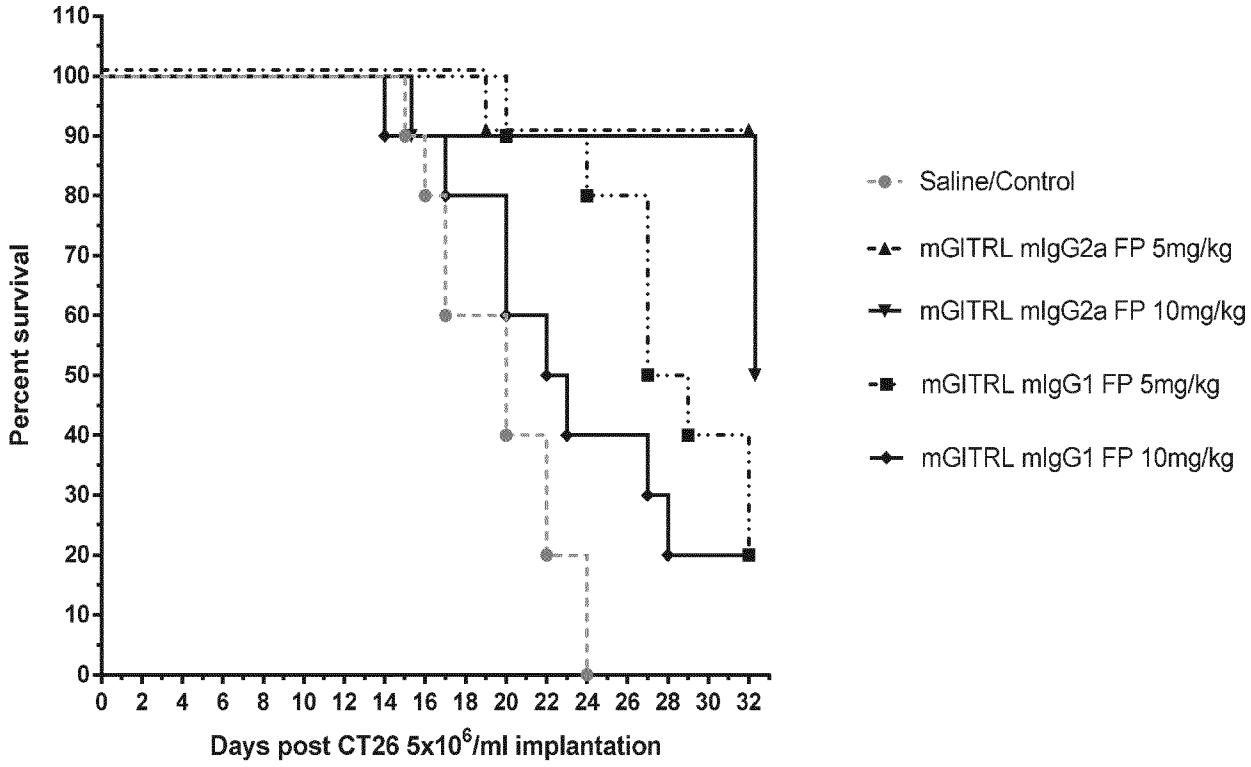


Figure 17

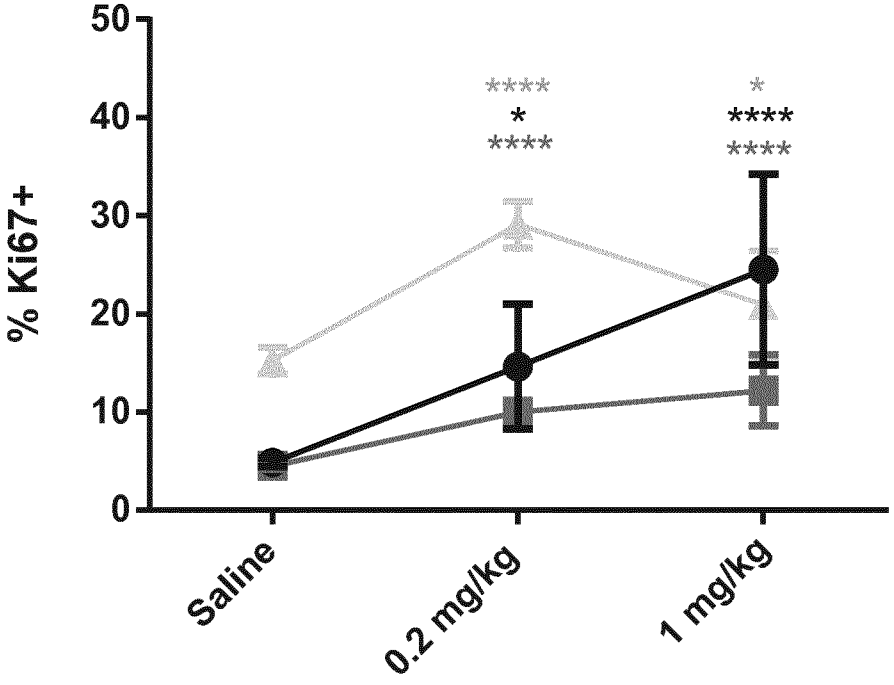


Figure 18

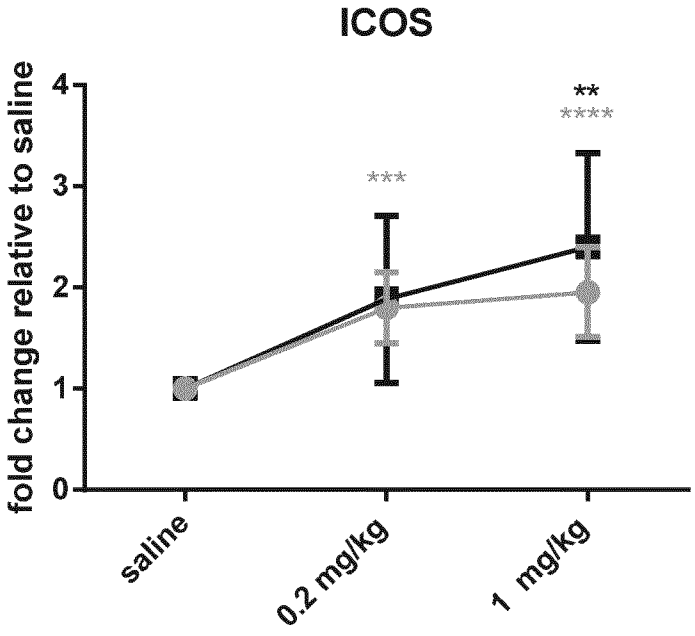


Figure 19

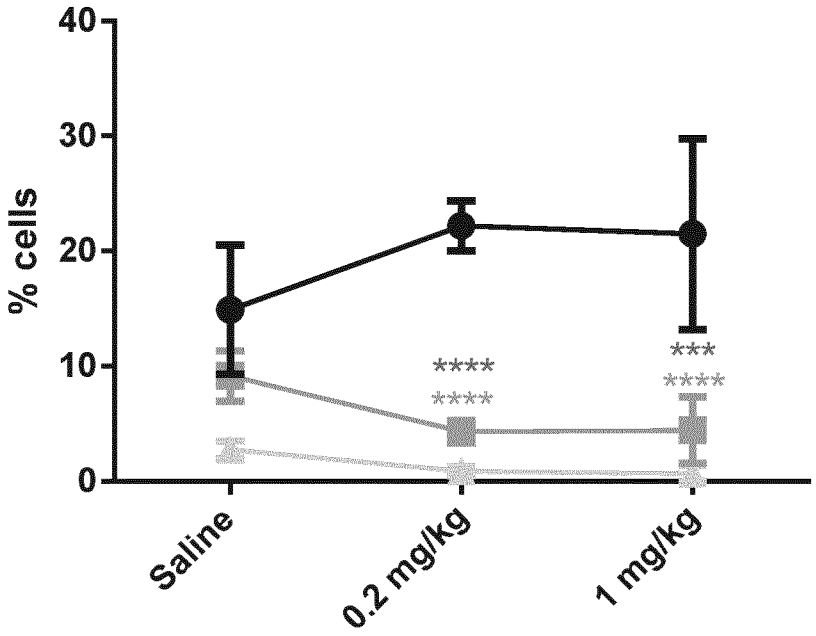


Figure 20

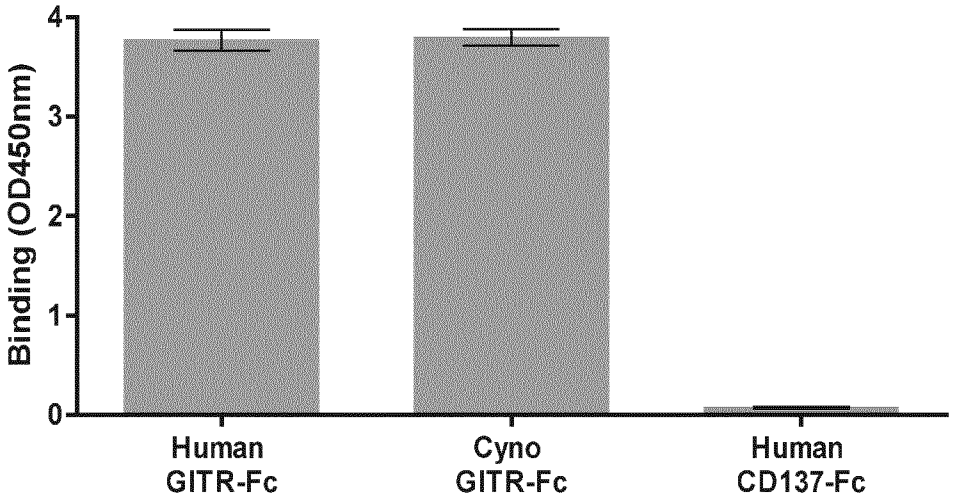


Figure 21

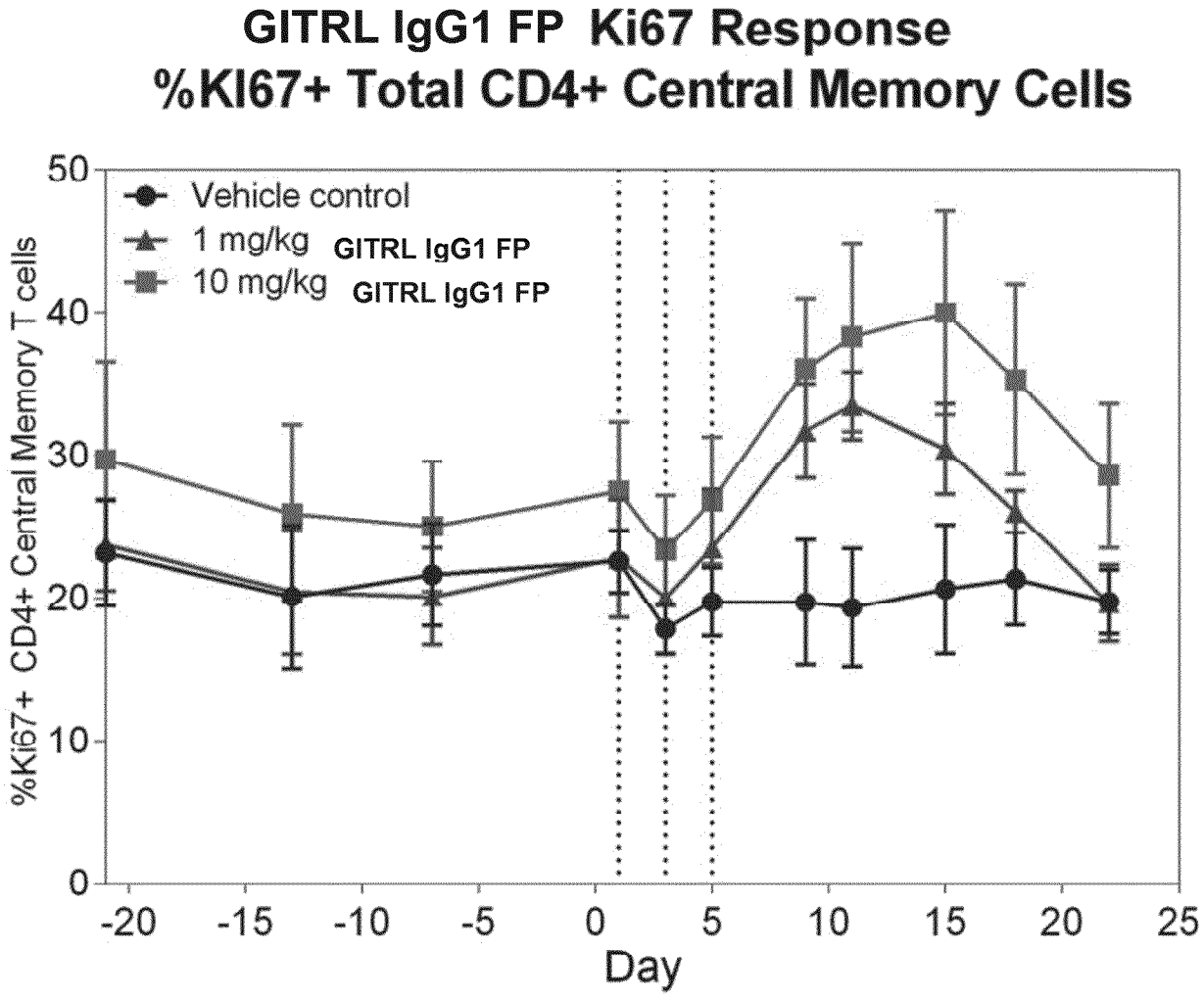

GITRL IgG1 FP Ki67 Response
%KI67+ Total CD4+ Central Memory Cells
Vehicle control
1 mg/kg GITRL IgG1 FP
10 mg/kg GITRL IgG1 FP
%Ki67+ CD4+ Central Memory T cells
Day
50
40
30
20
10
0
-20
-15
-10
-5
0
5
10
15
20
25

Figure 22

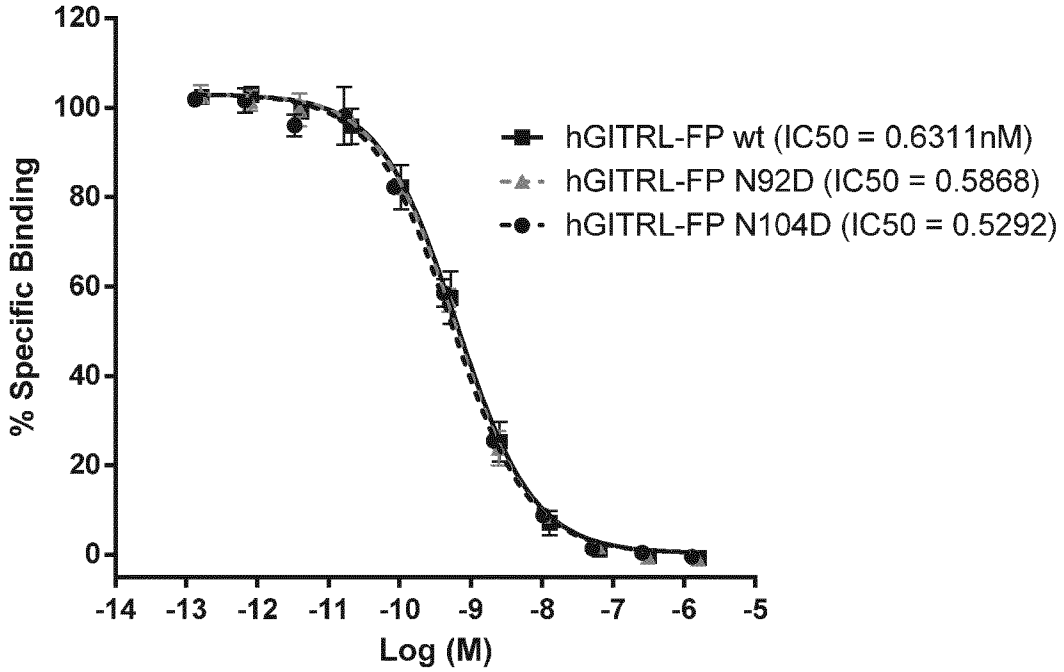


Figure 23A

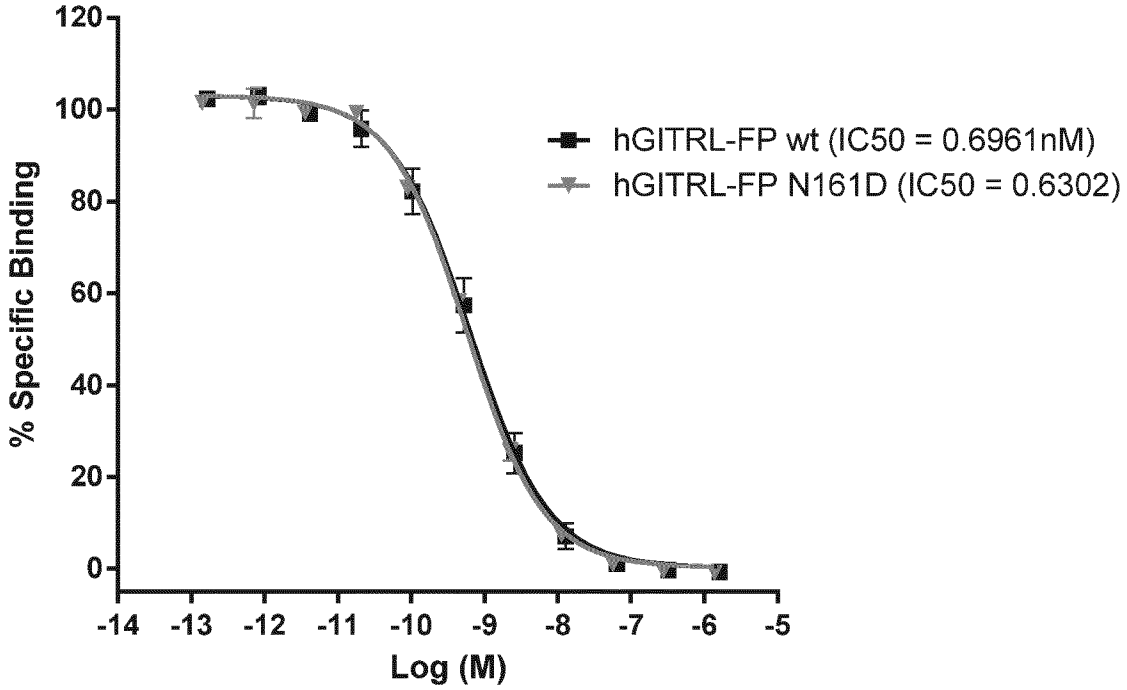


Figure 23B

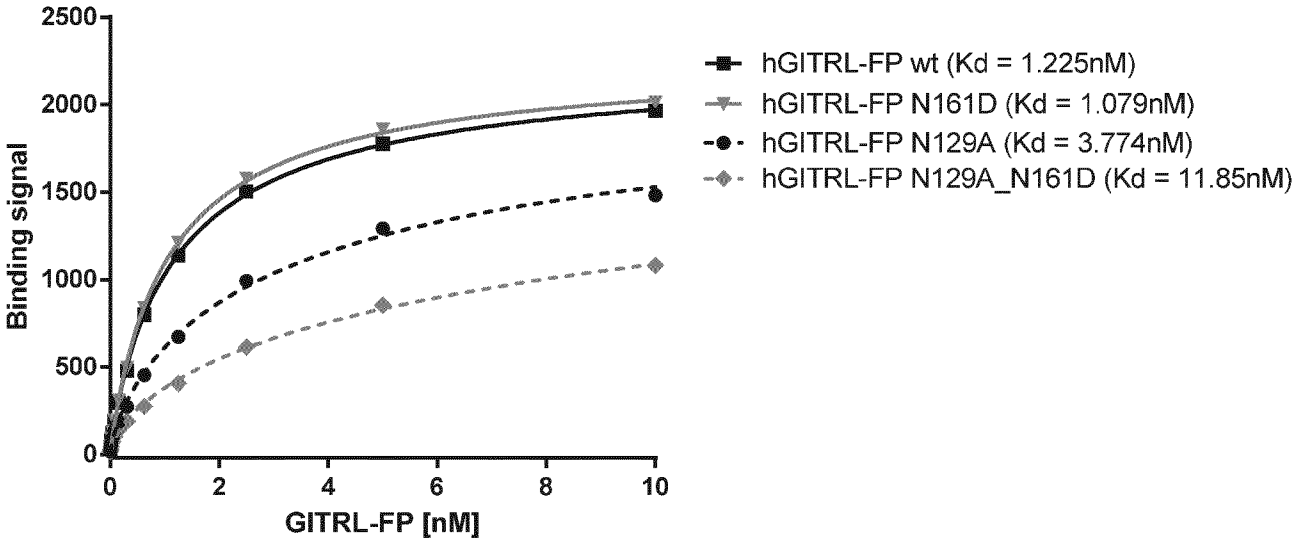


Figure 24

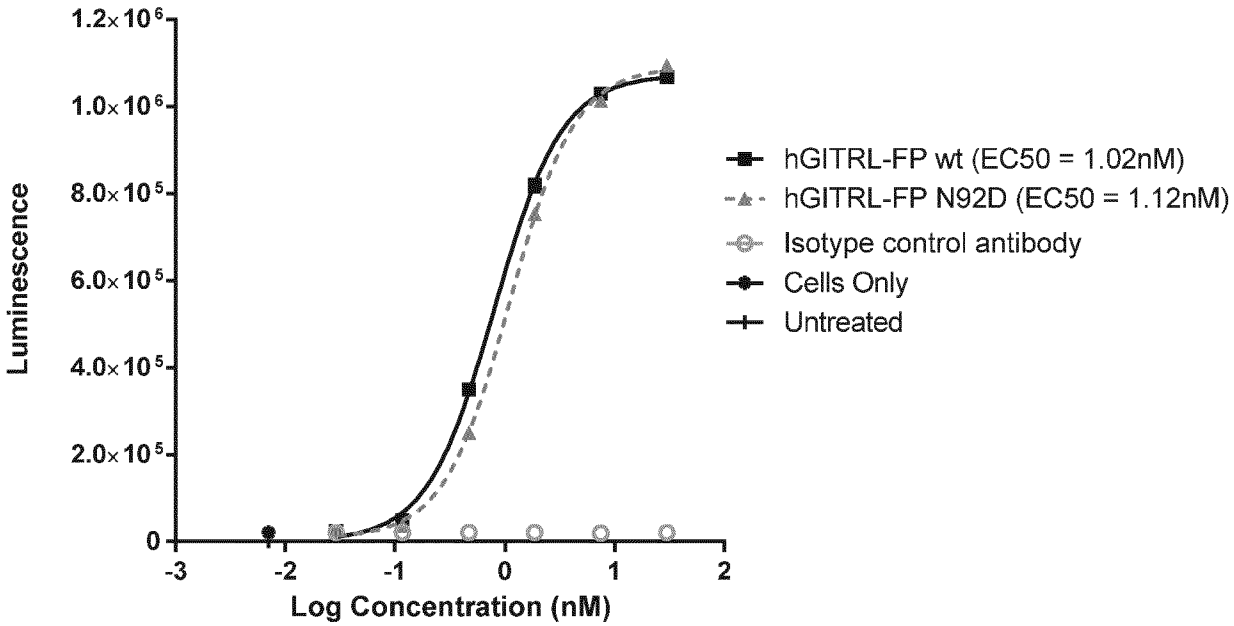


Figure 25A

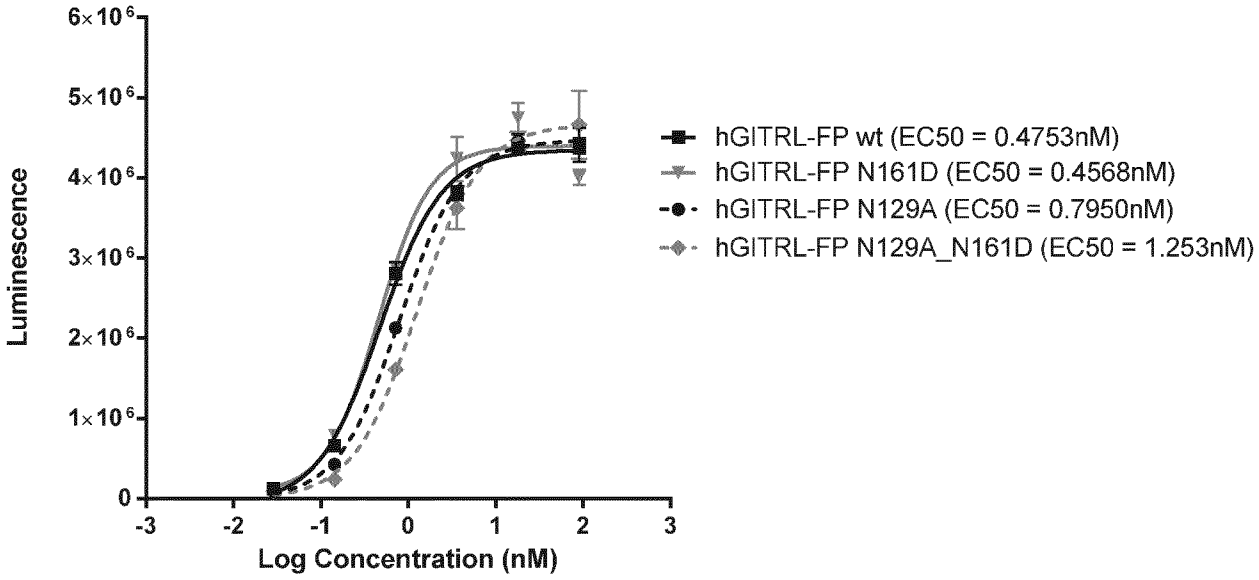


Figure 25B

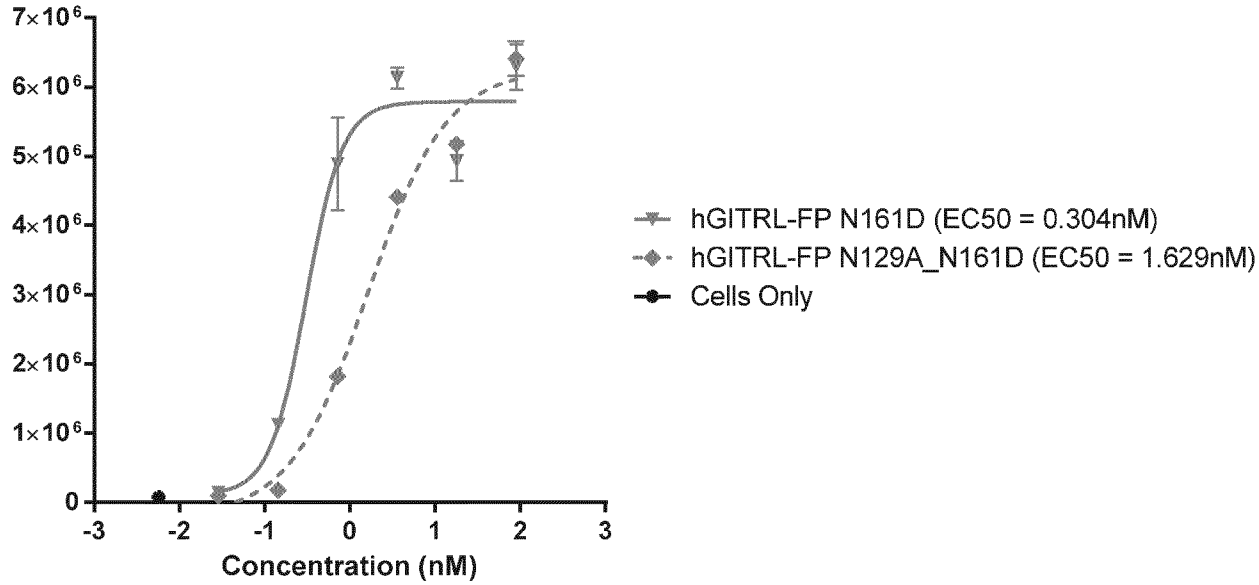
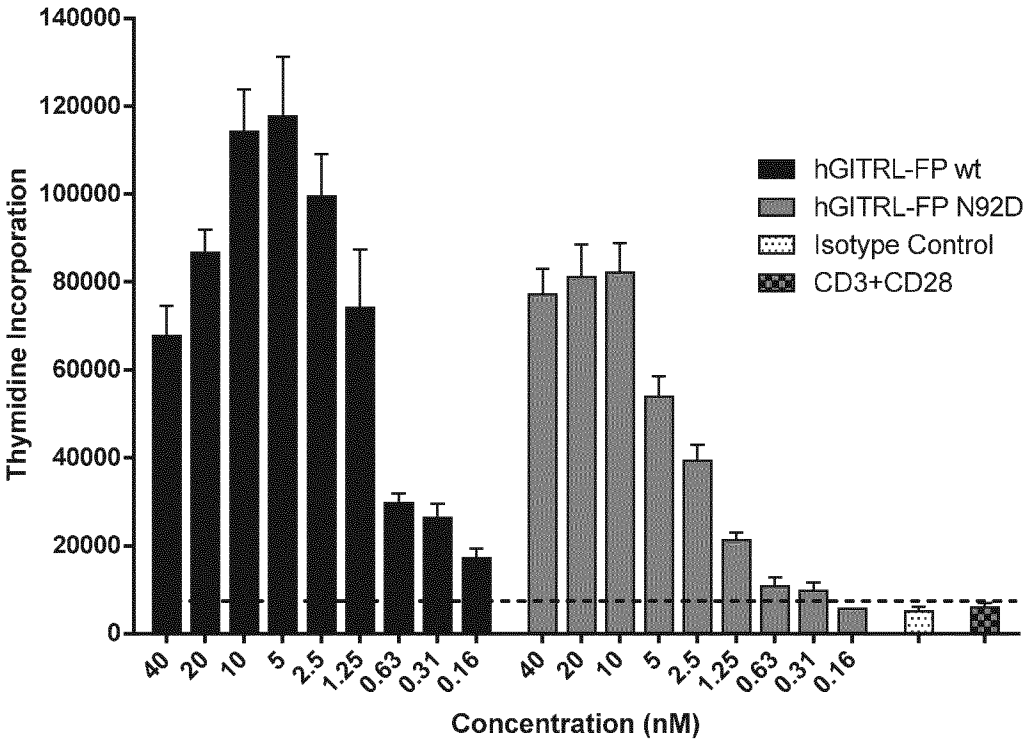


Figure 25C

Figure 26A


140000
120000
100000
80000
60000
40000
20000
0
Thymidine Incorporation
hGITRL-FP wt
hGITRL-FP N92D
Isotype Control
CD3+CD28
40
20
10
5
2.5
1.25
0.63
0.31
0.16
40
20
10
5
2.5
1.25
0.63
0.31
0.16
Concentration (nM)

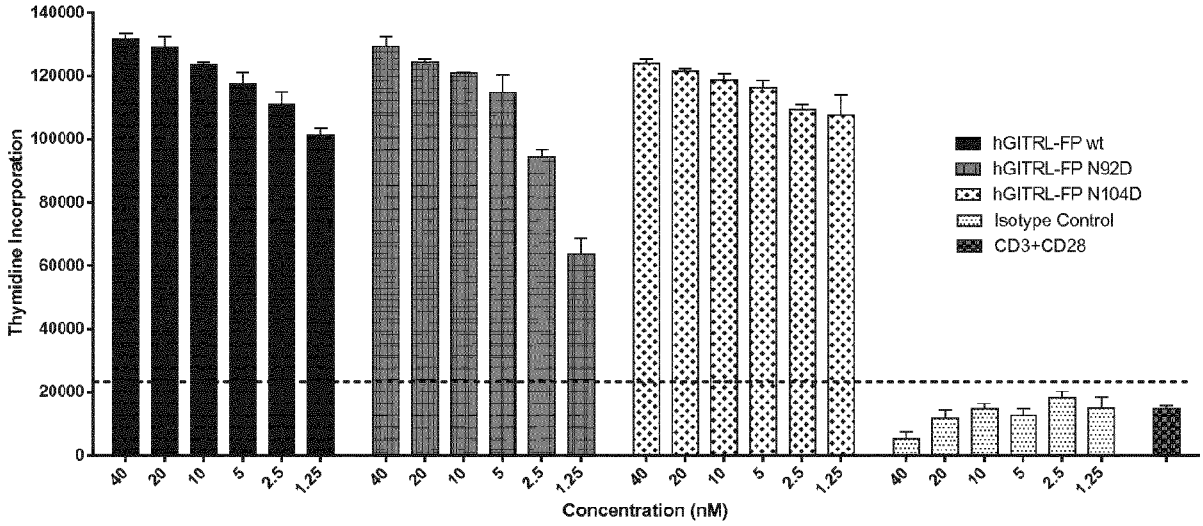

Thymidine Incorporation
140000
120000
100000
80000
60000
40000
20000
0
40
20
10
5
2.5
1.25
Concentration (nM)
hGITRL-FP wt
hGITRL-FP N92D
hGITRL-FP N104D
Isotype Control
CD3+CD28

Figure 26B

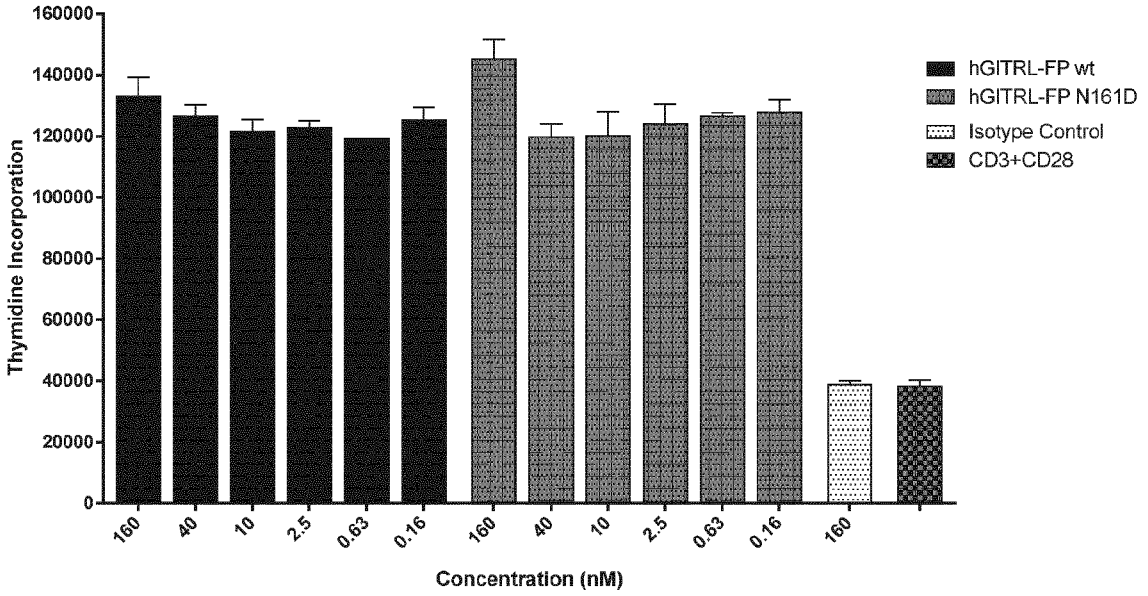


Figure 26C

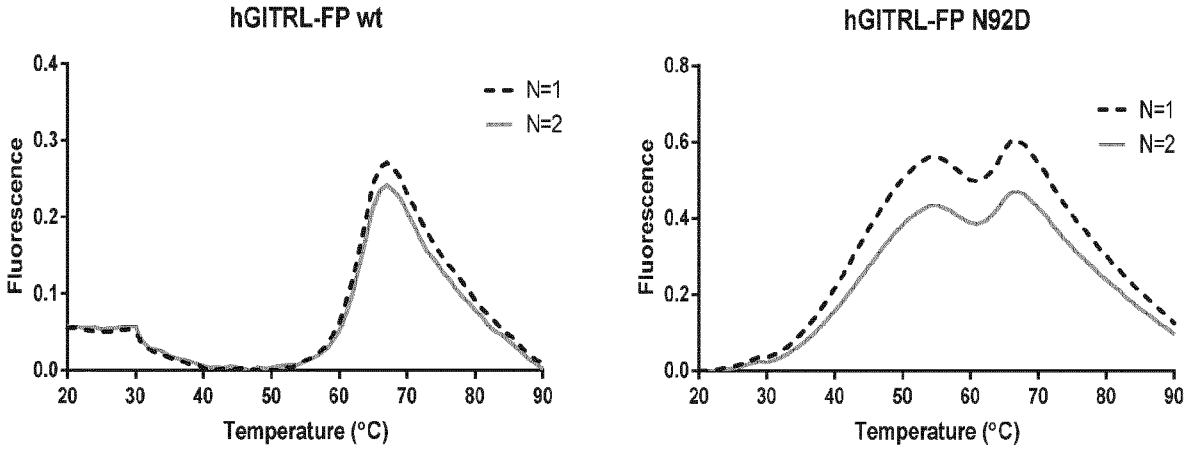


Figure 27

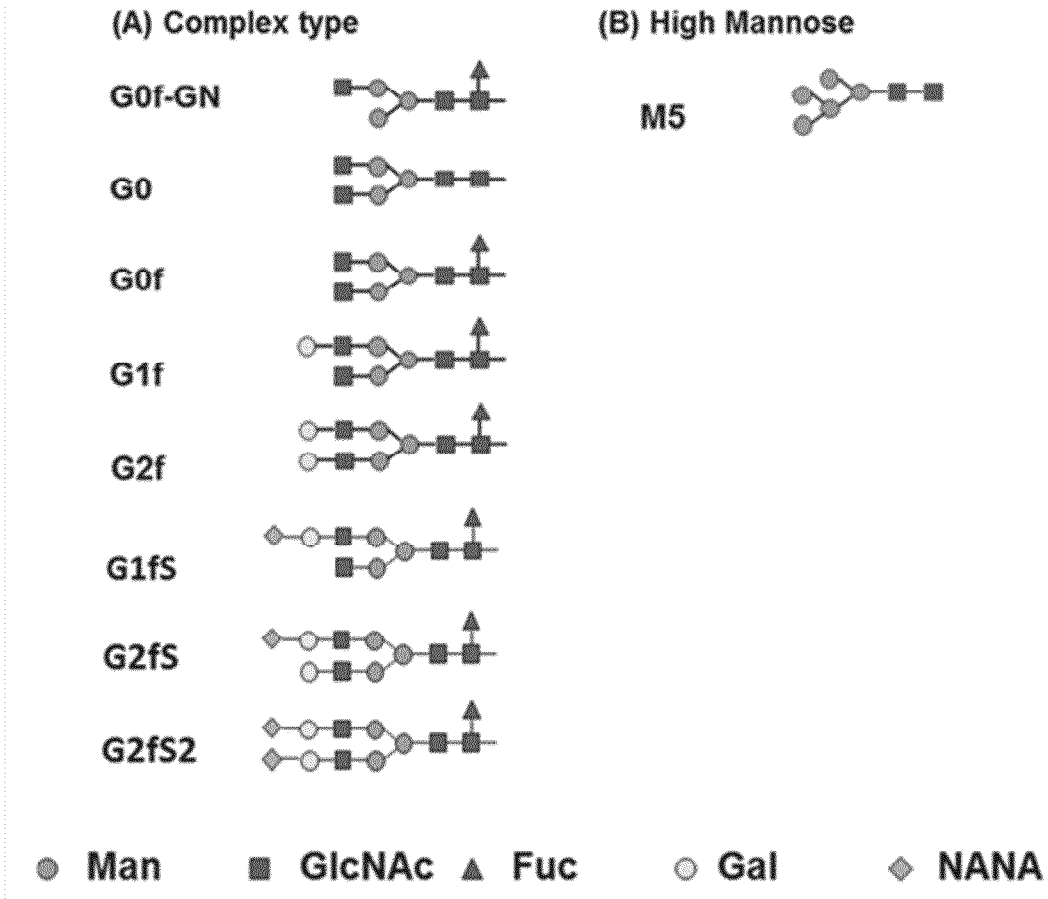

(A) Complex type
(B) High Mannose
G0f-GN
M5
G0
G0f
G1f
G2f
G1fS
G2fS
G2fS2
Man
GlcNAc
Fuc
Gal
NANA

Figure 28

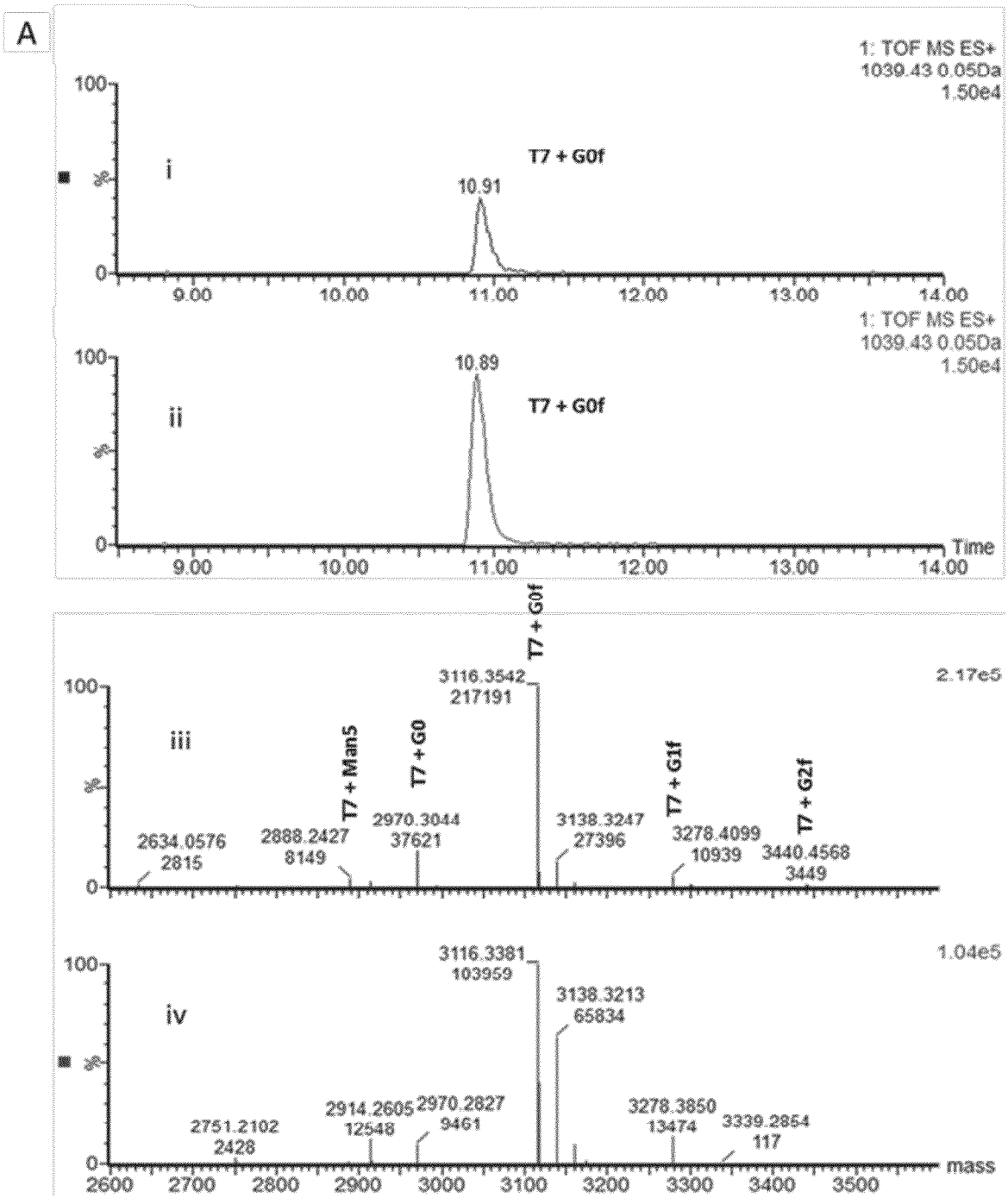

A
1: TOF MS ES+
1039.43 0.05Da
1.50e4
i
T7 + G0f
10.91
ii
10.89
T7 + G0f
Time
iii
T7 + G0f
3116.3542
217191
2.17e5
T7 + Man5
T7 + G0
T7 + G1f
T7 + G2f
2634.0576
2815
2888.2427
8149
2970.3044
37621
3138.3247
27396
3278.4099
10939
3440.4568
3449
iv
3116.3381
103959
1.04e5
3138.3213
65834
2751.2102
2428
2914.2605
12548
2970.2827
9461
3278.3850
13474
3339.2854
117
mass

Figure 29A

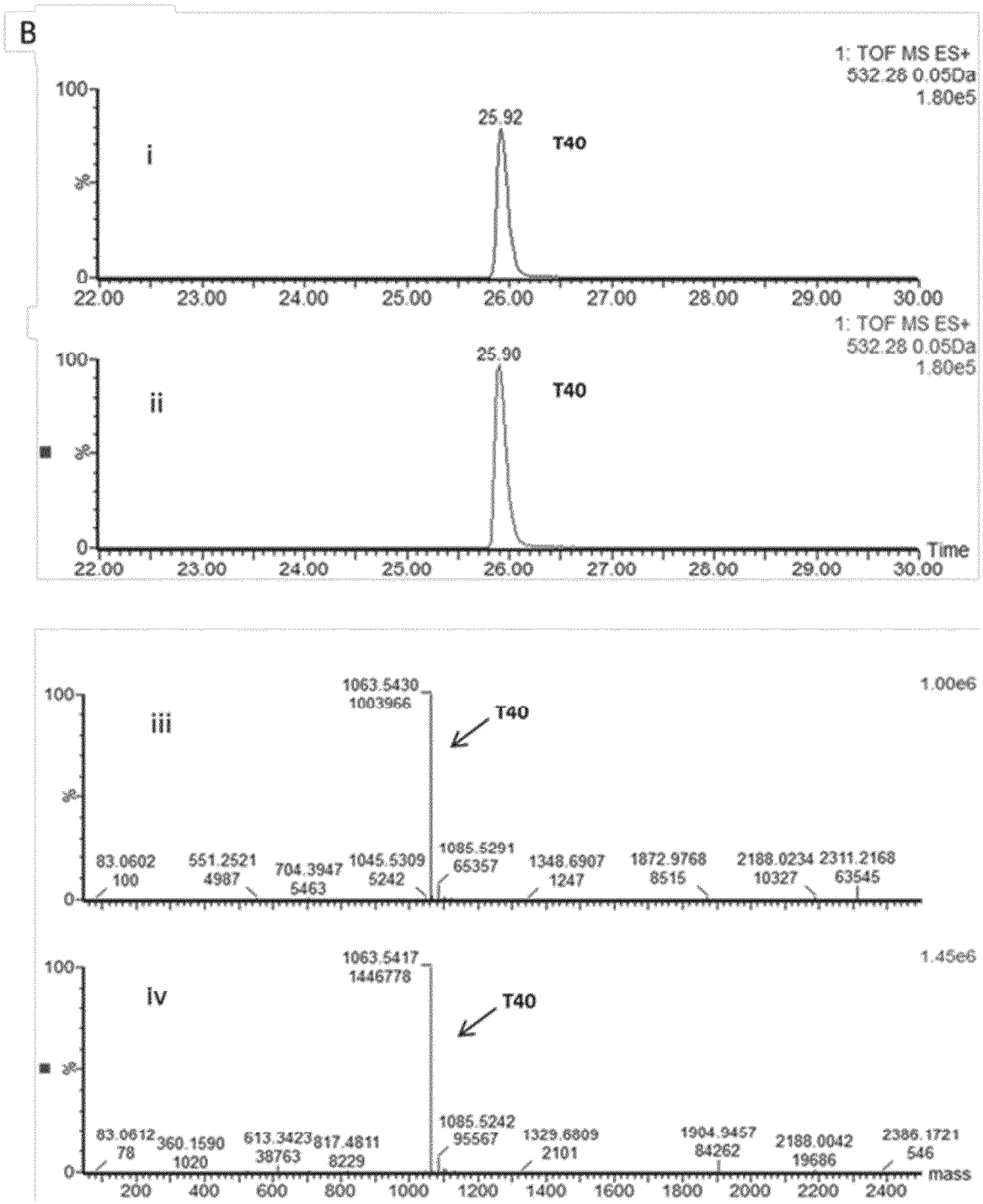

B
1: TOF MS ES+
532.28 0.05Da
1.80e5
i
25.92
T40
ii
25.90
T40
Time
iii
1063.5430
1003966
T40
1.00e6
83.0602 100
551.2521 4987
704.3947 5463
1045.5309 5242
1085.5291 65357
1348.6907 1247
1872.9768 8515
2188.0234 10327
2311.2168 63545
iv
1063.5417
1446778
T40
1.45e6
83.0612 78
360.1590 1020
613.3423 38763
817.4811 8229
1085.5242 95567
1329.6809 2101
1904.9457 84262
2188.0042 19686
2386.1721 546
mass

Figure 29B

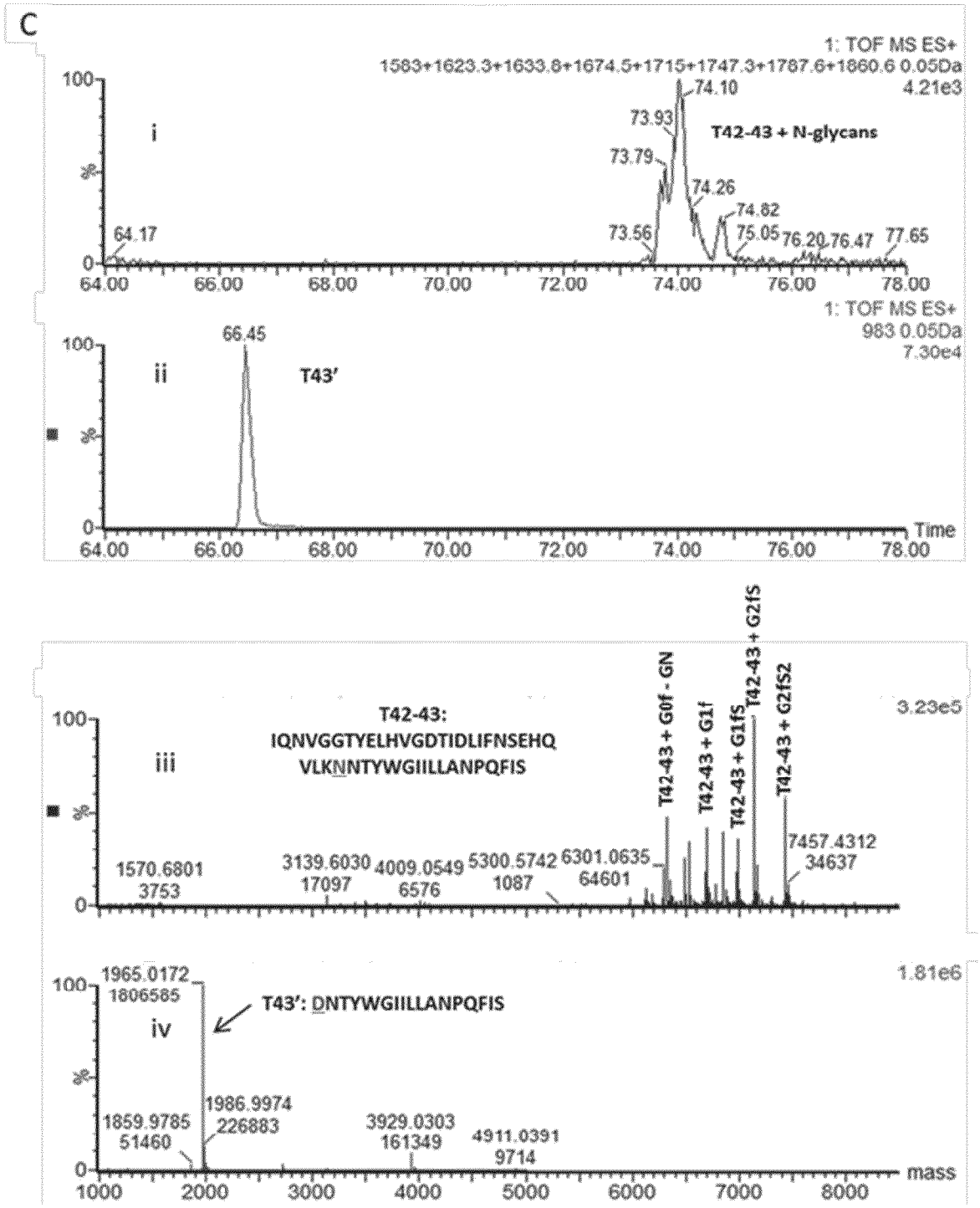

C
1: TOF MS ES+
1583+1623.3+1633.8+1674.5+1715+1747.3+1787.6+1860.6 0.05Da
4.21e3
i
T42-43 + N-glycans
74.10
73.93
73.79
74.26
74.82
75.05
76.20
76.47
77.65
73.56
64.17
1: TOF MS ES+
983 0.05Da
7.30e4
66.45
ii
T43'
Time
T42-43:
IQNVGGTYELHVGDTIDLIFNSEHQ
VLKNNTYWGIILLANPQFIS
iii
T42-43 + G0f - GN
T42-43 + G1f
T42-43 + G1fS
T42-43 + G2fS
T42-43 + G2fS2
3.23e5
1570.6801
3753
3139.6030
17097
4009.0549
6576
5300.5742
1087
6301.0635
64601
7457.4312
34637
1.81e6
1965.0172
1806585
iv
T43': DNTYWGIILLANPQFIS
1986.9974
226883
1859.9785
51460
3929.0303
161349
4911.0391
9714
mass

Figure 29C

Figure 30

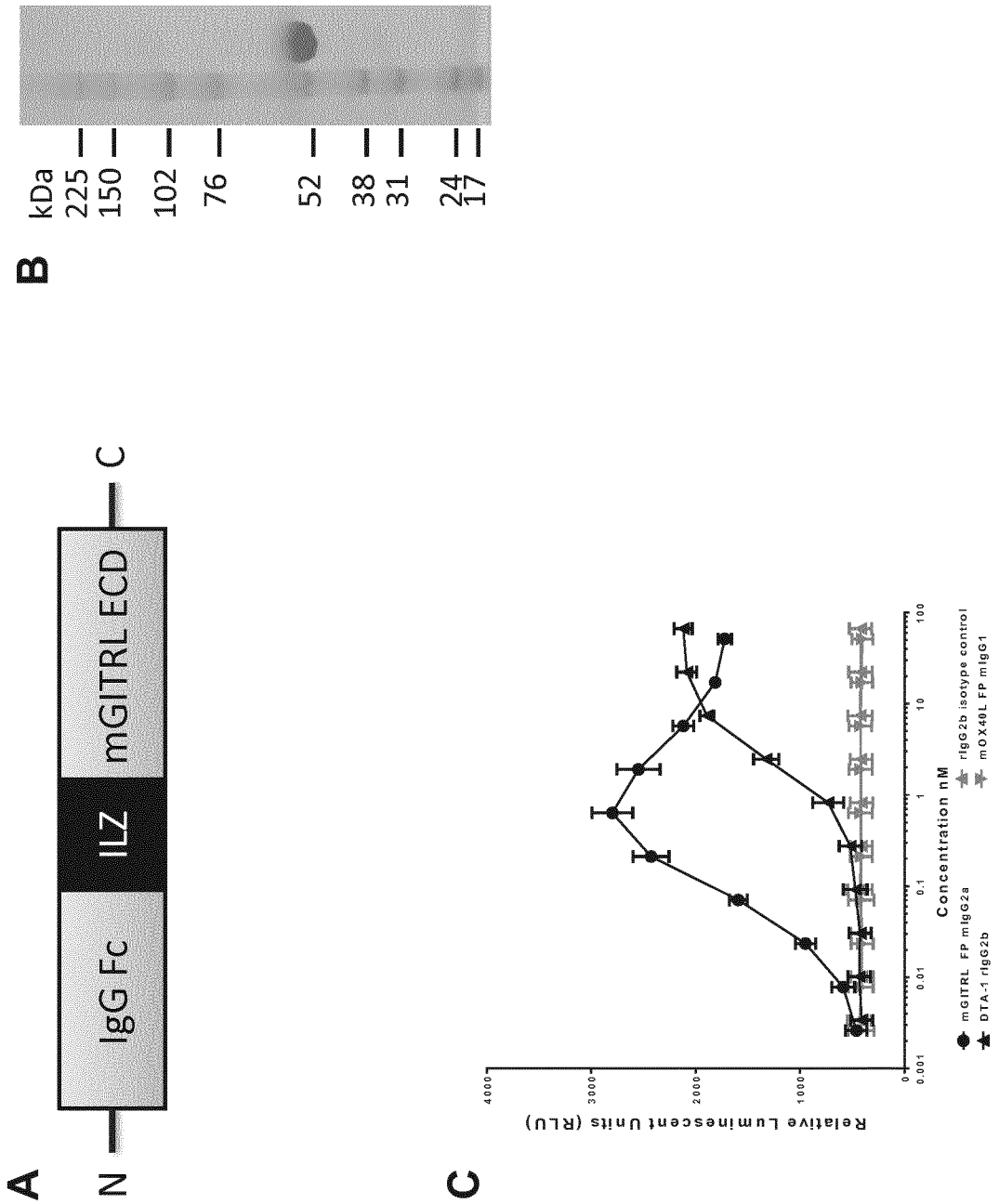

A
N
IgG Fc
ILZ
mGITRL ECD
C
B
kDa
225
150
102
76
52
38
31
24
17
C
Relative Luminescent Units (RLU)
4000
3000
2000
1000
0
0.001
0.01
0.1
1
10
100
Concentration nM
mGITRL FP mIgG2a
DTA-1 rIgG2b
rIgG2b isotype control
mOX40L FP mIgG1

Figure 31

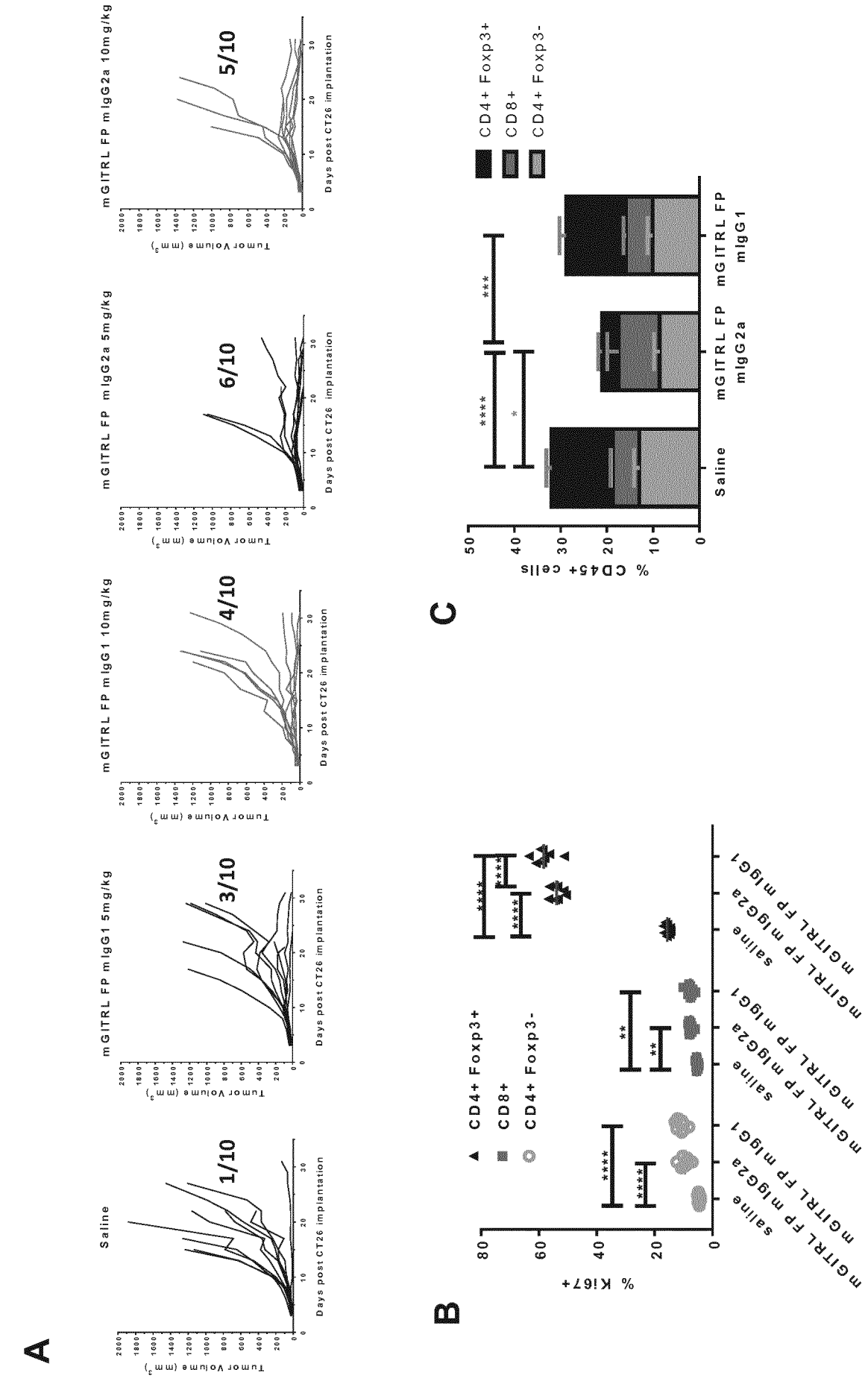

A
Saline
1/10
mGITRL FP mIgG1 5mg/kg
3/10
mGITRL FP mIgG1 10mg/kg
4/10
mGITRL FP mIgG2a 5mg/kg
6/10
mGITRL FP mIgG2a 10mg/kg
5/10
Tumor Volume (mm3)
Days post CT26 implantation
B
CD4+ Foxp3+
CD8+
CD4+ Foxp3-
% Ki67+
saline
mGITRL FP mIgG2a
mGITRL FP mIgG1
C
CD4+ Foxp3+
CD8+
CD4+ Foxp3-
% CD45+ cells
Saline
mGITRL FP mIgG2a
mGITRL FP mIgG1

Figure 31 Cont'd

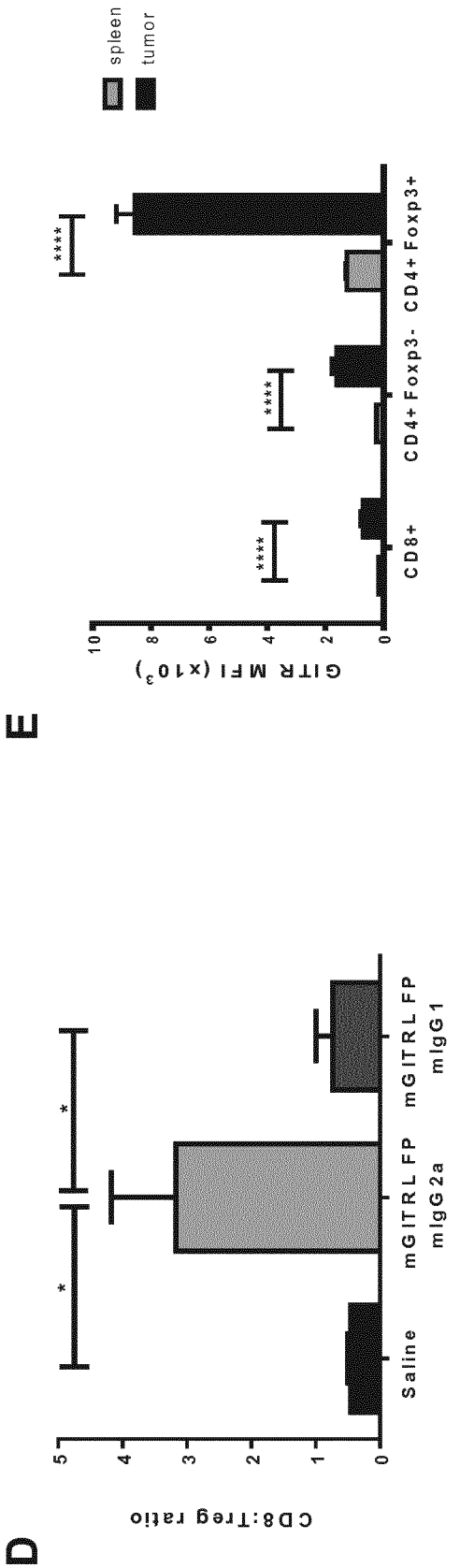


Figure 32

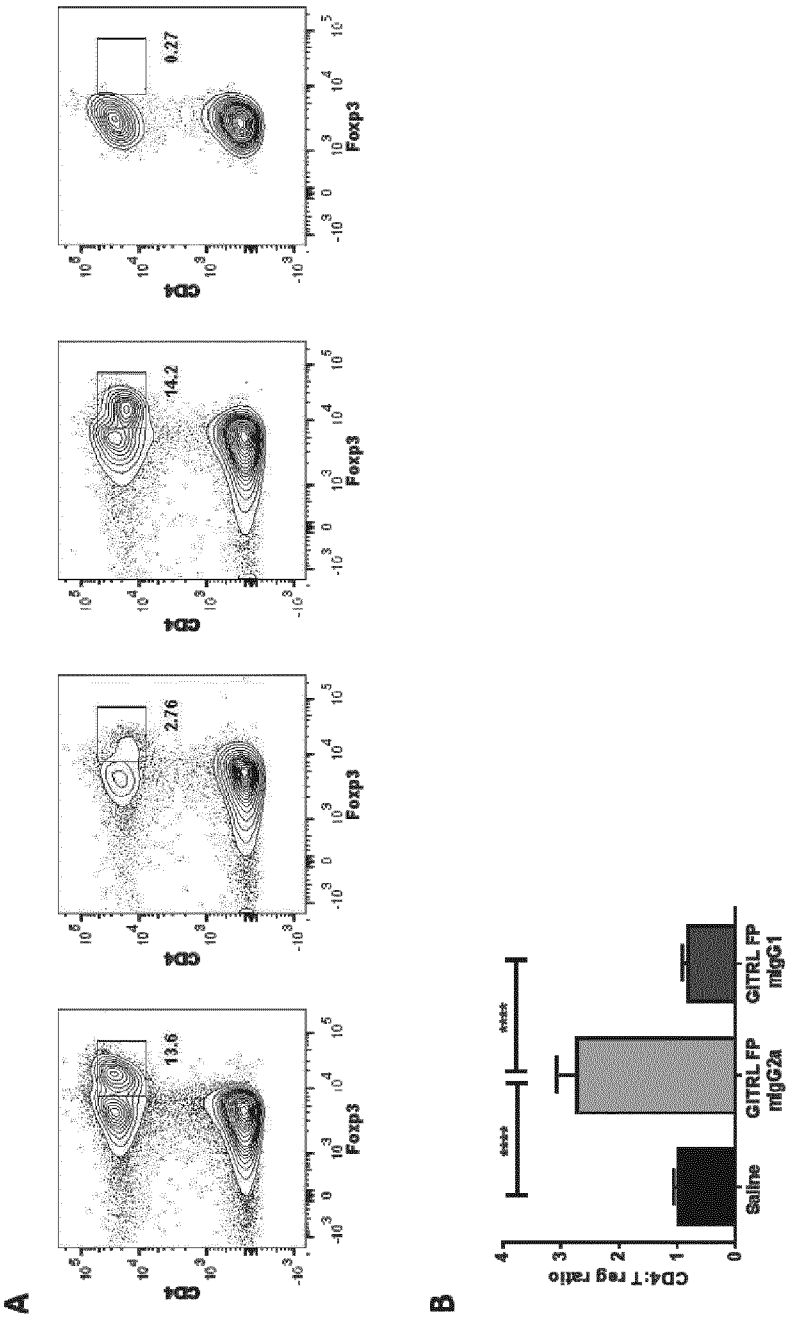

A
13.6
2.76
14.2
0.27
CD4
Foxp3
B
CD4:T reg ratio
Saline
GITRL FP mIgG2a
GITRL FP mIgG1
****
****

Figure 33

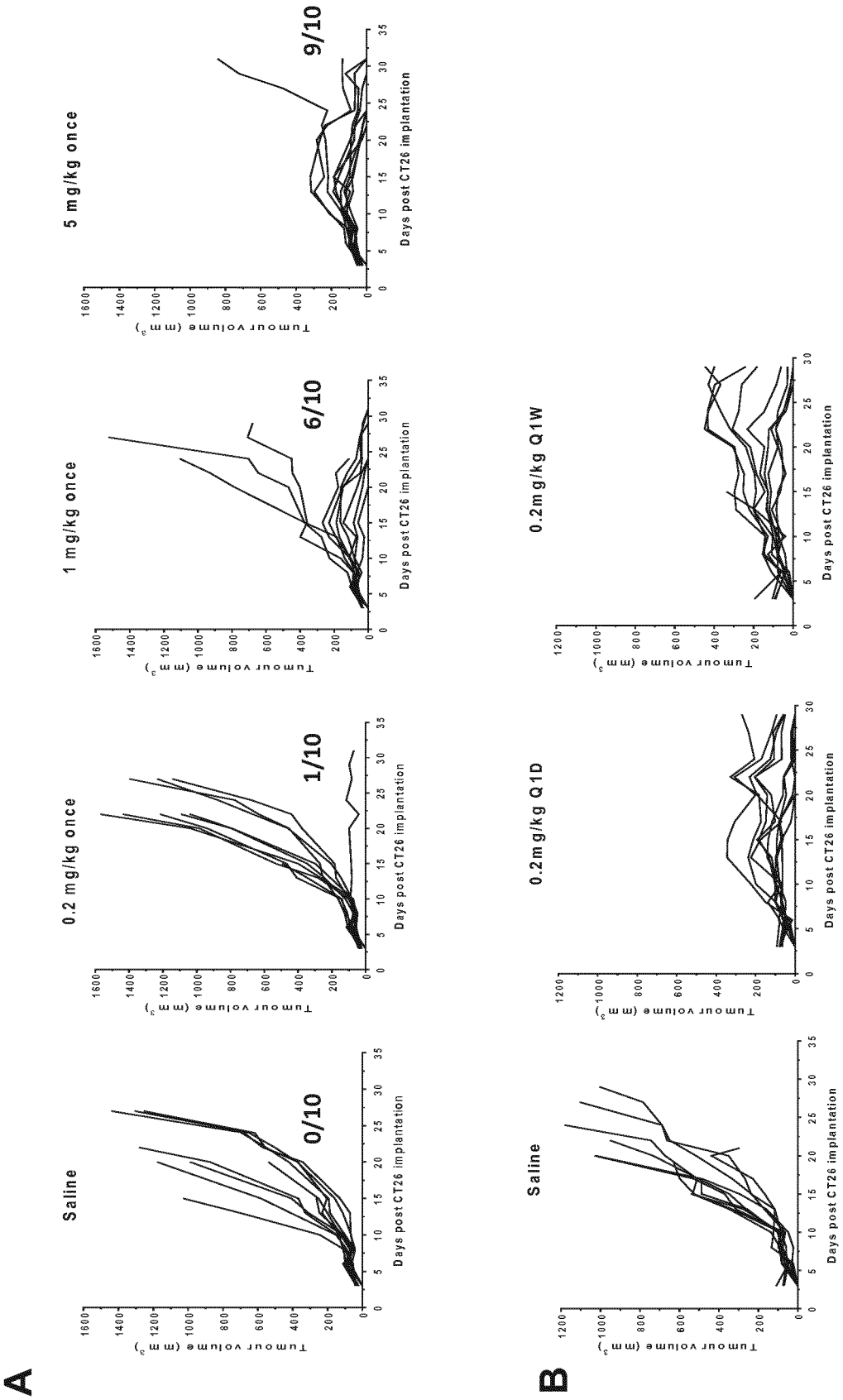
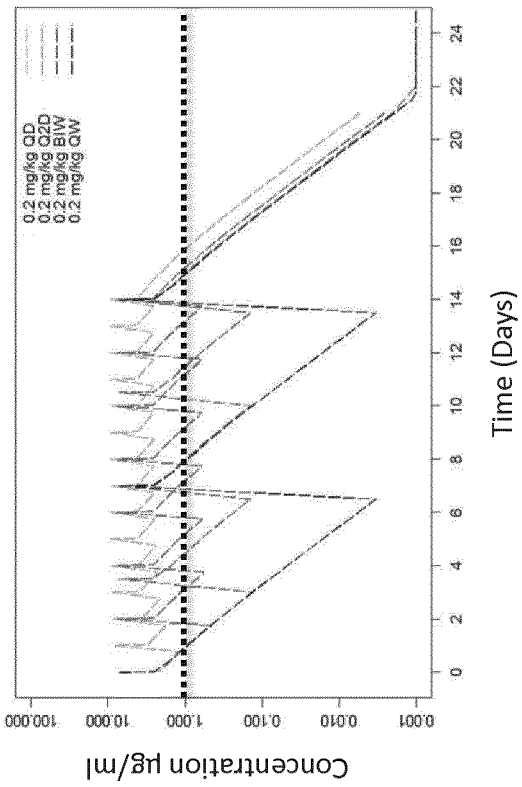

A
Saline
0/10
0.2 mg/kg once
1/10
1 mg/kg once
6/10
5 mg/kg once
9/10
B
Saline
0.2mg/kg Q1D
0.2mg/kg Q1W
Tumour volume (mm3)
Days post CT26 implantation

Figure 33 cont'd

C



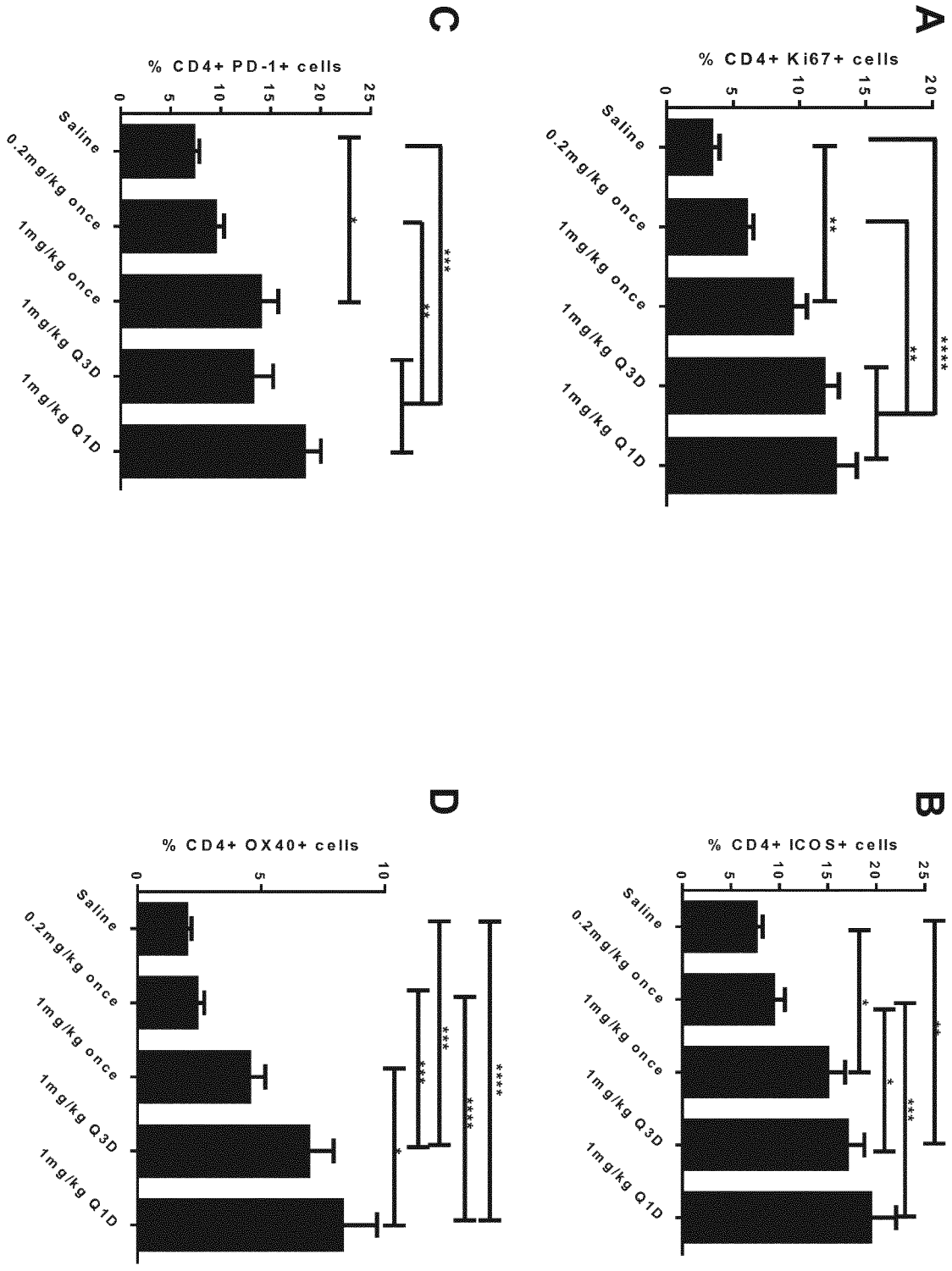

A
% CD4+ Ki67+ cells
0
5
10
15
20
Saline
0.2mg/kg once
1mg/kg once
1mg/kg Q3D
1mg/kg Q1D
****
**
**
B
% CD4+ ICOS+ cells
0
5
10
15
20
25
Saline
0.2mg/kg once
1mg/kg once
1mg/kg Q3D
1mg/kg Q1D
****
**
*
*
***
C
% CD4+ PD-1+ cells
0
5
10
15
20
25
Saline
0.2mg/kg once
1mg/kg once
1mg/kg Q3D
1mg/kg Q1D
***
**
*
D
% CD4+ OX40+ cells
0
5
10
Saline
0.2mg/kg once
1mg/kg once
1mg/kg Q3D
1mg/kg Q1D
****
****
***
***
*

Figure 34

Figure 35

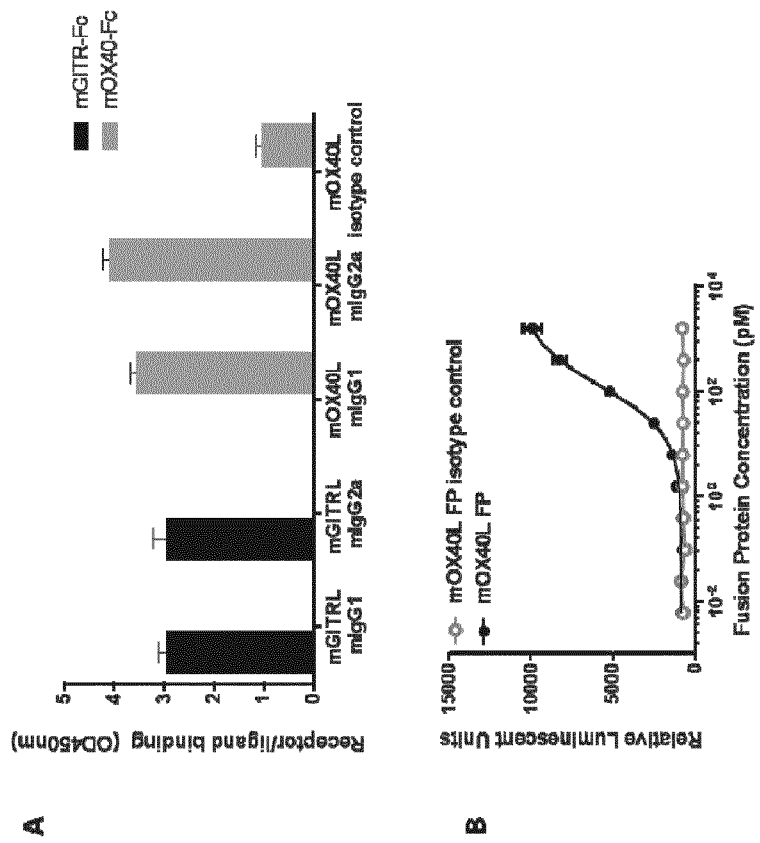

A
mGITR-Fc
mOX40-Fc
Receptor/ligand binding (OD450nm)
0
1
2
3
4
5
mGITRL mIgG1
mGITRL mIgG2a
mOX40L mIgG1
mOX40L mIgG2a
mOX40L isotype control
B
mOX40L FP isotype control
mOX40L FP
Relative Luminescent Units
0
5000
10000
15000
10⁻²
10⁰
10²
10⁴
Fusion Protein Concentration (pM)

Figure 36

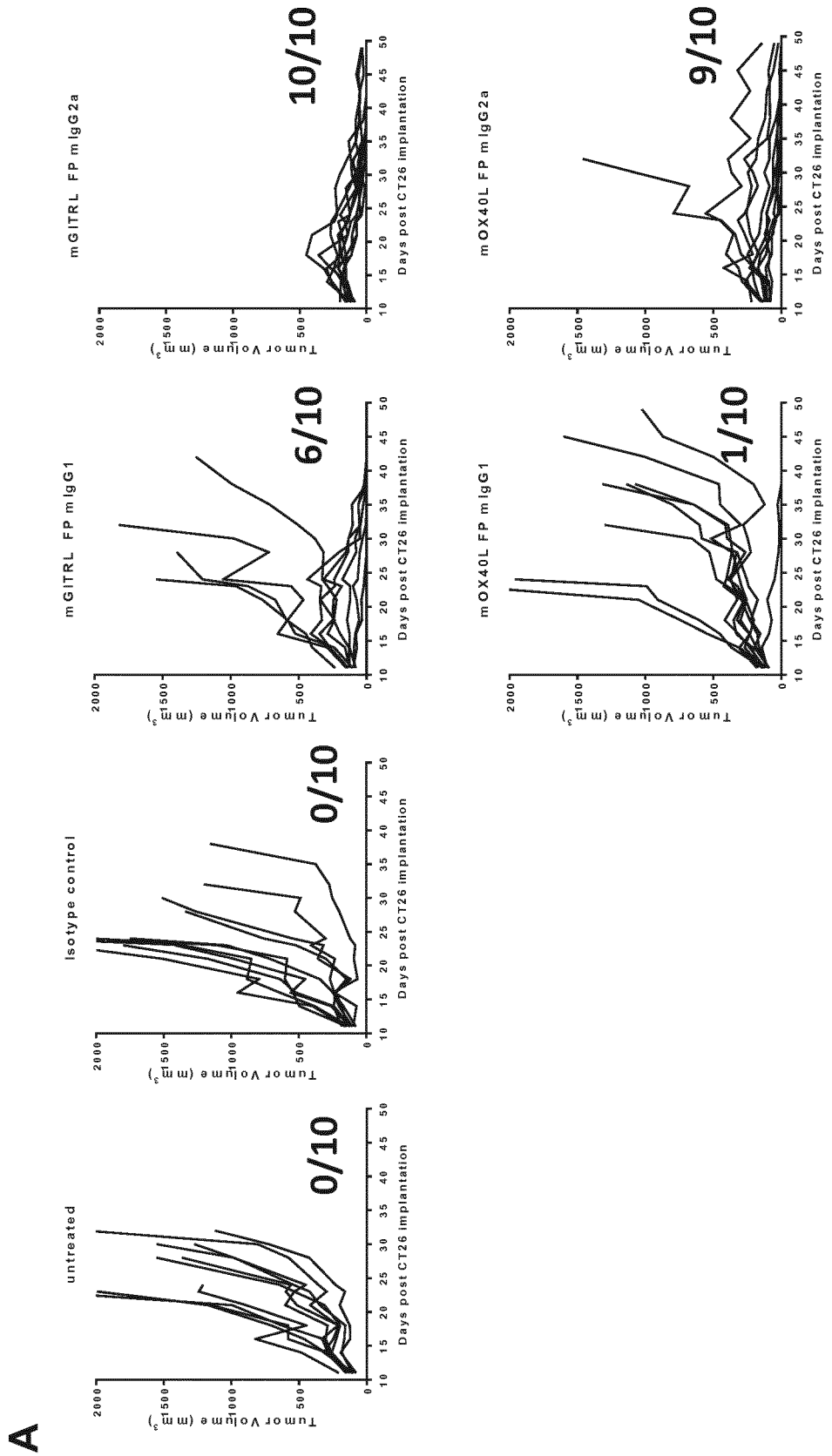

A
untreated
0/10
Isotype control
0/10
mGITRL FP mIgG1
6/10
mGITRL FP mIgG2a
10/10
mOX40L FP mIgG1
1/10
mOX40L FP mIgG2a
9/10
Tumor Volume (mm³)
Days post CT26 implantation

Figure 36 cont'd

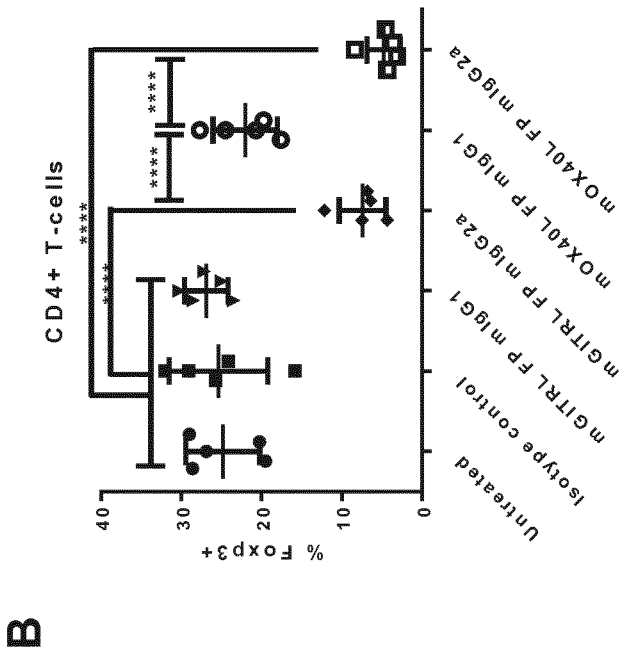


Figure 37

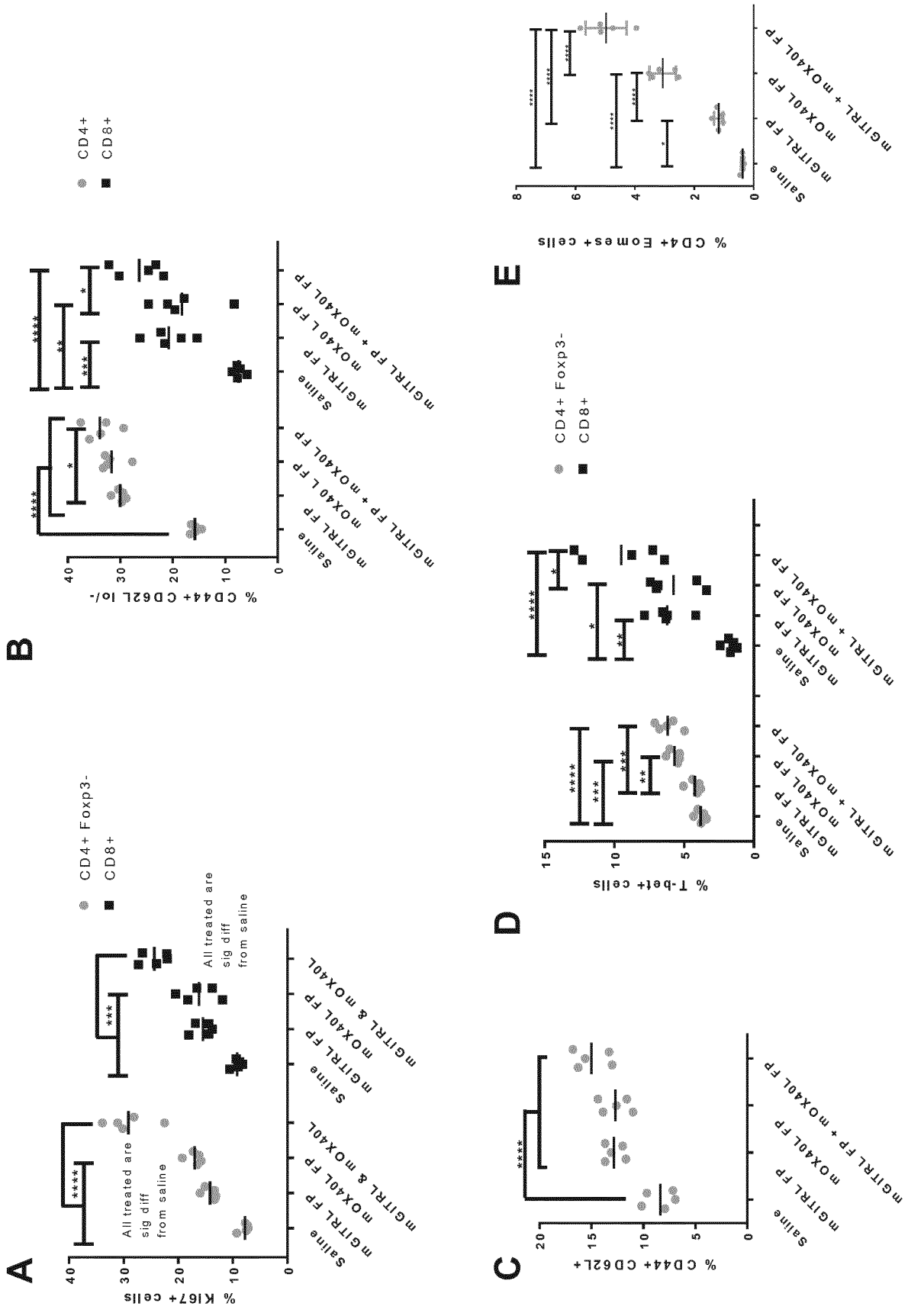

A
% Ki67+ cells
CD4+ Foxp3-
CD8+
All treated are sig diff from saline
All treated are sig diff from saline
Saline
mGITRL FP
mOX40L FP
mGITRL & mOX40L
B
% CD44+ CD62L lo/-
CD4+
CD8+
Saline
mGITRL FP
mOX40L FP
mGITRL FP + mOX40L FP
C
% CD44+ CD62L+
D
% T-bet+ cells
CD4+ Foxp3-
CD8+
E
% CD4+ Eomes+ cells

Figure 38

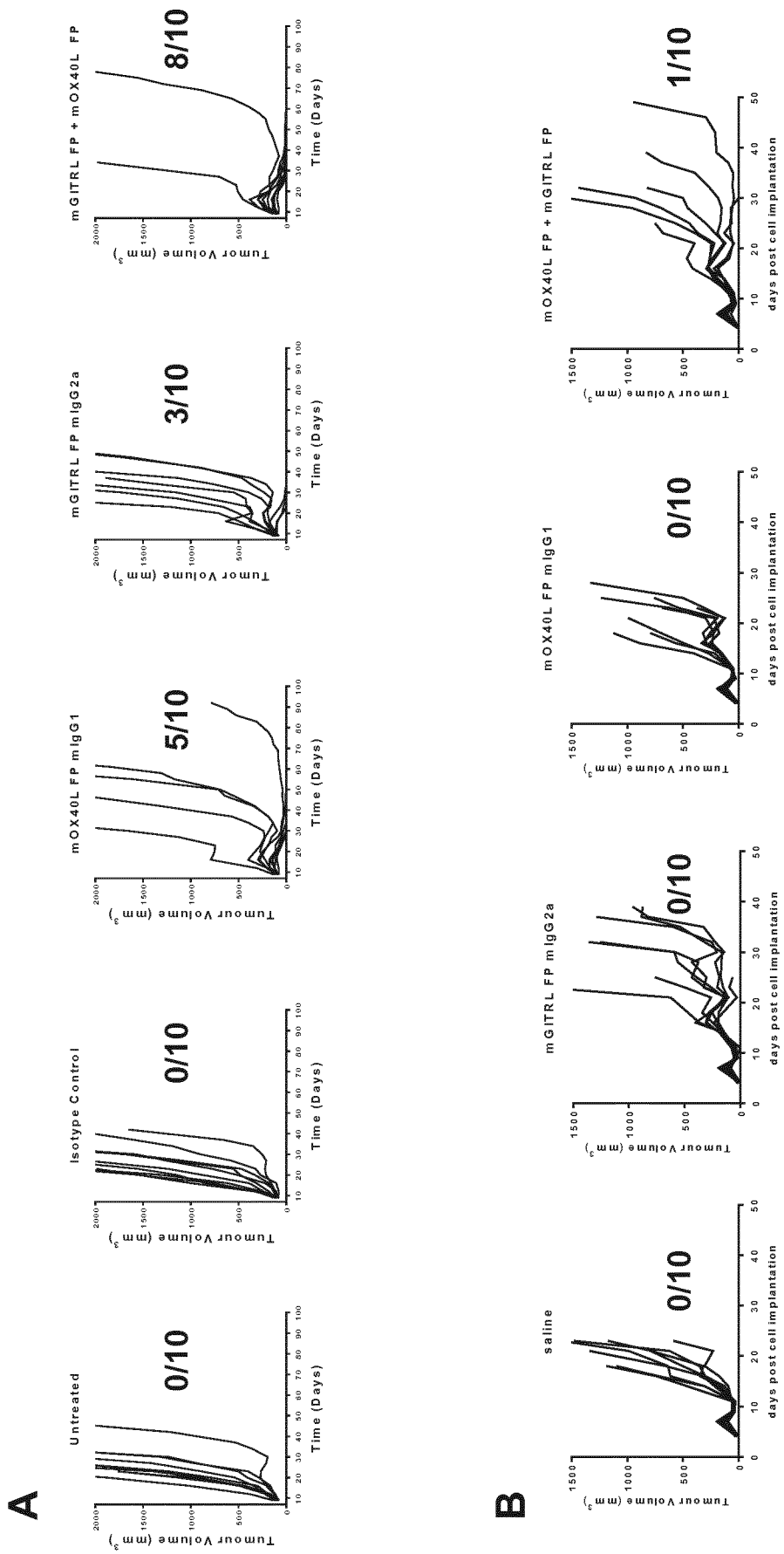
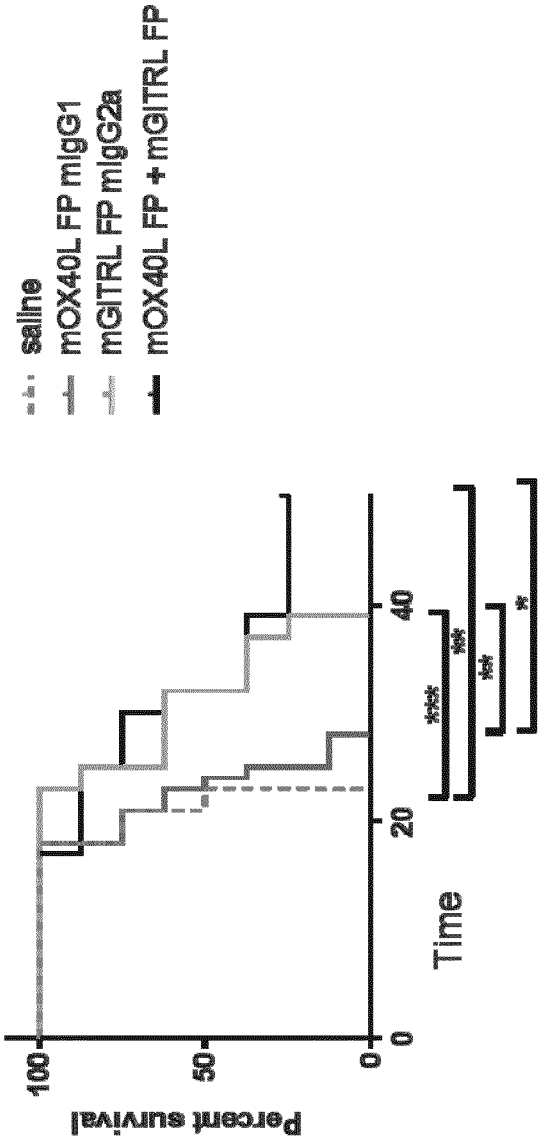

A
Untreated
0/10
Isotype Control
0/10
mOX40L FP mIgG1
5/10
mGITRL FP mIgG2a
3/10
mGITRL FP + mOX40L FP
8/10
Tumour Volume (mm3)
Time (Days)
B
saline
0/10
mGITRL FP mIgG2a
0/10
mOX40L FP mIgG1
0/10
mOX40L FP + mGITRL FP
1/10
Tumour Volume (mm3)
days post cell implantation

Figure 39


saline
mOX40L FP mIgG1
mGITRL FP mIgG2a
mOX40L FP + mGITRL FP
Percent survival
100
50
0
0
20
40
Time
***
**
**
*

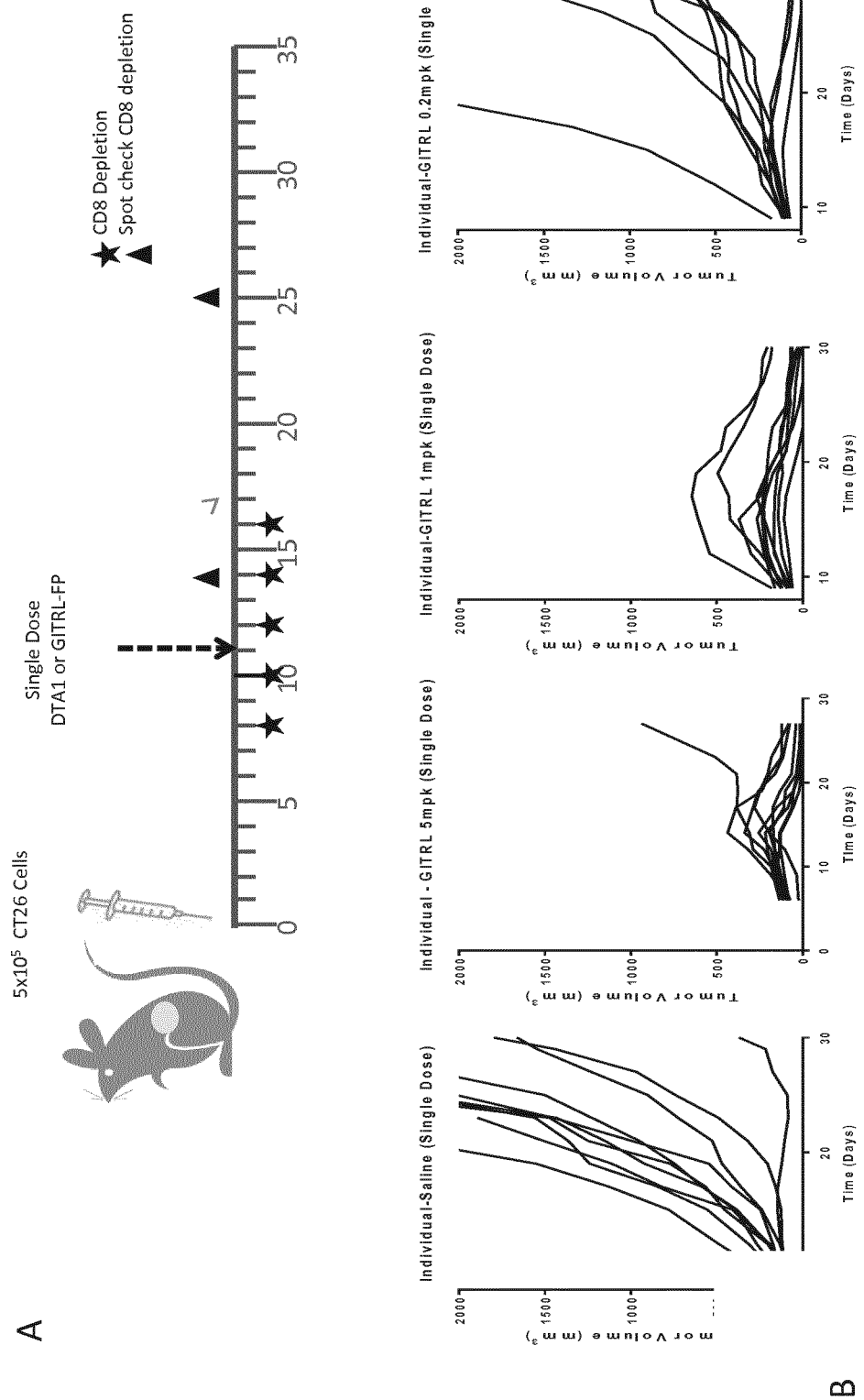

A
5x10^5 CT26 Cells
Single Dose
DTA1 or GITRL-FP
CD8 Depletion
Spot check CD8 depletion
0
5
10
15
20
25
30
35
B
Individual-Saline (Single Dose)
Individual - GITRL 5mpk (Single Dose)
Individual-GITRL 1mpk (Single Dose)
Individual-GITRL 0.2mpk (Single Dose)
Individual-GITRL 0.1mpk (Single Dose)
Tumor Volume (mm3)
Time (Days)

Figure 40

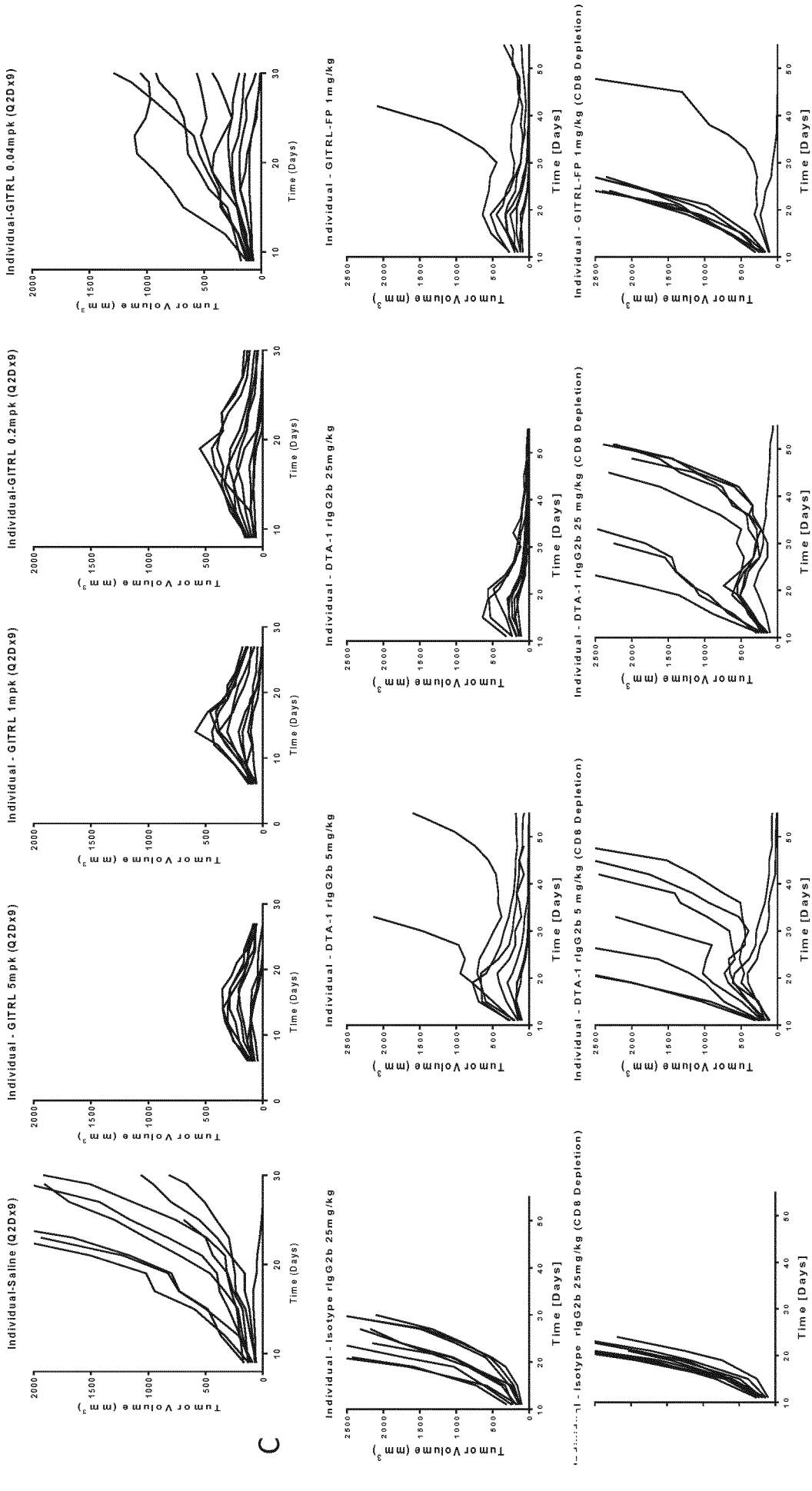


Figure 40 Cont'd

F

CD8 Depletion Group	Dose (mg/kg)	Median Survival (days)
Isotype	25	24
DTA-1	5	42
DTA-1	25	48
GITRL-FP	1	27

G

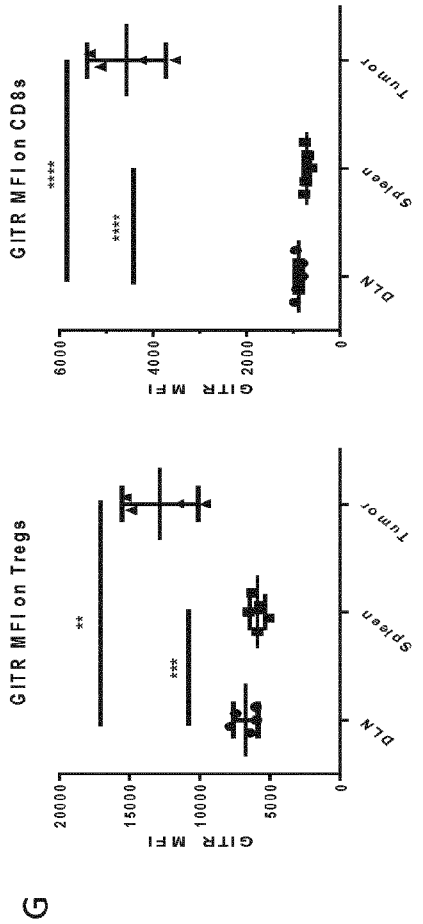


Figure 40 Cont'd

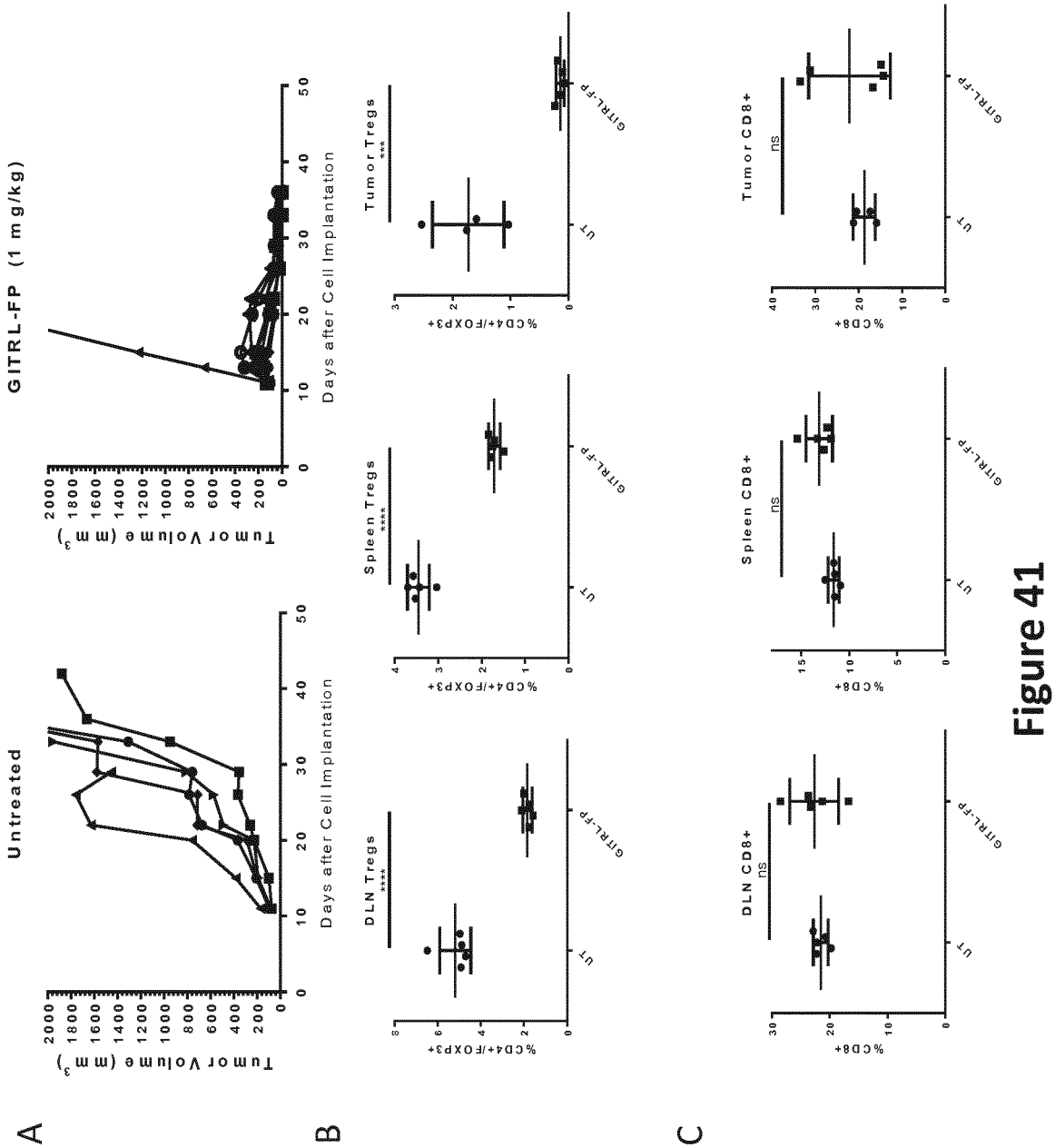

A
Untreated
GITRL-FP (1 mg/kg)
Tumor Volume (mm3)
Days after Cell Implantation
B
DLN Tregs
Spleen Tregs
Tumor Tregs
%CD4+/FOXP3+
UT
GITRL-FP
C
DLN CD8+
Spleen CD8+
Tumor CD8+
%CD8+
ns

Figure 41

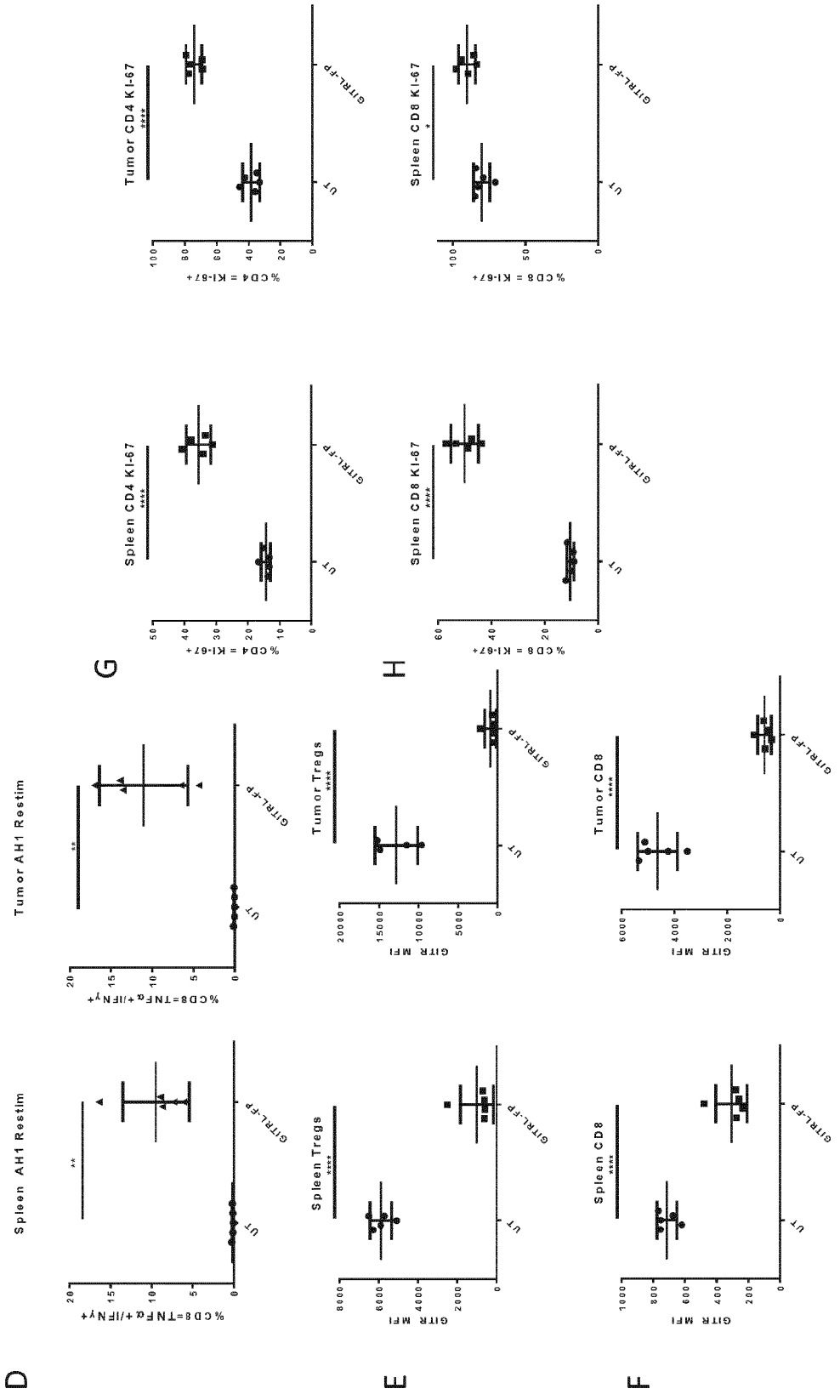


Figure 41 Cont'd

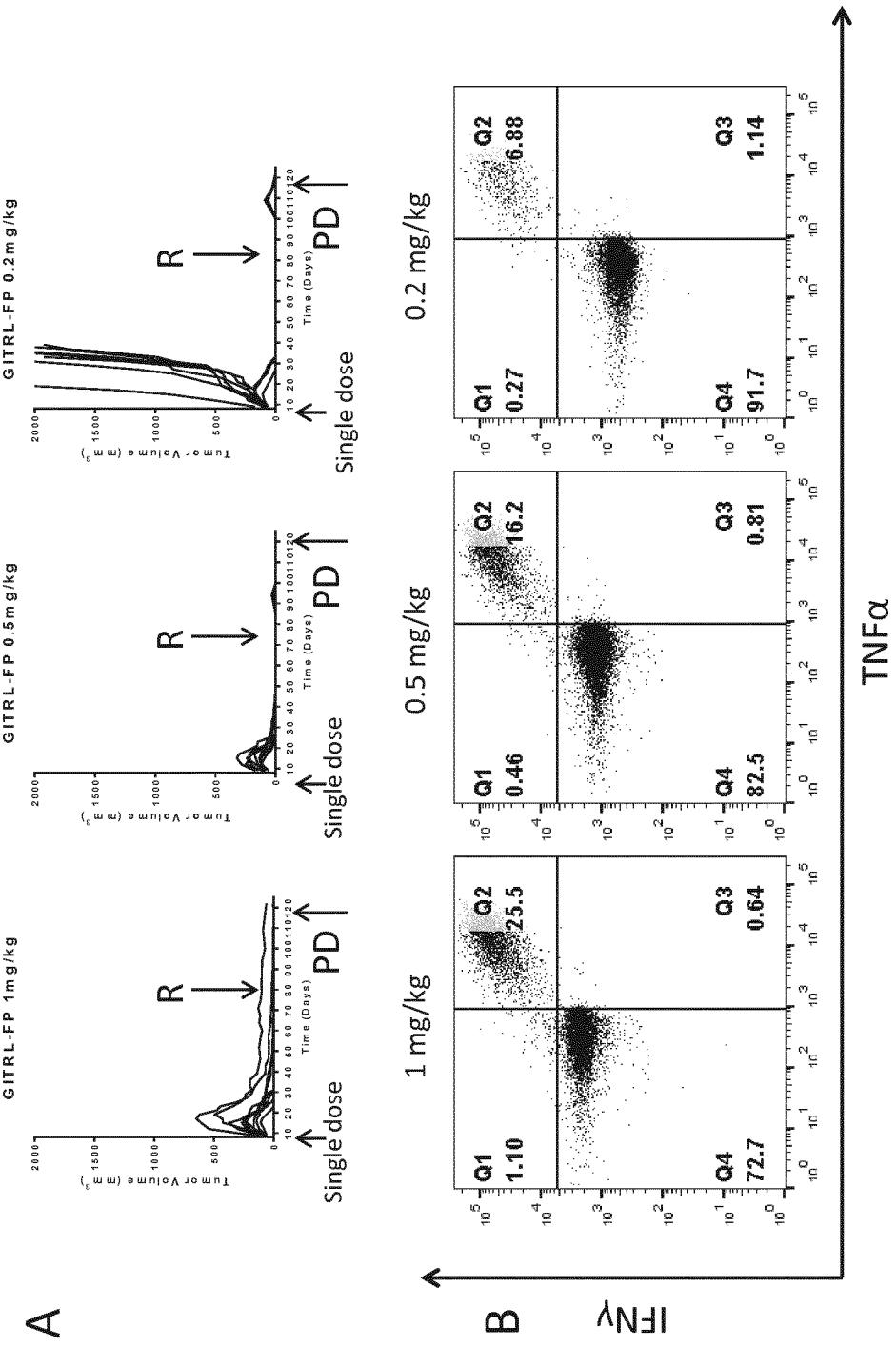

A
GITRL-FP 1mg/kg
GITRL-FP 0.5mg/kg
GITRL-FP 0.2mg/kg
Tumor Volume (mm³)
Time (Days)
R
PD
Single dose
B
1 mg/kg
0.5 mg/kg
0.2 mg/kg
IFNγ
TNFα
Q1 1.10
Q2 25.5
Q4 72.7
Q3 0.64
Q1 0.46
Q2 16.2
Q4 82.5
Q3 0.81
Q1 0.27
Q2 6.88
Q4 91.7
Q3 1.14

Figure 42

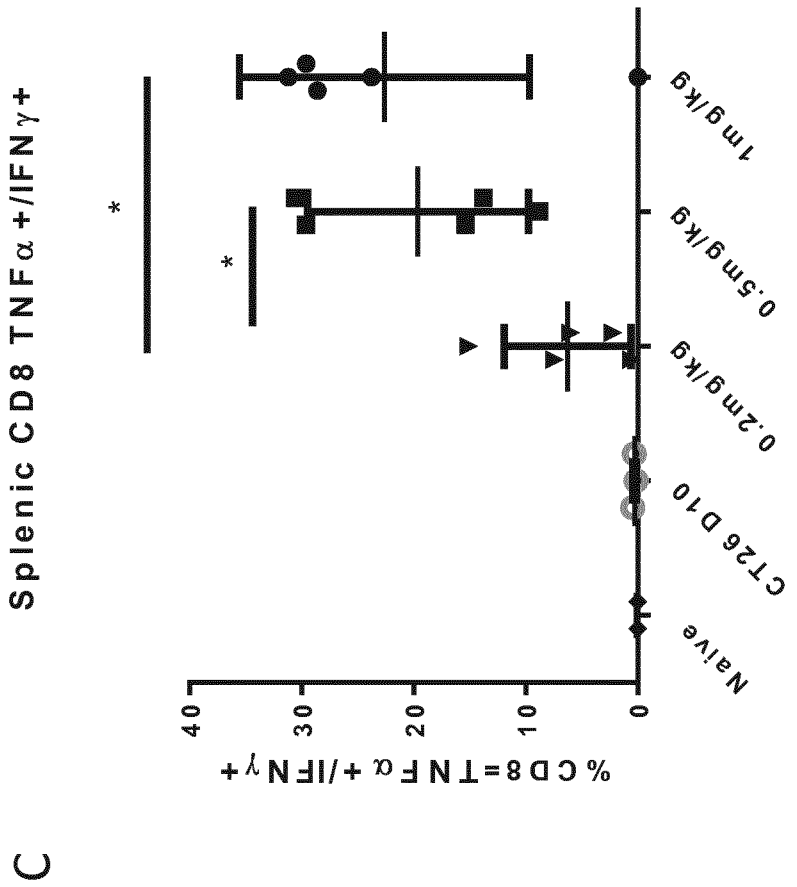


Figure 42 Cont'd

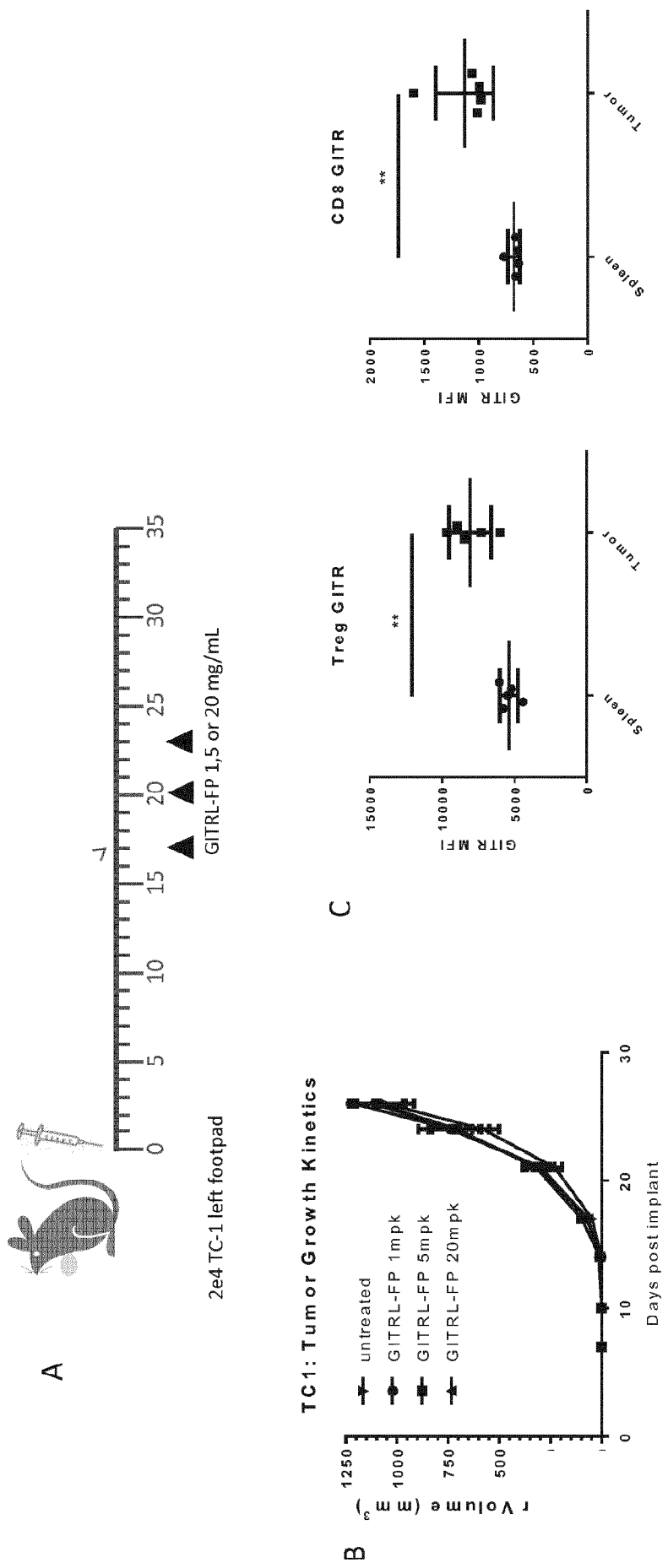

A
0
5
7
10
15
20
25
30
35
2e4 TC-1 left footpad
GITRL-FP 1,5 or 20 mg/mL
B
TC1: Tumor Growth Kinetics
untreated
GITRL-FP 1mpk
GITRL-FP 5mpk
GITRL-FP 20mpk
Tumor Volume (mm³)
1250
1000
750
500
0
10
20
30
Days post implant
C
Treg GITR
**
GITR MFI
15000
10000
5000
0
Tumor
Spleen
CD8 GITR
**
GITR MFI
2000
1500
1000
500
0
Tumor
Spleen

Figure 43

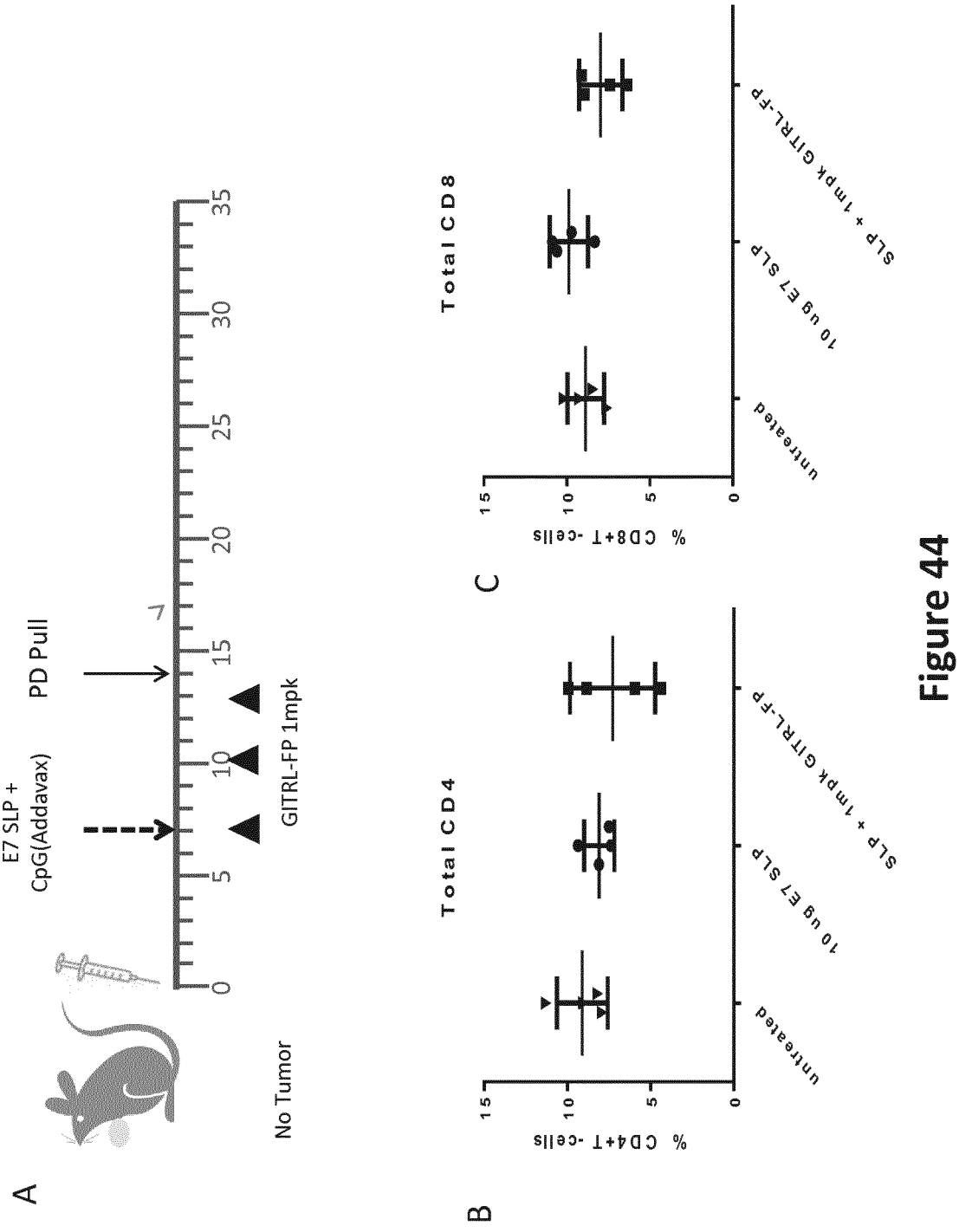

A
E7 SLP +
CpG(Addavax)
PD Pull
GITRL-FP 1mpk
No Tumor
0
5
10
15
20
25
30
35
B
Total CD4
% CD4+T -cells
C
Total CD8
% CD8+T -cells
untreated
10 ug E7 SLP
SLP + 1mpk GITRL-FP

Figure 44

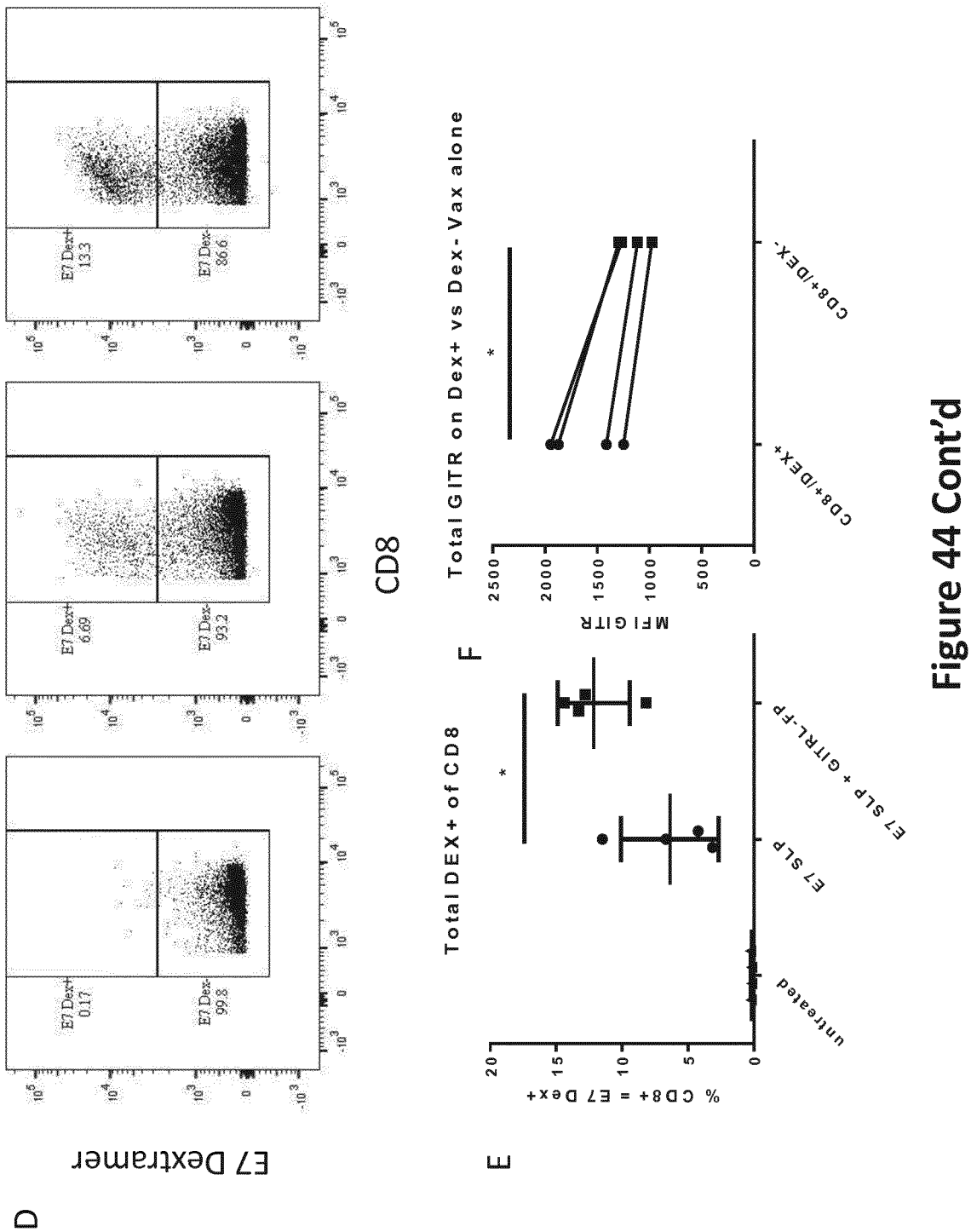


Figure 44 Cont'd

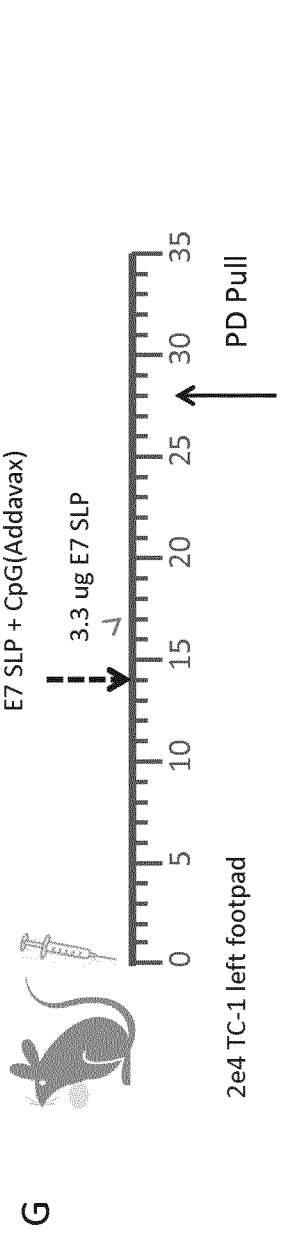


Figure 44 Cont'd

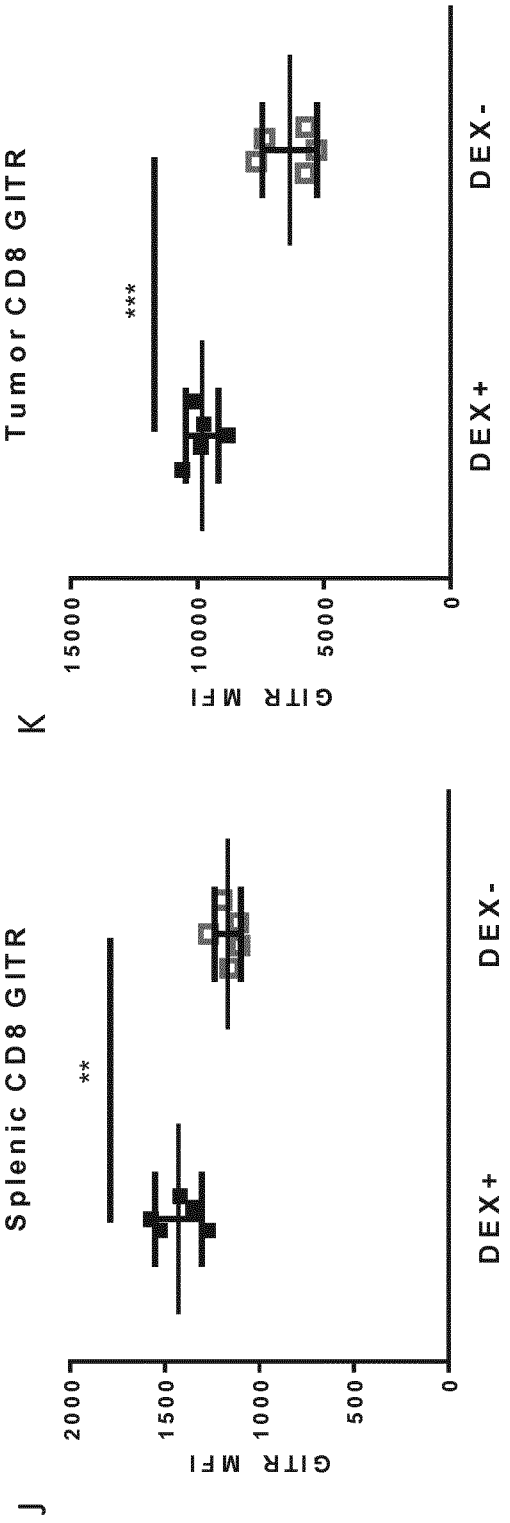


Figure 44 Cont'd

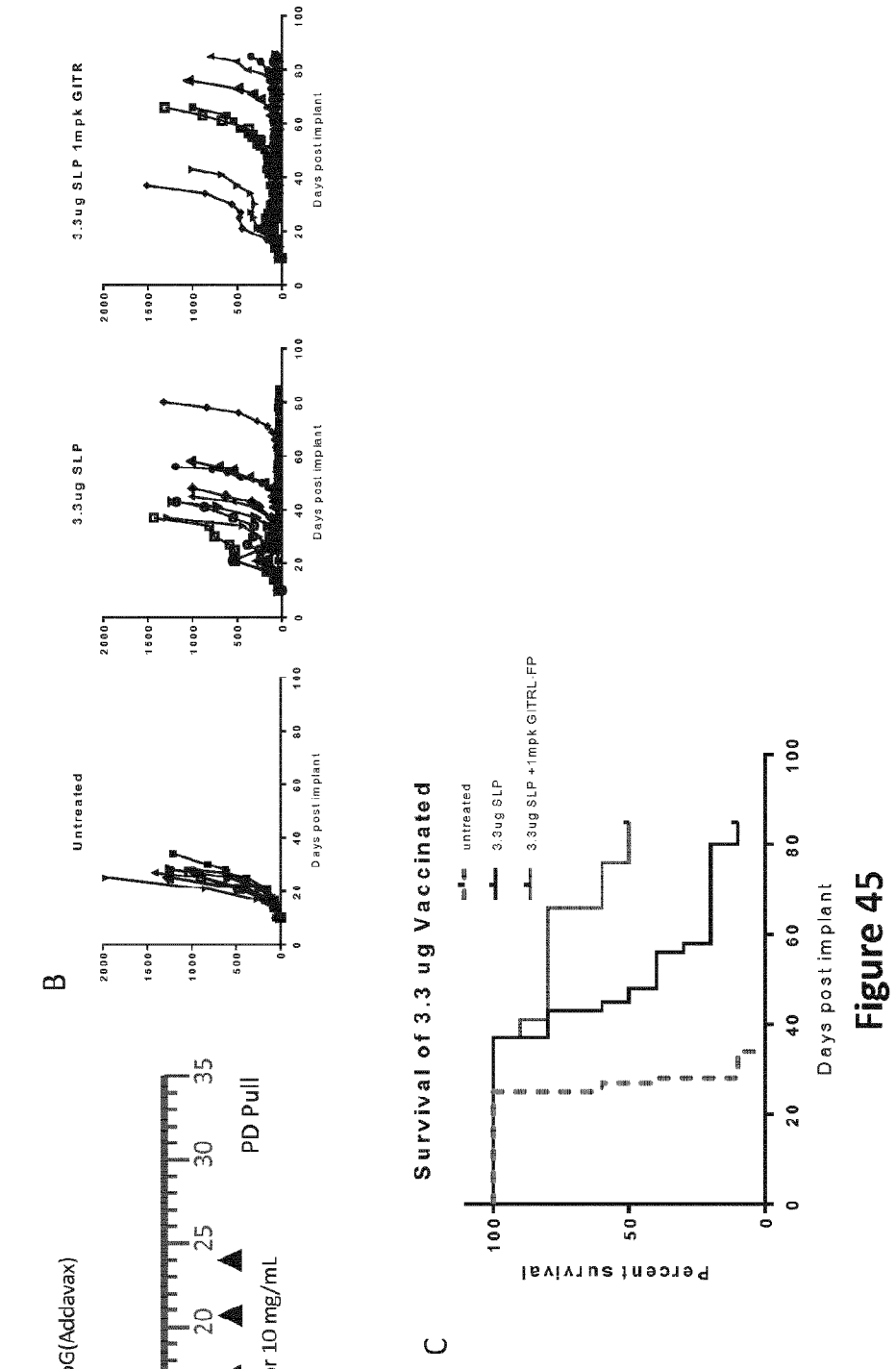
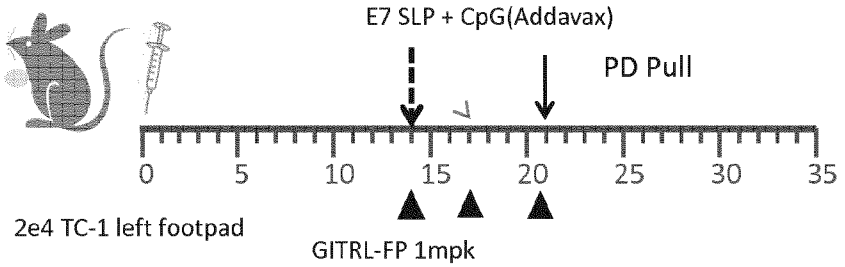

A
E7 SLP + CpG(Addavax)
2e4 TC-1 left footpad
GITRL-FP 1 or 10 mg/mL
PD Pull
0
5
10
15
20
25
30
35
B
Untreated
3.3ug SLP
3.3ug SLP 1mpk GITR
Days post implant
C
Survival of 3.3 ug Vaccinated
untreated
3.3ug SLP
3.3ug SLP +1mpk GITRL-FP
Percent survival
Days post implant

Figure 45

D

Group	Median Survival
Untreated	27
E7 SLP Alone	46.5
E7 SLP + GITRL-FP(1)	80.5
E7 SLP + GITRL-FP(10)	75.5

E



F

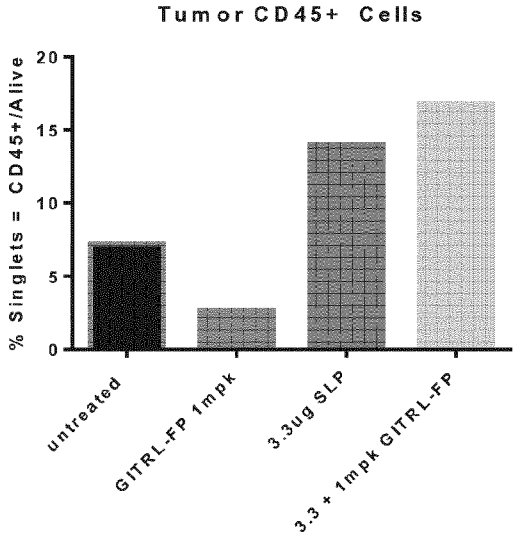


Figure 45 Cont'd.

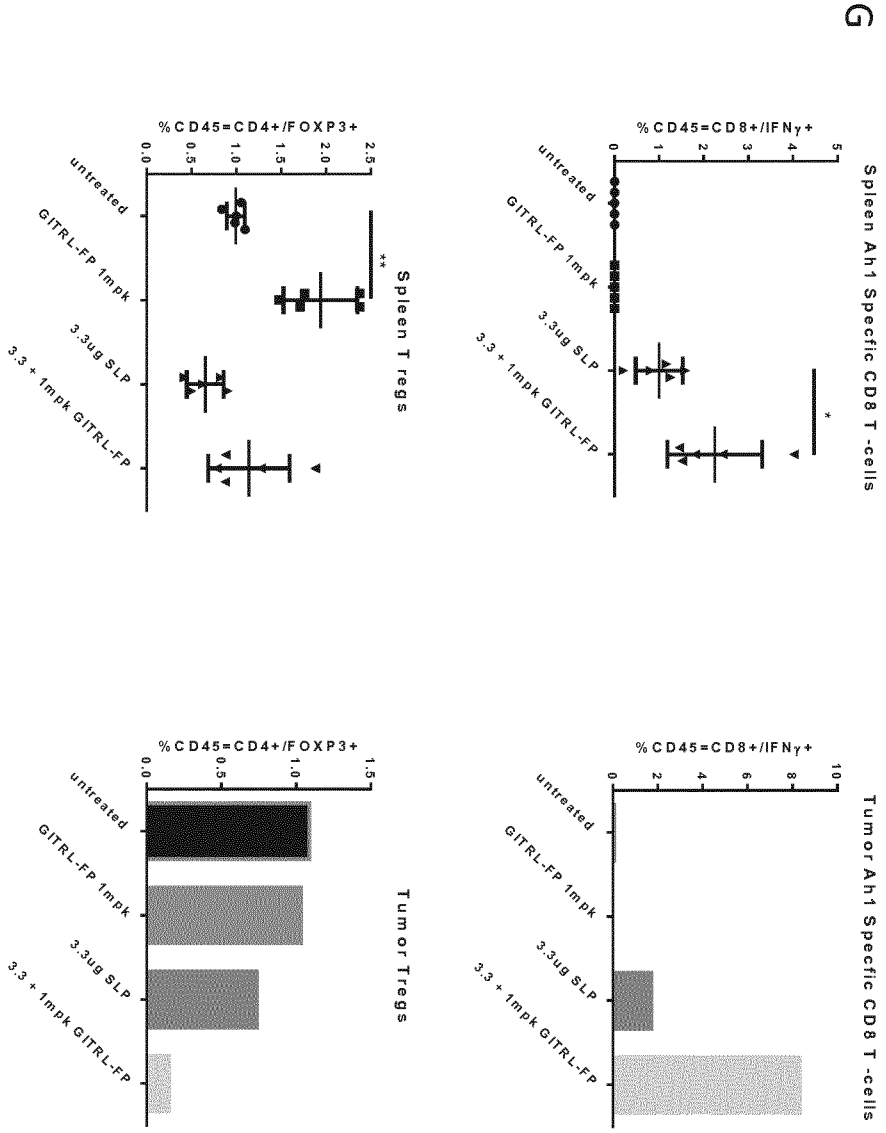


Figure 45 Cont'd.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/069175

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/705 A61K38/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. WYZGOL ET AL: "Trimer Stabilization, Oligomerization, and Antibody-Mediated Cell Surface Immobilization Improve the Activity of Soluble Trimers of CD27L, CD40L, 41BBL, and Glucocorticoid-Induced TNF Receptor Ligand", THE JOURNAL OF IMMUNOLOGY, vol. 183, no. 3, 1 August 2009 (2009-08-01), pages 1851-1861, XP055015511, ISSN: 0022-1767, DOI: 10.4049/jimmunol.0802597	1-85
Y	the whole document page 1859, column 1, paragraph 2 ----- -/--	86-136

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2016	30/11/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Voigt-Ritzer, Heike

2

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GIUSEPPE NOCENTINI ET AL: "Pharmacological modulation of GITRL/GITR system: therapeutic perspectives", BRITISH JOURNAL OF PHARMACOLOGY, vol. 165, no. 7, 9 March 2012 (2012-03-09) , pages 2089-2099, XP055150987, ISSN: 0007-1188, DOI: 10.1111/j.1476-5381.2011.01753.x	86-130, 135,136
A	the whole document	1-85, 131-134

Y	WO 2015/116178 A1 (UNIV JEFFERSON [US]; UNIV PENNSYLVANIA [US]) 6 August 2015 (2015-08-06)	131-134
A	page 62 - page 68 abstract	1-130, 135

A	WO 2009/009116 A2 (TOLERX INC [US]; ROSENZWEIG MICHAEL [US]; PONATH PAUL [US]; PONTE JOSE) 15 January 2009 (2009-01-15) examples 1-7 page 6, paragraph 2 page 1, paragraph 3	1-135

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/069175

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

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

   a. [ ] forming part of the international application as filed:

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

      [ ] on paper or in the form of an image file.

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

   c. [X] furnished subsequent to the international filing date for the purposes of international search only:

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

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

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/069175

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015116178 A1	06-08-2015	NONE	
WO 2009009116 A2	15-01-2009	AU 2008275589 A1	15-01-2009
		CA 2693677 A1	15-01-2009
		CN 101801413 A	11-08-2010
		DK 2175884 T3	26-09-2016
		EP 2175884 A2	21-04-2010
		JP 5932217 B2	08-06-2016
		JP 2010533184 A	21-10-2010
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		US 2009136494 A1	28-05-2009
		US 2014220002 A1	07-08-2014
		US 2016324963 A1	10-11-2016
		WO 2009009116 A2	15-01-2009

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(51)Int.Cl.
C07K 14/705(2006.01)
A61K 38/00(2006.01)

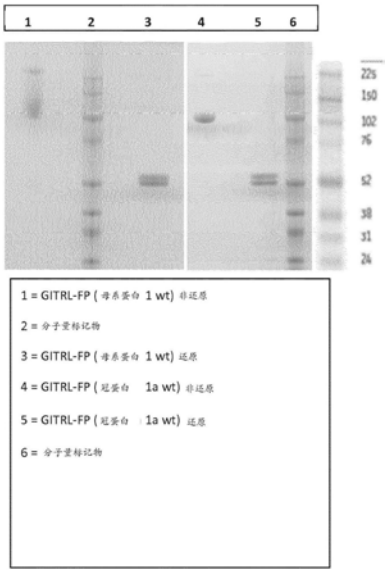
权利要求书8页 说明书69页
序列表32页 附图56页

(54)发明名称

GITRL融合蛋白及其用途

(57)摘要

本披露提供包括IgG Fc结构域、三聚结构域、和GITR配体的受体结合结构域的GITRL融合多肽亚基，其中这些融合多肽亚基可自组装成六聚体蛋白。还提供制造融合多肽亚基和六聚体蛋白的方法，以及例如用于治疗癌症的方法。



CN 108026158 A

1.一种分离的单链多肽亚基,包括:IgG Fc结构域;功能性多聚结构域;和糖皮质激素诱导的TNF受体配体(GITRL)的受体结合结构域,其中该多肽亚基可以自组装成三聚体或六聚体蛋白。

2.如权利要求1所述的多肽亚基,其中该多聚结构域是三聚结构域。

3.如权利要求1或2所述的多肽亚基,该多肽亚基从氨基末端到羧基末端包括该IgG Fc结构域,随后是该多聚或三聚结构域,随后是该GITRL受体结合结构域。

4.如权利要求1至3中任一项所述的多肽亚基,其中该IgG Fc结构域的羧基末端经由第一接头区与该多聚或三聚结构域的氨基末端融合。

5.如权利要求1至4中任一项所述的多肽亚基,其中该多聚或三聚结构域的羧基末端经由第二接头区与该GITRL受体结合结构域的氨基末端融合。

6.如权利要求1至4中任一项所述的多肽亚基,其中该IgG Fc结构域的羧基末端直接与该多聚或三聚结构域的氨基末端融合。

7.如权利要求5或6所述的多肽亚基,其中该IgG Fc结构域在其氨基末端包括IgG铰链区。

8.如权利要求5或6所述的多肽亚基,其中该IgG铰链区包括赋予完整的重链间二硫键形成的突变。

9.如权利要求7或8所述的多肽亚基,其中该IgG铰链区包括IgG1铰链区、IgG4铰链区或其变体。

10.如权利要求9所述的多肽亚基,其中该IgG4铰链区具有根据EU编号(IgG4P)的在位置228处的丝氨酸至脯氨酸突变(S228P)。

11.如权利要求1至10中任一项所述的多肽亚基,其中该IgG Fc结构域是人IgG Fc结构域。

12.如权利要求1至4或6至10中任一项所述的多肽亚基,其中该多聚或三聚结构域的羧基末端直接与该GITRL受体结合结构域的氨基末端融合。

13.如权利要求5至12中任一项所述的多肽亚基,其中当存在时,该第一接头区、该第二接头区、或者该第一接头区和该第二接头区独立地选自下组,该组由以下各项组成:包含(Gly_4)n基序、(Gly_4Ser)n基序(SEQ ID NO:19)、$Ser(Gly_4Ser)$n基序(SEQ ID NO:22)、GGGGSGGGGSGGGGSAL(SEQ ID NO:23)、GGGGSGGGGSGGGGSA(SEQ ID NO:24)及其组合的接头区,其中n是选自下组的正整数,该组由以下各项组成:1、2、3、4、5、6、7、8、9和10。

14.如权利要求13所述的多肽亚基,其中该第一接头区和该第二接头区独立地选自下组,该组由以下各项组成:GGGGSGGGGSGGGGS(SEQ ID NO:25)和GGGGSGGGGSGGGG(SEQ ID NO:26)。

15.如权利要求13或权利要求14所述的多肽亚基,其中该第一接头区是GGGGSGGGGSGGGGSGGGGSGGGGS(SEQ ID NO:20),并且该第二接头区是(Gly_4)基序。

16.如权利要求1至15中任一项所述的多肽亚基,其中该IgG Fc结构域包括IgG1、IgG2、IgG3、IgG4、IG4P Fc结构域或其变体。

17.如权利要求1至16中任一项所述的多肽亚基,其中该IgG Fc结构域含有选自下组的一个或多个氨基酸残基取代,该组由以下各项组成:252Y、254T、256E及其组合,其中这些残基是根据EU编号进行编号。

18.如权利要求1至17中任一项所述的多肽亚基，其中该IgG Fc结构域包含CH2区。

19.如权利要求18所述的多肽亚基，其中该IgG Fc结构域进一步包含CH3区。

20.如权利要求19所述的多肽亚基，其中该IgG Fc结构域包含与SEQ ID NO:21具有至少85％、至少90％、至少95％、或至少99％序列一致性的氨基酸序列。

21.如权利要求19所述的多肽亚基，其中该IgG Fc结构域包括SEQ ID NO:21的氨基酸序列。

22.如权利要求1至21中任一项所述的多肽亚基，其中该三聚结构域包括α-螺旋卷曲螺旋结构域、亮氨酸拉链结构域、或它们的组合。

23.如权利要求22所述的多肽亚基，其中该三聚结构域源自于母系蛋白1、冠蛋白1a、营养不良性肌强直激酶(DMPK)、朗格汉斯蛋白或其组合。

24.如权利要求23所述的多肽亚基，其中该三聚结构域源自于母系蛋白1或冠蛋白1a。

25.如权利要求24所述的多肽亚基，其中该三聚结构域源自于冠蛋白1a。

26.如权利要求25所述的多肽亚基，其中该三聚结构域包括与SEQ ID NO:11具有至少85％、至少90％、至少95％或至少99％序列一致性的冠蛋白1a三聚结构域。

27.如权利要求26所述的多肽亚基，其中该冠蛋白1a三聚结构域包括SEQ ID NO:11的氨基酸序列。

28.如权利要求26所述的多肽亚基，其中该三聚结构域包括SEQ ID NO:10的冠蛋白1a三聚结构域。

29.如权利要求26所述的多肽亚基，其中该冠蛋白1a三聚结构域包括SEQ ID NO:11、SEQ ID NO:12、SEQ ID NO:13、SEQ ID NO:14、SEQ ID NO:15、SEQ ID NO:16、SEQ ID NO:17、SEQ ID NO:18的氨基酸序列或其任何组合或变体。

30.如权利要求24所述的多肽亚基，其中该三聚结构域包括与SEQ ID NO:28具有至少80％、至少85％、至少90％、至少95％或至少99％序列一致性的母系蛋白1三聚结构域。

31.如权利要求23所述的多肽亚基，其中该三聚结构域包括与SEQ ID NO:30具有至少80％、至少85％、至少90％、至少95％或至少99％序列一致性的DMPK三聚结构域。

32.如权利要求23所述的多肽亚基，其中该三聚结构域包括与SEQ ID NO:32具有至少80％、至少85％、至少90％、至少95％或至少99％序列一致性的朗格汉斯蛋白三聚结构域。

33.如权利要求23所述的多肽亚基，其中该三聚结构域包括SEQ ID NO:28、SEQ ID NO:29、SEQ ID NO:30、SEQ ID NO:31、SEQ ID NO:32或SEQ ID NO:33的氨基酸序列。

34.如权利要求1至33中任一项所述的多肽亚基，其中该GITRL受体结合结构域包括与SEQ ID NO:34具有至少85％、至少90％、至少95％、或至少99％序列一致性的氨基酸序列。

35.如权利要求1至33中任一项所述的多肽亚基，其中该GITRL受体结合结构域包括与SEQ ID NO:37具有至少80％、至少85％、至少90％、至少95％、或至少99％序列一致性的氨基酸序列，其中残基161不是天冬酰胺酰残基。

36.如权利要求35所述的多肽亚基，其中该GITRL受体结合结构域包括SEQ ID NO:35的氨基酸序列。

37.如权利要求1至33中任一项所述的多肽亚基，其中该GITRL受体结合结构域包括SEQ ID NO:35的氨基酸序列，其中残基161是天冬氨酰残基。

38.如权利要求37所述的多肽亚基，其中该GITRL受体结合结构域包括SEQ ID NO:36的

氨基酸序列。

39.如权利要求38所述的多肽亚基，其中该GITRL受体结合结构域包括SEQ ID NO:37的氨基酸序列。

40.如权利要求1至39中任一项所述的多肽亚基，其中从六个该多肽亚基组装的六聚体蛋白可特异性地结合至人GITR。

41.如权利要求1至40中任一项所述的多肽亚基，其中该蛋白质是非糖基化的。

42.如权利要求1至41中任一项所述的多肽亚基，包括与SEQ ID NO:6具有至少80％、至少85％、至少90％、至少95％或至少99％序列一致性的氨基酸序列。

43.如权利要求42所述的多肽亚基，包括SEQ ID NO:6的氨基酸序列。

44.如权利要求1至43中任一项所述的多肽亚基，其中该亚基具有激动剂活性。

45.如权利要求1至44中任一项所述的多肽亚基，进一步包括相关联的异源剂。

46.如权利要求45所述的多肽亚基，其中该异源剂是异源多肽并经由肽键融合到该多肽亚基。

47.如权利要求46所述的多肽亚基，其中该异源多肽融合至该IgG-Fc结构域的N-末端、融合至GITRL的该受体结合结构域的C末端、融合至该IgG-Fc结构域的C-末端和该三聚结构域的N-末端，或融合至该三聚结构域的C-末端和GITRL的该受体结合结构域的N-末端。

48.如权利要求45所述的多肽亚基，其中该异源剂以化学方式轭合至该多肽亚基。

49.如权利要求45至48中任一项所述的多肽亚基，其中该异源剂包括细胞毒性分子、稳定剂、免疫应答调节剂、或可检测的药剂。

50.一种三聚体蛋白，包括三个如权利要求1至49中任一项所述的多肽亚基。

51.一种六聚体蛋白，包括六个如权利要求1至49中任一项所述的多肽亚基。

52.如权利要求51所述的六聚体蛋白，其可以特异性地结合在$CD4^+$或$CD8^+$T细胞、B细胞或NK细胞上表达的糖皮质激素诱导的TNF受体(GITR)，其中这些$CD4^+$或$CD8^+$T细胞或B细胞任选地是抗原处理的或这些NK细胞任选地是活化的，这些细胞来自人或非人灵长类动物，任选地食蟹猴、恒河猴，或其任何组合。

53.如权利要求52所述的六聚体蛋白，该六聚体蛋白可特异性地结合在来自人，或非人灵长类动物，任选地食蟹猴、恒河猴，或其任何组合的初级$CD4^+$或$CD8^+$T细胞上表达的GITR。

54.如权利要求52或53中任一项所述的六聚体蛋白，其中这些$CD4^+$或$CD8^+$T细胞是抗原处理的。

55.如权利要求51或权利要求54所述的六聚体蛋白，其中如通过动力学排除测定测量的，对人GITR的结合亲和力是约54nM至约111nM。

56.如权利要求55所述的六聚体蛋白，其中如在动力学排除测定(KinExA)中测量的，该结合亲和力是约82nM。

57.如权利要求51至56中任一项所述的六聚体蛋白，其可诱导剂量依赖性增殖和自GITR阳性免疫细胞的剂量依赖性细胞因子释放。

58.如权利要求57所述的六聚体蛋白，其中这些GITR阳性免疫细胞是基于板的测定中的T细胞。

59.如权利要求52至58中任一项所述的六聚体蛋白，其中这些细胞是$CD4^+$、$CD8^+$、NK、或B细胞。

60.如权利要求57至59中任一项所述的六聚体蛋白，其中这些GITR阳性免疫细胞、T细胞、$CD4^+$、$CD8^+$或B细胞是抗原处理的和/或NK细胞是活化的。

61.如权利要求60所述的六聚体蛋白，如通过胸苷掺入测量的，该六聚体蛋白可刺激抗原处理的初级人T细胞的增殖，EC_{50}是约0.1至约2.7nM。

62.如权利要求61所述的六聚体蛋白，其中该EC_{50}是约0.5nM。

63.如权利要求57所述的六聚体蛋白，其中该细胞因子是IFNγ、TNFα、IL-5、IL-10、IL-2、IL-4、IL-13、IL-8、IL-12p70、IL-1β、或其任何组合。

64.如权利要求51至63中任一项所述的六聚体蛋白，其可以活化表达GITR的细胞中的下游信号传导途径，任选地，其中该下游信号传导途径是NFκB途径或MAPK途径。

65.如权利要求64所述的六聚体蛋白，其中这些表达GITR的细胞是T细胞。

66.如权利要求64所述的六聚体蛋白，其中这些表达GITR的T细胞是表达GITR的Jurkat NFκB-荧光素酶报告细胞，该Jurkat NFκB-荧光素酶报告细胞响应于NFκB信号传导途径的刺激产生荧光素酶。

67.如权利要求51至66中任一项所述的六聚体蛋白，其中向需要癌症治疗的受试者给予有效剂量可抑制该受试者中的肿瘤生长。

68.如权利要求67所述的六聚体蛋白，其中该肿瘤生长抑制是在GITR阳性免疫细胞任选地T-细胞或NK细胞的存在下实现的。

69.如权利要求67或68所述的六聚体蛋白，其中与给予同种型匹配的对照相比，肿瘤生长被抑制至少10％、至少20％、至少30％、至少40％、至少50％、至少60％、至少70％、至少80％、至少90％、至少95％、至少98％或至少100％。

70.如权利要求51至69中任一项所述的六聚体蛋白，其中该多肽亚基包括IgG1、IgG2、IgG3、IgG4或IgG4P Fc结构域，并且可通过结合GITR而诱导表达GITR的$CD4^+$或$CD8^+$T细胞的细胞因子活化和增殖，但基本上不触发针对这些$CD4^+$或$CD8^+$T细胞的补体依赖性或抗体依赖性细胞毒作用。

71.如权利要求51至69中任一项所述的六聚体蛋白，其中该多肽亚基包括IgG1Fc结构域并且触发针对表达高水平GITR的细胞的Fc受体依赖性细胞毒作用。

72.如权利要求51至69中任一项所述的六聚体蛋白，其中该多肽亚基包括IgG2、IgG3、IgG4或IG4P Fc结构域并且可通过结合GITR而诱导表达GITR的细胞的增殖。

73.如权利要求71所述的六聚体蛋白，其中该Fc受体依赖性细胞毒作用是抗体依赖性细胞毒作用或抗体依赖性吞噬作用。

74.如权利要求71所述的六聚体蛋白，其中这些表达高水平GITR的细胞是肿瘤细胞。

75.如权利要求71所述的六聚体蛋白，其中这些表达高水平GITR的细胞是$CD4^+FOXP3^+$T细胞或抗原处理的$CD4^+FOXP3^-$T细胞。

76.如权利要求71所述的六聚体蛋白，其中该六聚体蛋白与对照相比提供了$CD4^+FOXP3^+$调节性T细胞频率的降低。

77.如权利要求71所述的六聚体蛋白，其中该六聚体蛋白与对照相比提供了肿瘤内$CD4^+FOXP3^+$调节性T细胞频率的降低。

78.一种组合物，包括如权利要求51至77中任一项所述的六聚体蛋白、以及载体。

79.一种多核苷酸，包括编码如权利要求1至49中任一项所述的多肽亚基或如权利要求

51至78中任一项所述的六聚体蛋白的核酸。

80.如权利要求79所述的多核苷酸，其中该多核苷酸进一步包括编码与该多肽亚基可操作连接的信号肽的核酸序列。

81.如权利要求80所述的多核苷酸，其中编码该信号肽的核酸与编码该多肽亚基的IgG Fc结构域的氨基末端的核酸可操作地连接。

82.如权利要求79所述的多核苷酸，包括SEQ ID NO:5。

83.一种载体，其包括如权利要求79至82中任一项所述的多核苷酸。

84.一种宿主细胞，其包括如权利要求79至权利要求82中任一项所述的多核苷酸或如权利要求83所述的载体。

85.一种生产如权利要求1至49中任一项所述的多肽亚基或如权利要求51至77中任一项所述的六聚体蛋白的方法，该方法包括在表达由该多核苷酸或载体编码的该多肽亚基或六聚体蛋白的条件下培养如权利要求84所述的宿主细胞，并且回收该多肽亚基或六聚体蛋白。

86.一种用以促进抗原处理的T细胞和/或NK细胞的存活或增殖的方法，该方法包括使抗原处理的T细胞和/或NK细胞与如权利要求51至77中任一项所述的六聚体蛋白或如权利要求78所述的组合物接触，其中该六聚体蛋白可以特异性地结合这些T细胞和/或NK细胞表面上的GITR。

87.一种诱导从活化的表达GITR的免疫细胞释放细胞因子的方法，该方法包括使这些细胞接触如权利要求51至77中任一项所述的六聚体蛋白或如权利要求78所述的组合物，其中该六聚体蛋白可特异性地结合这些细胞表面上的GITR。

88.如权利要求87所述的方法，其中该细胞因子是IFNγ、TNFα、IL-10、GM-CSF、或其任何组合。

89.如权利要求87或88中任一项所述的方法，其中这些表达GITR的免疫细胞是抗原处理的$CD4^+$T细胞、抗原处理的$CD8^+$T细胞、活化的NK细胞或其组合。

90.如权利要求86至89中任一项所述的方法，其中这些活化的NK细胞、抗原处理的$CD4^+$T细胞或抗原处理的$CD8^+$T细胞是人NK细胞、$CD4^+$或$CD8^+$T细胞，食蟹猴NK细胞、$CD4^+$或$CD8^+$T细胞，恒河猴NK细胞、$CD4^+$或$CD8^+$T细胞，或其组合。

91.一种促进T细胞或NK细胞活化的方法，该方法包括使T细胞或NK细胞接触如权利要求51至77中任一项所述的六聚体蛋白或如权利要求78所述的组合物，其中该六聚体蛋白可特异性地结合这些T细胞或NK细胞表面上的GITR。

92.如权利要求91所述的方法，进一步包括通过该Fc结构域与表达FcγR的细胞的相互作用使该六聚体蛋白交联。

93.如权利要求92所述的方法，其中该表达FcγR的细胞是B细胞、单核细胞、巨噬细胞、髓样树突状细胞或浆细胞样树突状细胞、滤泡树突状细胞、朗格汉斯细胞、内皮细胞、NK细胞、嗜中性粒细胞、嗜酸性粒细胞、血小板、肥大细胞、来自原发人肿瘤或肿瘤引流或非引流淋巴结的$CD45^+$细胞、来自其他二级或三级淋巴样结构的$CD45^+$细胞、或它们的组合。

94.如权利要求91至93中任一项所述的方法，其中NK细胞或T-细胞活化可以通过NFκB或MAPK信号传导途径的刺激来测量。

95.如权利要求91至94中任一项所述的方法，其中这些NK或T细胞是活化的NK细胞、抗

原处理的$CD4^+$T细胞、抗原处理的$CD8^+$T细胞、或其组合。

96.如权利要求95所述的方法，其中这些NK细胞、$CD4^+$或$CD8^+$T细胞是初级人NK细胞、$CD4^+$或$CD8^+$T细胞，食蟹猴NK细胞、$CD4^+$或$CD8^+$T细胞，恒河猴NK细胞、$CD4^+$或$CD8^+$T细胞，或其组合。

97.如权利要求86至96中任一项所述的方法，其中该接触包括向受试者给予有效量的该六聚体蛋白或包括该六聚体蛋白的组合物。

98.一种治疗受试者的癌症的方法，该方法包括向需要治疗的受试者给予有效量的如权利要求51至77中任一项所述的六聚体蛋白、或如权利要求78所述的组合物。

99.如权利要求98所述的方法，其中该受试者是人类受试者或犬受试者。

100.如权利要求98所述的方法，其中该癌症是实体瘤。

101.如权利要求100所述的方法，其中该实体瘤与选自下组的癌症相关联，该组由以下各项组成：结直肠癌、乳腺癌、肝细胞癌、非小细胞肺癌、小细胞肺癌、间皮瘤、头颈癌、胃癌、胰腺癌、黑色素瘤、葡萄膜黑色素瘤、肾癌、卵巢癌、宫颈癌、成胶质细胞瘤、睾丸癌、甲状腺癌、前列腺癌和食管癌。

102.如权利要求98所述的方法，其中该癌症是血癌。

103.如权利要求102所述的方法，其中该血癌选自下组，该组由以下各项组成：霍奇金氏淋巴瘤、非霍奇金氏淋巴瘤、多发性骨髓瘤、急性成淋巴细胞性白血病、急性骨髓性白血病、慢性淋巴细胞性白血病、以及慢性骨髓性白血病。

104.如权利要求98至103中任一项所述的方法，其中该六聚体蛋白或组合物的给予可抑制肿瘤生长，可促进肿瘤减小，或两者。

105.如权利要求98至104中任一项所述的方法，其中肿瘤生长抑制是在NK细胞或T细胞的存在下实现的。

106.如权利要求104或权利要求105所述的方法，其中与给予缺乏GITRL受体结合结构域的同种型匹配对照相比，肿瘤生长被抑制至少10％、至少20％、至少30％、至少40％、至少50％、至少60％、至少70％、至少80％、至少90％、至少95％、至少98％或100％。

107.如权利要求98至106中任一项所述的方法，其中该六聚体蛋白包括IgG1、IgG2、IgG3、IgG4或IgG4P Fc结构域，并且可以通过结合GITR而诱导抗原处理的表达GITR的免疫细胞的增殖，但基本上不触发针对活化的免疫细胞的补体依赖性或抗体依赖性细胞毒作用。

108.如权利要求107所述的方法，其中这些表达GITR的免疫细胞是$CD4^+$T细胞、NK细胞或$CD8^+$T细胞。

109.如权利要求98至106中任一项所述的方法，其中该六聚体蛋白包括IgG1Fc结构域并且触发表达高水平GITR的细胞的NK细胞介导的和/或巨噬细胞介导的抗体依赖性细胞毒作用(ADCC)或抗体依赖性细胞吞噬作用。

110.如权利要求109所述的方法，其中这些表达高水平GITR的细胞是肿瘤细胞。

111.如权利要求109所述的方法，其中这些表达高水平GITR的细胞是$CD4^+FOXP3^+$T细胞。

112.如权利要求111所述的方法，其中该六聚体蛋白与对照相比提供了$CD4^+FOXP3^+$调节性T细胞频率的降低。

113.如权利要求111所述的方法，其中该六聚体蛋白与对照相比提供了肿瘤内$CD4^+$

FOXP3$^+$调节性T细胞频率的降低。

114.一种增强受试者的免疫应答的方法，该方法包括向对其有需要的受试者给予治疗有效量的如权利要求51至77中任一项所述的六聚体蛋白、或如权利要求78所述的组合物。

115.如权利要求114所述的方法，其中该受试者是人类受试者或犬受试者。

116.如权利要求115所述的方法，其中该对其有需要的受试者患有癌症。

117.如权利要求116所述的方法，其中该癌症是实体瘤。

118.如权利要求114至117中任一项所述的方法，其中该受试者患有结直肠癌、乳腺癌、肝细胞癌、非小细胞肺癌(NSCLC)、小细胞肺癌、间皮瘤、头颈癌、胃癌、胰腺癌、黑色素瘤、葡萄膜黑色素瘤、肾癌、卵巢癌、宫颈癌、成胶质细胞瘤、睾丸癌、甲状腺癌、前列腺癌、食管癌、膀胱癌、头颈部鳞状细胞癌(SCCHN)、结直肠癌(CRC)、血癌或其组合。

119.如权利要求118所述的方法，其中该癌症是血癌。

120.如权利要求119所述的方法，其中该血癌选自下组，该组由以下各项组成：霍奇金氏淋巴瘤、非霍奇金氏淋巴瘤、多发性骨髓瘤、急性成淋巴细胞性白血病、急性骨髓性白血病、慢性淋巴细胞性白血病、以及慢性骨髓性白血病。

121.如权利要求114至120中任一项所述的方法，其中该六聚体蛋白或组合物的给予可抑制肿瘤生长，可促进肿瘤减小，或两者。

122.如权利要求114至121中任一项所述的方法，其中肿瘤生长抑制是在NK细胞或T细胞的存在下实现的。

123.如权利要求121或122中任一项所述的方法，其中与给予缺乏GITRL受体结合结构域的同种型匹配对照相比，肿瘤生长被抑制至少10％、至少20％、至少30％、至少40％、至少50％、至少60％、至少70％、至少80％、至少90％、至少95％、至少98％或100％。

124.如权利要求114至123中任一项所述的方法，其中该六聚体蛋白包括IgG1、IgG2、IgG3、IgG4或IgG4P Fc结构域，并且可以通过结合GITR而诱导活化的表达GITR的免疫细胞的增殖，但基本上不触发针对这些活化的免疫细胞的补体依赖性或抗体依赖性细胞毒作用。

125.如权利要求124所述的方法，其中这些表达GITR的免疫细胞是CD4$^+$T细胞、NK细胞或CD8$^+$T细胞。

126.如权利要求114至123中任一项所述的方法，其中该六聚体蛋白包括IgG1Fc结构域并且触发表达高水平GITR的细胞的NK细胞介导的和/或巨噬细胞介导的抗体依赖性细胞毒作用(ADCC)或抗体依赖性细胞吞噬作用。

127.如权利要求126所述的方法，其中这些表达高水平GITR的细胞是肿瘤细胞。

128.如权利要求126所述的方法，其中这些表达高水平GITR的细胞是CD4$^+$FOXP3$^+$T细胞。

129.如权利要求126所述的方法，其中该六聚体蛋白与对照相比提供了CD4$^+$FOXP3$^+$调节性T细胞频率的降低。

130.如权利要求126所述的方法，其中该六聚体蛋白与对照相比提供了肿瘤内CD4$^+$FOXP3$^+$调节性T细胞频率的降低。

131.一种治疗受试者的实体瘤的方法，该方法包括向该受试者给予如权利要求1所述的分离的单链多肽亚基和OX40激动剂。

132.如权利要求131所述的方法，其中该OX40激动剂是OX40配体融合蛋白或抗-OX40抗

体中的一种或多种。

133.如权利要求132所述的方法，其中该OX40配体融合蛋白是MEDI6383。

134.如权利要求132所述的方法，其中该抗OX40抗体是MEDI0562。

135.一种治疗受试者的癌症的方法，该方法包括向需要治疗的受试者给予有效量的与T-细胞引发剂组合的如权利要求51至77中任一项所述的六聚体蛋白或如权利要求78所述的组合物。

136.如权利要求136所述的方法，其中该T-细胞引发剂是E7合成长肽(SLP)和加CpG寡脱氧核苷酸。

GITRL融合蛋白及其用途

对以电子方式提交的序列表的引用

[0001] 与本申请一起提交的ASCII文本文件的以电子方式提交的序列表内容(名称:GITRLF-100P2_ST25.txt;大小:56,159字节;以及创建日期:2016年6月15日)通过引用以其全部内容结合在此。

背景技术

[0002] 糖皮质激素诱导的肿瘤坏死因子受体(TNFR)相关蛋白(GITR),也称为TNFRSF18、AITR或CD357,是在调节性T细胞上表达,并在抗原处理的$CD4^+$辅助细胞和$CD8^+$细胞毒性T细胞以及活化的NK细胞上上调(Stephens等人J.Immunol.[免疫学杂志](2004)173(8):5008-5020;Clothier和Watts,Cytokine Growth Factor Rev.[细胞因子生长因子评论](2014))。GITR是涉及通过抗原暴露控制T-细胞活化的受体和配体的复杂系统的一部分。GITR具有一个已知的内源性配体,GITR配体(GITRL),该GITR配体以松散三聚体的形式存在,并且可以使GITR聚集,在T细胞内导致有效的细胞信号传导事件(Chattopadhyay等人(2007)Proc.Natl.Acad.Sci.USA[美国国家科学院院刊]104(49):19452-19457)。GITR和GITRL之间的相互作用导致向T细胞递送正共刺激信号,这通过抗原暴露增强其增殖和活化,帮助促进记忆细胞产生并重编程调节性T细胞;降低其抑制功能(Clothier和Watts,Cytokine Growth FactorRev.[细胞因子生长因子评论](2014)1月4日;Schaer等人Curr Opin Immunol.[当前免疫学观点](2012))。

发明内容

[0003] 本披露涉及多肽亚基,每一个作为融合多肽都包括GITR配体(GITRL)的受体结合结构域、多聚结构域(例如三聚结构域)和IgG Fc结构域,这些多肽亚基能够形成稳定的多聚体(例如,六聚体蛋白)。提供了可用于癌症免疫疗法和病毒感染治疗的组合物和方法。

[0004] 在某些方面中,提供了分离的单链多肽亚基,其包括:IgG Fc结构域;功能性多聚结构域;和糖皮质激素诱导的TNF受体配体(GITRL)的受体结合结构域,其中该多肽亚基可以自组装成三聚体或六聚体蛋白。

[0005] 在某些方面中,提供了包括三个单链多肽亚基的三聚体蛋白,每一个单链多肽亚基包括:IgG Fc结构域;功能性多聚结构域;和糖皮质激素诱导的TNF受体配体(GITRL)的受体结合结构域。

[0006] 在某些方面中,提供了包括六个单链多肽亚基的六聚体蛋白,每一个单链多肽亚基包括:IgG Fc结构域;功能性多聚结构域;和糖皮质激素诱导的TNF受体配体(GITRL)的受体结合结构域。

[0007] 在某些方面中,提供了包括六聚体蛋白和载体的组合物。

[0008] 在某些方面中,提供了包括编码单链多肽亚基或六聚体蛋白的核酸的多核苷酸。

[0009] 在某些方面中,提供了包括编码单链多肽亚基或六聚体蛋白的多核苷酸的载体。

[0010] 在某些方面中,提供了包括编码单链多肽亚基或六聚体蛋白的多核苷酸的宿主细

胞或包括包含这些多核苷酸的载体的宿主细胞。

[0011] 在某些方面中,提供了产生多肽亚基或产生六聚体蛋白的方法,其中这些方法包括:在如下条件下培养包括编码这些多肽亚基或六聚体蛋白的多核苷酸或载体的宿主细胞,在这些条件中由该多核苷酸或载体编码的多肽亚基或六聚体蛋白得以表达;并回收多肽亚基或六聚体蛋白。

[0012] 在某些方面中,提供了用以促进抗原处理的T细胞和/或活化的NK细胞的存活或增殖的方法,其中这些方法包括使抗原处理的T细胞和/或活化的NK细胞与该六聚体蛋白或组合物接触,其中该六聚体蛋白可以特异性地结合T细胞和/或NK细胞表面上的GITR。

[0013] 在某些方面中,提供了诱导细胞因子从活化的表达GITR的免疫细胞释放的方法,其中这些方法包括使这些细胞与该六聚体蛋白或组合物接触,其中该六聚体蛋白可以特异性地结合这些细胞表面上的GITR。

[0014] 在某些方面中,提供了促进T细胞或NK细胞活化的方法,其中这些方法包括使T细胞或NK细胞与该六聚体蛋白或组合物接触,其中该六聚体蛋白可以特异性地结合T细胞或NK细胞表面上的GITR。

[0015] 在某些方面中,提供了治疗受试者的癌症的方法,其中这些方法包括向需要治疗的受试者给予有效量的六聚体蛋白或组合物。

[0016] 在某些方面中,提供了增强受试者的免疫应答的方法,其中这些方法包括向对其有需要的受试者给予治疗有效量的六聚体蛋白或组合物。

[0017] 在某些方面中,提供了治疗受试者的实体瘤的方法,包括向受试者给予以上披露的分离的单链多肽亚基和OX40激动剂。

[0018] 另一方面中,提供了治疗受试者的实体瘤的方法,包括向受试者给予以上披露的分离的单链多肽亚基和T-细胞引发剂。

附图说明

[0019] 图1.使用蛋白G和尺寸排阻色谱法纯化的重组GITRL融合蛋白(FP)(母系蛋白1wt)和GITRL FP(冠蛋白1a wt)蛋白的SDS-PAGE分析。

[0020] 图2A-D.显示六聚体GITRL FP变体与表达GITR的CHO细胞的结合特征曲线的图表。

[0021] 图3A-D.显示与三聚体GITRL竞争结合GITR-Fc的六聚体GITRL FP变体的抑制特征曲线的图表。

[0022] 图4A-D.显示使用人GITR转染的NF-κB荧光素酶基因报告细胞系,GITRL FP分子的相对效力的图表。

[0023] 图5A-D.GITRL FP(GCN4pII)、GITRL FP(冠蛋白1a wt)、GITRL FP(朗格汉斯蛋白wt)和GITRL FP(朗格汉斯蛋白变体)的去折叠转变。

[0024] 图6.洗脱的峰的摩尔质量组成。该图表显示溶液中的多聚体GITRL FP母系蛋白-1蛋白(虚线)形成重均摩尔质量(从左到右)为612、312和215kDa的三种物质,其中没有容易鉴别的主要种类。另一方面,>90%的多聚体GITRL FP(冠蛋白1a wt;虚线)和多聚体GITRL FP(GCN4pII;实线)蛋白质量洗脱为单一蛋白种类。这些峰虽然不是完全单分散的,但最可能是由于附接至蛋白质上的聚糖的异质性。

[0025] 图7.六聚体GITRL FP分子的示意图。

[0026] 图8.代表性GITRL IgG1融合多肽亚基的核苷酸和经翻译的蛋白质序列。各结构域被突出显示并注释。ECD＝胞外结构域;GITRL＝糖皮质激素诱导的肿瘤坏死因子受体配体;HA＝血凝素。图8的核酸序列提供为SEQ ID NO:7,并且编码的前体蛋白质序列提供为SEQ ID NO:8。

[0027] 图9.针对还原的GITRL IgG1 FP亚基的解卷积LC-QTOF MS谱。如通过与四极杆飞行时间(QTOF)质谱法(LC-QTOF MS)偶联的液相色谱法测定的,GITRL IgG1 FP单体亚基(SEQ ID NO:6)的精确质量与在Fc结构域中的典范糖基化位点处每个链加入一个双触角聚糖(主要为G0f)的预期氨基酸序列一致。

[0028] 图10.在均相时间分辨荧光测定中,与铕穴状化合物轭合的人GITR-Fc与hGITRL-HA结合。IgG1同种型对照抗体的滴定不抑制此结合。包括具有在SEQ ID NO:6中列出的氨基酸序列的单体亚基的六聚体GITRL IgG1 FP以0.562nM的半最大抑制浓度(IC50)抑制hGITR和hGITRL之间的结合。实验在一式两份的孔中进行。误差条代表均值的标准误差。GITR(L)＝糖皮质激素诱导的肿瘤坏死因子受体(配体)。

[0029] 图11.六聚体GITRL FP是GITR受体的有效激动剂。将测试品在溶液中以指示浓度添加到用hGITR和与NFκB启动子连接的荧光素酶报告基因转染的Jurkat细胞。在三小时后测定荧光素酶活性,测量为发光。包括具有在SEQ ID NO:6中列出的氨基酸序列的单体亚基的六聚体GITRL IgG1 FP或包括具有在SEQ ID NO:40中列出的氨基酸序列的单体亚基的六聚体GITRL IgG4P FP导致发光的浓度依赖性增加。关于此影响,六聚体GITRL IgG1 FP的EC_{50}为182pM。关于此影响,六聚体GITRL IgG4P FP的EC_{50}为289pM。同种型对照抗体没有影响。实验在一式三份的孔中进行。误差条代表均值的标准误差。

[0030] 图12.六聚体GITRL FP增强初级人T细胞响应于抗CD3和抗CD28的增殖。通过添加板结合的包括具有在SEQ ID NO:6中列出的氨基酸序列的单体亚基的六聚体GITRL IgG1 FP或包括具有在SEQ ID NO:40中列出的氨基酸序列的单体亚基的六聚体GITRL IgG4P FP,增加了初级人T细胞响应于抗CD3和抗CD28的增殖。该影响是浓度依赖性的,GITRL IgG1 FP的EC_{50}为0.3nM,并且GITRL IgG4P FP的EC_{50}为0.5nM。加入同种型对照抗体没有影响。实验在一式三份的孔中进行。误差条代表均值的标准误差。

[0031] 图13.通过添加板结合的包括具有在SEQ ID NO:6中列出的氨基酸序列的单体亚基的六聚体GITRL IgG1 FP或包括具有在SEQ ID NO:40中列出的氨基酸序列的单体亚基的六聚体GITRL IgG4P FP,增加了初级人T细胞响应于抗CD3和抗CD28的IFN-γ释放。该影响是浓度依赖性的,GITRL IgG1 FP的EC_{50}为0.6nM,并且GITRL IgG4P FP的EC_{50}为0.8nM。加入同种型对照抗体没有影响。实验在一式三份的孔中进行。误差条代表均值的标准误差。

[0032] 图14.六聚体GITRL IgG1 FP通过NK细胞介导初级人T细胞的ADCC。将抗原处理的初级人T细胞进行荧光标记,并以1个T细胞与32个NK细胞的比率与初级人NK细胞混合。按指示加入测试品,并在孵育24小时后计算T细胞的裂解%。包括具有在SEQ ID NO:6中列出的氨基酸序列的单体亚基的六聚体GITRL FP IgG1导致裂解百分比增加。该影响是浓度依赖性的,EC_{50}是239pM。包括具有在SEQ ID NO:40中列出的氨基酸序列的单体亚基的阴性对照六聚体GITRL IgG4P FP不会导致T细胞裂解百分比的任何增加。

[0033] 图15.由六聚体GITRL IgG1 FP介导的ADCC有利于产生增加的CD8:CD4 T细胞比率。将抗原处理的初级人T细胞进行荧光标记,并以1个T细胞与32个NK细胞的比率与初级人

NK细胞混合。按指示加入测试品，并在在24小时孵育后通过流式细胞术评估存在于总T细胞群中的$CD4^+$和$CD8^+$T细胞的百分比。包括具有在SEQ ID NO:6中列出的氨基酸序列的单体亚基的六聚体GITRL IgG1 FP导致CD8:CD4 T细胞比率的浓度依赖性转变，这有利于CD8 T细胞。

[0034] 图16.六聚体GITRL IgG1 FP克服了调节性T细胞介导的效应T细胞增殖抑制。在用抗CD3和抗CD28抗体刺激五天后，通过流式细胞术分析分裂的$CD4^+CD25^-$效应T细胞的百分比。在存在渐增数目的T-reg的情况下，分裂细胞的百分比降低。添加板结合的同种型对照进一步降低分裂细胞的百分比。在指示浓度添加板结合的包括具有在SEQ ID NO:6中列出的氨基酸序列的单体亚基的六聚体GITRL IgG1 FP将分裂细胞的百分比恢复到不存在T-reg时观察到的百分比。单独使用效应物的实验在单个孔中进行。所有其他的实验都在一式两份的孔中进行。误差条代表均值的标准误差。

[0035] 图17.用mGITRL FP处理的小鼠的存活是同种型依赖性的。在皮下植入CT26细胞后第6天至第23天，每天通过腹膜内给予mGITRL FP mIgG2a或mGITRL IgG1 FP(两者均为5或10mg/kg)对小鼠进行处理。盐水作为阴性对照给予。

[0036] 图18.mGITRL FP导致T细胞增殖增加。在用单剂量的0.2mg/kg或1mg/kg mGITRL FP处理后7天，通过流式细胞术在脾T细胞中测量Ki67的表达。带圆圈的黑色线＝CD8 T细胞；带正方形的深灰色线＝$CD4^+Foxp3^-$；带三角形的浅灰色线＝$CD4^+Foxp3^+$细胞。显著性是使用学生T检验(Student's T test)来计算，其中*p<0.05；**p<0.01，***p<0.001，****p<0.0001。

[0037] 图19.mGITRL FP导致T细胞上活化标记物ICOS的表达增加。在用单剂量的0.2mg/kg或1mg/kg mGITRL FP处理后7天，通过流式细胞术在脾T细胞中测量ICOS的表达。带圆圈的深灰色线＝CD8 T细胞；带正方形的黑色线＝$CD4^+Foxp3$。显著性是使用学生T检验(Student's T test)来计算，其中*p<0.05；**p<0.01，***p<0.001，****p<0.0001。用mGITRL FP处理导致肿瘤内$CD4^+FOXP3^+$调节性T细胞和$CD4^+FOXP3^-$辅助细胞的频率降低，但不改变$CD8^+$细胞毒性T细胞的频率。总体结果是肿瘤微环境内的CD8:CD4比率增加。

[0038] 图20.mGITRL FP导致肿瘤内CD8:CD4比率升高。在用单剂量的0.2mg/kg或1mg/kg mGITRL FP处理后7天，通过流式细胞术测量肿瘤内CD8 T细胞(带圆圈的黑色线)、$CD4^+Foxp3^-$细胞(带正方形的深灰色线)、$CD4^+Foxp3^+$细胞(带三角形的浅灰色线)的频率。显著性是使用学生T检验(Student's T test)来计算，其中*p<0.05；**p<0.01，***p<0.001，****p<0.0001。

[0039] 图21.ELISA数据证明六聚体hGITRL IgG1 FP结合人和食蟹猴GITR-Fc。包括具有在SEQ ID NO:6中列出的氨基酸序列的单体亚基的生物素化六聚体hGITRL IgG1 FP与重组人和食蟹猴(cyno)GITR的结合。使用CD137-Fc作为阴性对照以确定该测定中的背景信号。实验在一式三份的孔中进行。误差条代表标准偏差。CD137＝分化群137(TNFRSF9)；CD137-Fc＝与hIgG1的Fc结构域连接的分化群137胞外结构域；Cyno＝食蟹猴；ELISA＝酶联免疫吸附测定；GITR-Fc＝与hIgG1的Fc结构域连接的糖皮质激素诱导的肿瘤坏死因子受体胞外结构域；OD450nm＝在450nm波长下的光密度读数。

[0040] 图22.六聚体hGITRL IgG1 FP处理的食蟹猴中KI67阳性T细胞亚群％的测量。持续20天监测食蟹猴的KI67阳性T细胞亚群％的基线水平，在第0天用运载体对照(圆圈)、1mg/

kg hGITRL IgG1 FP(三角形)或10mg/kg hGITRL IgG1 FP(正方形)处理,并且然后在第1、3、5、9、11、15、18、22和29天监测KI67阳性T细胞亚群%。

[0041] 图23A-B.与三聚体hGITRL竞争结合hGITR-Fc的hGITRL FP蛋白的抑制特征曲线和IC_{50}值。hGITRL FP wt,N92D和N104D(A)以及hGITRL FP,N161D(B)

[0042] 图24.hGITRL FP蛋白结合hGITR-Fc的结合特征曲线和Kd值。

[0043] 图25A-C.显示使用人GITR转染的NF-κB荧光素酶基因报告细胞系,GITRL FP分子的相对效力的图表和EC_{50}值。hGITRL FP wt和N92D(A);hGITRL FP wt,N161D,N129A和N129A/N161D(B);N161D和N129A/N161D(C)

[0044] 图26A-C.显示使用具有胸苷掺入读出的人初级$CD3^+$T细胞再刺激测定,GITRL FP分子的相对效力的图表。hGITRL FP wt和N92D(A);hGITRL FP wt,N92D和N104D(B);wt和N161D(C)。

[0045] 图27.GITRL FP wt和N92D变体的去折叠转换。

[0046] 图28.在中国仓鼠卵巢细胞中产生的hGITRL FP中发现的主要寡糖结构;复合型(A);高甘露糖(B)。Man=甘露糖;GlcNAc=N-乙酰葡糖胺;Fuc=海藻糖;Gal=半乳糖;NANA=N-乙酰神经氨酸(唾液酸)。

[0047] 图29A-C.GITRL FP肽作图。对于GITRL FP wt(Ai)和GITRL FP N161D(Aii),含有Fc N-糖基化位点的胰蛋白酶水解的肽7(T7)的提取离子色谱图。对于GITRL FP wt(Aiii)和GITRL FP N161D(Aiv),T7的组合的解卷积质谱,显示了主要糖型。对于GITRL FP wt(Bi)和GITRL FP N161D(Bii),含有GITRL ECD N129N-糖基化共有序列的胰蛋白酶水解的肽40(T40)的提取离子色谱图。对于GITRL FP wt(Biii)和GITRL FP N161D(Biv),T40的组合的解卷积质谱,显示了与在N129处不存在N-糖基化的情况一致的质量。分别对于GITRL FP wt(Ci)和GITRL FP N161D(Cii),胰蛋白酶水解的肽42-43(T42-43)和43(T43)的提取离子色谱图。对于GITRL FP wt(Ciii),T42-43的组合的解卷积质谱,显示了GITRL ECD N161N-糖基化位点处的主要糖型。对于GITRL FP N161D(Civ),T43的组合的解卷积质谱,显示了证实N161D取代和N-糖基化不存在的质量。

[0048] 图30A-C.鼠GITR配体融合蛋白的结构和激动潜力。(A)从N-至C-末端,由免疫球蛋白G1(IgG1)或2a(IgG2a)的片段可结晶(Fc)区、多聚结构域(MD)和鼠GITR配体的胞外(GITR结合)结构域(ECD)组成的鼠GITRL-FP的示意图(B)经纯化的鼠GITRL-FP的SDS-PAGE。(C)在用mGITRL-FP、DTA-1rIgG2b同种型对照或mOX40L-FP处理后,在鼠GITR受体转导的Jurkat细胞系中的NF-κB相关发光。数据代表至少两个独立实验。

[0049] 图31A-E.全面FcγR接合增加抗肿瘤活性但不驱动GITR下游的T-细胞增殖的增加(A)Balb/c小鼠中的肿瘤生长。通过按指示将小鼠通过i.p.注射盐水对照、mGITRL-FP mIgG1或mGITRL-FP mIgG2a处理一次。在每个单独的图表上指示消退数(B)在治疗CT26荷瘤小鼠后4天,脾T-细胞中Ki67表达的频率。(C)按指示用10mg/kg的mGITRL-FP或盐水对照处理CT26荷瘤小鼠后4天肿瘤内T-细胞亚组的频率和(D)肿瘤内CD8+与CD4+FoxP3+细胞的比率。(E)处理后4天,脾脏和肿瘤内T-细胞亚组上GITR表达的荧光强度中值。误差条指示均值的标准误差;n=7-10只小鼠/组。对于(B)和(C),**p<0.005***p<0.001和****p<0.0001,如通过双向ANOVA计算的;C的显著性对于CD4+Foxp3+细胞的变化是黑色的,并且对于CD4+Foxp3-细胞的变化是灰色的;对于(D),*P<0.05,如通过单向ANOVA计算的;对于(E),****p<

0.0001,如通过学生T检验计算的。

[0050] 图32A-B.用mGITRL-FP mIgG2a或mIgG1处理后肿瘤内T-reg耗减和CD4+Foxp3-:T-reg比率。在CT26植入后6天,向CT26荷瘤小鼠i.p.注射盐水对照、mGITRL-FP mIgG1(10mg/kg)或mGITRL-FP mIgG2a(10mg/kg)一次。(A)显示处理后4天肿瘤中CD4+Foxp3+T-reg的比例的流式细胞术绘图。CD4+Foxp3+流式细胞术分析设门是基于Foxp3荧光减一(Foxp3fluorescence minus one)(FMO)对照定位的。(B)按指示处理后第4天测量的肿瘤内CD4+Foxp3-:T-reg比率。使用单向ANOVA进行统计分析,其中****指示P-值<0.0001。

[0051] 图33A-C.鼠GITRL-FP mIgG2a以剂量和方案依赖性方式介导抗肿瘤活性。在Balb/c小鼠中的肿瘤生长。通过i.p.注射以下项对小鼠进行处理:(A)指示剂量水平的单剂量mGITRL-FP mIgG2a或(B)每天[Q1D]或每周[Q1W]给予的多剂量的0.2mg/kg mGITRL-FP mIgG2a。(C)使用指示剂量和方案给予后mGITRL-FP的预测血清浓度。虚线指示实现最大抗肿瘤活性所需的mGITRL-FP mIgG2a的血液浓度阈值。

[0052] 图34A-D.鼠GITRL-FP mIgG2a以剂量和方案依赖性方式介导T-细胞增殖和活化的PD改变。在用0.2或1mg/kg mIgG2a mGITRL-FP一次、每三天[Q3D]或每天[Q1D]处理后7天CT26荷瘤小鼠的肿瘤引流淋巴结中的(A)Ki67、(B)ICOS、(C)PD-1和(D)OX40阳性CD4+ T-细胞的频率。误差条表示来自7-8只小鼠/组的均值的标准误差。*p<0.05,**p<0.01***p<0.001,****0<0.0001,如通过单向ANOVA计算的。

[0053] 图35A-B.小鼠OX40配体融合蛋白的结合和效力特征曲线。(A)结合ELISA,显示mGITRL-FP mIgG1和mIgG2a各自特异性地结合重组小鼠GITR-Fc(黑色条)而不是重组小鼠OX40-Fc,并且mOX40L-FP mIgG1和mIgG2a各自特异性地结合重组小鼠OX40(灰色条)而不是重组小鼠GITR。mOX40L-FP Y182A同种型对照最低限度地结合重组小鼠OX40-Fc。(B)mOX40L-FP mIgG1(黑色圆圈)或Y182A同种型对照(空心圆圈)与Jurkat人OX40NF-κB报告细胞系上的人OX40的结合。在报告物测定中,小鼠OX40L FP mIgG1诱导NFκB信号传导,但是这对于mOX40L-FP Y182A同种型对照不明显。

[0054] 图36A-B.mGITRL-FP的抗肿瘤活性优于CT26模型中的mOX40L-FP。(A)在Balb/c小鼠中的肿瘤生长。每周两次i.p.注射5mg/kg mIgG2a或mIgG1mGITRL-FP或mOX40L-FP、5mg/kg mIgG1融合蛋白同种型对照或盐水对小鼠进行处理。在每个单独的图表上指示总消退数。(B)按指示处理后第10天,CT26荷瘤Balb/c小鼠的肿瘤内CD4+,FoxP3+T-reg的频率。

[0055] 图37A-E.由mGITRL-FP mIgG2a和mOX40L-FP mIgG1介导的药效学(PD)变化是有差别的,并且可通过组合增强。在用25mg/kg mGITRL-FP mIgG2a、15mg/kg mOX40L-FP mIgG1或两种分子的组合每周两次处理后14天,在CT26荷瘤小鼠的脾脏中的(A)CD4+FoxP3-或CD8+,Ki67+,(B)CD4+或CD8+,CD44+CD62L Lo/-效应记忆,(C)CD4+CD44+CD62L+中枢记忆,(D)CD4+或CD8+,T-bet+和(E)CD4+EOMES+T-细胞的频率。*p<0.05,**p<0.01***p<0.001,****0<0.0001,如通过单向ANOVA计算的。

[0056] 图38A-B.mGITRL-FP mIgG2a和mOX40L-FP mIgG1的组合在B16F10-Luc2和CT26荷瘤小鼠中协同诱导增加的抗肿瘤活性。B16F10-Luc2和CT26荷瘤小鼠的肿瘤生长。(A)向B16F10-Luc2荷瘤小鼠i.p.给予生理盐水、25mg/kg mGITRL-FP mIgG2a(每两周一次持续两周)、15mg/kg mOX40L-FP mIgG1(每两周一次持续三周)或两种分子的组合并测量肿瘤生长。(B)CT26荷瘤小鼠未经处理或通过i.p.注射同种型对照、7.5mg/kg的mOX40L-FP mIgG1

(每周两次,持续两个剂量)、0.1mg/kg的单个次最佳剂量的mGITRL-FP mIgG2a或两种分子的组合进行处理。在每个单独的图表旁边指示总消退数。

[0057] 图39.与单一疗法治疗相比,mGITRL-FP mIgG2a和mOX40L-FP mIgG1的组合诱导B16F10-Luc2荷瘤小鼠的存活率增加。向B16F10-Luc2荷瘤小鼠i.p.给予生理盐水、25mg/kg mGITRL-FP mIgG2a(每两周一次持续两周)、15mg/kg mOX40L-FP mIgG1(每两周一次持续三周)或两种分子的组合并测量存活率。对数秩检验,其中***指示P-值<0.001,**指示P-值<0.01,并且*指示P-值<0.05。

[0058] 图40(A)-(G).(A)将CT26细胞皮下植入Balb/C小鼠中,$5x10^5$个细胞/小鼠。在第6天将小鼠按肿瘤体积随机化并开始给药(组n=10只小鼠)。向小鼠IP给予mGITRL FP IgG2a,(B)单次剂量或(C)每隔一天持续给予9个剂量,Q2Dx9。以5、1、0.5、0.2、0.1和0.04mg/kg向它们给药。显示的数据代表两个重复实验。(D)在第11天,将它们基于肿瘤大小随机化,并且用以下项进行处理:无、DTA-1(抗GITR mAb)或mGITRL-FP(组n=9)。(E)在第8、10、12、14和16天,小鼠耗减CD8T-细胞。在第11天,将它们基于肿瘤大小随机化,并且用以下项进行处理:无、DTA-1或mGITRL-FP IgG2a。(F)存活期中值。(G)在第18天,处死具有CT26肿瘤的未经处理的小鼠,以检查脾脏和肿瘤中的CD8 T-细胞以及Treg上的GITR表达。

[0059] 图41(A)-(H).(A)将CT26细胞皮下植入Balb/C小鼠中,$5x10^5$个细胞/小鼠。在第10天将小鼠按肿瘤体积随机化并开始给药。向小鼠IP给予mGITRL-FP IgG2a,每两周一次持续4次总剂量。在第18天处死小鼠以检查(B)Treg,(C)CD8 T-细胞。(D)小鼠脾脏和肿瘤用10μg/mL AH1肽/蛋白转运抑制剂再刺激5小时,并针对IFNγ和TNFα进行染色。(E)CD8细胞上的GITR表达。(F)脾脏、淋巴结和肿瘤中的Treg上的GITR表达。(G)CD4 T-细胞上的KI-67(H)CD8 T-细胞上的KI-67。

[0060] 图42(A)-(C).mGITRL FP以剂量依赖性方式扩增抗原特异T-细胞。(A)用单剂量的mGITRL FP IgG2a处理的CT26荷瘤小鼠清除肿瘤并在第85天免受5E5C T26细胞/小鼠的再激发[R箭头]。(B)用CT26再激发后,第120天,收获小鼠脾脏[PD箭头],加工成单细胞,并用10μg/mL AH1肽/蛋白转运抑制剂再刺激5小时。小鼠的AH1特异性T细胞有剂量依赖性的增加。(C)来自每个组的5只小鼠的代表图。为了比较,在第10天包括初试小鼠和未经处理的具有CT26肿瘤的小鼠。

[0061] 图43(A)-(D).(A)-(B)将TC-1细胞植入C57BL/6小鼠的足垫中,$2x10^4$个细胞/小鼠。在第14天将小鼠按肿瘤体积随机化并开始给药。向小鼠IP给予mGITRL-FP IgG2a,每两周一次持续4次总剂量。在第24天,处死未经处理的小鼠以检查(C)Treg上的GITR表达和CD8 T-细胞上的GITR表达。对小鼠进行针对E7特异性T-细胞的评价,并且通过E7 restim或dextramer未检测到。

[0062] 图44(A)-(K).(A)为了产生E7特异性T-细胞,将初试C57BL/6小鼠在尾根部注射CpG(Addavax)中的10ug E7 SLP。然后将小鼠用1mg/kg的mGITRL-FP IgG2a进行处理,持续3次剂量。对小鼠进行针对以下项的评价:脾(B)CD4 T-细胞,(C)CD8 T-细胞,(D)E7 Dextramer+T-细胞,(E)Treg,(F)抗原特异性细胞上的GITR水平。(G)将TC-1细胞植入C57BL/6小鼠的足垫中,$2x10^4$个细胞/小鼠。在第14天将小鼠按肿瘤体积随机化并开始给药。将C57BL/6小鼠在尾根部注射CpG(Addavax)中的10ug E7 SLP。在第28天,将小鼠处死。对脾脏和肿瘤进行针对(H)-(I)E7和特异性CD8 T-细胞(J)-(K)的评价。

[0063] 图45.(A)将TC-1细胞植入C57BL/6小鼠的足垫中,$2x10^4$个细胞/小鼠。在第14天将小鼠按肿瘤体积随机化并开始给药。(B)将接种疫苗的C57BL/6小鼠在尾根部注射CpG(Addavax)中的3.3ug E7 SLP。向经处理的小鼠IP给予GITRL-FP IgG2a,每两周一次持续4次总剂量。(C)TC-1植入后小鼠的Kaplan-Meier存活率,$P<0.05$。(D)各组的存活期中值。(E)为了检查药效学效应,处死(A)中相同处理的小鼠的组,并收获脾脏和肿瘤。合并肿瘤,并将脾脏按个体留下。(F)评价肿瘤中的CD45+细胞。(G)将小鼠脾脏和肿瘤用1μg/mL E7肽/蛋白转运抑制剂再刺激5小时,并针对IFNγ和TNFα进行染色,并测量脾脏和肿瘤Treg。

具体实施方式

[0064] 在由抗原引发的过程中或之后不久,在T细胞(例如,$CD4^+$T细胞或$CD8^+$T细胞)上的GITR受体的接合导致T细胞(例如,$CD4^+$T细胞或$CD8^+$T细胞)对抗原的应答增加。例如在活化信号(例如抗原暴露)引发期间或之后不久,在NK细胞或B细胞上的GITR受体的接合导致这些NK细胞或B细胞的应答增加。在本披露的上下文中,术语“接合”是指结合至GITR受体并刺激由GITR受体介导的至少一种活性。例如,与单独对抗原的应答相比,在抗原特异性物(例如,$CD4^+$T-细胞和/或$CD8^+$T-细胞)上的GITR受体的接合导致增加的T-细胞增殖和增加的细胞因子产生。对抗原的增加的应答可以比在没有GITR受体接合的情况下维持实质上更长的一段时间。因此,经由GITR受体的刺激通过提高非自身的例如肿瘤抗原或病毒抗原的T-细胞、NK细胞或B细胞识别来增强抗原特异性免疫应答。GITR牵涉入某些慢性病毒感染的T-细胞介导的控制(Pascutti等人,PLoS Pathog.[科学公共图书馆·病原学]2015年3月4日;11(3);Clouthier等人,PLoS Pathog.[科学公共图书馆·病原学]2015年1月15日;11(1))。

[0065] 当在由抗原引发T细胞的过程中或之后不久向受试者给予时,GITR激动剂可在受试者(如人受试者)中增强抗原特异性免疫应答。GITR激动剂包括GITR配体(“GITRL”)如可溶性GITRL融合蛋白和抗GITR抗体或其片段。具体的实例是融合多肽亚基,包含GITRL的受体结合结构域、多聚结构域(例如三聚结构域(例如源自冠蛋白1a的α-螺旋卷曲螺旋结构域(helical coiled coil domain)))和IgG Fc结构域,其中该多肽亚基自组装成多聚体(例如,三聚体或六聚体)融合蛋白。在此还描述了包括编码此类融合多肽的多核苷酸序列的核酸。本披露还提供了用于在受试者中使用多聚体GITRL融合蛋白来增强抗原特异性免疫应答的方法。更一般地,在此披露的关于GITRL融合蛋白的组合物和方法可以应用于多聚体(例如三聚体和六聚体)受体结合融合蛋白的生产和例如在治疗癌症的方法、治疗病毒感染的方法、或增强受试者的免疫应答的方法中对它的使用。

定义

[0066] 术语“一个/种(a或an)”实体是指一个/种或多个/种该实体;例如,“多肽亚基”应理解为代表一种或多种多肽亚基。因此,术语“一个”(或“一种”)、“一个或多个(一种或多种)”、以及“至少一个(至少一种)”在此可以互换地使用。

[0067] 除非另外定义,在此所使用的所有技术和科学术语具有与本披露涉及的领域所属的技术人员通常所理解的相同的意义。例如,Concise Dictionary ofBiomedicine and Molecular Biology[生物医学与分子生物学简明词典],Juo、Pei-Show,第2版,2002,CRC出版社;The Dictionary of Cell and Molecular Biology[细胞与分子生物学词典]第3版,1999,学术出版社(AcademicPress);以及Oxford Dictionary Of Biochemistry And

Molecular Biology[生物化学和分子生物学牛津词典],修订版,2000,牛津大学出版社(Oxford University Press)为技术人员提供在本披露中使用的许多术语的通用词典注释。

[0068] 单位、前缀和符号均以它们的国际单位系统(SI)接受形式表示。数值范围包括定义该范围的数字。除非另外指明,否则氨基酸序列以氨基到羧基的方向从左到右书写。在此提供的小标题不是本披露的不同方面或方面的限制,可以通过作为一个整体参考本说明书来获得这些方面。因此,通过以其全文参考说明书,更完全地定义了就在以下定义的术语。

[0069] 如在此使用,短语"抗原处理的"用于描述已经暴露于抗原的细胞,其中暴露于该抗原已在该细胞中引起应答。

[0070] 如在此使用,术语"多肽"旨在涵盖单数"多肽"和复数"多肽",并且是指由通过酰胺键(也称为肽键)线性连接的单体(氨基酸)组成的分子。术语"多肽"是指具有两个或更多个氨基酸的任何一个或多个链,并且不是指产物的具体长度。因此,肽、二肽、三肽、寡肽、"蛋白质"、"氨基酸链"或用于指具有两个或更多个氨基酸的一个或多个链的任何其他术语包含在"多肽"的定义中,并且术语"多肽"可以代替这些术语中的任何一个、或可与其互换使用。术语"多肽"还旨在指多肽在表达后修饰的产物,包括而不限于糖基化、乙酰化、磷酸化、酰胺化、通过已知保护/阻断基团来进行的衍生、蛋白水解裂解或通过非标准的氨基酸来进行的修饰。多肽可来自天然生物来源或通过重组技术来产生,但不是必然从一个指定的核酸序列翻译而来。它可以按任何方式、包括通过化学合成来产生。

[0071] 如在此使用的,"蛋白质"可指单个多肽,即,如上所定义的单个氨基酸链,而且还可以指相关联的两个或更多个多肽,例如,通过二硫键、氢键、或疏水相互作用来产生多聚体蛋白。如在此使用的,术语"多肽亚基"是指氨基酸的多肽链,该多肽链可以与其他多肽亚基(无论是相同或不同的)相互作用以形成多聚体蛋白,例如,如在此所描述的六聚体蛋白。

[0072] 如在此披露的多肽可以具有约3个或更多个、5个或更多个、10个或更多个、20个或更多个、25个或更多个、50个或更多个、75个或更多个、100个或更多个、200个或更多个、500个或更多个、1,000个或更多个,或2,000个或更多个氨基酸的大小。多肽可以具有一种限定的三维结构,但是它们不是必然具有这种结构。具有限定的三维结构的多肽称为折叠的,并且不具有限定的三维结构而可采用很多不同构象的多肽称为去折叠的。

[0073] "分离的"物质、组合物、实体,和/或物质、组合物或实体的任何组合,或其任何语法变体(例如分离的生物材料)是不处于其自然环境的物质。不需要特定的纯化水平。例如,分离的抗体是不会产生于或位于其天然或自然环境中的抗体。如在此所披露的,认为重组生产的生物材料是分离的,如在非天然细胞(如杂交瘤)中产生的材料一样。如果已经通过任何合适的技术分离、分馏或者部分地或基本上纯化,则该物质例如生物材料也被认为是"分离的"。在某些方面中,分离的物质(例如分离的生物材料)可以是"非天然存在的"。

[0074] 如在此所使用,术语"非天然存在的"物质、组合物、实体和/或物质、组合物或实体的任何组合或其任何语法变化形式是明确排除但仅排除本领域普通技术人员熟知为"天然存在"或由法官或行政机关(如美国专利及商标局)或司法机构确定或解释为或可能在任何时候确定或解释为"天然存在"的物质、组合物、实体和/或物质、组合物或实体的任何组合的那些形式的条件术语。例如,术语"非天然存在的抗体"明确排除天然存在的那些抗体,例如会天然存在于暴露于抗原刺激正常环境的小鼠的免疫系统中的抗体,或由行政机构(例

如美国专利及商标局)或司法机构(例如联邦法院)最终确定为“天然存在”的抗体。

[0075] 在此披露的其他多肽是前述多肽的片段、衍生物、类似物或变体,以及其任何组合。当提及如在此披露的多肽亚基或多聚体蛋白时,术语“片段”、“变体”、“衍生物”和“类似物”可包括保留完整多肽或蛋白质的至少一些活性的任何多肽或蛋白质,但其在结构上不同。多肽的片段包括,例如,蛋白水解片段、以及缺失片段。变体包括如上所述的片段,而且还有由于氨基酸取代、缺失或插入而具有改变的氨基酸序列的多肽。变体可自发产生或有意构造。有意构造的变体可以使用本领域已知的诱变技术来产生。变体多肽可以包括保守或非保守氨基酸取代、缺失或添加。衍生物是已被改变使得展现出在天然多肽上找不到的附加特征的多肽。实例包括融合蛋白。变体多肽在此还可以称为“多肽类似物”。如在此所使用的,“衍生物”是指具有通过官能侧基的反应来化学衍生的一个或多个氨基酸的主题多肽。含有二十种标准氨基酸的一种或多种标准或合成的氨基酸衍生物的那些肽也作为“衍生物”包括在内。例如,4-羟基脯氨酸可以取代脯氨酸;5-羟基赖氨酸可以取代赖氨酸;3-甲基组氨酸可以取代组氨酸;高丝氨酸可以取代丝氨酸;并且鸟氨酸可以取代赖氨酸。

[0076] “保守的氨基酸取代”是一个氨基酸被具有相似侧链的另一个氨基酸置换。在本领域中已经定义了具有相似侧链的氨基酸家族,包括碱性侧链(例如,赖氨酸、精氨酸、组氨酸)、酸性侧链(例如,天门冬氨酸、谷氨酸),不带电荷的极性侧链(例如,天冬酰胺、谷氨酰胺、丝氨酸、苏氨酸、酪氨酸、半胱氨酸)、非极性的侧链(例如,甘氨酸、丙氨酸、缬氨酸、亮氨酸、异亮氨酸、脯氨酸、苯丙氨酸、甲硫氨酸、色氨酸)、β-分支侧链(例如,苏氨酸、缬氨酸、异亮氨酸)、以及芳香族侧链(例如,酪氨酸、苯丙氨酸、色氨酸、组氨酸)。例如,苯丙氨酸对于酪氨酸的取代是保守取代。鉴定不消除蛋白质活性的核苷酸和氨基酸保守取代的方法是本领域公知的(参见,例如,Brummell等人,Biochem.[生物化学]32:1180-1187(1993);Kobayashi等人,Protein Eng.[蛋白质工程]12(10):879-884(1999);以及Burks等人,Proc.Natl.Acad.Sci.USA[美国国家科学院院刊]94:412-417(1997))。

[0077] 如在此使用的术语“抗体”(或其片段、变体、或衍生物)是指能够结合至抗原的抗体的至少最小部分,例如,在由B细胞产生的典型抗体的情况下,至少重链(VH)的可变结构域和轻链(VL)的可变结构域。脊椎动物系统中的基础抗体结构相对较好理解。参见,例如,Harlow等人,Antibodies:A Laboratory Manual,(Cold Spring Harbor Laboratory Press,2nd ed.1988)[抗体:实验室手册(冷泉港实验室出版社,第2版,1988)]。

[0078] 抗体或其抗原结合片段、变体或衍生物包括但不限于多克隆抗体、单克隆抗体、人抗体、人源化抗体或嵌合抗体、单链抗体、表位结合片段,例如Fab、Fab'和F(ab')2、Fd、Fvs、单链Fvs(scFv)、单链抗体、二硫化物连接的Fvs(sdFv)、包括VL或VH域的片段、由Fab表达文库产生的片段。ScFv分子在本领域中是已知的并且描述于例如美国专利5,892,019之中。本披露涵盖的免疫球蛋白或抗体分子可以是免疫球蛋白分子的任何类型(例如IgG、IgE、IgM、IgD、IgA、以及IgY)、类别(例如IgG1、IgG2、IgG3、IgG4、IgA1、以及IgA2)或子类。

[0079] 术语“多核苷酸”旨在涵盖单数核酸以及复数核酸,并且是指经分离的核酸分子或构建体,例如信使RNA(mRNA)或质粒DNA(pDNA)。多核苷酸可包括常规磷酸二酯键或非常规键(例如酰胺键,诸如在肽核酸(PNA)中所发现)。术语“核酸”是指存在于多核苷酸中的任何一个或多个核酸节段,例如DNA或RNA片段。“分离的”核酸或多核苷酸意指已经从其天然环境中去除的核酸分子DNA或RNA。例如,在一个载体中所包含的编码多肽亚基的重组多核苷

酸被视为是分离的，如在此披露。分离的多核苷酸的另外实例包括维持在异源宿主细胞中的重组多核苷酸或溶液中的纯化(部分地或基本上)多核苷酸。分离的RNA分子包括多核苷酸的体内或体外RNA转录物。分离的多核苷酸或核酸进一步包括合成产生的这类分子。另外，多核苷酸或核酸可以是或可以包括调节元件如启动子、核糖体结合位点或转录终止子。

[0080] 如在此使用，“编码区”是包含翻译成氨基酸的密码子的核酸的一部分。虽然“终止密码子”(TAG、TGA或TAA)未被翻译成氨基酸，它可被认为是编码区的一部分，但是任何侧翼序列(例如启动子、核糖体结合位点、转录终止子、内含子、以及类似序列)并非编码区的一部分。两个或更多个编码区可以存在于单一多核苷酸构建体中，例如在单一载体上，或在单独的多核苷酸构建体中，例如在单独(不同)载体上。此外，任何载体可以含有单一编码区，或可以包括两个或更多个编码区，例如单一载体可以单独地编码一个免疫球蛋白重链可变区和一个免疫球蛋白轻链可变区。另外，载体、多核苷酸、或核酸可以编码异源编码区，无论是融合或非融合至编码如在此提供的多肽亚基或融合蛋白的核酸。异源编码区包括但不限于特化的元件或基序，如分泌信号肽或异源功能域。

[0081] 在一些实施例中，多核苷酸或核酸是DNA。在DNA的情况下，包括编码多肽的核酸的多核苷酸通常可以包括启动子和/或可操作地与一个或多个编码区相关联的其他转录或翻译控制元件。可操作地关联或连接是针对基因产物(例如多肽)的编码区按以下这种方式与一个或多个调节序列相关联，该方式使得该基因产物的表达处于这种或这些调节序列的影响或控制之下。如果启动子功能的诱导导致编码所希望的基因产物的mRNA的转录，并且如果两个DNA片段之间的连接的性质不干扰表达调节序列引导基因产物的表达的能力或不干扰有待转录的DNA模板的能力，那么两个DNA片段(如多肽编码区和与其关联的启动子)“可操作地关联”或“可操作地连接”。因此，启动子区将可操作地与编码多肽的核酸相关联，只要启动子能够实现所述核酸的转录。启动子可以是只在预定细胞中引导DNA的实质性转录的细胞特异性启动子。除了启动子以外，其他转录控制元件例如增强子、操纵子、阻遏子以及转录终止信号可以可操作地与多核苷酸相关联以便引导细胞特异性转录。在此披露了合适的启动子和其他转录控制区。

[0082] 多种转录控制区是本领域技术人员已知的。这些转录控制区包括但不限于在脊椎动物细胞中起作用的转录控制区，诸如但不限于来自巨细胞病毒的启动子和增强子片段(立即早期启动子，它与内含子A相结合)、猿病毒40(早期启动子)和逆转录病毒(诸如劳斯(Rous)肉瘤病毒)。其他转录控制区包括得自脊椎动物基因如肌动蛋白、热休克蛋白、牛生长激素以及兔β-球蛋白的那些转录控制区域，连同能够控制真核细胞中的基因表达的其他序列。另外的合适转录控制区包括组织特异性启动子和增强子，连同淋巴因子可诱导的启动子(例如可由干扰素或白介素诱导的启动子)。

[0083] 类似地，多种翻译控制元件是本领域普通技术人员已知的。这些翻译控制元件包括但不限于核糖体结合位点、翻译起始和终止密码子，以及得自小核糖核酸病毒的元件(特别是内核糖体进入位点，或IRES，也称为CITE序列)。

[0084] 在其他实施例中，多核苷酸可以是例如处于信使RNA(mRNA)形式的RNA。

[0085] 多核苷酸和核酸编码区可与编码分泌肽或信号肽的另外的编码区相关联，这些另外的编码区引导由如在此披露的多核苷酸(例如编码在此提供的多肽亚基的多核苷酸)所编码的多肽的分泌。根据信号假设，由哺乳动物细胞分泌的蛋白质具有信号肽或分泌前导

序列，一旦已经开始将生长的蛋白质链输出横穿粗面内质网，该信号肽或分泌前导序列就从成熟蛋白质上裂解。本领域普通技术人员意识到由脊椎动物细胞分泌的多肽一般具有融合至多肽的N末端的信号肽，这种信号肽从完整或“全长”多肽上裂解以便产生分泌或“成熟”形式的多肽。在一些实施例中，使用天然信号肽，例如免疫球蛋白重链或轻链信号肽，或保持引导与其可操作关联的多肽的分泌的能力的所述序列的功能衍生物。可替代地，可以使用异源哺乳动物信号肽，或其功能衍生物。例如，野生型前导序列可以用甲型流感病毒血细胞凝集素、人组织纤溶酶原活化剂(TPA)或小鼠β-葡糖醛酸酶的前导序列来取代。

[0086] “载体”是导入宿主细胞的核酸分子，从而产生转化的宿主细胞。载体可包括允许其在宿主细胞中复制的核酸序列，如复制起点。载体也可以包括一种或多种可选择的标记基因和本领域已知的其他遗传元件。

[0087] “转化的”细胞或“宿主”细胞是已经通过分子生物学技术将核酸分子引入的细胞。如在此使用的，术语转化涵盖可将核酸分子引入这样的细胞的所有技术，这些技术包括转染病毒载体，用质粒载体转化，以及通过电穿孔、脂转染、和粒子枪加速引入裸DNA。转化的细胞或宿主细胞可以是细菌细胞或真核细胞。

[0088] “特异性地结合”一般是指分子(例如，GITRL或其受体结合片段)经由其受体结合结构域来结合至另一个分子(例如，GITR)，并且这种结合需要配体与其受体之间有某种互补性。根据此定义，当与配体将结合至随机、不相关的分子相比，该配体更容易经由其受体结合域来结合至那个受体时，该配体被认为是“特异性地结合”至该受体。术语“特异性”在此用于对某种配体结合至某种受体的相对亲和力进行定性。例如，与配体“B”相比，配体“A”可被视为针对给定的受体具有较高特异性，或与配体“A”针对相关受体“D”所具有的特异性相比，配体“A”可被认为以较高特异性结合至受体“C”。

[0089] “受体-结合结构域”意指包括在配体(例如，如在此所披露的GITRL)中的结合结构域。

[0090] 配体，例如，如在此所披露的GITRL融合多肽亚基或多聚体GITRL融合蛋白可以结合到受体，该受体例如是具有解离速率(k(解离))小于或等于5x $10^{-2}sec^{-1}$、$10^{-2}sec^{-1}$、5x $10^{-3}sec^{-1}$或$10^{-3}sec^{-1}$的GITR。配体，例如，如在此所披露的GITRL融合多肽亚基或多聚体GITRL融合蛋白可以结合到受体，该受体例如是具有解离速率(k(解离))小于或等于5x $10^{-4}sec^{-1}$、$10^{-4}sec^{-1}$、5x $10^{-5}sec^{-1}$或$10^{-5}sec^{-1}$5x $10^{-6}sec^{-1}$、$10^{-6}sec^{-1}$、5x $10^{-7}sec^{-1}$或$10^{-7}sec^{-1}$的GITR。

[0091] 术语“抑制”、“阻断”、以及“阻遏”在此可互换地使用，并且指生物活性的任何统计学显著的降低，包括活性的完全阻断。例如，“抑制”可以指生物活性大约10％、20％、30％、40％、50％、60％、70％、80％、90％或100％的降低。

[0092] 如在此使用，术语“亲和力”是指配体与其同源受体的结合强度的度量。如在此使用的，术语“亲合力”是指配体和受体的群体之间复合体的总体稳定性，即，配体和受体的组合的功能性结合强度，例如，六聚体GITRL IgG4融合蛋白(GITRL FP)与细胞表面GITR的相互作用。亲合力与群体中的单独受体结合域与特定受体的亲和力相关，并且还与配体和受体的效价相关。

[0093] 配体，例如，在此所披露的GITRL融合多肽亚基或多聚体GITRL融合蛋白也可以就其对配体的结合亲和力方面来描述或指定。例如，配体可以按不大于5x $10^{-2}M$、$10^{-2}M$、5x 10

$^{-3}$M、10^{-3}M、5x 10^{-4}M、10^{-4}M、5x10^{-5}M、10^{-5}M、5x 10^{-6}M、10^{-6}M、5x 10^{-7}M、10^{-7}M、5x 10^{-8}M、10^{-8}M、5x 10^{-9}M、10^{-9}M、5x 10^{-10}M、10^{-10}M、5x 10^{-11}M、10^{-11}M、5x 10^{-12}M、10^{-12}M、5x 10^{-13}M、10^{-13}M、5x 10^{-14}M、10^{-14}M、5x 10^{-15}M或10^{-15}M的解离常数或K_D来结合至受体。

[0094] 配体，例如，如在此所披露的GITRL融合多肽亚基或多聚体GITRL融合蛋白可以结合到受体，该受体例如是具有结合速率(k(结合))大于或等于$10^3M^{-1}sec^{-1}$、5X $10^3M^{-1}sec^{-1}$、$10^4M^{-1}sec^{-1}$或5X $10^4M^{-1}sec^{-1}$的GITR。配体，例如，如在此所披露的GITRL融合多肽亚基或多聚体GITRL融合蛋白可以结合到受体，该受体例如是具有结合速率(k(结合))大于或等于$10^5M^{-1}sec^{-1}$、5X $10^5M^{-1}sec^{-1}$、$10^6M^{-1}sec^{-1}$、或5X $10^6M^{-1}sec^{-1}$、或$10^7M^{-1}sec^{-1}$的GITR。

[0095] GITR或“GITR受体”是蛋白质，也称为糖皮质激素诱导的TNF相关蛋白、肿瘤坏死因子配体超家族成员18、TNSF18、活化诱导型TNF相关受体、AITR、CD357和RP5-902P8.2，在活化的NK细胞和抗原处理的T-细胞(例如$CD4^+$和$CD8^+$T-细胞)以及$CD4^+CD25^+FOXP3^+$调节性T-细胞(Treg)的表面上表达(Stephens等人J.Immunol.[免疫学杂志](2004)173(8):5008-5020)。GITR是例如SEQ ID NO:47的蛋白质。“GITR配体”(“GITRL”)，也称为糖皮质激素诱导的TNF相关配体、肿瘤坏死因子配体超家族成员18配体、TNFSF18配体、TL6、活化诱导型TNF相关配体、AITR配体、AITRL和RP1-15D23，主要在抗原呈递细胞(APC)上发现(Stephens等人J.Immunol.[免疫学杂志](2004)173(8):5008-5020))。GITRL表达于细胞的表面上，并包括细胞内、跨膜和胞外受体结合结构域。

[0096] 如在此使用，术语“GITRL”是指整个GITR配体、可溶性GITR配体和GITR配体的功能活性部分。GITRL的定义内还包括GITRL的天然存在的等位基因变体、与天然存在的GITR配体分子在氨基酸序列上不同的GITR配体变体、以及这样的变体的组合，其中这些变体保留特异性地结合GITR受体的能力。GITRL的包括氨基酸残基取代的某些变体在此以SEQ ID NO:1的成熟GITRL蛋白中的残基编号来鉴定。例如，N161D是指在SEQ ID NO:1的成熟人GITRL的位置161处天冬酰胺酰残基被天冬氨酰残基取代，并且也指在SEQ ID NO:6和SEQ ID NO:8的人GITRL的胞外结构域的等同位置中的相同取代。在提及SEQ ID NO:1的GITRL序列中的各个取代时，在此还提供了除了SEQ ID NO:1的GITRL多肽之外的GITRL多肽中的相应残基的等同取代。通过将SEQ ID NO:1序列与待取代的GITRL序列进行比对，可以容易地鉴定出这些相应的残基。例如，具有SEQ ID NO:1的单个氨基酸N-末端添加的GITRL肽可以具有在位置162处的天冬酰胺酰残基的取代，其将等同于在SEQ ID NO:1的位置161处的天冬酰胺酰残基的取代。

[0097] 如在此使用，术语“GITRL融合多肽亚基”或“GITRL FP亚基”是指单链多肽亚基，其包括：人IgG Fc结构域；功能性三聚结构域；和糖皮质激素诱导的TNF受体配体(GITRL)的受体结合结构域，其中该多肽亚基可以自组装成多聚体例如三聚体或六聚体蛋白。术语“多聚体GITRL融合蛋白”或“多聚体GITRL FP”是指GITRL融合多肽亚基的自组装多聚体，包括例如三聚体和六聚体。当在GITRL FP亚基中使用某个同种型的IgG Fc结构域时，将具有该同种型的GITRL FP描述为“GITRL IgGX FP”，其中X可以是1、2、2a、3、4或4P，例如GITRL IgG1 FP、GITRL IgG2 FP、GITRL IgG2a FP、GITRL IgG3 FP、GITRL IgG4 FP和GITRL IgG4P FP。

[0098] 如在此使用，“OX40多肽”意指与NCBI登录号NP_003318具有至少约85％氨基酸一致性的多肽或其片段。OX40是受体TNFR超家族中的在抗原活化的哺乳动物CD4+和CD8+T淋巴细胞的表面上表达的成员。参见，例如，Paterson等人，Mol Immunol[分子免疫学]24，

1281-1290(1987);Mallett等人,EMBO J.[欧洲分子生物学学会杂志]9,1063-1068(1990);以及Calderhead等人,J Immunol[免疫学杂志]151,5261-5271(1993))。OX40还被称为CD134、ACT-4以及ACT35。OX40受体序列是本领域中已知的并且例如以GenBank登录号:AAB33944或CAE11757提供。

[0099] 以下提供了示例性人OX40氨基酸序列:

```
1   mcvgarrlgr gpcaallllg lglstvtglh cvgdtypsnd rcchecrpgn gmvsrcsrsq
61  ntvcrpcgpg fyndvvsskp ckpctwcnlr sgserkqlct atqdtvcrcr agtqpldsyk
121 pgvdcapcpp ghfspgdnqa ckpwtnctla gkhtlqpasn ssdaicedrd ppatqpqetq
181 gpparpitvq pteawprtsq gpstrpvevp ggravaailg lglvlgllgp laillalyll
241 rrdqrlppda hkppgggsfr tpiqeeqada hstlaki (SEQ ID NO: 52)
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[0100] "OX40配体"意指与NCBI登录号NP_003317具有至少约85%氨基酸一致性并且特异性地结合OX40受体的多肽或其片段。参见,例如,Baum P.R.等人EMBOJ.[欧洲分子生物学学会杂志]13:3992-4001(1994))。术语OX40L包括整个OX40配体、可溶性OX40配体、以及包含OX40配体的共价地连接到第二部分例如蛋白结构域的功能活性部分的融合蛋白。还包括在OX40L的定义内的是与天然存在的OX40L在氨基酸序列上不同但是保留特异性地结合到OX40受体的能力的变体。还包括在OX40L的定义内的是增强OX40的生物活性的变体。OX40配体序列在本领域中是已知的并且例如以Genbank登录号:NP_003318提供。

[0101] 以下提供了示例性人OX40配体氨基酸序列:

MERVQPLEENVGNAARPRFERNKLLLVASVIQGLGLLLCFTYICLHFSALQVSHRYPRIQSIKVQFTEYKKEKGFILTSQKEDEIMKVQNNSVIINCDGFYLISLKGYFSQEVNISLHYQKDEEPLFQLKKVRSVNSLMVASLTYKDKVYLNVTTDNTSLDDFHVNGGELILIHQNPGEFCVL(SEQ ID NO:53)

[0102] 如在此使用,"OX40激动剂"意指与OX40受体特异性地相互作用并且增加OX40受体的生物活性的OX40配体。令人希望的是,该生物活性增加至少约10%、20%、30%、50%、70%、80%、90%、95%或甚至100%。在某些方面中,如在此所披露的OX40激动剂包括OX40结合多肽,诸如抗OX40抗体(例如,OX40激动剂抗体)、OX40配体、或这些分子的片段或衍生物。

[0103] 如在此使用,"OX40抗体"意指特异性地结合OX40的抗体。OX40抗体包括对OX40具有特异性的单克隆抗体和多克隆抗体以及其抗原结合片段。在某些方面,如在此所述的抗OX40抗体是单克隆抗体(或其抗原结合片段),例如鼠类、人源化或全人单克隆抗体。在一个具体实施例中,该OX40抗体是OX40受体激动剂,诸如由Weinberg等人,J Immunother[免疫学治疗杂志]29,575-585(2006)所描述的小鼠抗人OX40单克隆抗体(9B12)。在其他实施例中,特异性地结合到OX40的抗体或其抗原结合片段结合到与mAb 9B12相同的OX40表位。在另一个方面中,该抗体是MEDI0562。参见例如,美国公开号2016/0137740。

[0104] 如在此使用,"OX40配体融合蛋白(OX40L FP)"意指特异性地结合OX40受体并增加免疫应答的蛋白质。在一个实施例中,OX40配体融合蛋白结合到OX40受体通过加强T-细胞识别来增强肿瘤抗原特异性免疫应答。示例性OX40配体融合蛋白在标题为"三聚体OX40免疫球蛋白融合蛋白和使用方法"("Trimeric OX40Immunoglobulin Fusion Protein and Methods of Use")的美国专利7,959,925中有所描述。其他OX40配体融合蛋白例如在美国专利号6,312,700中有所描述。在一个实施例中,OX40配体融合蛋白增强肿瘤特异性T-细胞

免疫性。在一个实施例中，OX40配体融合蛋白是MEDI6383(SEQ ID NO:50)。参见例如，美国公开号2016/0024176。

[0105] “三聚结构域”是在多肽内促进该多肽装配成三聚体的氨基酸序列。例如，三聚结构域可经由与其他三聚结构域(具有相同或不同的氨基酸序列的另外的多肽的三聚结构域)关联来促进装配成三聚体。该术语也用于指编码这样的肽或多肽的多核苷酸。

[0106] 术语“Fc”结构域是指抗体恒定区的一部分。传统上，术语Fc结构域是指涵盖抗体的配对CH2、CH3和铰链区的蛋白酶(例如，木瓜蛋白酶)裂解产物。在本披露的上下文中，术语Fc结构域或Fc是指不管产生手段的任何多肽(或编码这样一种多肽的核酸)，其包括免疫球蛋白多肽的CH2、CH3和铰链区的全部或一部分。

[0107] 如在此使用，术语“IgG Fc结构域”是指IgG1、IgG2、IgG3或IgG4免疫球蛋白的Fc结构域，以及这样的Fc结构域的变体。IgG4 Fc结构域的变体包括但不限于IgG4P Fc结构域。

[0108] 如在此使用，术语“CH2结构域”包括重链分子的Fc结构域的部分，使用常规的编号方案该部分例如从抗体的约氨基酸244延伸至氨基酸360(氨基酸244至360，卡巴特编号系统；以及氨基酸231-340，EU编号系统)。还被文献充分证明的是CH3结构域从CH2结构域延伸至IgG分子的C末端并且包括近似108个氨基酸。

[0109] 如在此使用，术语“接头区”包括用于融合或连接两个蛋白质结构域的任何肽。这样的接头包括但不限于包括一个或多个(Gly_4)n基序、一个或多个(Gly_4Ser)n基序(SEQ ID NO:19)和一个或多个Ser(Gly_4Ser)n基序(SEQ ID NO:22)的肽。

[0110] 如在此使用，术语“IgG铰链区”包括重链IgG分子的Fc结构域的将CH1结构域接合至CH2结构域的那部分。这个铰链区包括近似25个氨基酸并且是柔性的，由此允许两个N末端抗原结合区独立地移动。铰链区可细分成三个相异的结构域：上、中和下铰链结构域(Roux等人，J.Immunol.[免疫学杂志]161:4083(1998))。

[0111] 如在此使用，术语“二硫键”包括在两个硫原子之间形成的共价键。氨基酸半胱氨酸包括可以与第二硫醇基形成二硫键或二硫桥的硫醇基。在在此提供的某些方面中，人IgG4 Fc结构域可以在铰链区进行突变以确保两个铰链区之间二硫键形成，具体地，在位置228处丝氨酸至脯氨酸(根据EU编号)的突变。包含S228P突变的人IgG4 Fc结构域在此被称为“IgG4P Fc结构域”。

[0112] 如在此使用，术语“连接”、“融合(fused)”或“融合(fusion)”可互换地使用。这些术语是指通过包括化学轭合或重组手段的任何手段将两个更多个元件或组分接合在一起。“框内融合”是指连接两个或更多个多核苷酸开放阅读框架(ORF)以便以维持原始ORF的正确翻译阅读框架的方式来形成连续更长的ORF。因此，重组融合蛋白是含有对应于由原始ORF编码的多肽的两个或更多个节段的单一蛋白质(这些节段天然地通常并不这样连接)，例如，如在此提供的GITRL融合多肽亚基。虽然阅读框架由此在整个融合节段上被致使连续，但是这些节段可以被例如框内接头序列在物理上或空间上分离。

[0113] 在多肽的情况下，“线性序列”或“序列”是多肽中的在氨基至羧基末端方向上的氨基酸顺序，其中在多肽的一级活化结构中，在序列上彼此邻接的氨基酸是连续的。

[0114] 如在此使用的术语“表达”是指基因产生生物化学物质例如多肽的过程。该过程包括基因在细胞内的功能性存在的任何表现，包括但不限于基因敲除和瞬态表达与稳定表达两者。它包括而不限于将基因转录成信使RNA(mRNA)，和将这种mRNA翻译成多肽。如果最终

所希望的产物是生物化学物质，那么表达包括所述生物化学物质和任何前体的产生。基因的表达产生“基因产物”。如在此使用，基因产物可以是核酸，例如通过基因转录来产生的信使RNA，或从转录物翻译的多肽。在此描述的基因产物进一步包括具有转录后修饰(例如多聚腺苷酸化)的核酸，或具有翻译后修饰(例如甲基化、糖基化、添加脂质、与其他蛋白质亚基相关联、蛋白裂解等)的多肽。

[0115] 如在此使用，在治疗癌症的上下文中使用时，术语“治疗(treat、treatment、或treatment of)”(例如，在短语“治疗癌症患者”中)是指减少疾病病理的可能性，减少疾病症状的发生，例如在一定程度上受试者具有更长的存活率或减少的不适。例如，治疗可以是指当向受试者给予疗法时该疗法减少疾病症状、体征或病因的能力。治疗还指缓和或减少至少一种临床症状和/或抑制或延迟病症的进展和/或预防或延迟疾病或疾患的发作。

[0116] 如在此使用，在治疗病毒感染的上下文中使用时，术语“治疗(treat、treatment、或treatment of)”(例如，在短语“治疗病毒感染”中)是指减少与病毒感染相关的病理状况和/或症状。

[0117] “受试者”或“个体”或“动物”或“患者”或“哺乳动物，”是指希望诊断、预后或治疗的任何受试者，特别是哺乳动物受试者。哺乳动物受试者包括人、家畜、农畜、体育动物、和动物园动物，包括例如人、非人灵长类、狗、猫、豚鼠、兔、大鼠、小鼠、马、牛、熊等。

[0118] 术语“药物组合物”是指如下制剂，该制剂处于允许该活性成分的生物活性有效的形式，并且不含有另外的、对其将要给予的受试者具有不可接受的毒性的组分。这样的组合物可以是无菌的。

[0119] 如在此披露的，抗体的“有效量”是足以进行具体阐述目的的量。关于阐述的目的，“有效量”可以经验为主地并且以常规方式来确定。

GITRL融合多肽亚基

[0120] 本披露涉及GITRL融合多肽亚基，其可以组装成具有改进的性质的多聚体(例如，三聚体或六聚体蛋白)，这些性质包括但不限于在经转染的哺乳动物细胞培养物中表达时改进的产量；改进的GITR结合亲和力；与先前披露的含有GITRL的多肽(参见例如Wyzgol等人，JImmunol[免疫学杂志]2009；183:1851-1861)相比，在各种生物学测定中改进的活性和/或在纯化或部分纯化时改进的同质性。在此提供的GITRL融合多肽亚基可以包括IgG Fc结构域(例如人IgG Fc结构域)、三聚结构域和人GITRL的受体结合结构域。示例性实施例示意性地在图7示出。典型地，IgG Fc结构域、三聚结构域和GITRL受体结合结构域以N-末端至C-末端方向排列。示例性GITRL IgG1融合多肽亚基由SEQ ID NO:6代表。

[0121] 在某些实施例中，GITRL受体结合结构域是人GITRL的胞外结构域。一个这样的结构域的序列是由SEQ ID NO:37代表。

[0122] 在某些方面中，GITRL受体结合结构域是GITRL变体胞外结构域。可以使用的GITRL变体胞外结构域包括但不限于在SEQ ID NO:37的整个长度上具有至少70％、75％、80％、85％、90％、95％、97％、98％、99％、或100％序列一致性的多肽。在某些方面中，该GITRL变体胞外结构域是在SEQ ID NO:34的整个长度上具有至少70％、75％、80％、85％、90％、95％、97％、98％、99％、或100％序列一致性的多肽。在某些方面中，对应于SEQ ID NO:1的天冬酰胺酰161的GITRL变体胞外结构域残基被任何氨基酸或被天冬氨酰残基取代。GITRL变体胞外结构域(其中对应于SEQ ID NO:1的天冬酰胺酰161的残基被任何氨基酸或被天冬

氨酰残基取代)包括但不限于在SEQ ID NO:35、SEQ ID NO:36和SEQ ID NO:37的整个长度上具有至少70％、75％、80％、85％、90％、、95％、97％、98％、或99％序列一致性的多肽。这样的取代可以减少或消除该GITRL变体胞外结构域中此位点的N联糖基化。在某些方面中，对应于SEQ ID NO:1的天冬酰胺酰106的GITRL变体胞外结构域残基被丙氨酰残基取代。在某些背景下，hGITRL的N106A取代可导致与GITR的结合增加和改进的T-细胞增殖应答(Chattopadhyay等人(2007)Proc.Natl.Acad.Sci.USA[美国国家科学院院刊]104(49):19452-19457)。在某些方面中，对应于Glu 52、Phe 62、Pro 66、Pro 67、Met 71、Pro 77、Val 79、Asn 92、Ser 83、Gly 99、Asn 104、Pro 112、Arg 116、Met 123、Asn 153、Val 158、Asn 161、Iso 167、Iso 168及其组合的GITRL变体胞外结构域残基独立地被非天然存在的氨基酸残基或等位基因变体的氨基酸残基取代。SEQ ID NO:35中提供了GITRL变体胞外结构域的非限制性实例，其中X_1＝Glu或Ala，X_2＝Ser或Phe，X_3＝Thr或Pro，X_4＝Leu或Ser，X_5＝Thr或Met，X_6＝Leu或Pro，X_7＝Met或Val，X_8＝Thr，Phe，或Ser，X_9＝Ser或Gly，X_{10}＝Arg或Pro，X_{11}＝Trp或Arg，X_{12}＝Leu或Met，X_{13}＝Ser或Asn，X_{14}＝Phe或Val，X_{15}＝除Asn或Asp外的任何氨基酸，X_{16}＝Val或Ile，X_{17}＝Leu或Ile，并且其中X_1-X_{17}是独立地选择的并且可以以任何组合存在。

[0123] 保留结合于GITR受体所希望的性质的任何GITRL多肽序列适用于在此所述的融合多肽和方法。

[0124] 毗连GITRL受体结合结构域的是三聚结构域。术语“毗连”包括，例如，经由接头区或异源剂邻接或关联。这样的结构域在彼此邻接时是直接彼此融合的结构域。三聚结构域的作用是促进单个GITRL融合多肽亚基自组装成三聚体蛋白或六聚体蛋白。在某些实施例中，具有三聚结构域的GITRL融合多肽亚基自组装成六聚体GITRL融合蛋白。在一个实施例中，该三聚结构域是螺旋卷曲螺旋结构域，例如，亮氨酸拉链结构域。示例性三聚体亮氨酸拉链结构域是工程化的酵母GCN4pII变体，被描述于Harbury等人(1993)Science[科学]262:1401-1407，将其披露内容结合在此用于所有目的。示例性的三聚结构域包括:TNF受体相关因子2(TRAF2)(GENBANK®登录号Q12933[gi:23503103];氨基酸310-349);血小板反应蛋白1(登录号P07996[gi:135717];氨基酸291-314);母系蛋白-4(登录号095460[gi:14548117];氨基酸594-618;软骨基质蛋白(母系蛋白-1)(登录号NP002370[gi:4505111];氨基酸463-496;热休克转录因子(HSF)(登录号AAX42211[gi:61362386];氨基酸165-191;和吞饮受体(登录号NP001072[gi:4557503];氨基酸104-138。

[0125] 在某些方面中，三聚结构域包括α-螺旋卷曲螺旋结构域。有用的α-螺旋卷曲螺旋结构域包括但不限于衍生自母系蛋白1、冠蛋白1a、营养不良性肌强直激酶(DMPK)、朗格汉斯蛋白及其组合的那些。这样的衍生物包括但不限于具有野生型序列的卷曲螺旋结构域以及在卷曲螺旋结构域野生型序列中包括一个或多个氨基酸取代的变体。含有冠蛋白1a三聚结构域的冠蛋白1a蛋白有时也被同义地称为冠蛋白样蛋白A、Clipin-A、冠蛋白样蛋白p57、含色氨酸天冬氨酸酯的外壳蛋白和HUGO名称CORO1A中的任一项。衍生自可以使用的各种蛋白质的野生型卷曲螺旋结构域的非限制性实例包括母系蛋白1(SEQ ID NO:28)、DMPK(SEQ ID NO:30)、朗格汉斯蛋白(SEQ ID NO:32)和冠蛋白1a(SEQ ID NO:11)。α-螺旋卷曲螺旋结构域的变体可以包括等位基因变体、工程变体及其组合。α-螺旋卷曲螺旋结构域典型地组织成七残基序列重复“hpphcpc”(或abcdefg)，其可在一个或多个重复的一个或多个“h”位

置(“a”和/或“d”位置)处被丙氨酸、亮氨酸、异亮氨酸或缬氨酸残基独立取代。在这样的“hpphcpc”七残基重复序列中,h代表疏水性残基,c典型地代表带电荷的残基,并且p代表极性(并且因此是亲水性的)残基。提供了母系蛋白1、冠蛋白1a、营养不良性肌强直激酶(DMPK)和朗格汉斯蛋白的α-螺旋卷曲螺旋结构域变体,其中那些三聚结构域的七残基重复的一个或多个中的一个或多个“a”和/或“d”位置被丙氨酸、亮氨酸、异亮氨酸或缬氨酸残基取代。可以使用的α-螺旋卷曲螺旋结构域变体的非限制性实例包括母系蛋白1(SEQ ID NO:29)、DMPK(SEQ ID NO:31)、朗格汉斯蛋白(SEQ ID NO:32)和冠蛋白1a(SEQ ID NO:12-18)。在某些方面中,用于GITRL融合多肽亚基中的变体母系蛋白1三聚结构域可以包括但不限于在SEQ ID NO:29的整个长度上具有至少70%、75%、80%、85%、90%、95%、97%、98%、或99%序列一致性的多肽。在某些方面中,用于GITRL融合多肽亚基中的变体DMPK三聚结构域可以包括但不限于在SEQ ID NO:31的整个长度上具有至少70%、75%、80%、85%、90%、95%、97%、98%、或99%序列一致性的多肽。在某些方面中,用于GITRL融合多肽亚基中的变体朗格汉斯蛋白三聚结构域可以包括但不限于在SEQ ID NO:33的整个长度上具有至少70%、75%、80%、85%、90%、95%、97%、98%、或99%序列一致性的多肽。在某些方面中,用于GITRL融合多肽亚基中的冠蛋白1a三聚结构域可以包括但不限于在SEQ ID NO:11的整个长度上具有至少70%、75%、80%、85%、90%、95%、97%、98%、或99%序列一致性的多肽。在某些方面中,以SEQ ID NO:10提供了可用于GITRL融合多肽亚基的冠蛋白1a三聚结构域变体共有序列,其中那些三聚结构域的七残基重复的一个或多个中的一个或多个“A”和/或“D”位置被丙氨酸、亮氨酸、异亮氨酸或缬氨酸残基取代。表A中呈现了可用于GITRL融合多肽亚基的示例性冠蛋白1a wt和变体序列。

表A冠蛋白1a卷曲螺旋结构域及其变体

三聚结构域	序列	SEQ ID NO:	ProCoil 预测评分	预测的寡聚体状态
七残基序列重复	hpphcpc-hpphcpc-hpphcpc-hpphcpc-hpph ；其中 h 代表疏水性残基，c 典型地代表带电荷的残基，并且 p 代表极性（并且因此是亲水性的）残基。七残基重复的位置也可以用小写字母 *abcdefg* 表示。	NA		
hCor1a 共有（X = Ala，Leu，Ile 或 Val）	XSRXEEEXRKXQATXQELQKRXDRLEETVQAK	10		
hCor1a wt	VSRLEEEMRKLQATVQELQKRLDRLEETVQAK	11	0.101544216	三聚体
hCor1a 变体 1	VSRLEEEIRKLQATVQELQKRLDRLEETVQAK	12	0.205919088	三聚体
hCor1a 变体 2	VSRIEEEIRKLQATVQELQKRLDRLEETVQAK	13	0.386363142	三聚体
hCor1a 变体 3	ISRIEEEIRKLQATVQELQKRLDRLEETVQAK	14	0.431631235	三聚体
hCor1a 变体 4	ISRIEEEIRKIQATVQELQKRLDRLEETVQAK	15	0.509021117	三聚体
hCor1a 变体 5	ISRIEEEIRKIQATVQELQKRIDRLEETVQAK	16	0.668080151	三聚体
hCor1a 变体 6	ISRIEEEIRKINATVQELQKRIDRLEETVQAK	17	0.785463418	三聚体
hCor1a 变体 7	ISRIEEEIRKINATIQELQKRIDRLEETVQAK	18	0.729421966	三聚体

[0126] 当与具有不同三聚结构域的其他GITRL融合多肽亚基相比时，具有在此提供的某些三聚结构域(例如，冠蛋白1a三聚结构域或其变体)的GITRL融合多肽亚基可以展现出改进的性质。更确切地说，具有冠蛋白1a三聚结构域或其变体的GITRL融合多肽亚基可以展现出改进的性质，这些性质包括但不限于在经转化的哺乳动物细胞培养物中表达时改进的产

量，例如CHO细胞；改进的GITR结合亲和力；在各种生物学测定中改进的活性(例如NF-κB信号传导途径的活化)；和/或在纯化或部分纯化时改进的同质性。不寻求受理论的限制，认为具有冠蛋白1a三聚结构域或其变体的GITRL融合多肽亚基的这些改进的性质可以促进GITRL融合多肽亚基的制造和/或改进多聚体GITRL融合蛋白质在各种治疗应用中的功效。

[0127] 除了GITRL受体结合结构域和三聚结构域之外，如在此提供的GITRL融合多肽亚基包括免疫球蛋白结构域，例如恒定区或“Fc”结构域。在某些方面中，本披露提供了包括至少铰链区的人IgG1和IgG4 Fc结构域。在某些方面中，人IgG1或IgG4 Fc结构域进一步包括CH2结构域。在某些方面中，人IgG1或IgG4 Fc结构域进一步包括CH3结构域。在某些方面中，Fc结构域是人IgG1 Fc结构域或其变体。人IgG Fc结构域可包括具有如下氨基酸序列的肽，该氨基酸序列与SEQ ID NO:21具有至少70％、80％、85％、90％、95％、96％、97％、98％、99％、或100％的序列一致性。可以使用的IgG Fc结构域(例如IgG1 Fc结构域)的变体包括但不限于含有独立选自下组的一个或多个氨基酸残基的IgG Fc结构域，该组由以下各项组成：252Y、254T、256E及其组合，其中这些残基是根据EU编号进行编号。在某些方面中，IgG4 Fc区的铰链区可包括在位置228(根据EU编号)处的丝氨酸至脯氨酸的突变，该突变赋予完整的重链间二硫键形成。在某些方面中，该IgG4铰链区包括SEQ ID NO:40的氨基酸1至12。在某些方面中，人IgG4 Fc结构域是SEQ ID NO:38的具有S228P突变的IgG4P-Fc结构域。

[0128] 与将三个GITRL受体结合结构域带到一起的三聚结构域结合，在两个IgG Fc结构域之间的二硫键形成导致六聚体蛋白的形成(图7)。因此，免疫球蛋白结构域用作二聚结构域，促进两个三聚体GITRL融合蛋白之间经由不配对的免疫球蛋白结构域之间的相互作用组装成稳定的六聚体(即包含六个GITRL融合多肽亚基的多聚体)。在某些方面中，人IgG4 Fc结构域为六聚体蛋白提供稳定性而不促进效应功能例如抗体依赖性细胞毒作用(ADCC)或补体依赖性细胞毒作用。在其他方面中，人IgG1 Fc结构域为六聚体蛋白提供稳定性，同时促进效应功能例如抗体依赖性细胞毒作用(ADCC)或补体依赖性细胞毒作用。

[0129] 在某些方面中，本披露提供自组装以形成能够特异性地结合GITR的六聚体蛋白的单链多肽亚基。示例性多肽亚基包括：人IgG Fc结构域、功能性三聚结构域、和GITRL的受体结合结构域。在某些方面中，该多肽亚基可以自组装成六聚体蛋白。在某些方面中，该多肽亚基(从氨基末端到羧基末端)被安排如下：人IgG Fc结构域，随后是三聚结构域，随后是GITRL受体结合结构域。三个结构域可以是直接毗连的。例如，在某些方面中，人IgG Fc结构域的羧基末端直接融合到三聚结构域的氨基末端，且三聚结构域的羧基末端直接融合到GITRL受体结合结构域的氨基末端。可替代地，两个或三个结构域可以被一个或多个接头、间隔子或其他异源多肽分离。有用的接头包括但不限于(Gly_4)n基序、(Gly_4Ser)n基序(SEQ ID NO:19)、Ser(Gly_4Ser)n基序(SEQ ID NO:22)、GGGGSGGGGSGGGGSAL(SEQ ID NO:23)或GGGGSGGGGSGGGGSA(SEQ ID NO:24)及其组合，其中n是选自下组的正整数，该组由以下各项组成：1、2、3、4、5、6、7、8、9和10。

[0130] 在某些方面中，如在此提供的GITRL融合多肽亚基可以特异性地结合至人GITR。在某些方面中，如在此提供的GITRL融合多肽亚基可以特异性地结合至非人灵长类GITR，例如，食蟹猴GITR或恒河猴GITR。在某些方面中，如在此提供的GITRL融合多肽亚基不结合小鼠GITR或大鼠GITR。

[0131] 如在此提供的GITRL融合多肽亚基可以包含一个或多个保守氨基酸改变，例如，多

达十个保守性变化(例如,两个取代的氨基酸、三个取代的氨基酸、四个取代的氨基酸、或五个取代的氨基酸等),只要可以在该多肽中进行该变化而不改变GITRL融合多肽亚基或多聚体GITRL FP的生化功能。

[0132] 例如,可以在GITRL受体结合结构域中进行一个或多个保守性改变,而不改变其结合GITR能力。同样地,可以在三聚结构域中进行一个或多个保守性变化,而不改变其进行三聚的能力。

[0133] 在此提供的GITRL融合多肽亚基还可以含有阻断或减少GITRL的天冬酰胺酰残基161的N联糖基化的一个或多个氨基酸取代、插入或缺失。在某些方面中,GITRL的天冬酰胺酰残基161被除了天冬酰胺酰残基以外的任何氨基酸取代以阻断或减少糖基化。在某些方面中,GITRL的天冬酰胺酰残基161被天冬氨酰残基取代,例如,如SEQ ID NO:4所示的GITRL的N161D变体。在某些方面中,通过破坏GITRL残基161-163的N联糖基化位点序列NNT的氨基酸取代、插入或缺失来阻断或减少GITRL的天冬酰胺酰残基161的N联糖基化,使得此序列不再符合于典范的N联糖基化位点序列NX(T、S或C)。在某些实施例中,用除了丝氨酰或半胱氨酰以外的氨基酸残基取代苏氨酰残基163可用于阻断或减少GITRL的天冬酰胺酰残基161的N联糖基化,条件是可以在多肽中进行改变而不改变GITRL融合多肽亚基或多聚体GITRL融合蛋白的生物化学功能。

[0134] 另外,一个多肽结构域的一部分可以被缺失而不损害或消除它的全部功能。同样地,如下所述,可以在多肽链中进行插入或添加,例如,添加表位标签,而不损害或消除其功能。可以进行的、不实质性损害多肽的一个或多个功能的其他修饰包括,例如,在体内或体外结合了不常见氨基酸的化学和生化修饰。这样的修饰包括,例如,乙酰化、羧化、磷酸化、糖基化、标记(例如,使用放射性核素)、以及各种酶修饰,如本领域的普通技术人员将很容易理解的。各种用于标记多肽的方法和对于这样的目的有用的标记在本领域中是公知的,并且包括放射性同位素如^{32}P、荧光团、化学发光剂、酶和抗配体。

[0135] 融合多肽亚基可进一步包括异源剂,例如,稳定剂、免疫应答调节剂、或可检测的药剂。在某些方面中,异源剂包括经由肽键融合到多肽亚基的一个或多个另外的多肽序列,例如信号序列(例如,分泌信号序列)、接头序列、氨基酸标签或标记、或有助于纯化的肽或多肽序列。在某些方面中,异源多肽可融合到IgG-Fc结构域的N-末端,异源多肽可融合至GITRL的受体结合结构域的C-末端,异源多肽可融合至IgG-Fc结构域的C-末端和三聚结构域的N-末端,或异源多肽可融合至三聚结构域的C-末端和GITRL的受体结合结构域的N-末端。可替代地,异源多肽可以在任何IgG Fc结构域、三聚结构域、或GITRL受体结合结构域内发生内部融合,只要保持该结构域的功能特性。

[0136] 在某些方面中,可以将异源剂化学轭合至多肽亚基。可以化学轭合至多肽亚基的示例性异源剂包括但不限于,接头、药物、毒素、显像剂、放射性化合物、有机和无机聚合物、以及能够提供不为多肽亚基本身所提供的希望的活性的任何其他组合物。具体的试剂包括但不限于聚乙二醇(PEG)、细胞毒性剂、放射性核素、显像剂、生物素。

[0137] 在某些方面中,GITRL融合多肽亚基以及包括那些亚基的任何三聚体或六聚体蛋白可用作对照、用于开发或执行诊断测定(例如用于给药确定)的参考标准或研究工具。例如,本披露提供了如上所述的GITRL融合多肽亚基,其中GITRL受体结合结构域包括SEQ ID NO:34、35、36或37中的任一个。在某些方面中,对照、参照标准或工具可以包括与SEQ ID

NO:6具有至少70％、80％、85％、90％、95％、96％、97％、98％、99％、99.5％、或100％序列一致性的GITRL融合多肽亚基。在另一个实例中，本披露提供了可以形成如上所述的多聚体蛋白的GITRL融合多肽亚基，但是其中GITRL受体结合结构域是小鼠或大鼠GITRL受体结合结构域，并且Fc结构域是人或鼠来源的Fc结构域，并且多聚结构域是例如三聚结构域，例如冠蛋白1a三聚结构域。这种融合蛋白可用于在啮齿类动物中进行体内实验。不寻求受理论的限制，小鼠中由不同的IgG同种型赋予的IgG Fc结构域效应子功能通常不同于人类中由相同IgG同种型赋予的那些。然而，之前已经显示，认为特异性小鼠IgG同种型与人类中的可选IgG同种型是类似的或可比较的，例如认为小鼠IgG2a与人IgG1是类似的，而认为小鼠IgG1与人IgG4是可比较的。这样，在具有给定小鼠IgG Fc结构域同种型的小鼠中获得的结果可用于预测在具有类似或可比较的人IgG同种型的人中获得的结果。

多聚体GITRL融合蛋白

[0138] 如上所述的GITRL融合多肽亚基可自组装成六聚体GITRL FP。因此，本披露提供包括六个如上所述的多肽亚基的六聚体蛋白。在实例中所描述的示例性多肽亚基自组装成在此指定为“六聚体GITRL FP”的六聚体蛋白。自组装成六聚体GITRL FP的GITRL融合多肽亚基的氨基酸序列的非限制性实例提供于SEQ ID NO:6中。尽管如此，鉴于本披露内容，本领域的普通技术人员将认识到，许多其他序列也满足在此阐述的关于六聚体GITRL FP的标准。在此提供了包括六个GITRL融合多肽亚基的六聚体GITRL FP，该多肽亚基与SEQ ID NO:6具有至少70％、80％、85％、90％、95％、96％、97％、98％、99％、99.5％或100％的序列一致性。

[0139] 在某些方面中，在此提供的某些GITRL融合多肽亚基还可以自组装成包括三个GITRL融合多肽亚基的三聚体GITRL FP。例如，在GITRL FP中使用不能二聚化的Fc结构域来产生三聚体蛋白时，可能发生这种情况。不能二聚化并因此适于生产三聚体GITRL FP的Fc结构域的实例包括但不限于单体IgG1 Fc分子(Ying等人J Biol Chem.Jun 1,2012[生物化学杂志2012年6月1日];287(23):19399-19408)和单价IgG4分子(Wilkinson等人MAbs.2013年5月1日;5(3):406-417)。

[0140] 当GITR在抗原处理的初级T-细胞(例如，来自人、食蟹猴、恒河猴或其任意组合的抗原处理的初级T-细胞)上表达时，如在此提供的多聚体GITRL融合蛋白(例如，六聚体GITRL FP)可以特异性地结合该GITR。

[0141] 如在此提供的六聚体蛋白，例如六聚体GITRL FP，可以特异性地结合重组GITR。在某些方面中，如在此提供的六聚体蛋白(例如六聚体GITRL FP)可以结合重组人GITR，结合亲和力为约1nM至约120nM，例如约10nM至约100nM，例如约20nM至约100nM，例如约60nM至约100nM，所有均如通过动力学排除测定测量的。例如，如在此提供的六聚体蛋白(例如，六聚体GITRL FP)可以结合至重组人GITR，结合亲和力为约0.1nM、约0.5nM、约1nM、约2nM、约3nM、约4nM、约5nM、约6nM、约7nM、约8nM、约9nM、约10nM、约20nM、约30nM、约40nM、约50nM、约60nM、约70nM、约80nM、约90nM、约100nM、约120nM、约250nM、或约500nM，所有均如通过动力学排除测定测量的。在某些方面中，如在此提供的六聚体蛋白(例如，六聚体GITRL FP)可以结合至重组人GITR，结合亲和力为约0.1nM、约0.5nM、约1nM、约2nM、约3nM、约4nM、约5nM、约6nM、约7nM、约8nM、约9nM、约10nM、约20nM、约30nM、约40nM、约50nM、约60nM、或约70nM中任一个至约90nM、约100nM、约120nM、约250nM、或约500nM中任一个，所有均如通过动力学排除

测定测量的。在某些方面中，如在此提供的六聚体蛋白(例如，六聚体GITRL FP)可以结合重组人GITR，结合亲和力为约82nM，如通过动力学排除测定测量的。结合亲和力可通过许多不同的方法和/或仪器进行测量，相对结合亲和力可以依赖于方法或仪器而变化，如本领域的技术人员或普通技术人员将很好地理解的。

[0142] 在另一个实例中，如在此提供的六聚体蛋白(例如GITRL FP)可以结合抗原处理的初级食蟹猴T细胞(例如$CD4^+$或$CD8^+$T细胞)上表达的食蟹猴GITR。

[0143] 在某些方面中，如在此提供的六聚体蛋白(例如，GITRL FP)在基于板的测定法中可诱导抗原处理的$CD3^+$T细胞的剂量依赖性增殖。例如，在体外测定中使用如在此提供的六聚体蛋白(例如，GITRL)，在抗原处理的初级人$CD3^+$T细胞中在约0.03nM至约0.2nM(例如，约0.16nM)的六聚体蛋白浓度下可实现20％最大增殖应答(EC_{20})，在抗原处理的初级人$CD3^+$T细胞中在约0.2nM至约1nM(例如，约0.4nM)的六聚体蛋白浓度下可实现50％最大增殖应答(EC_{50})，并且在抗原处理的初级人$CD3^+$T细胞中在约0.7nM至约5nM(例如，约1.8nM)的六聚体蛋白浓度下可实现90％最大增殖应答(EC_{90})，所有如通过胸苷掺入测量的。

[0144] 在某些方面中，如在此提供的六聚体蛋白(例如，GITRL IgG融合蛋白)可诱导细胞因子从抗原处理的$CD3^+$T细胞(例如，抗原处理的人初级$CD3^+$T细胞)的剂量依赖性释放。在某些方面中，释放的细胞因子是IFNγ、TNFα、IL-5、IL-10、IL-2、IL-4、IL-13、IL-8、IL-12p70、IL-1β、或其任何组合。在某些方面中，该细胞因子是IFNγ、TNFα、IL-5、IL-10、或其任何组合。类似地，如在此提供的六聚体蛋白(例如GITRL FP)可增强抗原处理的初级食蟹猴T细胞和抗原处理的初级恒河猴T细胞中的T细胞增殖和细胞因子释放。

[0145] 在另外的方面中，如在此提供的六聚体蛋白(例如GITRL FP)可以活化表达GITR的T细胞中的NFκB途径。例如，如在此提供的六聚体蛋白(例如，GITRL FP)可以活化表达GITR的Jurkat NFκB荧光素酶报告细胞(这些报告细胞响应于NFκB信号传导途径的刺激而产生荧光素酶)中的NFκB途径，EC_{50}为约20pm至约300pM，例如对于六聚体GITRL IgG1 FP，为约182pM，而对于六聚体GITRL IgG4FP为289pM。可替代地，如在此提供的六聚体蛋白(例如，GITRL FP)可在表达人GITR、食蟹猴GITR或恒河猴GITR的细胞中活化NFκB途径。

[0146] 在又另一个方面中，如在此提供的六聚体蛋白(例如，GITRL FP)，当作为有效剂量向需要癌症治疗的受试者给予时，可以促进癌症治疗，例如通过减缓肿瘤生长、停止肿瘤生长、或减小现有肿瘤的尺寸。在某些方面中，可以在T细胞的存在下实现癌症治疗的促进。在某些方面中，当作为有效剂量向需要治疗的受试者给予时，与给予同种型匹配的对照分子相比，如在此提供的六聚体蛋白(例如，GITRL FP)可以使肿瘤生长减少至少10％、至少20％、至少30％、至少40％、至少50％、至少60％、至少70％、至少80％、至少90％、至少95％、至少98％、或至少100％。

[0147] 在又另一方面中，如在此提供的六聚体蛋白(例如GITRL FP)当作为有效剂量给予需要治疗的受试者(例如感染病毒的受试者)时，可以例如通过减缓病毒增殖、停止病毒增殖或者减少感染复发或感染复发频率来促进病毒感染的治疗。这样的受试者可能具有慢性或潜伏性病毒感染。在某些方面中，这些治疗是对具有潜伏性病毒感染的受试者的治疗，并且与用安慰剂治疗的受试者相比，减少了感染复发或感染复发频率。在某些方面中，可以在T细胞的存在下实现病毒感染治疗的促进。在某些方面中，当作为有效剂量向需要治疗的受试者给予时，与给予同种型匹配的对照分子相比，如在此提供的六聚体蛋白(例如，GITRL

FP)可以使病毒载量减少至少10％、至少20％、至少30％、至少40％、至少50％、至少60％、至少70％、至少80％、至少90％、至少95％、至少98％、或至少100％。在某些方面中，当作为有效剂量向需要治疗的受试者给予时，与给予同种型匹配的对照分子相比，如在此提供的六聚体蛋白(例如，GITRL FP)可以使病毒感染复发率减少至少10％、至少20％、至少30％、至少40％、至少50％、至少60％、或至少70％、至少80％、至少90％、至少95％、至少98％、或至少100％。

[0148] 在又进一步方面中，如在此提供的六聚体蛋白(例如，GITRL IgG4融合蛋白)，可通过结合至GITR来诱导抗原处理的表达GITR的T细胞的增殖，但基本上不触发针对抗原处理的T细胞的补体依赖性或抗体依赖性细胞毒作用。此外，在某些方面中，如在此提供的六聚体蛋白(例如多聚体GITRL IgG1融合蛋白)可通过结合GITR来诱导抗原处理的表达GITR的T细胞的增殖，但确实结合C1q并触发抗原处理的$CD4^+$T细胞(特别是$FOXP3^+CD4^+$调节性T细胞)的Fc受体介导的抗体依赖性细胞毒作用或吞噬作用。

编码GITRL IgG融合多肽亚基的多核苷酸

[0149] 本披露进一步提供了包含编码GITRL融合多肽亚基、或如在此提供的六聚体蛋白(例如，GITRL FP)的核酸的多核苷酸。编码GITRL融合多肽亚基的示例性多核苷酸序列由SEQ ID NO:5表示。在某些方面中，编码IgG Fc结构域、三聚结构域和GITRL受体结合结构域的核酸序列以5'至3'方向连接，例如，以5'至3'方向连续地连接起来。在其他方面中，所提供的多核苷酸可进一步包括信号序列，该信号序列编码，例如，分泌信号肽或膜定位序列。本披露提供了编码任何和所有GITRL融合多肽亚基或多聚体(例如，包含该亚基的六聚体蛋白)的多核苷酸。

[0150] 在某些方面，本披露提供了包含编码GITRL融合多肽亚基的核酸的多核苷酸。在某些方面中，核酸序列包含SEQ ID NO:5。

[0151] 编码如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如GITRL FP)的多核苷酸包括编码在此描述的融合多肽的脱氧核糖核苷酸(DNA，cDNA)或核糖核苷酸(RNA)序列、或任一核苷酸的修饰形式。该术语包括单链和双链形式的DNA和/或RNA。

[0152] 还提供包含含有一个或少数个缺失、添加和/或取代的核酸序列的多核苷酸。这样的改变可以是连续的，或者可以分布在该核酸的不同位置。基本上相同的核酸序列可以，例如，具有1个、或2个、或3个、或4个、或甚至更多的核苷酸缺失、添加和/或取代。在某些方面中，一个或更多个缺失、添加和/或取代不改变由多核苷酸序列编码的阅读框，使得修饰的(“突变体”)但基本上相同的多肽经核酸的表达而产生。

[0153] 氨基酸(和/或核酸)的序列之间的相似性按照序列之间的相似性来表达，否则被称为序列一致性。序列一致性经常按照一致性(或相似性)百分比来测量；百分比越高，两个序列的初级活化结构越相似。“一致性百分比(％)”在此被定义为在比对序列并且(必要时)在候选和/或选择的序列中引入空位以便获得最大的序列一致性百分比之后，并且不考虑将任何保守氨基酸取代作为序列一致性的一部分，在候选序列中与所选择的序列中的氨基酸残基相同的氨基酸残基的百分比。

[0154] 因此，包括含有编码如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如，GITRL FP)的核酸的多核苷酸可以是至少约70％、至少约75％、至少约80％、至少约85％、至少约90％、至少约95％、或至少96％、经常至少97％、98％、或99％相同于SEQ ID NO:5或相同于

其中的至少一个子序列。出于确定百分比同源性(即,序列相似性)或一致性百分比的目的的比对可以用在本领域的技术范围内的各种方式(例如,使用公众或专有算法)来实现。例如,可使用成对比对方法来确定序列相似性,这些成对比对方法例如是BLAST、BLAST-2、ALIGN、或ALIGN-2、或多重序列比对方法,如Megalign(DNASTAR)、ClustalW或T-咖啡(T-Coffee)软件。本领域的技术人员可确定适当的计分函数,例如,缺口罚分或得分矩阵用于测量比对,包括在被比较序列的全长上实现最佳比对质量所需的任何算法。此外,可以用结构比对方法(例如,使用二级或三级结构信息来对齐两个或更多个序列的方法),或组合序列、结构和系统发育信息来鉴定和最佳对齐候选蛋白质序列的混合法来实现序列比对。

[0155] NCBI基本局部比对搜索工具(BLAST)(Altschul等人,J.Mol.Biol.[分子生物学杂志](1990)215:403)可从若干来源获得,这些来源包括国家生物技术信息中心(NCBI,贝塞斯达,马里兰州)和在互联网上,用于与序列分析程序blastp、blastn、blast、tblastn和tblastx连接使用。如何使用这个程序确定序列一致性的描述在互联网的NCBI网站上可获得。

[0156] 因此,基本上相同或基本上相似于SEQ ID NO:5的核酸序列被涵盖在本披露之内。如果序列是以逐个核苷酸为基础与参比序列(例如,SEQ ID NO:4)的至少一个子序列相同,则序列基本上与SEQ ID NO:5相同。这样的核酸可以包括,例如,相对于SEQ ID NO:4的插入、缺失和取代。例如,这些核酸可以与参比核酸至少约70%、80%、90%、95%、96%、97%、98%、或甚至99%相同,或可以编码与参考多肽序列(例如,SEQ ID NO:4)至少70%、80%、90%、95%、96%、97%、98%、或甚至99%相同的多肽。

[0157] 另外,包括编码如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如,GITRL FP)的核酸的多核苷酸也可以包括多核苷酸序列,例如,促进核酸的表达或复制的表达调节序列和/或载体序列。同样地,包括编码如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如,GITRL FP)的核酸的多核苷酸可以包括对所编码的多肽赋予功能属性的另外的编码序列。这样的序列包括但不限于分泌信号序列和膜定位信号。SEQ ID NO:7中提供了编码如下信号肽的核酸的非限制性实例,该信号肽与编码GITRL融合多肽亚基的核酸是可操作连接的。

[0158] 可以通过常规技术将包括编码如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如,GITRL FP)的核酸的多核苷酸引入载体,例如真核表达载体。因此,本披露提供了包含在此提供的多核苷酸的载体。将表达载体设计来通过提供启动和增强cDNA的转录和保证其正确的剪接和聚腺苷酸化的调节序列允许编码如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如,GITRL FP)的多核苷酸序列在细胞中的转录。许多表达载体是对本领域的普通技术人员而言已知的,并且是商业上可获得的,或可以根据传统的分子生物学程序从个别组件组装的。

[0159] 表达控制序列和表达载体的选择将取决于宿主细胞的选择。可以采用多种多样的表达宿主/载体组合。对于真核宿主有用的表达载体包括例如包含来自SV40、牛乳头瘤病毒、腺病毒、以及巨细胞病毒的表达控制序列的载体。对于细菌宿主有用的表达载体包括已知细菌质粒,诸如来自大肠杆菌的质粒,包括pCR 1、pBR322、pMB9以及其衍生物,更广泛的宿主范围质粒,诸如M13和丝状单链DNA噬菌体。

[0160] 对于表达如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如,GITRL FP)的合

适的宿主细胞包括在适当的启动子控制下的原核生物、酵母、昆虫或高等真核细胞。原核细胞包括革兰阴性或革兰阳性生物体，例如大肠杆菌或杆菌。高等真核细胞包括如下所述哺乳动物起源的已建立的细胞系。还可以采用无细胞翻译系统。关于蛋白质产生(包括抗体产生)方法的其他信息可以在例如美国专利公开号2008/0187954、美国专利号6,413,746和6,660,501、以及国际专利公开号WO 2004/009823中找到，这些专利各自通过引用以其全文结合在此。

[0161] 还提供的是包括如在此提供的多核苷酸或载体的宿主细胞。还可以有利地采用不同的哺乳动物或昆虫细胞培养系统，以表达在此提供的多肽亚基或六聚体蛋白。可以在哺乳动物细胞中进行重组蛋白质的表达，因为此类蛋白质总体上被正确地折叠，适当地修饰并且完全起作用。适合的哺乳动物宿主细胞系的实例包括HEK-293和HEK-293T、由格卢兹曼(Gluzman)(细胞23:175,1981)描述的猴肾细胞的COS-7系、以及包括例如L细胞、C127、3T3、中国仓鼠卵巢(CHO)、HeLa以及BHK细胞系的其他细胞系。哺乳动物表达载体可以包括非转录元件(如复制起点)、连接至待表达的基因上的合适的启动子和增强子以及其他5'或3'侧翼非转录序列和5'或3'非翻译序列，如必要的核糖体结合位点、聚腺苷酸化位点、剪接供体和受体位点以及转录终止序列。用于产生昆虫细胞中的异源蛋白质的杆状病毒系统通过Luckow和Summers，BioTechnology[生物技术]6:47(1988)评价。

[0162] 蛋白质例如如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如，GITRL FP)的表达和纯化可以使用标准的实验室技术进行。在此讨论或参考了这样的方法的实例。表达后，纯化的蛋白质有许多用途，包括例如功能分析、抗体产生和诊断，以及如下所述的预防和治疗用途。例如，在此提供的多肽亚基或六聚体蛋白可被用于生产药物组合物，包括适合用于预防和/或治疗给药的疫苗组合物。

[0163] 通过转化的宿主产生的如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如，GITRL FP)可以按照任何合适的方法来纯化。此类标准方法包括色谱法(例如，离子交换色谱、亲和色谱以及尺寸分级柱色谱)、离心、差别溶解度，或者通过用于蛋白质纯化的任何其他标准技术。亲和标签诸如六组氨酸、麦芽糖结合结构域、流感外壳序列以及谷胱甘肽-S-转移酶可以附接至蛋白质，以便允许蛋白质通过合适的亲和柱后较容易地得到纯化。还可以使用诸如蛋白质水解、核磁共振和x射线结晶的此类技术来物理性表征分离的蛋白质。

[0164] 例如，可以首先使用商业上可获得的蛋白浓缩过滤器例如艾美康恩(Amicon)或密理博皮里康恩(Millipore Pellicon)超滤单元浓缩来自将重组蛋白分泌到培养基中的系统的上清液。浓缩步骤之后，浓缩物可以施加到适合的纯化基质。可替代地，可以采用阴离子交换树脂，例如具有侧接的二乙氨基乙基(DEAE)基团的基质或基底。基质可以是丙烯酰胺、琼脂糖、右旋糖酐、纤维素或在蛋白质纯化中常用的其他类型。可替代地，可以采用阳离子交换步骤。适合的阳离子交换剂包括含有磺丙基或羧甲基的各种不可溶基质。最后，可以使用采用疏水性RP-HPLC介质(例如，具有侧接甲基或其他脂族基团的硅胶)的一个或多个反相高效液相色谱(RP-HPLC)步骤，以便进一步纯化流感B/山形病毒结合分子。还可以采用不同组合的一些或所有上述纯化步骤以便提供均质的重组蛋白。

[0165] 在细菌培养物中产生的如在此提供的GITRL融合多肽亚基或六聚体蛋白(例如，GITRL FP)可以例如通过以下方法分离：最初从细胞沉淀提取，然后进行一次或多次浓缩、盐析、水性离子交换或尺寸排阻色谱步骤。可以采用高效液相色谱(HPLC)进行最终纯化步

骤。用于重组蛋白表达的微生物细胞可以通过任何常规方法来破坏，这些方法包括冻融循环、超声处理、机械破坏或使用细胞溶解剂。

药物组合物和给药方法

[0166] 制备如在此提供的六聚体蛋白(例如，如在此提供的GITRL FP)的方法和将其给予至对其有需要的受试者例如以便增强在癌症患者中的免疫应答的方法，例如以便抑制或减少肿瘤生长的方法，或给予至具有病毒感染的患者例如以便减少病毒载量或减少病毒感染复发率的方法，是本领域的技术人员熟知的或可以容易地确定的。如在此提供的六聚体蛋白(例如，GITRL FP)的给药途径可以是，例如，口服、肠胃外、通过吸入或局部。如在此所用的术语肠胃外包括例如静脉内、动脉内、腹膜内、肌内、皮下、直肠或经阴道给药。虽然所有这些给药形式可清楚地考虑为适合的形式，但对于给予形式的另一个实例是用于注射、特别是用于静脉内或动脉内注射或滴注的溶液。通常，合适的药物组合物可以包括但不限于缓冲液(例如乙酸盐、磷酸盐或柠檬酸盐缓冲液)、表面活性剂(如聚山梨酯)、稳定剂试剂(例如人白蛋白)等。在与在此的传授内容相容的其他方法中，如在此提供的六聚体蛋白(例如，如在此提供的GITRL FP)可直接递送到有害细胞群的位点，从而增加患病组织对治疗剂的暴露。

[0167] 在此提供的某些药物组合物可以按一种可接受的剂型(包括例如胶囊、片剂、水性混悬液或溶液)来口服给予。某些药物组合物也可以通过鼻气雾剂或吸入来给予。使用苄醇或其他合适的防腐剂、提高生物利用度的吸收促进剂、和/或其他常规增溶剂或分散剂，这样的组合物可以作为盐水中的溶液来制备。

[0168] 可与载体材料相组合以便产生单一剂型的如在此提供的六聚体蛋白(例如，GITRL FP)的量将取决于所治疗的受试者和具体给予模式而变化。该组合物可以作为单次剂量，多次剂量或在一个既定时间段中的输注给药。也可以调整剂量方案以便提供最佳期望应答(例如治疗性或预防性应答)。

[0169] “治疗有效剂量或量”或“有效量”意指如在此提供的六聚体蛋白(例如，GITRL FP)当被给予时引起对患有待治疗疾病或病症的治疗的积极治疗应答的量。

试剂盒

[0170] 本披露进一步提供包含如在此提供的六聚体蛋白(例如，在此描述的GITRL FP)和可用于执行在此描述的方法的试剂盒。在某些实施例中，试剂盒在一个或多个容器中包含至少一种纯化的如在此提供的六聚体蛋白(例如，GITRL FP)。本领域的技术人员将容易地认识到所披露的、如在此提供的六聚体蛋白(例如，GITRL FP)可以容易地与在本领域中熟知的已建立的试剂盒形式之一结合。

免疫测定

[0171] 如在此提供的六聚体蛋白(例如，GITRL FP)可以通过本领域中已知的任何方法测定特异性和/或选择性结合。可使用的免疫测定包括但不限于使用如以下技术的竞争性和非竞争性测定系统，例如：蛋白质印迹、放射免疫测定、ELISA(酶联免疫吸附测定)、荧光集落测定(FFA)、微量中和测定、血凝抑制测定(HAI)、“夹心”免疫测定、免疫沉淀测定、沉淀素反应、凝胶扩散沉淀素反应、免疫扩散测定、凝集测定、补体固定测定、免疫放射测定、荧光免疫测定、仅举几个例子。此类测定是常规的并且在本领域是熟知的(参见例如，Ausubel等人编辑(1994)Current Protocols in Molecular Biology(John Wiley&Sons,Inc.,NY)

Vol.1[当前分子生物学方案(约翰威利父子公司,纽约)第1卷],其通过引用以整体结合在此)。FFA,微量中和测定,和HAI将在以下实例中进行详细讨论。

[0172] 适合于确定在此提供的六聚体蛋白的结合特性的方法和试剂是在本领域中已知的和/或可商购的。设计用于此类动力学分析的设备和软件是可商购的(例如,BIAcore®, BIAevaluation®软件,GE医疗集团(GE Healthcare);KENEXA®软件,Sapidyne仪器)。

免疫增强和治疗的方法

[0173] 在抗原活化过程中或之后通过在抗原处理的T细胞(例如,抗原处理的$CD4^+$或$CD8^+$T细胞)上接合GITR在受试者(例如,哺乳动物受试者,如人受试者)内增强抗原特异性免疫应答可以利用各种各样的方法来实现。选择的方法将主要取决于期望增强免疫应答的抗原的类型,并且可用的各种方法将在下面讨论。无论哪种方法被选中,可向受试者(例如人类受试者)给予如在此提供的六聚体蛋白(例如,GITRL FP)使得在T细胞被抗原致敏过程中或之后不久它呈现于受试者的T细胞。使用OX40六聚体蛋白活化受试者(例如人受试者)中的免疫应答的示例方法在US公开号2016/0024176(将其通过引用以其全文结合在此)中呈现,并且可以适用于在此提供的GITRL FP。

[0174] 在某些方面中,本披露提供了促进抗原处理的T细胞(例如,抗原处理的$CD4^+$或$CD8^+$T细胞)存活或增殖的方法,该方法包括在该六聚体蛋白可特异性地结合在T细胞(例如,抗原处理的$CD4^+$或$CD8^+$T细胞)表面上的GITR的条件下使抗原处理的T细胞(例如,抗原处理的$CD4^+$或$CD8^+$T细胞)接触如在此提供的六聚体蛋白(例如,GITRL FP)。在某些方面中,该接触是体外的。在某些方面中,该接触是体内的,例如,经由向需要治疗的受试者给予有效剂量的六聚体蛋白。在某些方面中,该接触与T-细胞活化(例如,抗原活化)同时发生,在某些方面中,该接触在T-细胞活化后发生。

[0175] 在另外的方面中,本披露提供了增强自抗原处理的T细胞(例如,抗原处理的$CD4^+$或$CD8^+$T细胞)的细胞因子释放的方法,该方法包括使抗原处理的T细胞(例如,抗原处理的$CD4^+$或$CD8^+$T细胞)接触如在此提供的六聚体蛋白(例如,FP),其中该六聚体蛋白可特异性地结合在抗原处理的T细胞(例如,抗原处理的$CD4^+$或$CD8^+$T细胞)表面上的GITR。在某些方面中,该接触是体外的。在某些方面中,该接触是体内的,例如,经由向需要治疗的受试者给予有效剂量的六聚体蛋白。在某些方面中,该接触与T-细胞活化(例如,抗原活化)同时发生,在某些方面中,该接触在T-细胞活化后发生。在某些方面中,该细胞因子可以是IFNγ、TNFα、IL-5、IL-10、IL-2、IL-4、IL-13、IL-8、IL-12p70、IL-1β、GM-CSF或其任何组合。在某些方面中,该细胞因子是IFNγ、TNFα、IL-5、IL-10、IL-4、IL-13、GM-CSF或其任何组合。

[0176] 在某些方面中,抗原处理的T细胞(例如,抗原处理的$CD4^+$或$CD8^+$T细胞)是人$CD4^+$或$CD8^+$T细胞、食蟹猴$CD4^+$或$CD8^+$T细胞、恒河猴$CD4^+$或$CD8^+$T细胞或其组合。

[0177] 本披露进一步提供促进T-细胞活化的方法,该方法包括使T-细胞接触如在此提供的六聚体蛋白(例如,GITRL融合蛋白),其中该六聚体蛋白可以特异性地结合T-细胞表面上的GITR。在某些方面中,在抗原,例如,肿瘤抗原的存在下接触发生。在某些方面中,该方法进一步包括通过GITRL FP的IgG-Fc结构域与表达FcγR的细胞的相互作用使六聚体GITRL融合蛋白交联,这些表达FcγR的细胞例如是:B细胞、单核细胞、巨噬细胞、髓样树突状细胞或浆细胞样树突状细胞、滤泡树突状细胞、朗格汉斯细胞、内皮细胞、NK细胞、嗜中性粒细

胞、嗜酸性粒细胞、血小板、肥大细胞、来自原发人肿瘤或肿瘤引流或非引流淋巴结的$CD45^+$细胞、来自其他二级或三级淋巴样结构的$CD45^+$细胞，或它们的组合。在某些方面中，T-细胞活化可以按NFκB信号传导途径的刺激进行测量。在某些方面中，促进T-细胞活化的GITRL FP是GITRL IgG1 FP、GITRL IgG4 FP或其变体。在某些方面中，该接触是体外的。在某些方面中，该接触是体内的，例如，经由向需要治疗的受试者给予有效剂量的六聚体蛋白。

[0178] 还在此提供了用于治疗癌症的方法，这些方法包括给予GITRL FP和OX40激动剂(例如，OX40配体融合蛋白或OX40激动剂抗体)。在小鼠肿瘤模型中，给予GITRL FP和OX40配体融合蛋白导致肿瘤体积的减小和存活期的增加。在某些方面中，对呈现有实体瘤的患者给予GITRL FP和OX40配体融合蛋白(例如，MEDI6383)。

[0179] 使用癌症疗法(包括GITRL FP和OX40激动剂)的有效治疗包括例如降低癌症的进展速率，阻滞或稳定化原发性肿瘤部位处或一个或多个转移中的肿瘤或转移生长、肿瘤收缩、和/或肿瘤消退。在一些方面中，肿瘤生长的降低或阻滞可以是统计学上显著的。可以通过与基线处的患者肿瘤的生长、针对预期的肿瘤生长、针对基于大的患者群体的预期肿瘤生长或针对对照群体的肿瘤生长进行比较来测量肿瘤生长的减少。在其他实施例中，本发明的这些方法增加存活。

[0180] 对给予癌症疗法(包括GITRL FP和OX40激动剂)的临床应答可以使用临床医生已知的诊断技术来评估，这些诊断技术包括但不限于磁共振成像(MRI)扫描、x-射线照相成像、计算机断层照相(CT)扫描、流式细胞术或荧光活化的细胞分选器(FACS)分析、组织学、宏观病理学、以及血液化学，包括但不限于可通过ELISA、RIA和色谱法检测到的变化。

[0181] 本披露进一步提供在受试者中治疗癌症或病毒感染的方法，该方法包括向需要治疗的受试者给予有效量的在此提供的六聚体蛋白(例如，GITRL FP)或包含六聚体蛋白的组合物或配制品。在某些方面中，该癌症是实体瘤。根据这个方法，该六聚体蛋白或组合物的给予可以抑制肿瘤生长；可以促进肿瘤缩小、或两者。在某些方面中，在T细胞存在下实现肿瘤生长抑制。

[0182] 术语“癌症”、“肿瘤”、“癌性”、和“恶性”指或描述在哺乳动物中典型地是特征为不受控制的细胞生长的生理病症。癌症的实例包括但不限于上皮癌，包括腺癌、淋巴瘤、胚细胞瘤、黑色素瘤、肉瘤、和白血病。此类癌症的更具体的实例包括鳞状细胞癌、小细胞肺癌、非小细胞肺癌、胃肠癌、霍奇金氏淋巴瘤和非霍奇金氏淋巴瘤、胰腺癌、成胶质细胞瘤、神经胶质瘤、宫颈癌、卵巢癌、肝癌(诸如肝癌和肝细胞瘤)、膀胱癌、乳腺癌(包括激素介导的乳腺癌，参见例如，Innes等人(2006)Br.J.Cancer[英国癌症杂志]94:1057-1065)、结肠癌、结直肠癌、子宫内膜癌、骨髓瘤(诸如多发性骨髓瘤)、唾液腺癌、肾癌(诸如肾细胞癌和维尔姆斯氏瘤)、基底细胞癌、黑色素瘤、前列腺癌、外阴癌、甲状腺癌、睾丸癌、食道癌、血癌(包括但不限于急性髓细胞性白血病(AML)和多发性骨髓瘤(MM))、各种类型的头颈癌(包括但不限于鳞状细胞癌)、以及粘液性起源的癌症(诸如粘液性卵巢癌)、胆管癌(肝)以及肾乳头状癌。在某些实施例中，该血癌选自下组，该组由以下各项组成：霍奇金氏淋巴瘤、非霍奇金氏淋巴瘤、多发性骨髓瘤、急性成淋巴细胞性白血病、急性骨髓性白血病、慢性淋巴细胞性白血病、以及慢性骨髓性白血病。

[0183] 一些实施例针对在有需要的受试者中预防或治疗癌症或病毒感染的方法，该方法包括向受试者给予有效量的如在此提供的六聚体蛋白(例如，GITRL FP)、包含该六聚体蛋

白的组合物或配制品、或如在此所述的多核苷酸、载体、或宿主细胞。

[0184] 提供了在有需要的受试者中治疗病毒感染的方法，该方法包括向该受试者给予有效量的六聚体蛋白或包含该六聚体蛋白的组合物。在某些实施例中，病毒感染可以是慢性或潜伏性病毒感染。这种慢性病毒感染是由发生病毒体增殖的数周、数月或数年的病毒感染表征的病毒感染。这种潜伏性病毒感染是由病毒体不增殖的一段时间表征的病毒感染。在某些实施例中，可以通过在受试者或从受试者获得的样品中进行针对病毒存在的分析诊断来鉴定有需要的受试者。在某些实施例中，该治疗可以提供病毒载量的减少、病毒再活化的减少、与所述感染相关的症状的改善或其组合。在某些实施例中，病毒载量、再活化或症状的减少是与用安慰剂治疗的对照受试者相比。在任何上述方法的某些实施例中，病毒感染是由选自下组的病毒造成，该组由以下各项组成：人免疫缺陷病毒(HIV)、乙型肝炎病毒、丙型肝炎病毒、麻疹、埃-巴二氏病毒(EBV)、巨细胞病毒(CMV)、腺病毒(AdV)、人乳头瘤病毒(HPV)、单纯疱疹病毒(HSV)、水痘-带状疱疹病毒(VZV)及其组合。

[0185] 本披露的组合物可以通过任何合适方法来给予，例如肠胃外、心室内、口服、通过吸入喷雾剂、局部、直肠、经鼻、经颊、经阴道或经由植入的储槽。如在此使用的术语“肠胃外”包括皮下、静脉内、肌内、关节内、滑膜内、胸骨内、鞘内、肝内、病灶内以及颅内注射或输注技术。

[0186] 本披露进一步提供增强受试者的免疫应答的方法，该方法包括向对其有需要的受试者给予治疗有效量的如在此提供的六聚体蛋白(例如，GITRL FP)或包含六聚体蛋白的组合物或配制品。

[0187] 待治疗的受试者可以是需要治疗的任何动物，例如，哺乳动物，在某些方面中，受试者是人受试者。

[0188] 在其最简单的形式中，将向受试者给予的制剂是以常规的剂量形式给予的如在此提供的六聚体蛋白(例如，GITRL FP)，并且优选地，与如在此别处描述的药用赋形剂、载体或稀释剂组合。

[0189] 如在此提供的六聚体蛋白(例如，GITRL FP)可以通过如在此别处所述的任何合适的方法给予，例如，通过静脉内输注给予。在某些方面中，如在此提供的六聚体蛋白(例如，GITRL FP)可被引入到肿瘤中或引入到肿瘤细胞附近。

[0190] 所有类型的肿瘤都潜在适合于通过这个方法来治疗，这些肿瘤包括但不限于乳腺癌、肺癌、胰腺癌、卵巢癌、肾癌、结肠癌和膀胱癌、以及黑色素瘤、肉瘤和淋巴瘤。

T细胞引发剂

[0191] 提供了在有需要的受试者中治疗癌症的方法，该方法包括向该受试者给予有效量的六聚体蛋白或包括与T细胞引发剂组合的六聚体蛋白的组合物。在某些方面中，该T-细胞引发剂是DNA疫苗加佐剂。在具体的方面中，这种组合是例如E7合成长肽(SLP)和CpG寡脱氧核苷酸。在另外的方面中，该T细胞引发剂是表观遗传修饰剂，例如5-氮杂-2'-脱氧胞苷或组蛋白修饰剂，例如HDAC抑制剂。在另外的方面中，该T细胞引发剂是病毒，例如牛痘、李斯特菌属或新城疫病毒。

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[0192] 除非另外指示，否则本披露采用细胞生物学、细胞培养、分子生物学、转基因生物学、微生物学、重组DNA以及免疫学的常规技术，这些技术在本领域的技能范围内。此类技术

在文献中得到充分解释。参见，例如，Sambrook等人编辑(1989)，Molecular Cloning A Laboratory Manual[分子克隆：实验室手册](第2版，冷泉港实验室出版社(Cold Spring Harbor Laboratory Press))；Sambrook等人编辑(1992)Molecular Cloning:A Laboratory Manual[分子克隆：实验室手册]，(冷泉港实验室(Cold Spring Harbor Laboratory)，纽约)；D.N.Glover编辑(1985)DNA Cloning[DNA克隆]，第I卷和第II卷；Gait编辑(1984)Oligonucleotide Synthesis[寡核苷酸合成]；Mullis等人，美国专利号4,683,195；Hames和Higgins编辑(1984)Nucleic Acid Hybridization[核酸杂交]；Hames和Higgins编辑(1984)Transcription And Translation[转录与翻译]；Freshney(1987)Culture Of Animal Cells[动物细胞的培养](Alan R.Liss公司)；Immobilized Cells And Enzymes[固定化细胞与酶](IRL出版社)(1986)；Perbal(1984)A Practical Guide To Molecular Cloning[分子克隆实用指南]；专题论文，Methods In Enzymology[酶学方法]，(学术出版社公司，纽约州)；Miller和Calos编辑(1987)Gene Transfer Vectors For Mammalian Cells[哺乳动物细胞的基因转移载体]，(冷泉港实验室)；Wu等人编辑，Methods In Enzymology[酶学方法]，第154卷和第155卷；Mayer和Walker编辑(1987)Immunochemical Methods In Cell And Molecular Biology[细胞与分子生物学中的免疫化学方法](学术出版社，伦敦)；Weir和Blackwell编辑(1986)Handbook Of Experimental Immunology[实验免疫学手册]，第I-IV卷；Manipulating the Mouse Embryo[操纵小鼠胚胎]，冷泉港实验室出版社，冷泉港，纽约州，(1986)；以及Ausubel等人(1989)Current Protocols in Molecular Biology[当代分子生物学方案](John Wiley and Sons(约翰威利父子公司)，巴尔的摩，马里兰州)。

[0193] 在Borrebaeck编辑(1995)Antibody Engineering(2nd ed.;Oxford Univ.Press)[抗体工程(第二版；牛津大学出版社)]中，提出了抗体工程的普遍原理。蛋白质工程化的一般原理列举在Rickwood等人编辑(1995)Protein Engineering,A Practical Approach(IRL Press at Oxford Univ.Press,Oxford,Eng.)[蛋白质工程化，实用方法(牛津大学出版社的IRL出版社，牛津，英国)]中。在Nisonoff(1984)Molecular Immunology(2nd ed.;Sinauer Associates,Sunderland,Mass.)[分子免疫学(第2版；西诺埃联合公司，桑德兰，马萨诸塞州)]；以及Steward(1984)Antibodies,Their Structure and Function(Chapman and Hall,New York,N.Y.)[抗体，它们的结构和功能(Chapman和Hall，纽约，纽约州)]中阐明了抗体和抗体半抗原结合的普遍原理。另外，本领域中已知的免疫学标准方法，不再做具体的描述，大体上遵循以下：Current Protocols in Immunology,John Wiley&Sons,New York[免疫学现代方法，约翰威利父子公司，纽约]；Stites等人编辑(1994)Basic and Clinical Immunology(8th ed;Appleton&Lange,Norwalk,Conn.)[基础和临床免疫学(第8版；阿普尔顿和兰格公司，诺瓦克，康涅狄格州)]；以及Mishell和Shiigi(编辑)(1980)Selected Methods in Cellular Immunology(W.H.Freeman and Co.,NY)[细胞免疫学中所选择的方法(W.H.弗里曼与公司，纽约州)]。

[0194] 列举免疫学的一般原理的标准参考工作包括Current Protocols in Immunology,John Wiley&Sons,New York[当代免疫学实验技术，约翰威利父子公司，纽约]；Klein(1982)J.,Immunology:The Science of Self-Nonself Discrimination(John Wiley&Sons,NY)[免疫学：自我-非自身辨别的科学(约翰威利父子公司，纽约)]；Kennett等

人编辑(1980)Monoclonal Antibodies,Hybridoma:A New Dimension in Biological Analyses(Plenum Press,NY)[单克隆抗体,杂交瘤:生物分析的一个新维度(普莱南出版社,纽约)];Campbell(1984)"Monoclonal Antibody Technology"in Laboratory Techniques in Biochemistry and Molecular Biology,ed.Burden et al.,(Elsevier,Amsterdam)["单克隆抗体技术",生物化学和分子生物学实验室技术,Burden等人编辑(爱思唯尔,阿姆斯特丹)];Goldsby等人编辑(2000)Kuby Immunology(4th ed.;H.Freemand&Co.)[Kuby免疫性(第4版;H.弗里曼出版社)];Roitt等人(2001)Immunology(6th ed.;London:Mosby)[免疫学(第6版;伦敦:莫斯比出版社)];Abbas等人(2005)Cellular and Molecular Immunology(5th ed.;Elsevier Health Sciences Division)[细胞与分子免疫学(第5版;爱思唯尔健康科学部)];Kontermann和Dubel(2001)Antibody Engineering(Springer Verlag)[抗体工程化(施普林格维拉格出版社)];Sambrook和Russell(2001)Molecular Cloning:A Laboratory Manual(Cold Spring Harbor Press)[分子克隆:实验室手册(冷泉港出版社)];Lewin(2003)Genes VIII(Prentice Hall 2003)[基因VIII(普伦蒂斯·霍尔出版社2003)];Harlow和Lane(1988)Antibodies:A Laboratory Manual(Cold Spring Harbor Press)[抗体:实验室手册(冷泉港出版社)];Dieffenbach和Dveksler(2003)PCR Primer(Cold Spring Harbor Press)[PCR引物(冷泉港出版社)]。

[0195] 以上引用的所有参考文献,连同在此引用的所有参考文献,是通过引用以其全文结合在此。

[0196] 以说明的方式而不是以限制的方式提供以下实例。

实例

表B:缩略词列表与术语定义

缩略词或术语	定义
A	丙氨酸
ADCC	抗体依赖性细胞毒作用

°C	摄氏度
CDC	补体依赖性细胞毒作用
CR	完全应答
Cyno	食蟹猴
F	苯丙氨酸
FACS	荧光激活细胞分选
FBS	牛胎儿血清
LC-QTOF MS	液相色谱与四重飞行时间质谱联用
M	摩尔浓度
mAb	单克隆抗体
hGITRL	人 GITRL
μg	微克
MFI	荧光强度均值
min	分钟
mL	毫升
多聚体 mGITRL FP	多聚体小鼠 GITR 配体小鼠 IgG 融合蛋白
NIP228	针对 4-羟基-3-碘代-5-硝基苯乙酸的人单克隆抗体
NK	自然杀伤
PBS	磷酸盐缓冲盐水
pM	皮摩尔
RBD	受体结合结构域
Rh	重组人
ROA	给药途径
rpm	每分钟转动次数
RT	室温

SC	皮下
SD	标准偏差
TCR	T细胞受体
TGI	肿瘤生长抑制
TNFR	肿瘤坏死因子受体
Treg	T调节性
V	体积

实例1:GITRL IgG Fc融合蛋白的工程化

可用于产生多聚体人GITRL-FP分子的合适的人三聚化基序的鉴定

[0197] 对产生人六聚体GITRL FP的努力最初集中于使用GCN4pII三聚化基序来稳定GITRL三聚体并形成六聚体蛋白。然而,此基序源自酵母蛋白质,并且将含有非人类基序的GITRL FP给予人可导致免疫原性。因此,认为理想的是产生含有源自人蛋白质的三聚化基序的等同GITRL FP。通过蛋白质数据库(PDB;可通过互联网经由万维网站"wwpdb.org"访问)鉴定来自形成三聚体卷曲螺旋基序的51个蛋白质的氨基酸序列,如其三维晶体结构或直向同源物的那些所证明的,并且对于经验性实验测试,选择4个卷曲螺旋基序的子组并入GITRL FP分子中。

方法

[0198] 使用PyMol Visualization Graphics软件分析了冠蛋白-1A(PDB代码:2akf)、母系蛋白1(1aq5)、朗格汉斯蛋白(3kqg)和营养不良性肌强直激酶DMPK(PDB代码:1wt6)卷曲螺旋基序的三维晶体结构。

结果

[0199] 在蛋白质数据库(PDB)搜索了鉴定51个候选序列的三聚体卷曲螺旋蛋白质序列之后,选择了四个序列用于产生多聚体GITRL FP(表1-1)。这四个序列是来自人蛋白质冠蛋白1a、母系蛋白1、朗格汉斯蛋白和DMPK的卷曲螺旋序列。据报道来自小鼠冠蛋白1a(PDB代码:2akf)的卷曲螺旋基序的结构在溶液中是稳定的三聚体(Kammerer,R.A.等人PNAS,卷:102,第13891-13896页(2005))。人冠蛋白1a卷曲螺旋序列显示与小鼠蛋白的高度序列一致性(78.1%序列一致性),所以预测此序列形成三聚体卷曲螺旋结构。类似地,据报道来自鸡母系蛋白1(PDB代码:1aq5)的卷曲螺旋基序的结构是三聚体(Dames,S.A.等人NAT.STRUCT.BIOL.[自然结构生物学]5:687-691(1998);PDB代码:1aq5)。预测人母系蛋白1卷曲螺旋序列基于其与鸡直向同源物的高度序列一致性(60.0%序列一致性)形成三聚体

卷曲螺旋结构。据报道人朗格汉斯蛋白(PDB代码:3kqg)和人DMPK(PDB代码:1wt6)卷曲螺旋序列是三聚体(Feinberg,H.等人,J.BIOL.CHEM.[生物化学杂志]285:13285-13293(2010)),并被选择用于产生多聚体人GITRL融合蛋白。

[0200] 为了提供用于GITRL融合蛋白的另外的卷曲螺旋序列,产生来自人冠蛋白-1A、母系蛋白-1、朗格汉斯蛋白和DMPK的野生型卷曲螺旋序列的变体。为了产生这些变体序列,使用ProCoil算法的在线实现(可通过互联网经由万维网站"bioinf.jku.at/software/procoil/"访问)。此算法预测给定序列形成二聚体和三聚体卷曲螺旋结构的概率。与野生型序列相比,上面提到的野生型卷曲螺旋序列的几种变体被预测具有形成三聚体的更高概率评分。选择具有成为三聚体的最高可能性的变体序列用于产生六聚体GITRL FP。

[0201] 上述四种人蛋白质的变体卷曲螺旋序列示于表1-1。

表1-1:在GITRL FP构建体中用作三聚体卷曲螺旋基序的序列

三聚化基序	序列和 SEQ ID NO
GCN4 pII	IKQIEDKIEEILSKIYHIENEIARIKKL (SEQ ID NO: 27)
母系蛋白 1 wt	CACESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVV (SEQ ID NO: 28)
母系蛋白 1 变体	CACESLVKFQAKVEGLIQALTRKLEAVSKRIAILENTVV (SEQ ID NO: 29)
冠蛋白 1a wt	VSRLEEEMRKLQATVQELQKRLDRLEETVQAK (SEQ ID NO: 11)
冠蛋白 1a 变体	ISRIEEEIRKINATVQELQKRIDRLEETVQAK (SEQ ID NO: 17)
DMPK wt	EAEAEVTLRELQEALEEEVLTRQSLSREMEAIRTDNQNFASQLREAEARNRDLEAHVRQLQERMELLQAE (SEQ ID NO: 30)
DMPK 变体	IAEIEVTIRELQEAIEEEVLTRQSLSREIEAIRTDIQNIASQLREIEARIRDLEAHVRQLQERMELLQAE (SEQ ID NO: 31)

三聚化基序	序列和 SEQ ID NO
朗格汉斯蛋白 wt	ASALNTKIRALQGSLENMSKLLKRQNDILQVVS (SEQ ID NO: 32)

用以对三聚化基序进行排序的计算机模拟分析

[0202] 通过用算法(LOGICOIL)分析表1-1中的序列以预测其寡聚状态而进行对将产生更稳定的GITRL三聚体并因此产生更稳定的多聚体GITRL FP的基序的计算机模拟预测。此外,通过两种算法(MARCOIL和LOGICOIL)的组合,将针对形成平行三聚体获得的评分与来自从整个人蛋白质组中鉴定的卷曲螺旋的那些进行比较。

方法

[0203] 在从ftp站点"ftp.ncbi.nlm.nih.gov/refseq/H_sapiens/mRNA_Prot/human.protein.faa.gz"下载的人蛋白质组上进行全基因组预测。此版本包括总数71861个蛋白质序列。扫描这些序列,并通过执行从万维网站"bcf.isb-sib.ch/Delorenzi/

Marcoil/Marcoilcode.tar.gz”下载的软件MARCOIL来鉴定卷曲螺旋基序。使用从互联网上经由万维网站“coiledcoils.chm.bris.ac.uk/LOGICOIL/LOGICOIL_Source.zip”下载的LOGICOIL算法进一步分析了所有通过某些特定阈值(即0.01、0.10、0.50、0.90和0.99)的基序,来预测卷曲螺旋序列的寡聚状态(平行或反平行二聚体、三聚体或四聚体)。此软件包是用R语言实现的(版本3.0.2-2013-09-25)。ad-hoc perl脚本是为了将MARCOIL的输出转换成LOGICOIL预期的文件格式而开发的。使用格点软件包(lattice package,版本0.20-23)在R中绘制直方图。

结果

[0204] 预测了来自Refseq的所有人序列中的卷曲螺旋基序(使用MARCOIL)。通过MARCOIL算法对不同阈值预测的卷曲螺旋基序的数目显示于表1-2中。然后使用LOGICOIL预测0.99阈值的所有基序的寡聚状态,以及表1-2中的八个选定的三聚体卷曲螺旋结构域。

这些评分的分布和在此项研究中使用的基序的等级显示于表1-3中。

表1-2:通过MARCOIL算法对不同阈值预测的卷曲螺旋基序的数目

阈值	基序的数目
0.01	168583
0.10	68793
0.50	41762
0.90	34714
0.99	27240

表1-3:整个蛋白质组的背景下八个卷曲螺旋基序的等级,包括LOGICOIL TRIM评分

蛋白质组等级	子组等级	序列	LOGICOIL TRIM
20	1	GCN4 pII	2.43
201	2	hDMPK-突变体	1.74
219	3	h 朗格汉斯蛋白-wt	1.71
241	4	h 朗格汉斯蛋白-变体	1.68
427	5	hCor1a-wt	1.58
1065	6	hCor1a-突变体	1.44
3620	7	hDMPK-wt	1.18
12473	8	h 母系蛋白-变体	0.96
18993	9	h 母系蛋白-wt	0.81

含有不同人三聚化基序的GITRL FP变体的产生

[0205] 除了GCN4 pII序列之外,将每个选择的三聚化基序的序列以适当的构型克隆到编码GITRL FP的其他元件的DNA载体中,即在人Fc结构域和人GITRL的胞外结构域之间,由短

的柔性氨基酸接头序列分开。这些载体被用于瞬时转染哺乳动物细胞，使重组六聚体GITRL FP蛋白的分泌和随后的纯化成为可能。

方法

[0206] 使用PEI将悬浮CHO细胞用编码不同六聚体GITRL FP的DNA载体瞬时转染并使其在37℃下生长八天，以80％湿度在140rpm下振荡。通过在1600x g离心并过滤，将40毫升含有分泌蛋白的条件培养基与细胞和细胞碎片分离。使用Mab SelectSure™树脂纯化蛋白质，并通过还原性SDS-PAGE分析其大小和完整性。

随后对六聚体GITRL FP(冠蛋白1a wt)和六聚体GITRL FP(母系蛋白1wt)采用较大规模表达和两步纯化方案。在此，使较大体积的CHO条件培养基(400ml)经受蛋白G色谱，随后尺寸排阻色谱步骤(S20016/60)。将这些纯化的蛋白质用于SEC-MALLS分析。

结果

[0207] 所有重组六聚体GITRL FP蛋白质以相似水平表达，但含有冠蛋白1a wt基序的六聚体GITRL-FP显现比其他蛋白质提供更高的产量(表1-4:图1)。

表1-4:针对八种GITRL FP蛋白来自在CHO细胞中瞬时表达的蛋白质产量

GITRL FP	浓度(mg/ml)
母系蛋白1wt	0.51
母系蛋白1变体	0.57
冠蛋白1awt	0.9
冠蛋白1a变体	0.63
DMPKwt	0.66
DMPK变体	0.34
朗格汉斯蛋白wt	0.37
朗格汉斯蛋白变体	0.48

含有不同人三聚化基序的GITRL FP变体的表征

GITRL FP变体与表达GITR的细胞的结合

[0208] 为了确定六聚体GITRL FP分子是否能结合细胞表面表达的GITR，将它们与过表达GITR的细胞一起孵育，并通过其Fc结构域检测它们的结合。

方法

[0209] 以一式两份的孔将固定浓度的DyLight-649轭合的抗人IgG抗体缓冲液、随后是以上在1.3节中所述的经纯化的六聚体GITRL FP蛋白的2倍稀释液和同种型对照抗体(NIP228)添加到384孔黑色壁透明底板。向所有孔中加入稳定表达GITR的CHO细胞，并将该板在室温下孵育4小时。使用具有640nm激光的Mirrorball™平板细胞计数器测定与CHO-GITR细胞的结合，并测量荧光。

结果

[0210] 结果呈现在图2A-D中。所获得的结合特征曲线证明了当六聚体GITRL FP的浓度超过DyLight-649轭合的检测抗体的浓度时观察到的“钩效应”，导致在较高浓度的六聚体GITRL FP下检测到较少的结合；因此产生钟形的结合特征曲线。尽管有这种现象，不同的六聚体GITRL FP分子产生非常相似的结合特征曲线。

GITRL的竞争GITRL FP变体与重组三聚体配体竞争结合GITR

[0211] 使用均相时间分辨荧光(HTRF)测定来确定六聚体GITRL FP分子对人GITRL与人GITR的结合的影响。

方法

[0212] 将六聚体GITRL FP分子滴定到HTRF测定中,其中测量了GITRL-HA(血凝素标签)与GITR-Fc的结合。将人GITR Fc与铕穴状化合物轭合,并将与XL665轭合的抗HA抗体用于检测GITRL-HA蛋白。通过用Prism 5.01软件(GraphPad)将分析的数据曲线拟合到四参数逻辑方程来确定IC_{50}值。

结果

[0213] 结果呈现于图3A-D和表1-5中。具有不同三聚化基序的六聚体GITRL FP分子都是三聚体GITRL-HA与GITR-Fc结合的有效抑制剂。除GITRL FP(朗格汉斯蛋白wt)证明比GITRL FP(GCN4)低8倍的效力外,大部分GITRL FP变体产生相似的抑制曲线和IC_{50}值。GITRL FP(冠蛋白1a)在0.61nM处具有最低的IC_{50}值。

表1-5:与三聚体GITRL竞争结合GITR-Fc的GITRL FP蛋白的IC_{50}(nM)值

测试样品	IC50 (nM)
GITRL FP (GCN4 pII)	1.06
GITRL FP (母系蛋白 1 wt)	1.36
GITRL FP (母系蛋白 1 变体)	0.83
GITRL FP (冠蛋白 1a wt)	0.61
GITRL FP (冠蛋白 1a 变体)	1.44

测试样品	IC50 (nM)
GITRL FP (DMPK wt)	2.73
GITRL FP (DMPK 变体)	2.43
GITRL FP (朗格汉斯蛋白 wt)	8.05
GITRL FP (朗格汉斯蛋白变体)	2.0
NIP228 IgG-TM	无抑制

在报告物测定中GITRL FP变体的活性

[0214] 在使用稳定表达GITR的NFκB荧光素酶报告细胞的测定中确定不同GITRL FP分子(六聚体GITRL IgG1 FP(SEQ ID NO:6)和六聚体GITRL IgG4 FP(SEQ ID NO:40))的功能活性。测量了由GITR的激动和随后的NFκB途径的活化驱动的发光。

方法

[0215] 针对六点数据曲线将GITRL FP蛋白以4倍连续稀释并一式三份加入到96孔板中。然后将用人GITR转染的Jurkat NF-κB荧光素酶报告细胞加入到测定板的所有孔中，并在37℃孵育3小时。通过将Steady-Glo试剂添加到测定板的所有孔中来检测荧光素酶表达。将各板在室温下孵育5分钟，并且然后测量发光，并在GraphPad Prism 5.01(GraphPad)中使用log(激动剂)与响应可变斜率非线性曲线拟合产生EC_{50}值。

结果

[0216] 结果呈现于图4A-D和表1-6中。所有的六聚体GITRL FP蛋白都能触发NF-κB信号传导。蛋白质产生与GITRL FP(GCN4)相似的效力特征曲线和EC_{50}值。

表1-6:使用人GITR转染的NF-κB荧光素酶基因报告细胞系，GITRL FP变体的EC_{50}(nM)值

	测试样品	EC_{50} (nM)
	GITRL FP（GCN4 pII）	1.02
	GITRL FP（母系蛋白 1 wt）	1.12

	测试样品	EC_{50} (nM)
	GITRL FP（母系蛋白 1 变体）	0.90
	GITRL FP（冠蛋白 1a wt）	0.95
	GITRL FP（冠蛋白 1a 变体）	0.74
	GITRL FP（DMPK wt）	1.37
	GITRL FP（DMPK 变体）	1.39
	GITRL FP（朗格汉斯蛋白 wt）	1.54
	GITRL FP（朗格汉斯蛋白变体）	0.75

GITRL FP变体的解链温度

[0217] 使用荧光染料(Sypro Orange)测定GITRL FP蛋白的解链温度，在去折叠蛋白的存在下该荧光染料的发射特性改变。

方法

[0218] 使用基于Sypro Orange的测定来评估多聚体GITRL FP变体的热稳定性以计算解链温度(Tm)。在分配到96孔PCR板中之前，首先将不同的蛋白质在2×PBS中稀释至0.5mg/mL。将Sypro Orange™加入到板上的每个孔中，然后将其密封。使用Chromo4™连续荧光检测器在Real-Time™ PCR仪上读板。温度设定为从20℃增加到90℃，每1℃读取一次，并且保持

时间为1s。通过绘制荧光强度和荧光衍生物作为温度的函数来确定去折叠转变。一式两份对每个多聚体GITRL-FP蛋白进行分析。

结果

[0219] 结果呈现在图5A-D中。下表1-7中总结了九个多聚体GITRL-FP蛋白中每一个的解链温度。除GITRL FP(DMPK wt)外,所有变体都具有类似的特征曲线,GITRL FP(DMPK wt)在59℃展示单一转变峰,相比之下其他大多数为62℃-64℃,GITRL FP(朗格汉斯蛋白wt)和GITRL FP(朗格汉斯蛋白变体)都在低温(45℃-50℃)下显示另外的转变峰,这表明一些结构不稳定性。

表1-7:九种GITRL FP蛋白的转变温度

	测试样品	Tm1 (℃)	Tm2 (℃)
	GITRL FP (GCN4 pII)	/	63
	GITRL FP (母系蛋白 1 wt)	/	62.5
	GITRL FP (母系蛋白 1 变体)	/	63
	GITRL FP (冠蛋白 1a wt)	/	63
	GITRL FP (冠蛋白 1a 变体)	/	63
	GITRL FP (DMPK wt)	/	59
	GITRL FP (DMPK 变体)	/	63.5
	GITRL FP (朗格汉斯蛋白 wt)	47.5	63
	GITRL FP (朗格汉斯蛋白变体)	45-50	62

GITRL FP变体的计算机模拟免疫原性分析

[0220] 使用ProPred算法确定8个卷曲螺旋结构域和其周围序列的预测的免疫原性。

方法

[0221] 使用ProPred[Singh&Raghava(2001)Bioinformatics[生物信息学]17(12)],评价8个选定的人三聚化基序和酵母GCN4pII的T评分。相对于氨基酸序列的总长度,任意氨基酸序列的T评分定量强结合MHC II类表位的数目。如果ProPred返回的结合评分高于通过评价一组10,000个随机产生的九-mer序列获得的评分的95个百分点,则九-mer子序列被认为是强结合表位。针对最常见的八个人等位基因计算每个基序的T-评分,包括基序之前的2x[G4S]序列以及基序之后GITRL结构域的[G4]序列和前4个氨基酸。然后总体T评分形成个体等位基因T评分的总和。

结果

[0222] 每个三聚化基序及其周围序列的总体T评分示于表1-8中。注意,最高评分等于8,这对应于针对所有测试的八个等位基因,序列中每个九-mer都是强结合表位的情况。有趣的是,这个全局分析预测,除了DMPK wt之外,几乎所有候选物都具有比GCN4略强的免疫原性特征曲线。母系蛋白wt和变体都具有最高的总体T评分。

表1-8:八个人三聚化基序和GCN4pII的总体T评分

#	卷曲螺旋基序	T评分
1	GCN4 pII	0.131579
2	母系蛋白1	0.270833
3	母系蛋白1变体	0.270833
4	冠蛋白1a wt	0.142857
5	冠蛋白1a变体	0.166667
6	DMPK wt	0.0875
7	DMPK变体	0.175
8	朗格汉斯蛋白wt	0.209302
9	朗格汉斯蛋白变体	0.186047

GITRL FP变体的SEC-MALLS分析

方法

[0223] 在BioSep-Sec-S™ 4000(空隙体积,V0=5.7ml)上分析蛋白质,并且所有运行都以0.5ml/min的流速进行30min。用UV280、折射率和多角度激光散射检测器监测洗脱。将样品(1-2mg/ml)加载到柱上,并且然后计算摩尔质量和粒径。

结果

多聚体GITRL FP(母系蛋白1wt)

[0224] 具有母系蛋白-1三聚化基序的GITRL FP在凝胶过滤柱上产生三个不同的峰:峰2具有312kDa的重均摩尔质量,与糖基化六聚体的预期摩尔质量一致,并且构成总蛋白质质量的40.9%,而峰1具有对应于六聚体(约610kDa)的二聚体的重均摩尔质量并且构成注入总质量的41.3%。峰3的质量低于预期(约215kDa),这可能是来自六聚体的二聚体碎裂或损失的结果。它构成总蛋白质质量的17.7%。因此这种蛋白质制剂的组成是非均匀的,没有单一主要寡聚种类。

多聚体GITRL FP(冠蛋白1a wt)

[0225] UV280、折射率和900-激光散射迹线的叠加分析表明,此蛋白质的93.9%在16.4min洗脱为300kDa的单分散物质。这个观察到的质量与六聚体GITRL FP多肽的预测质量(274.95kDa)一致。计算的和观察到的质量之间的25kDa的差异最可能是由糖基化引起的。在14.7min处的次要峰具有690kDa的重均摩尔质量,这可归因于六聚体的二聚体。

多聚体GITRL FP(GCN4 pII)

[0226] 类似地,具有GCN4 pII三聚化基序的GITRL FP的激光散射和折射率色谱的分析证明,此蛋白质的93.75%洗脱为330kDa的单分散物质,与糖基化六聚体一致。在15分钟洗脱的峰仅含有总蛋白质的6.25%,并且具有大约700kDa的重均摩尔质量,可能反映了六聚体的二聚体的存在。

摩尔质量与时间特征曲线

[0227] 图6显示了三种多聚体GITRL FP蛋白各自的洗脱峰的摩尔质量组成。对于所有三种蛋白质，保留时间约15min的早期峰的摩尔质量是高度可变的，特别是在GITRL FP(母系蛋白1wt)的情况下，其中在相同峰开始和结束时洗脱的分子的质量相差超过100kDa。在约17分钟时洗脱的峰在其分子组成上较不均匀，GITRL FP(冠蛋白1a wt)中的最大摩尔质量变化小于20kDa。重均摩尔质量的变化最有可能是由于在相同峰内洗脱的糖蛋白的聚糖含量差异引起的。

表征数据的总结

[0228] 在分析如

[0229] 表1-9中所示的编译数据中，比较了多聚体GITRL FP中各卷曲螺旋三聚结构域的性能，冠蛋白1a卷曲螺旋结构域提供了改进的表达产量。然而，冠蛋白-1a卷曲螺旋基序与来自分类为三聚体卷曲螺旋的人类序列的基序的预测评分的比较显示，冠蛋白-1a wt的LOGICOIL等级为5。

表1-9:表征数据的总结

三聚化基序	LOGICOIL 等级	表达产量（mg/ml）	Tm1 (Sypro Orange)	受体/配体抑制 IC50 (HTRF)	细胞结合（Mirror ball）	激动 EC_{50}（NFkB报告物）	计算机模拟免疫原性
GCN4	1		63	1.06	良好	0.89	0.132
母系蛋白 1 wt	9	0.51	62.5	1.36	良好	0.92	0.271
母系蛋白 1 变体	8	0.57	63	0.83	良好	0.89	0.271
冠蛋白 1a wt	5	0.9	63	0.61	良好	0.75	0.143
冠蛋白 1a 变体	6	0.63	63	1.44	良好	0.71	0.167
DMPK wt	7	0.66	59	2.73	良好	0.96	0.088
DMPK 变体	2	0.34	63.5	2.43	良好	1.38	0.175
朗格汉斯蛋白 wt	3	0.37	47.5	8.05	良好	1.17	0.209
朗格汉斯蛋白变体	4	0.48	45-50	2	良好	0.57	0.186

实例2:GITRL融合蛋白的产生和表征，相同物的表征，以及小鼠GITRL融合蛋白的表征

衍生物和组成

[0230] 构建了六聚体GITRL IgG1融合蛋白，其包括具有SEQ ID NO:6的氨基酸序列的单体亚基。SEQ ID NO:6的每个GITRL IgG1 FP单体亚基包括3个不同的结构域:1)人IgG1 Fc

结构域;2)源自人冠蛋白1A蛋白的α螺旋卷曲螺旋三聚结构域和3)人GITRL ECD,其中人GITRL ECD中的单点突变(N161D)消除了唯一被占据的糖基化位点。每个结构域由富含甘氨酸和丝氨酸或富含甘氨酸的(例如$(Gly)_4$)柔性接头分开(图8)。每个单体中的人GITRL ECD结构域与溶液中的另外两个单体形成弱的非共价三聚体,并且通过这些人冠蛋白1A三聚结构域之间的相互作用增强了这种缔合,导致稳定的三聚体结构。存在于每个三聚体中的IgG1 Fc结构域的相互作用导致随后形成GITRL三聚体的二聚体,得到最终的六聚体构象。

[0231] 合成在SEQ ID NO:7中列出的DNA分子,其编码具有SEQ ID NO:8的氨基酸序列的前体GITRL IgG1 FP多肽亚基,并使用标准分子生物学技术将其克隆到表达载体中,使得有效的瞬时重组蛋白表达成为可能。

[0232] 在SEQ ID NO:6中列出的GITRL IgG1 FP多肽亚基的亚基单体的核苷酸和推导的氨基酸序列显示在图8中。

重组蛋白的表达和纯化

[0233] 在波袋生物反应器中,将生长于类似于CD-CHO™(生命技术有限公司(Life Technologies Ltd),佩斯利,英国)的化学限定培养基的悬浮液中的CHO细胞用编码不同六聚体GITRL FP的DNA载体进行转染。使用类似于CHO CD-Efficient Feed A™(生命技术有限公司,佩斯利,英国)的营养物饲料,将培养物以补料分批维持10-12天,此时使用过滤以除去细胞来收获它们。然后在纯化之前将该条件培养基冷藏。

[0234] 使用MabSelectSure™树脂从条件培养基纯化蛋白质。在羟基磷灰石1型树脂上进一步纯化之前,将洗脱的峰中和并过滤。用盐梯度进行洗脱。在整个过程中通过SEC HPLC监测纯度。

GITRL IgG1 FP多肽亚基的糖基化分析

[0235] 使用与四极杆飞行时间(QTOF)质谱(LC-QTOF MS)偶联的液相色谱法测定SEQ ID NO:6的GITRL IgG1 FP多肽亚基的糖基化状态,并用质谱进行肽作图。结果显示于图9中。

[0236] 通过对还原的多肽亚基的LC-QTOF MS分析获得的SEQ ID NO:6的GITRL IgG1 FP多肽亚基的精确质量与每个链加入一个双触角聚糖(主要为G0f)的预期氨基酸序列一致。如通过肽作图所证实的,该质量特征曲线与在SEQ ID NO:6的GITRL IgG1 FP多肽亚基的GITRL ECD(融合蛋白中的N369)中N129处无N-聚糖占据的Fc结构域糖基化一致。这些数据还证实GITRL ECD中的N161D突变(融合蛋白中的N401)导致未糖基化的GITRL ECD,其中N-糖基化仅存在于Fc结构域中的典范糖基化位点处。

六聚体GITRL IgG1 FP的体外表征

六聚体GITRL IgG1 FP对配体-受体结合的影响

[0237] 使用均相时间分辨荧光(HTRF)测定来确定具有SEQ ID NO:6的单体GITRL IgG1 FP亚基序列的六聚体GITRL IgG1 FP对人GITRL与人GITR的结合的影响。将GITRL IgG1 FP滴定到HTRF测定中,其中测量了GITRL-HA(血凝素标签)与GITR-Fc的结合。将人GITR-Fc与铕穴状化合物轭合,并将与XL665轭合的抗HA抗体用于检测GITRL-HA蛋白。通过用Prism 6.0x软件(GraphPad)将分析的数据曲线拟合到四参数逻辑方程来确定IC_{50}值。图10中显示的代表性结果证明GITRL IgG1 FP以0.562nM的IC_{50}抑制GITRL-HA与GITR的结合。对同种型对照抗体NIP228没有观察到抑制。

GITR激动作用

[0238] 本实验的目的是确定GITRL融合蛋白(FP)通过GITR受体递送信号的能力。

方法

[0239] 将六聚体GITRL FP在溶液中添加到转染了hGITR和与核因子κB(NFκB)启动子连接的荧光素酶报告基因的Jurkat细胞。在本测定中,GITR受体的活化导致经由NFκB途径的信号传导,其继而导致可以通过发光测量的荧光素酶活性的增加。

结果

[0240] 在测定系统中加入六聚体GITRL FP导致发光增加。观察到的影响是浓度依赖性的,EC_{50}是大约180pM。相比之下,加入同种型对照抗体没有影响。结果示于图11中。这些数据证明六聚体GITRL FP是GITR受体的有效激动剂。

初级T细胞活化

[0241] 本实验旨在评估由六聚体GITRL FP介导的GITR激动作用对人T细胞的增殖和功能的影响。

方法

[0242] 从健康人的血液分离总人T细胞并经抗原处理,通过在板结合的抗CD3的存在下培养4天,以上调GITR的表达。在六聚体GITRL FP存在下用次最佳浓度的抗CD3和抗CD28再刺激之前,通过仅在培养基中培养使抗原处理的细胞静止2天。通过在18小时内定量掺入细胞的胸苷来评估细胞的增殖。使用中等规模的发现来定量干扰素γ(IFN-γ)的释放。

结果

[0243] 一起加入抗CD3与抗CD28导致最小水平的增殖和IFN-γ的最小释放。加入板结合的六聚体GITRL FP与板结合的抗CD3和抗CD28导致增殖水平和IFN-γ释放的浓度依赖性增加。代表性数据示于图12和图13中。同种型对照抗体对增殖或细胞因子的释放没有影响。

ADCC

[0244] 小鼠六聚体GITRL IgG1 FP已经在体内显示耗减肿瘤内CD4阳性T细胞,包括FOXP3阳性调节性T细胞,导致肿瘤内$CD8^+$与$CD4^+$T细胞的比率增加。进行本实验是来确定六聚体GITRL FP通过ADCC介导人T细胞耗减的能力,并评估存活细胞群体中所得的CD8:CD4比率改变。

方法

[0245] 从健康人血液中分离的初级人T细胞是用植物凝集素(PHA)和IL-2进行抗原处理的,以上调GITR表达。然后用荧光染料标记它们,并按指示比率将它们与初级NK细胞连同指示浓度的六聚体GITRL FP孵育24小时。使用流式细胞术定量测定结束时存在的活T细胞的百分比,并将此用于计算经处理的孔相对于未经处理的孔的裂解百分比。还通过流式细胞术定量CD4和CD8细胞的比率。

结果

[0246] 单独的NK细胞介导初级T细胞的少量溶解,其通过添加包括在SEQ ID NO:6中列出的GITRL融合多肽亚基(含有IgG1 Fc结构域)的六聚体GITRL FP而以浓度依赖性方式显著增强。包括SEQ ID NO:40的GITRL融合多肽亚基(含有不能结合NK细胞上的Fcγ受体的IgG4 Fc结构域)的六聚体GITRL FP用作阴性对照,并且对所测量的溶解水平没有影响(图14)。

在测定结束时存在的$CD8^+$与$CD4^+$T细胞的百分比的流式细胞分析指示,由六聚体GITRL FP介导的ADCC有利于产生增加的CD8:CD4 T细胞比率(图15)。

调节性T-细胞测定

[0247] GITR在调节性T细胞(T-reg)上以增加的水平表达,并且已经提出通过GITR的信号传导影响T-reg抑制其他T细胞的能力。进行本实验是为了评估六聚体GITRL FP对T-reg功能的影响。

方法

[0248] 从健康人供体的外周血中分离$CD4^+CD25^-$效应T细胞和$CD4^+CD25^+$T-reg。在存在抗CD3抗体、抗CD28抗体、指示比率的T-reg和测试品的情况下培养之前,用CFSE标记效应T细胞。通过流式细胞术分析增殖型效应T细胞的百分比。

结果

[0249] 响应于添加抗CD3和抗CD28,观察到效应T细胞增殖。添加增加数目的T-reg导致在测定过程中已分裂的效应T细胞的百分比降低。板结合的同种型对照的添加进一步降低了分裂细胞的百分比,而添加板结合的六聚体GITRL FP则将分裂的百分比恢复到在不存在T-reg时观察到的百分比。结果示于图16中。

[0250] 本研究证明了六聚体GITRL FP克服调节性T-细胞介导的对其他T-细胞的抑制作用的能力。

体内生物学/功能活性的测试

同种型依赖性抗肿瘤活性

[0251] 因为人GITRL不与小鼠GITR交叉反应,所以人GITRL FP不能在免疫感受态小鼠癌症模型中测试。为了使这个测试成为可能,产生了替代品小鼠mGITRL IgG1 FP和mGITRL IgG2a FP。进行本研究是为了评价mGITRL FP在CT26癌症模型中的抗肿瘤活性,并确定Fc同种型对此活性大小的影响。

方法

[0252] 用CT26小鼠结直肠癌细胞系植入Balb/c小鼠。植入后第6天,向动物通过腹膜内(i.p.)注射一次或每天(持续17天)给予mGITRL FP。测试了两种不同形式的mGITRL FP;一个含有mIgG2a Fc结构域,而另一个含有mIgG1 Fc结构域测试了两个不同剂量水平的每种mGITRL FP;5mg/kg和10mg/kg。盐水处理用为阴性对照。

结果

[0253] 盐水处理组的生存期中值为22天,且本组中的小鼠都没有存活到研究结束。用10mg/kg的mGITRL FP处理将存活期中值延长至32天,并且在研究结束时得到50%的存活率。在用5mg/kg的mGITRL FP处理的组中,10只小鼠中的9只在研究结束时存活并且不能定义存活期中值。用5或10mg/kg的mGITRL FP mIgG1处理分别使存活期中值延长至28天和22.5天,并导致10只小鼠中有2只存活至研究结束。结果示于图17中。

本研究证明了mGITRL FP介导抗肿瘤活性的潜力,并且指示FP的同种型可以影响观察到的抗肿瘤活性的水平。

药效学效应

[0254] 进行本研究是为了确定mGITRL FP对荷瘤小鼠脾脏和肿瘤中T-细胞活化状态和比例的影响。

方法

[0255] 如所示以0.2或1mg/kg腹膜内注射mGITRL FP处理7-9周龄的CT26荷瘤Balb/c雌性

小鼠。盐水处理用为阴性对照。处理开始7天后处死小鼠，并使用流式细胞术来评估脾脏和肿瘤中细胞的频率和表型。

结果

[0256] 用mGITRL FP处理导致脾脏中所有T-细胞亚组中增殖标记物Ki67的表达增加，表明这些细胞的增殖增加。参见图18。

[0257] 用mGITRL FP处理导致脾脏中所有T-细胞亚组中活化标记物ICOS的表达增加，表明这些细胞的活化增加。参见图19。

[0258] 用mGITRL FP处理导致肿瘤内CD4+FOXP3+调节性T-细胞和CD4+FOXP3-辅助细胞的频率降低，但不改变CD8+细胞毒性T-细胞的频率。总体结果是肿瘤微环境内的CD8:CD4比率增加。参见图20。

方法

细胞系和试剂

[0259] TC-1肿瘤系获自ATCC(目录号CRL 6475，马纳萨斯，弗吉尼亚州)并保持在DMEM+10％FBS+1％青霉素/链霉素中。CT26肿瘤系获自ATCC(马纳萨斯，弗吉尼亚州)并维持在补充有10％胎牛血清的RPMI 1640培养基中。DTA-1和同种型抗体购自Bio X Cell(西黎巴嫩，新罕布什尔州)。

肿瘤模型

[0260] TC-1实验使用获自杰克逊实验室(Jackson Labs)(巴港，缅因州)的雌性C57BL/6小鼠(目录号000664)小鼠。CT26实验使用获自Envigo(弗雷德里克，马里兰州)的雌性Balb/C小鼠。在肿瘤植入时小鼠在6-8周龄之间。所有动物实验均按照研究机构动物护理和使用委员会制定的指导方针进行。对于TC-1肿瘤植入，将2x 10^4个活TC-1细胞皮下植入左后足垫。对于CT26肿瘤植入，将5x 10^5个细胞植入右侧腹。通过用卡尺1直接测量来评价肿瘤生长。每2-4天收集双向测量，并使用体积＝(长度•宽度2)/2计算肿瘤体积。允许肿瘤发展6-14天，并且然后将荷瘤小鼠按肿瘤体积随机分配到处理组。按照IACUC方案，当TC-1/足垫的原发性肿瘤超过1000mm^3和CT 26/侧腹的原发性肿瘤超过2000mm^3时使小鼠安乐死。对于PD研究，使小鼠安乐死，并收集肿瘤和脾脏，通过70μM滤器(Corning，康宁公司(Corning)，纽约)粉碎，并加工成单细胞悬浮液。

功能性T-细胞响应和流式细胞术

[0261] 对于抗原特异性刺激，每孔铺板1-2x10^6个活细胞和1ug AH1肽序列SPSYVYHQF(SEQ ID NO:56)(Anaspec，弗里蒙特，加利福尼亚州)。对于所有染色剂，染色顺序为live/dead blue(生命技术公司)、胞外蛋白、FOXP3 Fix/Perm Kit(E生物科学公司(Ebioscience))并且随后是细胞内细胞因子。抗体包括GITR(克隆DTA-1)。所有样品都在LSR-II或Fortessa(BD，圣何塞，加利福尼亚州)上运行。使用FlowJo(树星(Treestar)，阿什兰，俄勒冈州)来分析所有数据。

结果

mGITRL-FP是一种高效的GITR激动剂，并且这导致CT26肿瘤的排斥

[0262] 先前已经显示，GITR激动作用可有效引起Bablc小鼠中CT26肿瘤的消退和消除。给Balb/c小鼠植入CT26肿瘤细胞，并且6天后按肿瘤体积随机化(组n＝10)，并且以单次给予或重复给予mGITRL-FP IgG2a(Q2Dx9)进行处理。剂量范围从5mg/kg体重降至0.04mg/kg体

重。对于单次给予，剂量降至1mg/kg的mGITRL-FP到第30天时消除所有CT26小鼠肿瘤，除一个肿瘤外(图40B)。最低剂量在0.2mg/kg组产生3次消除，并且在0.1mg/kg组产生2次。增加mGITRL-FP的额外给予增加了疗效，并且除0.04mg/kg的最低剂量组外，所有组均可见100％的清除率(图40C)。最低的重复给予组可见4次消除。

[0263] 为了评价mGITRL-FP功能，将其与已知的单克隆GITR激动剂DTA-1进行比较。之前已经描述了使用100ug至500ug/小鼠范围的剂量。估计每只小鼠大约20克，因此剂量范围从5mg/kg到25mg/kg。给Balb/c小鼠植入CT26肿瘤细胞，并且10天后按肿瘤体积随机化(组n＝10)，并且然后通过单次给予1mg/kg的mGITRL-FP IgG2a或者5mg/kg或25mg/kg的DTA-1进行处理(图40D)。

[0264] 增加DTA-1的剂量增加了功效，然而1mg/kg的mGITRL-FP显示出与5mg/kg的DTA-1类似的功效和肿瘤生长动力学。为了确定CD8 T-细胞是否对我们的药物效应是必需的，评价相同组，但使用单克隆耗减抗体选择性地耗减CD8 T-细胞(图40E)。在没有CD8 T-细胞的情况下，DTA-1或mGITRL-FP完全消除CT26肿瘤的能力显著降低。每组仅有1-2只小鼠无瘤存活。

[0265] 此外，在没有CD8 T-细胞的情况下，mGITRL-FP在增加总体存活期中值方面不如DTA-1有效(图40F)。为了解mGITRL-FP如何与CD8相互作用，评价了单个淋巴样细胞群体的GITR表达。CD4+ Treg比CD8 T-细胞表达更高水平的GITR;然而，肿瘤中的Treg和CD8 T-细胞均比脾脏中其各自群体表达更高水平的GITR(图40G)。

mGITRL-FP耗减Treg并增加肿瘤抗原特异性T-细胞

[0266] 先前已经显示GITR激动剂减少Treg以及增加高亲合力T-细胞应答。为了解GITRL-FP能够介导什么药效学效应，在肿瘤消退期间评价CT26模型。用mGITRL-FP IgG2a处理具有CT26的Balb/c小鼠，并且在给药开始后8天，收获脾脏和肿瘤。包括两个研究组，一个TGI(肿瘤生长抑制)组(n＝5)和一个PD(药效)组(n＝5)。所有用mGITRL-FP处理的小鼠(除单个小鼠外)的CT26都被治愈(图41A)。在第18天，PD组被处死并评价脾脏和肿瘤二者中存在的免疫细胞的表型。然后通过用AH1(CT26免疫显性表位)再刺激来评价CD8 T-细胞功能。mGITRL-FP显著增加减少脾脏和肿瘤中的Treg数目(图41B)。虽然mGITRL-FP没有改变CD8的百分比，但其显著增加了对AH1具有抗原特异性的CD8的数目(图41C-D)。在脾脏中，总CD8的5％-10％是抗原特异性的并且能够产生IFNγ和TNFα。在肿瘤中，数目是5％-15％。这是两种肿瘤浸润淋巴细胞以及外周库的显著扩增。为了评价mGITRL-FP是否与CD4 Treg和CD8 T-细胞结合，使用DTA-1抗体对GITR进行染色(图41E-F)。Treg显示GITR MFI(荧光强度均值)超过75％的减少。在CD8 T-细胞上观察到这种相同的影响，显示mGITRL-FP改变CD8 T-细胞上的DTA-1结合。基于这个数据，假设T-细胞会显示增加的KI-67增殖。治疗后第8天进行评价后，CD4和CD8 T-细胞在肿瘤和脾脏中都显示出显著更多的KI-67阳性细胞(图41G-H)。

mGITRL-FP以剂量依赖性方式扩增抗原特异性CD8 T-细胞

[0267] 接下来研究治愈CT26的小鼠是否免于受到相同肿瘤的再激发。为了评价这一点，从用单剂量1.0、0.5或0.2mg/kg的mGITRL-FP IgG2a处理的组将治愈的小鼠再激发(图42A)。显示从起始剂量开始的所有小鼠，但仅在D85治愈的小鼠受到再激发。所有原来治愈CT26的小鼠均免于该肿瘤的再激发，尽管在最低剂量组中测量到一小块，但很快消除。为了

进一步查询这个结果，在第120天将小鼠处死，取出脾细胞，并针对AH1肽进行再刺激。AH1特异性T-细胞的数目存在剂量依赖性增加(图42B-C)。在1mg/kg mGITRL-FP组中，25％的脾CD8 T-细胞对CT26的单个表位是特异性的。这些小鼠在再激发期间没有显示肿瘤大小增加。最低剂量0.2mg/kg具有6％抗原特异性CD8，并且在再激发时显示出生长中的小块，该小块在第120天被清除。

六聚体GITRL IgG1 FP与人GITR的结合亲和力

[0268] 使用KinExA 3200仪器(萨比戴恩仪器公司(Sapidyne Instruments)，博伊西，爱达荷州)，使用动力学排除测定(KinExA)确定包括SEQ ID NO:6的GITRL IgG1 FP单体亚基的六聚体GITRL IgG1 FP针对重组人GITR的溶液KD(解离常数)。

[0269] 用重组人GITR滴定在D-PBS、0.02％钠和1mg mL^{-1}牛血清白蛋白缓冲液中六聚体GITRL IgG1 FP的2nM溶液，并在25℃平衡过夜。将样品转移到温度控制在25℃的KinExA 3200仪器中。平衡的混合物的取样是使用已用最少的胺-生物素化的人GITR滴定的、吖内酯珠结合的链霉亲和素来实现。使用的二级检测试剂是Fc特异性试剂DyLight 650标记的蛋白G'(可得自西格玛公司(Sigma)的蛋白G的片段)。使用KinExAPro软件(版本3.6.8)处理和解释数据。针对六聚体GITRL IgG1 FP与人GITR的结合获得82nM的K_D。

通过KinExA将六聚体GITRL IgG1 FP与重组食蟹猴GITR结合

[0270] 使用KinExA，以与6.1节中针对人GITR所述的相同方式确定包括SEQ ID NO:6的GITRL IgG1 FP单体亚基的六聚体GITRL IgG1 FP针对重组食蟹猴GITR的溶液K_D。针对六聚体GITRL IgG1 FP与食蟹猴GITR的结合获得107nM的K_D。

通过ELISA将六聚体GITRL IgG1 FP与重组食蟹猴GITR结合

[0271] 使用ELISA确定包括SEQ ID NO:6的GITRL IgG1 FP单体亚基的六聚体GITRL IgG1 FP与食蟹猴GITR的交叉反应性。将六聚体GITRL IgG1 FP生物素化，并且使用链霉亲和素-HRP和TMB底物检测其与固定的食蟹猴GITR-Fc的结合。图21显示六聚体GITRL IgG1 FP与食蟹猴GITR的结合非常类似于人GITR，并且没有观察到与阴性对照CD137-Fc蛋白的结合。

实例3:在食蟹猴中静脉内GITRL IgG1 FP处理的体内药效学效应

[0272] 为了确定食蟹猴中六聚体GITRL IgG1融合蛋白(FP)的药效学参数，通过静脉内(IV)推注向雄性食蟹猴组(5只/组)给予包括SEQ ID NO:6的GITRL IgG1 FP单体亚基的六聚体GITRL IgG1 FP。

方法

[0273] 在第1天、第3天和第5天用1或10mg/kg六聚体GITRL IgG1 FP注射单独的动物组。对照动物接受运载体(20mM磷酸钠，230mM蔗糖，0.02％P80，pH 7.5)。在给药前阶段期间和在第1、3、5、9、11、15、18、22和29天采集血样以测量药效学终点。

使用标准流式细胞术方法，从全血中测量Ki67阳性T-细胞群体。

结果

[0274] 观察到Ki67阳性T-细胞亚群％的增加，例如(CD3+CD4+CD95高CD28+/dim/-总记忆CD4+和CD3+CD8+CD95高CD28+/dim/-总记忆CD8+T-细胞)，指示细胞增殖(图22)。这个观察在给药阶段的第11或15天达到最大值，并且然后在研究结束时下降。

实例4:GITRL FP内的氨基酸突变及其对受体结合、激动剂活性和热稳定性的影响

[0275] 在GITRL受体结合结构域中具有突变的hGITRL FP变体的产生产生hGITRL FP变体

并测试其结合和激动GITR的能力。在一些情况下，也评估了它们的热稳定性。

方法

[0276] 使用基因合成和标准DNA克隆技术，产生编码GITRL FP变体的DNA载体。使用PEI将悬浮CHO细胞用编码不同六聚体hGITRL融合蛋白的DNA载体瞬时转染并使其在37℃下以80％湿度在140rpm振荡下生长八天。通过在1600x g离心并过滤，将40毫升含有分泌蛋白的条件培养基与细胞和细胞碎片分离。使用Mab SelectSure™树脂纯化蛋白质，并通过还原性SDS-PAGE分析其大小和完整性。

GITRL FP变体与重组三聚体配体竞争结合GITR

[0277] 使用均相时间分辨荧光(HTRF)测定来确定GITRL FP分子对人GITRL与人GITR的结合的影响。

方法

[0278] 将GITRL FP分子滴定到HTRF测定中，其中测量了GITRL-HA(血凝素标签)与GITR-Fc的结合。将人GITR Fc与铕穴状化合物轭合，并将与XL665轭合的抗HA抗体用于检测GITRL-HA蛋白。通过用Prism 5.01软件(GraphPad)将分析的数据曲线拟合到四参数逻辑方程来确定IC_{50}值。

结果

[0279] 突变的六聚体GITRL FP分子(hGITRL-FP wt、N92D、N104D和N161D)全是三聚体GITRL-HA与GITR-Fc结合的有效抑制剂并产生类似的抑制特征曲线和IC_{50}值(图23A、B)。

GITRL FP变体与重组GITR-Fc的结合

[0280] 在使用稳定表达hGITR的NFκB-荧光素酶报告细胞的测定中确定不同hGITRL FP分子的功能活性。测量了由hGITR的激动和随后的NFκB途径的活化驱动的发光。

方法

[0281] 将hGITRL FP分子滴定到HTRF测定中，其中测量了hGITRL FP与hGITR-Fc的结合。将人hGITR Fc与铕穴状化合物轭合，并将与XL665轭合的抗FLAG抗体用于检测hGITRL FP蛋白。通过用Prism 5.01软件(GraphPad)将分析的数据曲线拟合到单点饱和结合方程来确定KD值。

结果

[0282] hGITRL FP wt和hGITRL FP N161D的结合特征曲线非常类似(KD分别为1.225nM和1.079nM)，而N129A突变的hGITRL FP显示与hGITRFc的结合降低(KD＝3.774nM)。当N129A突变与N161D突变组合时，hGITRL FP分子的结合能力进一步受到负面影响(KD＝11.85nM)(参见图24)。

在报告物测定中GITRL FP变体的活性

[0283] 在使用稳定表达hGITR的NFκB-荧光素酶报告细胞的测定中确定不同hGITRL-FP分子的功能活性。测量了由hGITR的激动和随后的NFκB途径的活化驱动的发光。

方法

[0284] 针对6点数据曲线将hGITRL FP蛋白以4倍连续稀释并一式三份加入到96孔板中。然后将用人GITR转染的Jurkat NF-κB荧光素酶报告细胞加入到测定板的所有孔中，并在37℃孵育3小时。通过将Steady-Glo试剂添加到测定板的所有孔中来检测荧光素酶表达。将各板在室温下孵育5分钟，并且然后测量发光，并在GraphPad™ Prism 5.01(GraphPad软件公司，拉荷

亚,加利福尼亚州,美国)中使用log(激动剂)与响应可变斜率非线性曲线拟合产生EC_{50}值。

结果

[0285] 所有GITRL FP蛋白均触发NF-κB信号传导。N92D和N161D突变型hGITRL FP蛋白产生与GITRL FP(wt)类似的效力特征曲线和EC_{50}值。在本测定中,N129A和N129A/N161D突变负面影响了hGITRL FP激动GITR的能力(图25A-C)。

在T细胞再刺激测定中GITRL FP变体的活性

[0286] 在使用初级人T细胞和胸苷掺入读出的共刺激测定中确定不同hGITRL FP分子的功能活性。

方法

[0287] 人外周血单核细胞(PBMC)衍生的$CD3^+$T细胞是通过在37℃下在用小鼠抗人CD3抗体包被的TC处理的板中孵育4天来刺激。然后通过离心使它们沉淀,重悬于测定培养基中,加入到TC处理的板中并在37℃孵育两天(静止期)。将hGITRL FP分子在测定培养基中经10点以2倍连续稀释,并一式三份加入预先用小鼠抗人CD3包被的TC处理的板中。两小时后,将测定板洗涤并将预先制备的$CD3^+$T细胞加入到每个孔中,并在37℃下孵育4天。四天后,将测定培养基中的氚标记胸苷加入到每个孔中,并将板在37℃进一步孵育18小时。孵育后,使用Topcount™测量胸苷的掺入。

结果

[0288] 除了在两个独立实验中具有降低的活性的N92D以外,所有测试的hGITRL FP突变体均证明与hGITRL FP wt等同的活性(图26A-C)。

GITRL FP变体的解链温度

[0289] 使用荧光染料(Sypro™ Orange)测定GITRL FP wt和N92D蛋白的解链温度,在去折叠蛋白的存在下该荧光染料的发射特性改变。

方法

[0290] 使用基于Sypro Orange的测定来评估wt和N92D GITRL FP变体的热稳定性以计算解链温度(Tm)。在分配到96孔PCR板中之前,首先将蛋白质在2×PBS中稀释至0.5mg/mL。将Sypro™ Orange加入到板上的每个孔中,然后将其密封。使用Chromo4™连续荧光检测器在实时PCR仪上读板。温度设定为从20℃增加到90℃,每1℃读取一次,并且保持时间为1s。通过绘制荧光强度和荧光衍生物作为温度的函数来确定去折叠转变。一式两份对每种GITRL FP蛋白进行分析。

结果

[0291] 两种GITRL FP蛋白的解链温度总结在下表4-1中。两种变体在67℃均有转变峰,然而,hGITRL FP N92D在低温(54℃)下显示出另外一个宽的转变峰,表明结构不稳定(图27)。

表4-1:2种GITRL FP蛋白的转变温度

	测试样品	Tm1(℃)	Tm2(℃)
	GITRLFPwt	/	67
	GITRLFPN92D	54	67

数据总结和结论

[0292] 在GITR/GITRL竞争结合测定中,N104D GITRL FP蛋白证明与野生型hGITRL FP对应物等同的活性,并且初级T细胞再刺激测定表明N104在hGITRL FP的活性中不起关键作

用。Asn161至Asp的突变表示N联糖基化位点的去除(参见实例4中的数据),并且此突变体(hGITRL FP N161D)在所有测定(GITR/GITRL竞争结合、直接结合、报告物和初级T细胞再刺激测定)中保持活性,表明Asn161以及此位点上的聚糖不涉及重要功能,如与GITR的结合和激动作用。

[0293] N129A突变体证明与hGITR的结合降低,并且在报告物测定中活性降低,表明它在结合hGITR和随后的hGITR激动作用中起作用。有趣的是,当这种突变与N161D突变(单独不影响活性)组合时,GITR的结合和激动作用甚至进一步降低。

[0294] N92D突变显现不影响hGITRL FP在报告物测定中测试时的活性,然而在初级T细胞再刺激测定中观察到hGITRL FP N92D活性降低。当研究N92D突变体的热稳定性时,与hGITRL FP相比,观察到额外的非常宽的较低解链温度转变峰,表明在较低温度(包括37℃)下一定程度的结构不稳定性。由于初级T细胞测定(4天)的更长时间过程,可以设想hGITRL FP N92D分子变得不稳定并且去折叠,与报告物测定(3小时)相比,导致在此测定中活性降低。因此,就结构稳定性而言,Asn92至Asp的突变显现是不利的。

实例5:hGITRL FP内糖基化的氨基酸残基的鉴定。

[0295] GITR结合结构域(N161和N129)内有两个潜在的N-糖基化共有位点。通过质谱法确定在这些位点处以及在Fc结构域内的典范N-糖基化位点(在IgG背景下为N297;成熟hGITRL FP序列中的N78)处聚糖的存在和结构。

方法

重组蛋白质表达和纯化

[0296] 使用亲和色谱法和随后的尺寸排阻色谱法从用编码相关蛋白的载体瞬时转染的CHO细胞的条件培养基中纯化重组hGITRL FP wt和N161D蛋白。

胰蛋白酶水解肽作图

[0297] 将hGITRL FP wt和hGITRL FP N161D的样品变性、还原,并且将还原的半胱氨酸烷基化。然后用胰蛋白酶消化样品。在37℃下4小时后,通过添加酸淬灭消化。在UPLC上通过反相分离肽并使用UV检测器和质谱仪测量。所得的胰蛋白酶水解的肽序列在表5-1中提供。

结果:

表5-1

ID	序列	样品
T7	EEQYNSTYR (SEQ ID NO:43)	hGITRL FP wt & hGITRL FP N161D

ID	序列	样品
T40	DMIQTLTNK (SEQ ID NO:44)	hGITRL FP wt & hGITRL FP N161D
T42-43	IQNVGGTYELHVGDTIDLIFNSEHQVLKNNTYWGIILLANPQFIS (SEQ ID NO:45)	hGITRL FP wt
T43'	DNTYWGIILLANPQFIS (SEQ ID NO:46)	hGITRL FP N161D

N＝天冬酰胺部分N-糖基化共有序列。

D＝在hGITRL FP N161D中的N161D取代

[0298] 在图28中提供了在各hGITRL FP中发现的寡糖结构类型的示意图。在图29A、B和C中提供了质谱数据。

结论

[0299] 使用肽作图分析来确定GITRL和Fc结构域中的N-糖基化位点占据以及每个位点处主要寡糖的结构。在hGITRL FP wt和hGITRL FP N161D蛋白中，典范的Fc N-糖基化位点(N78)被糖基化，并且主要的碳水化合物结构是中性的双触角复合型寡糖；针对在CHO细胞中表达的IgG Fc区是典型的。在hGITRL FP wt或hGITRL FP N161D中GITRL RBD内的N129处的N-糖基化共有位点未被任何寡糖结构占据。在hGITRL FP wt中GITRL RBD内的N161处的N-糖基化共有位点被占据，并且发现主要的碳水化合物结构是中性和带电的双触角复合型寡糖。在hGITRL FP N161D蛋白中，肽作图证实N161D氨基酸取代，其从GITRL RBD中去除N161N-糖基化共有位点。正如所料，在D161处没有检测到N-糖基化。

实例6:鼠模型和OX40组合研究

[0300] 为了更好地理解GITR的靶向，将鼠GITRL-FP的活性和药效学效应与靶向OX40的激动性鼠OX40L FP的情况进行比较。

材料和方法

NF-κB报告物测定

[0301] 以50,000个细胞(Jukat mGITR)或200,000个细胞(Jurkat人OX40)/孔，将Jurkat mGITR或人OX40NFκB细胞，与抗GITR抗体DTA-1(生物传奇公司(Biolegend))、NIP rIgG2b同种型对照、mGITRL-FP mIgG2a或板固定的mOX40L-FP mIgG1或同种型对照(包括在位置(Y182A)的单个氨基酸突变，使蛋白质不能经由OX40发信号)一起在37℃、5％CO_2和85％湿度下在96孔板中进行培养。针对含有mOX40L-FP mIgG1的测定，在5小时后或16小时后将含有荧光素底物的Steady Glo®试剂(普洛麦格公司(Promega))添加至板并在板振荡器上在暗处将板孵育30min。使用Envision酶标仪(珀金埃尔默公司(Perkin Elmer))检测测定信号。

SDS PAGE

[0302] 将5微克蛋白质与上样缓冲液和还原剂混合，在80℃变性10分钟，并加载到4％-20％Tris-甘氨酸SDS-PAGE凝胶(Thermo Fisher)上，连同蛋白质分子量标记物(彩虹标记物(Rainbow Marker)，GE医疗集团(GE Healthcare))。将蛋白质在200V下电泳45分钟，并使用瞬时蓝(Instant Blue)蛋白染色剂(西格玛公司(Sigma))染色。

小鼠和肿瘤模型

[0303] 8-10周龄的BALB/c或C57BL/6雌性小鼠获自查尔斯河实验室(英国)(Charles River UK Ltd.)或哈兰实验室公司(Harlan Laboratories Inc)。将CT26或B16F10-Luc2细胞在PBS中的100μL悬浮液以5×10^6个细胞/mL或5×10^4个细胞/mL 100μl的细胞密度皮下注射到每只动物的右侧腹。B16F10-Luc2细胞系在CAG启动子的控制下掺入荧光素酶报告基因，并将细胞植入50％PBS和50％生长因子(还原)及不含酚红的基质胶(康宁公司)中。基于肿瘤体积将可测量的肿瘤随机化。每个肿瘤的长度(mm)和宽度(mm)用电子卡尺每周测量3次。基于公式(长度[mm]×宽度[mm]^2)/2计算肿瘤体积(mm^3)。如果不存在可测量的肿瘤或持

续的肿瘤生长抑制，使得在研究结束时体积小于200mm^3，则将肿瘤生长应答分类为应答。每个蜘蛛图上指示的消退数目是相对于植入肿瘤的总数的比例。在同基因肿瘤植入后第6天，或当它们达到200mm^{3}的体积时向小鼠i.p.给予mGITRL-FP或mOX40L-FP。在荷B16F10-Luc2小鼠中给予25mg/kg的mGITRL-FP只能耐受四次剂量。

流式细胞术

[0304] 将肿瘤、脾脏或肿瘤引流淋巴结解剖并置于冰上的RPMI-1640培养基中。将组织分别通过40或100μm尼龙细胞过滤器(Falcon)进行解聚，并且通过离心沉淀细胞并使其重悬于红细胞裂解缓冲液(西格玛公司)中。在室温下孵育2分钟后，将细胞洗涤并重悬于流式细胞术缓冲液(E生物科学公司)中。使用温和的MACS解离器和肿瘤解离试剂盒(美天旎生物技术公司(Miltenyi Biotec))按照制造商的说明处理肿瘤组织样品。

[0305] 使用live dead fixable blue(生命技术公司)按照制造商的说明对样品进行活力染色，并且然后在用荧光染料轭合的抗体染色之前用抗CD16/32(e生物科学公司)阻断。CD4(Rm4.5)、Foxp3(FJK-16S)、Ki67(Sol A15)、ICOS(7E.17G9)、Eomes(Dan11mag)、T-bet(Apr-46)和GITR(DTA-1)购自e生物科学公司。CD45(30-F11)、CD44(IM7)、CD62L(MEL-14)、PD-1(29F.1A12)和OX40(OX86)购自生物传奇公司。CD8(53-6.7)购自BD Pharmingen。对于细胞内抗原的染色，根据制造商的说明使用Foxp3染色试剂盒(e生物科学公司)。在使用Fortessa(贝迪公司(Becton Dickinson))采集样品之前，将样品固定在3.7％福尔马林中。用FlowJo软件(阿什兰，俄勒冈州)来分析数据。

药代动力学建模

[0306] 向Balb/c小鼠给予5或15mg/kg mGITRL-FP mIgG2a一次。处理后5分钟、0.5、1、2、6、24、72、144和240小时，将每组三只小鼠处死。收集血清样品，并使用抗鼠GITRL mAb(R&D系统公司(R&D Systems))，作为捕获和检测，在夹心ELISA中评估存在的循环mGITRL-FP mIgG2a的水平。

[0307] 将从2个给药组获得的药代动力学(PK)数据合并并同时使用群体方法建模。使用药理统计软件包NONMEM(7.2版本，ICON开发解决方案(ICON Development Solutions)，埃利科特城，马里兰州)进行群体分析。使用具有交互选项的FOCE方法。鼠GITRL-FP mIgG2a PK由二室模型充分描述。

细胞系

[0308] 将HEK293T-17细胞(ATCC，CRL-11268)保持在DMEM(英杰公司)加10％v/v热灭活的胎牛血清(HI FBS，HyClone)和1X非必需氨基酸(NEAA，英杰公司(Invitrogen))中。将Jurkat细胞(ATCC，TIB-152)保持在RPMI 1640(英杰公司)加10％v/v HI FBS中。将Jurkat mGITR NFκB细胞保持在补充有10％HI FBS、5μg/mL杀稻瘟素(英杰公司)和5μg/mL嘌呤霉素(英杰公司)的RPMI 1640中。

细胞系产生

[0309] 使用第三代慢病毒系统(系统生物科学公司(Systems Biosciences))通过慢病毒转导产生Jurkat mGITR NFκB细胞系。将鼠GITR基因(NM_009400.2)克隆到在CAG启动子的控制下且含有嘌呤霉素抗性基因的表达质粒中。NFκB报告基因表达质粒被设计为在萤火虫荧光素酶报告基因(luc2，普洛麦格公司)的最小启动子下和上游，与杀稻瘟素抗性基因一起，表达5个拷贝的NFκB应答元件。使用脂质转染胺2000(英杰公司)将每个表达质粒与包装

质粒(系统生物科学公司)一起共转染到HEK293T-17中。在转染后48小时收集含有病毒粒子的上清液并用于转导Jurkat细胞。从转导后48小时,将病毒转导的Jurkat细胞在加选择性抗生素5μg/mL杀稻瘟素和5μg/mL嘌呤霉素的培养基中培养。使用类似的程序产生Jurkat人OX40NFκB细胞系,除了转导被设计为组成型表达人OX40的慢病毒载体。小鼠OX40L结合人OX40;因此,可以使用表达OX40的人NFκB荧光素酶报告Jurkat细胞系来评估鼠OX40L融合蛋白的活性。

ELISA

[0310] 将重组小鼠GITR-Fc和OX40-Fc(R&D系统公司)糖蛋白以PBS中1μg/mL包被在96孔板(格雷内尔公司(Greiner))上过夜。将板用PBST(PBS+0.01%Tween-20)洗涤,在室温下用含有1%(w/v)BSA的PBS阻断1小时,并再次在PBST中洗涤。将在测定缓冲液[PBS+1%牛血清白蛋白(BSA)]中稀释的25微升的1μg/mL小鼠GITRL或OX40LFP加入到孔中,并且将板在室温下孵育2小时。洗涤3次后,向各孔中加入25μL在测定缓冲液中稀释的1μg/mL辣根过氧化物酶轭合的抗小鼠Fc抗体(西格玛公司),并将板孵育1小时。孵育后,将板在PBST中洗涤3次,向各孔中加入25μL四甲基联苯胺底物溶液(KPL),并将板孵育5分钟。孵育后,将15μL 0.5M硫酸终止液加入所有孔中。使用EnVision酶标仪(珀金埃尔默公司)测量450nm处的光密度。

统计

[0311] 使用Prism统计软件版本6进行所有统计学分析。

结果

mGITRL-FP体外效力

[0312] 为了研究GITR受体信号传导对体内免疫细胞活化和抗肿瘤活性的影响,产生四聚体mGITRL-FP。mGITRL-FP被设计为引起与效应细胞上的GITR受体和FcγR的热烈结合。该分子从N-末端至C-末端由免疫球蛋白G(IgG)的片段可结晶(Fc)区、异亮氨酸拉链结构域(ILZ)和鼠GITR配体的胞外(GITR结合)结构域(ECD)组成(图30A)。当通过SDS-PAGE(图30B)可视化经纯化的变性mGITRL-FP时,证明了高度均一性和略高于预期的48kDa分子量的分子量(大概归因于Fc和mGITRL结构域的糖基化)。在GITR依赖性NF-κB报告基因细胞测定中,mGITRL-FP mIgG2a和抗GITR抗体(DTA-1)二者均能够诱导NFκB信号传导,而鼠OX40L FP mIgG1(mOX40L-FP mIgG1)和同种型对照缺少任何可检测的信号(图30C)。重要的是,在本测定中,四聚体mGITRL-FP证明针对GITR激动作用EC_{50}为0.05nM,其比证明EC_{50}为2.31nM的DTA-1有效近50倍。

用mIgG2a Fc工程化的mGITRL-FP的抗肿瘤活性

[0313] 先前的研究显示活化FcγR对于DTA-1抗体的抗肿瘤活性是必需的,并且当携带mIgG2a Fc时,与缺少FcγR结合的rIgG2b Fc或N297A突变体Fc相比较,此抗体的抗肿瘤活性增加。为了确定Fc同种型对mGITRL-FP的抗肿瘤活性的影响,将CT26荷瘤小鼠用具有mIgG2a或mIgG1 Fc同种型的mGITRL-FP进行处理。如通过减少的肿瘤体积所证明的,与盐水处理的对照相比,用5或10mg/kg的任一个同种型处理小鼠产生显著的抗肿瘤活性(图31A)。然而,与mGITRL-FP mIgG1(7/20总消退,图31A)相反,用mGITRL-FP mIgG2a(11/20总消退)处理后整体抗肿瘤活性更大。

[0314] 为了确定Fc变体的这种活性差异是否是归因于它们介导T-细胞活化和GITR激动作用下游的后续事件的能力差异,使用$CD4^+FoxP3^-$(效应T-细胞)、$CD8^+$(效应T-细胞)和$CD4^+$

FoxP3$^+$(T-reg)细胞上的Ki67表达的流式细胞术分析来评估脾T-细胞群的增殖。与盐水对照相比时,mGITRL-FP的两个同种型都引起所有三个脾脏T-细胞亚群的增殖的可比较的显著增加(图31B)。脾T-reg细胞群在处理后展现最高Ki67表达(mGITRL-FP mIgG2a后为53.95%±0.75且mGITRL-FP mIgG1处理后为58.43%±1.26),随后是CD4$^+$FoxP3$^-$(用mGITRL-FP mIgG1时为9.52%±0.58且用mGITRL-FP mIgG1时为10.89%±0.56)和CD8$^+$T-细胞(用mGITRL-FP mIgG2a时为7.60%±0.27且用mGITRL-FP mIgG1时为7.79±0.43)。此数据表明单单脾脏T-细胞活化不能说明mGITRL-FP的mIgG1和mIgG2a Fc同种型之间在抗肿瘤免疫方面观察到的差异的原因。

[0315] 接下来研究了T-细胞群体中肿瘤内变化是否可以解释用mGITRL-FP的mIgG2a与mIgG1变体时观察到的抗肿瘤活性增加。用mGITRL-FP mIgG2a处理小鼠诱导肿瘤内T-reg的频率从盐水处理的动物中的13.31±1.06显著降低至3.60%±0.79(显著性值$p<0.0001$)(图31C和图32),并且随后CD8:T-reg(图31D)和CD4:T-reg(图32B)比率与对照处理的动物相比增加。相比之下,对于mGITRL-FP的mIgG1 Fc变体(12.95%±1.34),肿瘤内T-reg减少不明显。也有证据表明,与对照动物相比(13.01%±1.12),用mGITRL-FP mIgG2a处理后肿瘤内CD4$^+$Foxp3$^-$T-细胞的比例显著降低(8.64%±1.21),但这没有到对于T-reg观察到的程度。mGITRL-FP mIgG2a优先耗减T-reg可能归因于GITR在肿瘤内T-reg上的高表达(图31E)和CT26肿瘤的肿瘤微环境中活化Fc γ R的高表达,并且表明通过抗体依赖性细胞毒作用(ADCC)或抗体依赖性细胞吞噬作用(ADCP)进行的清除。总之,这些数据表明,对于mGITRL-FP处理后的最佳抗肿瘤活性,与肿瘤内T-reg减少一致的外周CD4$^+$和CD8$^+$T-细胞增殖是必需的。

mGITRL-FP mIgG2a介导的抗肿瘤活性

[0316] 因为mGITRL-FP mIgG2a引起与mGITRL-FP mIgG1相比增加的抗肿瘤活性,接下来表征了mGITRL-FP mIgG2a在血液中的暴露水平和暴露时间与CT26荷瘤小鼠中的抗肿瘤应答如何相关。首先,通过用单剂量盐水对照或0.2、1或5mg/kg mGITRL-FP mIgG2a处理小鼠,测量增加剂量水平对肿瘤生长的影响。用0.2mg/kg mGITRL-FP mIgG2a处理导致仅1/10完全消退,然而有证据表明当剂量从0.2mg/kg增加到1mg/kg或5mg/kg时抗肿瘤免疫性的剂量依赖性增加(分别导致6/10和9/10消退)(图33A)。此外,抗肿瘤活性也可以通过将0.2mg/kg给药频率增加到每天一次(Q1D)或每周一次(Q1W)来改进(图33B)。这些结果指示,如通过完全肿瘤消退和最小剩余肿瘤体积(其仅在使用Q1D方案时达到)所定义的,存在诱导最佳抗肿瘤活性需要的mGITRL-FP mIgG2a的必需暴露水平。为了确定此阈值,在用2个可替代剂量水平的mGITRL-FP处理的小鼠的血液中测量mGITRL-FP mIgG2a的浓度,并且然后将这些结果并入PK模型中。基于这个模型,计算出由Q1D方案保持的mGITRL-FP mIgG2a的血液浓度且认为是维持最佳抗肿瘤活性所需的血液浓度等于或大于1μg/mL(图33C)。

[0317] 接下来研究在mGITRL-FP mIgG2a处理之后抗肿瘤活性的PD生物标记物。使用Ki67的表达来评估增殖,同时也分析了所有已知在活化后在T-细胞上表达的ICOS、PD-1和OX40。通过增加剂量水平或增加给药频率来增加mGITRL-FP mIgG2a的暴露导致表达Ki67、ICOS、PD-1和OX40的外周CD4$^+$T-细胞的频率逐渐更大增加(图34A-D)。

mGITRL-FP和mOX40L-FP抗肿瘤活性的比较分析

[0318] 为了理解GITR的相对机制,与OX40靶向相反,将mGITRL-FP的抗肿瘤活性直接与mOX40L-FP相比较,该mOX40L-FP包括类似蛋白质结构,不与GITR受体交叉反应并且能够在

OX40报告细胞测定中诱导NFκB表达(图35A和B)。

[0319] 将CT26荷瘤小鼠每周用5mg/kg mIgG1或mIgG2a Fc同种型的mGITRL-FP或mOX40L-FP处理,并测量肿瘤生长。如在用mGITRL-FP的情况下所见,与用mIgG1处理后(1/10消退)相比在用mIgG2a同种型处理后用mOX40L-FP观察到的抗肿瘤活性较高(9/10消退)(图36A)。然而,相对于用mOX40L-FP mIgG1处理后所见,用mGITRL-FP mIgG1处理引起抗肿瘤活性增加(6/10消退),并且mIgG2a同种型(10/10)相比mOX40L-FP mIgG2a略微更好(图36A)。在用mGITRL-FP和mOX40L-FP两者以mIgG2a Fc同种型进行处理后观察到肿瘤内T-reg的类似耗减,但是这对任一分子的mIgG1 Fc同种型都不明显(图5B)。此数据指示在CT26荷瘤小鼠中,对于诱导GITR和OX40激动剂二者的抗肿瘤活性,mIgG2a同种型优于mIgG1。

mGITRL-FP mIgG2a和mOX40L-FP mIgG1组合研究

[0320] 考虑到用mGITRL-FP mIgG2a处理后$CD4^+$T-细胞上OX40受体的上调(图34D),进一步研究了使T-细胞活化最大化的潜力。使用mGITRL-FP mIgG2a和mOX40L-FP mIgG1来评估将靶向GITR和OX40途径的药物相组合的潜在益处。

[0321] 用mGITRL-FP mIgG2a和mOX40L-FP mIgG1组合每两周一次处理导致相比于盐水对照(7.82%±0.30和9.22%±0.31)或用任一单一疗法进行的处理(mGITRL-FP处理的CD4和CD8 T-细胞为14.2%±0.45和15.52%±0.66,且mOX40L-FP处理的CD4和CD8T-细胞为17.02%±0.49和16.22%±1.22),脾$CD4^+$(29.16%±1.51)和$CD8^+$(24.4%±0.87)T-细胞中的Ki67表达显著增加(图37A),显示了将两个分子组合的累加效应。对脾$CD4^+$和$CD8^+$T-细胞群体的另外分析表明,与单一疗法处理相比,组合处理增加了定义为$CD44^+$和$CD62^{lo}$的$CD4^+$和$CD8^+$效应记忆区室(图37B)及定义为$CD44^+$和$CD62^+$的$CD4^+$中枢记忆群体(图37C)二者的频率。在$CD4^+$和$CD8^+$T-细胞上,在组合中已显示IFNγ产生冗余性和$CD8^+$T-细胞中的细胞毒作用并且在Th1分化中也具有作用的转录因子T-bet(图37D)和Eomes(图37E)的表达也增加到比任一单一疗法更大的程度。GITR和OX40途径之间的机械差异是:与用mGITRL-FP mIgG2a处理的动物相比,在mOX40L-FP mIgG1处理的动物中$CD4^+$T-细胞上T-bet和Eomes的表达显著更高。

[0322] 基于这些发现,确定了将mGITRL-FP mIgG2a与mOX40L-FP mIgG1相组合对肿瘤生长的影响。接下来研究用mOX40L-FP mIgG1处理小鼠是否可以通过与单个次最佳剂量的mGITRL-FP mIgG2a的组合而进一步改进。用mOX40L-FP mIgG1单一疗法处理诱导5/10消退,mGITRL-FP mIgG2a单一疗法处理诱导3/10消退,而mGITRL-FP mIgG2a和mOX40L-FP mIgG1两者的组合导致抗肿瘤活性增强,显示8/10消退(图38A)。

[0323] 考虑到高剂量的mGITRL-FP mIgG2a的单一疗法处理在CT26模型的大多数小鼠中诱导完全肿瘤消退,针对在B16F10-Luc 2模型增加益处研究了高剂量的mOX40L-FP mIgG1和mGITRL-FP mIgG2a的组合。在这个模型中用单一疗法给药没有诱导任何肿瘤消退,然而两种分子的组合导致改进的抗肿瘤活性;与单一疗法处理相比,延迟肿瘤生长并且增加存活率(图38B和图39)。

实例7:T细胞引发剂与mGITRL-FP组合

材料和方法

细胞系和试剂

[0324] TC-1肿瘤系获自ATCC(目录号CRL 6475,马纳萨斯,弗吉尼亚州)并保持在DMEM+10%FBS+1%青霉素/链霉素中。CT26肿瘤系获自ATCC(马纳萨斯,弗吉尼亚州)并维持在补

充有10％胎牛血清的RPMI 1640培养基中。DTA-1和同种型抗体购自Bio X Cell(西黎巴嫩，新罕布什尔州)。

E7 SLP和疫苗接种

[0325] 由新英格兰肽(New England Peptide)(加德纳，马萨诸塞州)合成由45-mer HPV16-E7序列SSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVD(SEQ ID NO:57)组成的E7合成长肽(SLP)。将E7 SLP以10或3.3μg的剂量给予，并且在Addavax(生命技术公司，卡尔斯巴德，加利福尼亚州)和PBS中用20μg CpG ODN 2395(曲林克公司(TriLink)，圣地亚哥，加利福尼亚州)配制，总体积为50μL。将疫苗皮下给予到尾根部的背面。

肿瘤模型

[0326] TC-1实验使用获自杰克逊实验室(Jackson Labs)(巴港，缅因州)的雌性C57BL/6小鼠(目录号000664)小鼠。CT26实验使用获自Envigo(弗雷德里克，马里兰州)的雌性Balb/C小鼠。在肿瘤植入时小鼠在6-8周龄之间。所有动物实验均按照研究机构动物护理和使用委员会制定的指导方针进行。对于TC-1肿瘤植入，将2x 10^4个活TC-1细胞皮下植入左后足垫。对于CT26肿瘤植入，将5x 10^5个细胞植入右侧腹。通过用卡尺1直接测量来评价肿瘤生长。每2-4天收集双向测量，并使用体积＝(长度•宽度2)/2计算肿瘤体积。允许肿瘤发展6-14天，并且然后将荷瘤小鼠按肿瘤体积随机分配到处理组。按照IACUC方案，当TC-1/足垫的原发性肿瘤超过1000mm^3和CT 26/侧腹的原发性肿瘤超过2000mm^3时使小鼠安乐死。对于PD研究，使小鼠安乐死，并收集肿瘤和脾脏，通过70μM滤器(Corning™，康宁公司，纽约)粉碎，并加工成单细胞悬浮液。

功能性T-细胞响应和流式细胞术

[0327] 对于抗原特异性刺激，以1-2x10^6个活细胞/孔进行铺板，每孔具有1ug AH1肽序列SPSYVYHQF(SEQ ID NO:56)(Anaspec，弗里蒙特，加利福尼亚州)或10μg E7肽序列RAHYNIVTF(SEQ ID NO:58)(Anaspec)和蛋白质转运抑制剂(E生物科学公司，圣克拉拉(Santa Clara)，加利福利亚州)。5小时后，将细胞染色。对于所有染色剂，染色顺序为live/dead blue(生命技术公司)、胞外蛋白、FOXP3 Fix/Perm Kit(E生物科学公司(Ebioscience))并且随后是细胞内细胞因子。抗体包括CD45(克隆30-F11)、CD4(克隆RM4-5)CD8(克隆53-6.7)、GITR(克隆DTA-1)、IFNγ(克隆XMG 1.2)、TNFα(克隆MP6-XT22)、FOXP3(克隆FJK-16s)、KI-67(克隆SolA15)。装载有RAHYNIVTF的H2-Db E7 Dextramer购自Immudex(费尔法克斯郡，弗吉尼亚州)，并且我们遵循其染色方案进行。所有样品都在LSR-II或Fortessa(BD，圣何塞，加利福尼亚州)上运行。使用FlowJo(树星(TreeStar)，阿什兰，俄勒冈州)来分析所有数据。

mGITRL-FP需要抗原特异性细胞

[0328] CT26是一种非常有免疫原性的肿瘤模型，具有高的肿瘤内CD45和基础CTL水平。已经显示CT26能自引发低水平的AH1特异性T-细胞。为了评估mGITRL-FP是否能够导致抗原特异性应答的从头生成，使用E6/E7转化的TC-1肿瘤模型。该模型具有已知的CD8 T-细胞表位，并且E7特异性免疫应答可导致针对携带E7的肿瘤的保护。将TC-1肿瘤细胞植入C57/b6小鼠的足垫中。在第14天测量小鼠，基于肿瘤大小随机化并用1mg/kg、5mg/kg和20mg/kg的mGITRL-FP IgG2a处理(图43A)。在任何测试剂量下都有肿瘤生长的延迟或抑制(图43B)。在第24天，将未经处理和1mg/kg处理的小鼠的子组处死，并收获脾脏和肿瘤。评估T-细胞群和

E7抗原特异性应答。GITR水平存在于CD4和CD8 T-细胞中，并且在肿瘤中显著更高(图43C)。没有E7引发的情况下，mGITRL-FP不能延迟TC-1肿瘤生长或产生抗肿瘤免疫应答。

mGITRL-FP扩增了引发的抗原特异性CD8 T-细胞

[0329] DNA疫苗的疫苗接种已显示可有效产生E7特异性应答，其导致预防和抑制TC-1肿瘤模型的肿瘤生长。本研究假设mGITRL-FP可需要引发的抗原特异性T-细胞来驱动抗肿瘤应答。为了产生这种应答，在尾根部用10ug E7合成长肽(SLP)与CpG(addavax)接种初试的C57/b6小鼠。在此期间，将小鼠用3个剂量的mGITRL-FP IgG2a处理。7天后，取出脾细胞并用E7 dextramer评价T-细胞的抗原特异性(图44A)。未看到CD4和CD8百分比的差异，但是用mGITRL-FP处理时观察到抗原特异性细胞的大量增加(图44B-D)。另外，评价单独接种的小鼠的GITR水平，抗原特异性Dex+细胞具有比Dex-CD8 T-细胞更高的GITR MFI(图44E-F)。假设在荷瘤小鼠中这种差异甚至更高，因为在肿瘤微环境中GITR水平似乎是最高的。将C57/b6小鼠植入TC-1肿瘤并用E7 SLP/CpG(Addavax)接种(图44G)。通过dextamer测量，观察在脾脏和肿瘤二者中的抗原特异性E7细胞的引发(图44H-1)。在脾脏和肿瘤中，用dextramer呈染色阳性的细胞的GITR也较高(图44J-K)。在有或没有肿瘤存在的情况下，E7 SLP能够成功引发E7特异性应答，并且引发的抗原特异性细胞比其他CD8 T-细胞表达更高水平的GITR。

mGITRL-FP可扩增由E7 SLP引发的抗原特异性细胞并产生对TC-1肿瘤的保护性免疫应答

[0330] 为了评价mGITRL-FP是否可扩增对TC-1肿瘤细胞的E7特异性应答，将TC-1肿瘤细胞植入足垫并在第14天将小鼠按肿瘤体积随机化。在尾根部给予单剂量的E7 SLP与CpG(Addavax)，以及mGITRL-FP IgG2a，以四种剂量开始疫苗接种日(图45A)。包括两个研究组，一个TGI(肿瘤生长抑制)组(n＝10)和一个PD(药效)组(n＝5)。对照小鼠肿瘤迅速生长，并且所有小鼠死亡，存活期中值为27天。单独的E7 SLP为具有TC-1肿瘤的小鼠提供了存活优势，其中10只小鼠中的1只在第85天活着并且无肿瘤，且存活期中值为46.5天(图45B)。E7 SLP+mGITRL-FP显著延迟肿瘤生长，其中3/10只小鼠无肿瘤，并且5/10在第85天活着，且存活期中值为80.5天(图45C-D)。单独的疫苗接种提供了肿瘤进展的延迟，并且在上面添加mGITRL-FP进一步延迟进展。假设这是由于肿瘤特异性CD8的选择扩增而发生的。在第21天，处死PD小鼠组并收获脾脏和肿瘤用于药效学分析。归因于收获的组织量，合并来自足垫的肿瘤(图45E)。单独接种或与mGITRL-FP组合导致进入肿瘤的CD45+细胞显著增加(图45F)。为了评价抗原特异性功能，用1ug/mL的E7肽再刺激肿瘤或脾脏的单细胞悬浮液。疫苗接种提供了抗原特异性细胞的基础增加，并且添加mGITRL-FP进一步将此提高至在脾脏和肿瘤二者中均更高的水平(图45G)。mGITRL-FP显现不会耗减脾脏中的Treg，并且当E7 SLP疫苗存在时仅能够耗减肿瘤中的Treg(图45G)。

实例8:核苷酸和蛋白质序列。

[0331] 在此披露的核苷酸和蛋白质序列的注释版本在表6-1中提供。

表6-1.

SEQ ID NO.	描述	序列
1	全长 WT GITRL 的氨	MCLSHLENMPLSHSRTQGAQRSSWKLWLFCSIVMLLF LCSFSWLIFIFLQLETAKEPCMAKFGPLPSKWQMASSE PPCVNKVSDWKLEILQNGLYLIYGQVAPNANYNDVAP

	基酸序列	FEVRLYKNKDMIQTLTNKSKIQNVGGTYELHVGDTID LIFNSEHQVLKNNTYWGIILLANPQFIS
2	全长 GITRL 变体的氨基酸序列	MCLSHLENMPLSHSRTQGAQRSSWKLWLFCSI**X1X2**LL FLCSFSWLIFIFLQL**X3**TAKEPCMAK**X4**GPL**X5X6**KWQ**X7** ASSEP**X8**C**X9**NKV**X10**DWKLEILQNGLYLIY**X11**QVAPNA NYNDVA**X12**FEV**X13**LYKNKD**X14**IQTLTNKSKIQNVGGT YELHVGDTIDLIF**X15**SEHQ**X16**LK**X17**NTYWG**X18X19**LLA NPQFIS X_1 = Gly 或 Val，X_2 = Thr、Met、或 Val；X_3 = Glu 或 Ala，X_4 = Ser 或 Phe，X_5 =Thr 或 Pro，X_6 =Leu 或 Ser，X_7 =Thr 或 Met，X_8 = Leu 或 Pro，X_9 = Met 或 Val，X_{10} = Thr、Phe、或 Ser，X_{11} = Ser 或 Gly，X_{12} = Arg 或 Pro，X_{13} = Trp 或 Arg，X_{14} = Leu 或 Met，X_{15} = Ser 或 Asn，X_{16} =Phe 或 Val，X_{17} =除 Asn 外的任何氨基酸，X_{18} = Val 或 Ile，并且 X_{19} = Leu 或 Ile
3	全长 GITRL N161X 变体的氨基酸序列	MCLSHLENMPLSHSRTQGAQRSSWKLWLFCSIVMLLF LCSFSWLIFIFLQLETAKEPCMAKFGPLPSKWQMASSE PPCVNKVSDWKLEILQNGLYLIYGQVAPNANYNDVAP FEVRLYKNKDMIQTLTNKSKIQNVGGTYELHVGDTID LIFNSEHQVLK**X**NTYWGIILLANPQFIS X 是除了 Asn 外的任何氨基酸。
4	全长 GITRL N161D 变体的氨基酸序列	MCLSHLENMPLSHSRTQGAQRSSWKLWLFCSIVMLLF LCSFSWLIFIFLQLETAKEPCMAKFGPLPSKWQMASSE PPCVNKVSDWKLEILQNGLYLIYGQVAPNANYNDVAP FEVRLYKNKDMIQTLTNKSKIQNVGGTYELHVGDTID LIFNSEHQVLK**D**NTYWGIILLANPQFIS
5	编码成熟 GITRL IgG1 FP 亚基的核酸序列（无信号肽编码区）	CTGGACAAGACCCATACCTGTCCTCCATGCCCTGCC CCCGAACTGCTGGGAGGCCCTTCTGTGTTCCTGTTCC CCCCAAAGCCCAAGGACACCCTGATGATCTCCCGGA CCCCCGAAGTGACCTGCGTGGTGGTGGATGTGTCCC ACGAGGACCCTGAAGTGAAGTTCAATTGGTACGTGG ACGGCGTGGAAGTGCACAACGCCAAGACCAAGCCC AGAGAGGAACAGTACAACTCCACCTACCGGGTGGT GTCCGTGCTGACCGTGCTGCACCAGGATTGGCTGAA CGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAGG CCCTGCCTGCCCCCATCGAAAAGACCATCTCCAAGG CCAAGGGCCAGCCCCGGGAACCCCAGGTGTACACA CTGCCCCCTAGCCGGGAAGAGATGACCAAGAACCA GGTGTCCCTGACCTGTCTCGTGAAGGGCTTCTACCC CTCCGATATCGCCGTGGAATGGGAGTCCAACGGCCA GCCTGAGAACAACTACAAGACCACCCCCCCTGTGCT GGACTCCGACGGCTCATTCTTCCTGTACTCCAAGCT

		GACAGTGGACAAGTCCCGGTGGCAGCAGGGCAACG TGTTCTCCTGCTCCGTGATGCACGAGGCCCTGCACA ACCACTACACCCAGAAGTCCCTGTCCCTGAGCCCTG GAAAAGGCGGCGGAGGATCTGGCGGAGGCGGTTCT GGTGGTGGCGGATCTGGGGGCGGAGGTAGCGGAGG TGGTGGCTCTGTGTCTCGGCTGGAAGAGGAAATGCG GAAGCTGCAGGCCACCGTGCAGGAACTGCAGAAGC GGCTGGACAGACTGGAAGAGACAGTGCAGGCTAAG GGCGGTGGCGGACAGCTCGAGACAGCCAAAGAACC CTGCATGGCCAAGTTCGGCCCCCTGCCTTCCAAGTG GCAGATGGCCTCTTCCGAGCCCCCCTGCGTGAACAA AGTGTCCGACTGGAAGCTGGAAATCCTGCAGAACG GCCTGTACCTGATCTACGGCCAGGTGGCCCCCAACG CCAACTACAACGATGTGGCCCCCTTCGAAGTGCGGC TGTACAAGAACAAGGACATGATCCAGACCCTGACC AACAAGAGCAAGATCCAGAACGTGGGCGGCACCTA CGAGCTGCACGTGGGCGATACCATCGACCTGATCTT CAACTCCGAGCACCAGGTGCTGAAGGACAACACCT ACTGGGGCATCATCCTGCTGGCCAACCCCCAGTTCA TCTCC
6	成熟 GITRL IgG1 FP 亚基的氨基酸序列（接头 1 和接头 2 加下划线）	LDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK<u>GGGGSGGGGSGGGGSGGGGSGGGGS</u>VSRLEEE MRKLQATVQELQKRLDRLEETVQAK<u>GGGG</u>QLETAKE PCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQNG LYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQTLTNK SKIQNVGGTYELHVGDTIDLIFNSEHQVLK**D**NTYWGII LLANPQFIS
7	编码前体 GITRL IgG1 FP 亚基的核酸（包括信号肽编码区）	ATGGCCATCATCTACCTGATCCTGCTGTTCACCGCCG TGCGGGGCCTGGACAAGACCCATACCTGTCCTCCAT GCCCTGCCCCCGAACTGCTGGGAGGCCCTTCTGTGT TCCTGTTCCCCCCAAAGCCCAAGGACACCCTGATGA TCTCCCGGACCCCCGAAGTGACCTGCGTGGTGGTGG ATGTGTCCCACGAGGACCCTGAAGTGAAGTTCAATT GGTACGTGGACGGCGTGGAAGTGCACAACGCCAAG ACCAAGCCCAGAGAGGAACAGTACAACTCCACCTA CCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGA TTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGT CCAACAAGGCCCTGCCTGCCCCCATCGAAAAGACCA TCTCCAAGGCCAAGGGCCAGCCCCGGGAACCCCAG GTGTACACACTGCCCCCTAGCCGGGAAGAGATGACC AAGAACCAGGTGTCCCTGACCTGTCTCGTGAAGGGC TTCTACCCCTCCGATATCGCCGTGGAATGGGAGTCC

		AACGGCCAGCCTGAGAACAACTACAAGACCACCCC CCCTGTGCTGGACTCCGACGGCTCATTCTTCCTGTAC TCCAAGCTGACAGTGGACAAGTCCCGGTGGCAGCA GGGCAACGTGTTCTCCTGCTCCGTGATGCACGAGGC CCTGCACAACCACTACACCCAGAAGTCCCTGTCCCT GAGCCCTGGAAAAGGCGGCGGAGGATCTGGCGGAG GCGGTTCTGGTGGTGGCGGATCTGGGGGCGGAGGTA GCGGAGGTGGTGGCTCTGTGTCTCGGCTGGAAGAGG AAATGCGGAAGCTGCAGGCCACCGTGCAGGAACTG CAGAAGCGGCTGGACAGACTGGAAGAGACAGTGCA GGCTAAGGGCGGTGGCGGACAGCTCGAGACAGCCA AAGAACCCTGCATGGCCAAGTTCGGCCCCCTGCCTT CCAAGTGGCAGATGGCCTCTTCCGAGCCCCCCTGCG TGAACAAAGTGTCCGACTGGAAGCTGGAAATCCTGC AGAACGGCCTGTACCTGATCTACGGCCAGGTGGCCC CCAACGCCAACTACAACGATGTGGCCCCCTTCGAAG TGCGGCTGTACAAGAACAAGGACATGATCCAGACC CTGACCAACAAGAGCAAGATCCAGAACGTGGGCGG CACCTACGAGCTGCACGTGGGCGATACCATCGACCT GATCTTCAACTCCGAGCACCAGGTGCTGAAGGACAA CACCTACTGGGGCATCATCCTGCTGGCCAACCCCCA GTTCATCTCC
8	前体 GITRL IgG1 FP 亚基的氨基酸序列（包括信号肽编码区；接头 1 和接头 2 加下划线）	MAIIYLILLFTAVRGLDKTHTCPPCPAPELLGGPSVFLFP PKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLSPGKGGGGSGGGGSGGGGSGGGG SGGGGSVSRLEEEMRKLQATVQELQKRLDRLEETVQA KGGGGQLETAKEPCMAKFGPLPSKWQMASSEPPCVN KVSDWKLEILQNGLYLIYGQVAPNANYNDVAPFEVRL YKNKDMIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSE HQVLKDNTYWGIILLANPQFIS
9	人冠蛋白 1a（hCor1a）的氨基酸序列	MSRQVVRSSKFRHVFGQPAKADQCYEDVRVSQTTWD SGFCAVNPKFVALICEASGGGAFLVLPLGKTGRVDKN APTVCGHTAPVLDIAWCPHNDNVIASGSEDCTVMVW EIPDGGLMLPLREPVVTLEGHTKRVGIVAWHTTAQNV LLSAGCDNVIMVWDVGTGAAMLTLGPEVHPDTIYSV DWSRDGGLICTSCRDKRVRIIEPRKGTVVAEKDRPHEG TRPVRAVFVSEGKILTTGFSRMSERQVALWDTKHLEEP LSLQELDTSSGVLLPFFDPDTNIVYLCGKGDSSIRYFEIT SEAPFLHYLSMFSSKESQRGMGYMPKRGLEVNKCEIA RFYKLHERRCEPIAMTVPRKSDLFQEDLYPPTAGPDPA LTAEEWLGGRDAGPLLISLKDGYVPPKSRELRVNRGL DTGRRRAAPEASGTPSSDAVSRLEEEMRKLQATVQEL QKRLDRLEETVQAK
10	hCor1a 三聚	XSRXEEEXRKXQATXQELQKRXDRLEETVQAK

	结构域共有的氨基酸序列	X= Ala、Leu、Ile、或 Val
11	hCor1a wt 三聚结构域的氨基酸序列	VSRLEEEMRKLQATVQELQKRLDRLEETVQAK
12	hCor1a 变体 1 三聚结构域的氨基酸序列	VSRLEEEIRKLQATVQELQKRLDRLEETVQAK
13	hCor1a 变体 2 三聚结构域的氨基酸序列	VSRIEEEIRKLQATVQELQKRLDRLEETVQAK
14	hCor1a 变体 3 三聚结构域的氨基酸序列	ISRIEEEIRKLQATVQELQKRLDRLEETVQAK
15	hCor1a 变体 4 三聚结构域的氨基酸序列	ISRIEEEIRKIQATVQELQKRLDRLEETVQAK
16	hCor1a 变体 5 三聚结构域的氨基酸序列	ISRIEEEIRKIQATVQELQKRIDRLEETVQAK
17	hCor1a 变体 6 三聚结构域的氨基酸序列	ISRIEEEIRKINATVQELQKRIDRLEETVQAK
18	hCor1a 变体 7 三聚结构域的氨基酸序列	ISRIEEEIRKINATIQELQKRIDRLEETVQAK
19	Gly(4)Ser 基序（其中 n =	GGGGS

	1）的氨基酸序列	
20	接头 1 的氨基酸序列	GGGGSGGGGSGGGGSGGGGSGGGGS
21	用于 SEQ ID NO: 6 的 GITRL IgG1 FP 中的 IgG1 Fc 区的氨基酸序列	LDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
22	Ser(Gly_4Ser)n 基序（其中 n = 1）的氨基酸序列	SGGGGS
23	接头区的氨基酸序列	GGGGSGGGGSGGGGSAL
24	接头区的氨基酸序列	GGGGSGGGGSGGGGSA
25	Gly(4)Ser 基序（其中 n = 3）的氨基酸序列	GGGGSGGGGSGGGGS
26	接头区的氨基酸序列	GGGGSGGGGSGGGG
27	酵母 GCN4 pII 的氨基酸序列	IKQIEDKIEEILSKIYHIENEIARIKKL
28	母系蛋白 1 wt 三聚结构域的氨基酸序列	CACESLVKFQAKVEGLLQALTRKLEAVSKRLAILENT VV
29	母系蛋白 1 变体三聚结构域的氨基酸序列	CACESLVKFQAKVEGLIQALTRKLEAVSKRIAILENTV V

30	DMPK wt 三聚结构域的氨基酸序列	EAEAEVTLRELQEALEEEVLTRQSLSREMEAIRTDNQN FASQLREAEARNRDLEAHVRQLQERMELLQAE
31	DMPK 变体三聚结构域的氨基酸序列	IAEIEVTIRELQEAIEEEVLTRQSLSREIEAIRTDIQNIASQ LREIEARIRDLEAHVRQLQERMELLQAE
32	朗格汉斯蛋白 wt 三聚结构域的氨基酸序列	ASALNTKIRALQGSLENMSKLLKRQNDILQVVS
33	朗格汉斯蛋白变体三聚结构域的氨基酸序列	ISALNTKIRAIQGSIENMSKLIKRQNDIIQVVS
34	成熟 WT GITRL 胞外结构域的氨基酸序列	QLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDW KLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNKD MIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLK NNTYWGIILLANPQFIS
35	成熟 GITRL 变体胞外结构域的氨基酸序列	QL**X**$_1$TAKEPCMAK**X**$_2$GPL**X**$_3$**X**$_4$KWQ**X**$_5$ASSEP**X**$_6$C**X**$_7$NK V**X**$_8$DWKLEILQNGLYLIY**X**$_9$QVAPNANYNDVA**X**$_{10}$FEV **X**$_{11}$LYKNKD**X**$_{12}$IQTLTNKSKIQNVGGTYELHVGDTIDLI F**X**$_{13}$SEHQ**X**$_{14}$LK**X**$_{15}$NTYWG**X**$_{16}$**X**$_{17}$LLANPQFIS X_1 = Glu 或 Ala，X_2 = Ser 或 Phe，X_3 =Thr 或 Pro，X_4 =Leu 或 Ser，X_5 = Thr 或 Met，X_6 = Leu 或 Pro，X_7 = Met 或 Val，X_8 = Thr、Phe、或 Ser，X_9 = Ser 或 Gly，X_{10} = Arg 或 Pro，X_{11} = Trp 或 Arg，X_{12} = Leu 或 Met，X_{13} = Ser 或 Asn，X_{14} =Phe 或 Val，X_{15} =除 Asn 外的任何氨基酸，X_{16} = Val 或 Ile，并且 X_{17} = Leu 或 Ile
36	成熟 GITRL N161X 变体胞外结构域的氨基酸序列	QLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDW KLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNKD MIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLK **X**NTYWGIILLANPQFIS X 是除了 Asn 外的任何氨基酸。
37	成熟 GITRL	QLETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDW

	N161D 变体胞外结构域的氨基酸序列	KLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNKD MIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLK **D**NTYWGIILLANPQFIS
38	用于 GITRL IgG4P FP 中的 IgG1 Fc 区的氨基酸序列（S228P 突变加下划线）	ESKYGPPCP**P**CPAPEFLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPS SIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLS LSLGK
39	编码成熟 GITRL IgG4P FP 亚基的核酸（无信号肽编码区）	GAGTCTAAGTACGGCCCTCCTTGTCCTCCTTGCCCTG CCCCTGAGTTTCTGGGCGGACCTTCCGTGTTCCTGTT CCCCCCAAAGCCCAAGGACACCCTGATGATCTCCCG GACCCCCGAAGTGACCTGCGTGGTGGTGGATGTGTC CCAGGAAGATCCCGAGGTGCAGTTCAATTGGTACGT GGACGGCGTGGAAGTGCACAACGCCAAGACCAAGC CCAGAGAGGAACAGTTCAACTCCACCTACCGGGTGG TGTCCGTGCTGACCGTGCTGCACCAGGATTGGCTGA ACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAG GGCCTGCCCTCCAGCATCGAAAAGACCATCTCCAAG GCCAAGGGCCAGCCCCGGGAACCCCAGGTGTACAC ACTGCCTCCAAGCCAGGAAGAGATGACCAAGAACC AGGTGTCCCTGACCTGTCTCGTGAAGGGCTTCTACC CCTCCGATATCGCCGTGGAATGGGAGTCCAACGGCC AGCCTGAGAACAACTACAAGACCACCCCCCCTGTGC TGGACTCCGACGGCTCCTTCTTCCTGTACTCCCGCCT GACCGTGGACAAGTCCAGATGGCAGGAAGGCAACG TGTTCTCCTGCTCCGTGATGCACGAGGCCCTGCACA ACCACTACACCCAGAAGTCCCTGTCCCTGTCTCTGG GCAAGGGCGGCGGAGGATCTGGCGGAGGCGGTTCT GGTGGTGGTGGATCTGGTGGCGGAGGAAGTGGGGG AGGGGGATCTGTGTCTCGGCTGGAAGAGGAAATGC GGAAGCTGCAGGCCACCGTGCAGGAACTGCAGAAG CGGCTGGACAGACTGGAAGAGACAGTGCAGGCTAA GGGCGGTGGCGGACAGCTCGAGACAGCCAAAGAAC CCTGCATGGCCAAGTTCGGCCCCCTGCCTTCCAAGT GGCAGATGGCCTCTTCCGAGCCCCCCTGCGTGAACA AAGTGTCCGACTGGAAGCTGGAAATCCTGCAGAAC GGCCTGTACCTGATCTACGGCCAGGTGGCCCCCAAC GCCAACTACAACGATGTGGCCCCCTTCGAAGTGCGG CTGTACAAGAACAAGGACATGATCCAGACCCTGACC AACAAGAGCAAGATCCAGAACGTGGGCGGCACCTA CGAGCTGCACGTGGGCGATACCATCGACCTGATCTT CAACTCCGAGCACCAGGTGCTGAAGGACAACACCT ACTGGGGCATCATCCTGCTGGCCAACCCCCAGTTCA

		TCTCC
40	成熟 GITRL IgG4P FP 亚基的氨基酸序列	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPS SIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLS LSLGKGGGGSGGGGSGGGGSGGGGSGGGGSVSRLEEE MRKLQATVQELQKRLDRLEETVQAKGGGGQLETAKE PCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQNG LYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQTLTNK SKIQNVGGTYELHVGDTIDLIFNSEHQVLKDNTYWGII LLANPQFIS
41	编码前体 GITRL IgG4P FP 亚基的核酸（包括信号肽编码区）	ATGGCCATCATCTACCTGATCCTGCTGTTCACCGCCG TGCGGGGCGAGTCTAAGTACGGCCCTCCTTGTCCTC CTTGCCCTGCCCCTGAGTTTCTGGGCGGACCTTCCGT GTTCCTGTTCCCCCCAAAGCCCAAGGACACCCTGAT GATCTCCCGGACCCCCGAAGTGACCTGCGTGGTGGT GGATGTGTCCCAGGAAGATCCCGAGGTGCAGTTCAA TTGGTACGTGGACGGCGTGGAAGTGCACAACGCCA AGACCAAGCCCAGAGAGGAACAGTTCAACTCCACC TACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAG GATTGGCTGAACGGCAAAGAGTACAAGTGCAAGGT GTCCAACAAGGGCCTGCCCTCCAGCATCGAAAAGAC CATCTCCAAGGCCAAGGGCCAGCCCCGGGAACCCC AGGTGTACACACTGCCTCCAAGCCAGGAAGAGATG ACCAAGAACCAGGTGTCCCTGACCTGTCTCGTGAAG GGCTTCTACCCCTCCGATATCGCCGTGGAATGGGAG TCCAACGGCCAGCCTGAGAACAACTACAAGACCAC CCCCCCTGTGCTGGACTCCGACGGCTCCTTCTTCCTG TACTCCCGCCTGACCGTGGACAAGTCCAGATGGCAG GAAGGCAACGTGTTCTCCTGCTCCGTGATGCACGAG GCCCTGCACAACCACTACACCCAGAAGTCCCTGTCC CTGTCTCTGGGCAAGGGCGGCGGAGGATCTGGCGG AGGCGGTTCTGGTGGTGGTGGATCTGGTGGCGGAGG AAGTGGGGGAGGGGGATCTGTGTCTCGGCTGGAAG AGGAAATGCGGAAGCTGCAGGCCACCGTGCAGGAA CTGCAGAAGCGGCTGGACAGACTGGAAGAGACAGT GCAGGCTAAGGGCGGTGGCGGACAGCTCGAGACAG CCAAAGAACCCTGCATGGCCAAGTTCGGCCCCCTGC CTTCCAAGTGGCAGATGGCCTCTTCCGAGCCCCCCT GCGTGAACAAAGTGTCCGACTGGAAGCTGGAAATC CTGCAGAACGGCCTGTACCTGATCTACGGCCAGGTG GCCCCCAACGCCAACTACAACGATGTGGCCCCCTTC GAAGTGCGGCTGTACAAGAACAAGGACATGATCCA GACCCTGACCAACAAGAGCAAGATCCAGAACGTGG GCGGCACCTACGAGCTGCACGTGGGCGATACCATCG ACCTGATCTTCAACTCCGAGCACCAGGTGCTGAAGG ACAACACCTACTGGGGCATCATCCTGCTGGCCAACC

		CCCAGTTCATCTCC
42	前体 GITRL IgG4P FP 亚基的氨基酸序列（具有信号肽编码区）	MAIIYLILLFTAVRGESKYGPPCPPCPAPEFLGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVD GVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQ EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK TTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH EALHNHYTQKSLSLSLGKGGGGSGGGGSGGGGSGGG GSGGGGSVSRLEEEMRKLQATVQELQKRLDRLEETVQ AKGGGGQLETAKEPCMAKFGPLPSKWQMASSEPPCV NKVSDWKLEILQNGLYLIYGQVAPNANYNDVAPFEVR LYKNKDMIQTLTNKSKIQNVGGTYELHVGDTIDLIFNS EHQVLKDNTYWGIILLANPQFIS
43	hGITRL-FP wt & hGITRL-FP N161D 多肽亚基胰蛋白酶水解片段	EEQY**N**STYR
44	hGITRL-FP wt & hGITRL-FP N161D 多肽亚基胰蛋白酶水解片段	DMIQTLT**N**K
45	hGITRL-FP wt 多肽亚基胰蛋白酶水解片段	IQNVGGTYELHVGDTIDLIFNSEHQVLK**N**NTYWGIILL ANPQFIS
46	hGITRL-FP N161D 多肽亚基胰蛋白酶水解片段	DNTYWGIILLANPQFIS
47	hGITR（人GITR）	MAQHGAMGAFRALCGLALLCALSLGQRPTGGPGCGP GRLLLGTGTDARCCRVHTTRCCRDYPGEECCSEWDC MCVQPEFHCGDPCCTTCRHHPCPPGQGVQSQGKFSFG FQCIDCASGTFSGGHEGHCKPWTDCTQFGFLTVFPGN KTHNAVCVPGSPPAEPLGWLTVVLLAVAACVLLLTSA QLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFP EEERGERSAEEKGRLGDLWV
48	mGITRL-FP	MAIIYLILLFTAVRGIKPCPPCKCPAPNLLGGPSVFIFPP KIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVE VHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEF

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49	mOX40L-FP	VPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVT CVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFN STFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTI SKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFP EDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKL NVQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSPGK RLDQDKIEALSNKVQQLERSIGLKDLAMADLEQKVSE LEVSTSSPAKDPPIQRLRGAVTRCEDGQLFISSYKNEYQ TMEVQNNSVVIKCDGLYIIYLKGSFFQEVKIDLHFRED HNPISIPMLNDGRRIVFTVVASLAFKDKVYLTVNAPDT LCEHLQINDGELIVVQLTPGYCAPEGSYHSTVNQVPL
50	MEDI6383	LATDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL SLSPGKELLGGGSIKQIEDKIEEILSKIYHIENEIARIKKLI GERGHGGGSNSQVSHRYPRFQSIKVQFTEYKKEKGFIL TSQKEDEIMKVQNNSVIINCDGFYLISLKGYFSQEVNIS LHYQKDEEPLFQLKKVRSVNSLMVASLTYKDKVYLN VTTDNTSLDDFHVNGGELILIHQNPGEFCVL
51	MEDI0562 VH	QVQLQESGPGLVKPSQTLSLTCAVYGGSFSSGYWNWI RKHPGKGLEYIGYISYNGITYHNPSLKSRITINRDTSKN QYSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYW GQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLS SVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
52	MEDI0562 VL	DIQMTQSPSSLSASVGDRVTITCRASQDISNYLNWYQQ KPGKAPKLLIYYTSKLHSGVPSRFSGSGSGTDYTLTISS LQPEDFATYYCQQGSALPWTFGQGTKVEIKRTVAAPS VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD

		NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGEC
53	人 OX40	MCVGARRLGR GPCAALLLLG LGLSTVTGLH CVGDTYPSND RCCHECRPGN GMVSRCSRSQNTVCRPCGPG FYNDVVSSKP CKPCTWCNLR SGSERKQLCTATQDTVCRCR AGTQPLDSYK PGVDCAPCPP GHFSPGDNQA CKPWTNCTLA GKHTLQPASN SSDAICEDRD PPATQPQETQGPPARPITVQ PTEAWPRTSQ GPSTRPVEVP GGRAVAAILG LGLVLGLLGP LAILLALYLL RRDQRLPPDA HKPPGGGSFR TPIQEEQADA HSTLAKI
54	人 OX40 配体	MERVQPLEENVGNAARPRFERNKLLLVASVIQGLGLL LCFTYICLHFSALQVSHRYPRIQSIKVQFTEYKKEKGFI LTSQKEDEIMKVQNNSVIINCDGFYLISLKGYFSQEVNI SLHYQKDEEPLFQLKKVRSVNSLMVASLTYKDKVYLN VTTDNTSLDDFHVNGGELILIHQNPGEFCVL
55	mOX40L-FP Y182A	VPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVT CVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFN STFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTI SKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFP EDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKL NVQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSPGK RLDQDKIEALSNKVQQLERSIGLKDLAMADLEQKVSE LEVSTSSPAKDPPIQRLRGAVTRCEDGQLFISSYKNEYQ TMEVQNNSVVIKCDGLYIIYLKGSFFQEVKIDLHFRED HNPISIPMLNDGRRIVFTVVASLAFKDKVYLTVNAPDT LCEHLQINDGELIVVQLTPGACAPEGSYHSTVNQVPL
56	AH1 肽	SPSYVYHQF
57	E7 合成长肽（SLP）	SSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRLC VQSTHVD
58	E7 肽	RAHYNIVTF

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[0332] 本披露的宽度和范围应当不限于以上描述的示例性实施例中的任一个，而应当仅根据以下权利要求书和它们的等效物来限定。

<110> Stewart, Ross A
Tigue, Natalie J
Young, Lesley L
Higazi, Daniel R
Bamber, Lisa
Sridharan, Sudharsan
Leyland, Rebecca
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Phe Glu Val Xaa Leu Tyr Lys Asn Lys Asp Xaa Ile Gln Thr Leu Thr
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Leu Pro Ser Lys Trp Gln Met Ala Ser Ser Glu Pro Pro Cys Val Asn
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gcggtggcgg acagctcgag acagccaaag aaccctgcat ggccaagttc ggcccccctgc 960
cttccaagtg gcagatggcc tcttccgagc ccccctgcgt gaacaaagtg tccgactgga 1020
agctggaaat cctgcagaac ggcctgtacc tgatctacgg ccaggtggcc cccaacgcca 1080
actacaacga tgtggccccc ttcgaagtgc ggctgtacaa gaacaaggac atgatccaga 1140
ccctgaccaa caagagcaag atccagaacg tgggcggcac ctacgagctg cacgtgggcg 1200
ataccatcga cctgatcttc aactccgagc accaggtgct gaaggacaac acctactggg 1260
gcatcatcct gctggccaac ccccagttca tctcc 1295

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<211> 432
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 8

Met Ala Ile Ile Tyr Leu Ile Leu Leu Phe Thr Ala Val Arg Gly Leu
1 5 10 15
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly
20 25 30
Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
35 40 45
Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
50 55 60
Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val
65 70 75 80
His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
85 90 95
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
100 105 110
Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile
115 120 125
Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val

130 135 140

Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser
145 150 155 160
Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
165 170 175
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
180 185 190
Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
195 200 205
Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
210 215 220
His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
225 230 235 240
Pro Gly Lys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly
245 250 255
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Val Ser Arg Leu
260 265 270
Glu Glu Glu Met Arg Lys Leu Gln Ala Thr Val Gln Glu Leu Gln Lys
275 280 285
Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys Gly Gly Gly Gly
290 295 300
Gln Leu Glu Thr Ala Lys Glu Pro Cys Met Ala Lys Phe Gly Pro Leu
305 310 315 320
Pro Ser Lys Trp Gln Met Ala Ser Ser Glu Pro Pro Cys Val Asn Lys
325 330 335
Val Ser Asp Trp Lys Leu Glu Ile Leu Gln Asn Gly Leu Tyr Leu Ile
340 345 350
Tyr Gly Gln Val Ala Pro Asn Ala Asn Tyr Asn Asp Val Ala Pro Phe
355 360 365
Glu Val Arg Leu Tyr Lys Asn Lys Asp Met Ile Gln Thr Leu Thr Asn
370 375 380
Lys Ser Lys Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His Val Gly
385 390 395 400
Asp Thr Ile Asp Leu Ile Phe Asn Ser Glu His Gln Val Leu Lys Asp
405 410 415
Asn Thr Tyr Trp Gly Ile Ile Leu Leu Ala Asn Pro Gln Phe Ile Ser
420 425 430

<210> 9
<211> 461

<212> PRT
<213> 智人
<400> 9

Met Ser Arg Gln Val Val Arg Ser Ser Lys Phe Arg His Val Phe Gly
1 5 10 15
Gln Pro Ala Lys Ala Asp Gln Cys Tyr Glu Asp Val Arg Val Ser Gln
20 25 30
Thr Thr Trp Asp Ser Gly Phe Cys Ala Val Asn Pro Lys Phe Val Ala
35 40 45
Leu Ile Cys Glu Ala Ser Gly Gly Gly Ala Phe Leu Val Leu Pro Leu
50 55 60
Gly Lys Thr Gly Arg Val Asp Lys Asn Ala Pro Thr Val Cys Gly His
65 70 75 80
Thr Ala Pro Val Leu Asp Ile Ala Trp Cys Pro His Asn Asp Asn Val
85 90 95
Ile Ala Ser Gly Ser Glu Asp Cys Thr Val Met Val Trp Glu Ile Pro
100 105 110
Asp Gly Gly Leu Met Leu Pro Leu Arg Glu Pro Val Val Thr Leu Glu
115 120 125
Gly His Thr Lys Arg Val Gly Ile Val Ala Trp His Thr Thr Ala Gln
130 135 140
Asn Val Leu Leu Ser Ala Gly Cys Asp Asn Val Ile Met Val Trp Asp
145 150 155 160
Val Gly Thr Gly Ala Ala Met Leu Thr Leu Gly Pro Glu Val His Pro
165 170 175
Asp Thr Ile Tyr Ser Val Asp Trp Ser Arg Asp Gly Gly Leu Ile Cys
180 185 190
Thr Ser Cys Arg Asp Lys Arg Val Arg Ile Ile Glu Pro Arg Lys Gly
195 200 205
Thr Val Val Ala Glu Lys Asp Arg Pro His Glu Gly Thr Arg Pro Val
210 215 220
Arg Ala Val Phe Val Ser Glu Gly Lys Ile Leu Thr Thr Gly Phe Ser
225 230 235 240
Arg Met Ser Glu Arg Gln Val Ala Leu Trp Asp Thr Lys His Leu Glu
245 250 255
Glu Pro Leu Ser Leu Gln Glu Leu Asp Thr Ser Ser Gly Val Leu Leu
260 265 270
Pro Phe Phe Asp Pro Asp Thr Asn Ile Val Tyr Leu Cys Gly Lys Gly
275 280 285

Asp Ser Ser Ile Arg Tyr Phe Glu Ile Thr Ser Glu Ala Pro Phe Leu
290 295 300
His Tyr Leu Ser Met Phe Ser Ser Lys Glu Ser Gln Arg Gly Met Gly
305 310 315 320
Tyr Met Pro Lys Arg Gly Leu Glu Val Asn Lys Cys Glu Ile Ala Arg
325 330 335
Phe Tyr Lys Leu His Glu Arg Arg Cys Glu Pro Ile Ala Met Thr Val
340 345 350
Pro Arg Lys Ser Asp Leu Phe Gln Glu Asp Leu Tyr Pro Pro Thr Ala
355 360 365
Gly Pro Asp Pro Ala Leu Thr Ala Glu Glu Trp Leu Gly Gly Arg Asp
370 375 380
Ala Gly Pro Leu Leu Ile Ser Leu Lys Asp Gly Tyr Val Pro Pro Lys
385 390 395 400
Ser Arg Glu Leu Arg Val Asn Arg Gly Leu Asp Thr Gly Arg Arg Arg
405 410 415
Ala Ala Pro Glu Ala Ser Gly Thr Pro Ser Ser Asp Ala Val Ser Arg
420 425 430
Leu Glu Glu Glu Met Arg Lys Leu Gln Ala Thr Val Gln Glu Leu Gln
435 440 445
Lys Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
450 455 460

<210> 10
<211> 32
<212> PRT
<213> 人工
<220>
<223> 合成
<220>
<221> 尚未归类的特征
<222> (1)..(1)
<223> Xaa 是 Ala、Leu、Ile、或 Val
<220>
<221> 尚未归类的特征
<222> (4)..(4)
<223> Xaa 是 Ala、Leu、Ile、或 Val
<220>
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<222> (8)..(8)

<223> Xaa 是 Ala、Leu、Ile、或 Val
<220>
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<222> (11)..(11)
<223> Xaa 是 Ala、Leu、Ile、或 Val
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<221> 尚未归类的特征
<222> (15)..(15)
<223> Xaa 是 Ala、Leu、Ile、或 Val
<220>
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<222> (22)..(22)
<223> Xaa 是 Ala、Leu、Ile、或 Val
<400> 10

Xaa Ser Arg Xaa Glu Glu Glu Xaa Arg Lys Xaa Gln Ala Thr Xaa Gln
1 5 10 15
Glu Leu Gln Lys Arg Xaa Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30

<210> 11
<211> 32
<212> PRT
<213> 智人
<400> 11

Val Ser Arg Leu Glu Glu Glu Met Arg Lys Leu Gln Ala Thr Val Gln
1 5 10 15
Glu Leu Gln Lys Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30

<210> 12
<211> 32
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 12

Val Ser Arg Leu Glu Glu Glu Ile Arg Lys Leu Gln Ala Thr Val Gln
1 5 10 15
Glu Leu Gln Lys Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30

<210> 13

<211> 32
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 13
Val Ser Arg Ile Glu Glu Glu Ile Arg Lys Leu Gln Ala Thr Val Gln
1 5 10 15
Glu Leu Gln Lys Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30
<210> 14
<211> 32
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 14
Ile Ser Arg Ile Glu Glu Glu Ile Arg Lys Leu Gln Ala Thr Val Gln
1 5 10 15
Glu Leu Gln Lys Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30
<210> 15
<211> 32
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 15
Ile Ser Arg Ile Glu Glu Glu Ile Arg Lys Ile Gln Ala Thr Val Gln
1 5 10 15
Glu Leu Gln Lys Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30
<210> 16
<211> 32
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 16

Ile Ser Arg Ile Glu Glu Glu Ile Arg Lys Ile Gln Ala Thr Val Gln
1 5 10 15
Glu Leu Gln Lys Arg Ile Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30

<210> 17
<211> 32
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 17

Ile Ser Arg Ile Glu Glu Glu Ile Arg Lys Ile Asn Ala Thr Val Gln
1 5 10 15
Glu Leu Gln Lys Arg Ile Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30

<210> 18
<211> 32
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 18

Ile Ser Arg Ile Glu Glu Glu Ile Arg Lys Ile Asn Ala Thr Ile Gln
1 5 10 15
Glu Leu Gln Lys Arg Ile Asp Arg Leu Glu Glu Thr Val Gln Ala Lys
20 25 30

<210> 19
<211> 5
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 19

Gly Gly Gly Gly Ser
1 5

<210> 20
<211> 25
<212> PRT
<213> 人工

<220>
<223> 合成
<400> 20
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15
Gly Gly Gly Ser Gly Gly Gly Gly Ser
20 25
<210> 21
<211> 228
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 21
Leu Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
1 5 10 15
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
20 25 30
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
35 40 45
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
50 55 60
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
65 70 75 80
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
85 90 95
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
100 105 110
Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
115 120 125
Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
130 135 140
Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145 150 155 160
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
165 170 175
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
180 185 190
Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val

195 200 205

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu

210 215 220

Ser Pro Gly Lys

225

<210> 22

<211> 6

<212> PRT

<213> 人工

<220>

<223> 合成

<400> 22

Ser Gly Gly Gly Gly Ser

1 5

<210> 23

<211> 17

<212> PRT

<213> 人工

<220>

<223> 合成

<400> 23

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ala

1 5 10 15

Leu

<210> 24

<211> 16

<212> PRT

<213> 人工

<220>

<223> 合成

<400> 24

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ala

1 5 10 15

<210> 25

<211> 15

<212> PRT

<213> 人工

<220>

<223> 合成

<400> 25
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15
<210> 26
<211> 14
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 26
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
1 5 10
<210> 27
<211> 28
<212> PRT
<213> 酿酒酵母
<400> 27
Ile Lys Gln Ile Glu Asp Lys Ile Glu Glu Ile Leu Ser Lys Ile Tyr
1 5 10 15
His Ile Glu Asn Glu Ile Ala Arg Ile Lys Lys Leu
20 25
<210> 28
<211> 39
<212> PRT
<213> 智人
<400> 28
Cys Ala Cys Glu Ser Leu Val Lys Phe Gln Ala Lys Val Glu Gly Leu
1 5 10 15
Leu Gln Ala Leu Thr Arg Lys Leu Glu Ala Val Ser Lys Arg Leu Ala
20 25 30
Ile Leu Glu Asn Thr Val Val
35
<210> 29
<211> 39
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 29

Cys Ala Cys Glu Ser Leu Val Lys Phe Gln Ala Lys Val Glu Gly Leu
1 5 10 15
Ile Gln Ala Leu Thr Arg Lys Leu Glu Ala Val Ser Lys Arg Ile Ala
20 25 30
Ile Leu Glu Asn Thr Val Val
35

<210> 30
<211> 70
<212> PRT
<213> 智人
<400> 30
Glu Ala Glu Ala Glu Val Thr Leu Arg Glu Leu Gln Glu Ala Leu Glu
1 5 10 15
Glu Glu Val Leu Thr Arg Gln Ser Leu Ser Arg Glu Met Glu Ala Ile
20 25 30
Arg Thr Asp Asn Gln Asn Phe Ala Ser Gln Leu Arg Glu Ala Glu Ala
35 40 45
Arg Asn Arg Asp Leu Glu Ala His Val Arg Gln Leu Gln Glu Arg Met
50 55 60
Glu Leu Leu Gln Ala Glu
65 70

<210> 31
<211> 70
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 31
Ile Ala Glu Ile Glu Val Thr Ile Arg Glu Leu Gln Glu Ala Ile Glu
1 5 10 15
Glu Glu Val Leu Thr Arg Gln Ser Leu Ser Arg Glu Ile Glu Ala Ile
20 25 30
Arg Thr Asp Ile Gln Asn Ile Ala Ser Gln Leu Arg Glu Ile Glu Ala
35 40 45
Arg Ile Arg Asp Leu Glu Ala His Val Arg Gln Leu Gln Glu Arg Met
50 55 60
Glu Leu Leu Gln Ala Glu
65 70

<210> 32

<211> 33
<212> PRT
<213> 智人
<400> 32
Ala Ser Ala Leu Asn Thr Lys Ile Arg Ala Leu Gln Gly Ser Leu Glu
1 5 10 15
Asn Met Ser Lys Leu Leu Lys Arg Gln Asn Asp Ile Leu Gln Val Val
20 25 30
Ser
<210> 33
<211> 33
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 33
Ile Ser Ala Leu Asn Thr Lys Ile Arg Ala Ile Gln Gly Ser Ile Glu
1 5 10 15
Asn Met Ser Lys Leu Ile Lys Arg Gln Asn Asp Ile Ile Gln Val Val
20 25 30
Ser
<210> 34
<211> 128
<212> PRT
<213> 智人
<400> 34
Gln Leu Glu Thr Ala Lys Glu Pro Cys Met Ala Lys Phe Gly Pro Leu
1 5 10 15
Pro Ser Lys Trp Gln Met Ala Ser Ser Glu Pro Pro Cys Val Asn Lys
20 25 30
Val Ser Asp Trp Lys Leu Glu Ile Leu Gln Asn Gly Leu Tyr Leu Ile
35 40 45
Tyr Gly Gln Val Ala Pro Asn Ala Asn Tyr Asn Asp Val Ala Pro Phe
50 55 60
Glu Val Arg Leu Tyr Lys Asn Lys Asp Met Ile Gln Thr Leu Thr Asn
65 70 75 80
Lys Ser Lys Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His Val Gly
85 90 95
Asp Thr Ile Asp Leu Ile Phe Asn Ser Glu His Gln Val Leu Lys Asn

100 105 110

Asn Thr Tyr Trp Gly Ile Ile Leu Leu Ala Asn Pro Gln Phe Ile Ser

115 120 125

<210> 35

<211> 128

<212> PRT

<213> 人工

<220>

<223> 合成

<220>

<221> 尚未归类的特征

<222> (3)..(3)

<223> Xaa 是 Glu 或 Ala

<220>

<221> 尚未归类的特征

<222> (13)..(13)

<223> Xaa 是 Ser 或 Phe

<220>

<221> 尚未归类的特征

<222> (17)..(17)

<223> Xaa 是 Thr 或 Pro

<220>

<221> 尚未归类的特征

<222> (18)..(18)

<223> Xaa 是 Leu 或 Ser

<220>

<221> 尚未归类的特征

<222> (22)..(22)

<223> Xaa 是 Thr 或 Met

<220>

<221> 尚未归类的特征

<222> (28)..(28)

<223> Xaa 是 Leu 或 Pro

<220>

<221> 尚未归类的特征

<222> (30)..(30)

<223> Xaa 是 Met 或 Val

<220>

<221> 尚未归类的特征

<222> (34)..(34)
<223> Xaa 是 Thr、Phe、或 Ser
<220>
<221> 尚未归类的特征
<222> (50)..(50)
<223> Xaa 是 Ser 或 Gly
<220>
<221> 尚未归类的特征
<222> (63)..(63)
<223> Xaa 是 Arg 或 Pro
<220>
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<222> (67)..(67)
<223> Xaa 是 Trp 或 Arg
<220>
<221> 尚未归类的特征
<222> (74)..(74)
<223> Xaa 是 Leu 或 Met
<220>
<221> 尚未归类的特征
<222> (104)..(104)
<223> Xaa 是 Ser 或 Asn
<220>
<221> 尚未归类的特征
<222> (109)..(109)
<223> Xaa 是 Phe 或 Val
<220>
<221> 尚未归类的特征
<222> (112)..(112)
<223> Xaa 是 Xaa 是除了Asn外的任何氨基酸
<220>
<221> 尚未归类的特征
<222> (118)..(118)
<223> Xaa 是 Val 或 Ile
<220>
<221> 尚未归类的特征
<222> (119)..(119)
<223> Xaa 是 Leu 或 Ile
<400> 35

Gln Leu Xaa Thr Ala Lys Glu Pro Cys Met Ala Lys Xaa Gly Pro Leu
1 5 10 15
Xaa Xaa Lys Trp Gln Xaa Ala Ser Ser Glu Pro Xaa Cys Xaa Asn Lys
20 25 30
Val Xaa Asp Trp Lys Leu Glu Ile Leu Gln Asn Gly Leu Tyr Leu Ile
35 40 45
Tyr Xaa Gln Val Ala Pro Asn Ala Asn Tyr Asn Asp Val Ala Xaa Phe
50 55 60
Glu Val Xaa Leu Tyr Lys Asn Lys Asp Xaa Ile Gln Thr Leu Thr Asn
65 70 75 80
Lys Ser Lys Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His Val Gly
85 90 95
Asp Thr Ile Asp Leu Ile Phe Xaa Ser Glu His Gln Xaa Leu Lys Xaa
100 105 110
Asn Thr Tyr Trp Gly Xaa Xaa Leu Leu Ala Asn Pro Gln Phe Ile Ser
115 120 125

<210> 36
<211> 128
<212> PRT
<213> 人工
<220>
<223> 合成
<220>
<221> 尚未归类的特征
<222> (112)..(112)
<223> Xaa 是除了Asn外的任何氨基酸

<400> 36
Gln Leu Glu Thr Ala Lys Glu Pro Cys Met Ala Lys Phe Gly Pro Leu
1 5 10 15
Pro Ser Lys Trp Gln Met Ala Ser Ser Glu Pro Pro Cys Val Asn Lys
20 25 30
Val Ser Asp Trp Lys Leu Glu Ile Leu Gln Asn Gly Leu Tyr Leu Ile
35 40 45
Tyr Gly Gln Val Ala Pro Asn Ala Asn Tyr Asn Asp Val Ala Pro Phe
50 55 60
Glu Val Arg Leu Tyr Lys Asn Lys Asp Met Ile Gln Thr Leu Thr Asn
65 70 75 80
Lys Ser Lys Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His Val Gly
85 90 95

Asp Thr Ile Asp Leu Ile Phe Asn Ser Glu His Gln Val Leu Lys Xaa
100 105 110
Asn Thr Tyr Trp Gly Ile Ile Leu Leu Ala Asn Pro Gln Phe Ile Ser
115 120 125

<210> 37
<211> 128
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 37

Gln Leu Glu Thr Ala Lys Glu Pro Cys Met Ala Lys Phe Gly Pro Leu
1 5 10 15
Pro Ser Lys Trp Gln Met Ala Ser Ser Glu Pro Pro Cys Val Asn Lys
20 25 30
Val Ser Asp Trp Lys Leu Glu Ile Leu Gln Asn Gly Leu Tyr Leu Ile
35 40 45
Tyr Gly Gln Val Ala Pro Asn Ala Asn Tyr Asn Asp Val Ala Pro Phe
50 55 60
Glu Val Arg Leu Tyr Lys Asn Lys Asp Met Ile Gln Thr Leu Thr Asn
65 70 75 80
Lys Ser Lys Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His Val Gly
85 90 95
Asp Thr Ile Asp Leu Ile Phe Asn Ser Glu His Gln Val Leu Lys Asp
100 105 110
Asn Thr Tyr Trp Gly Ile Ile Leu Leu Ala Asn Pro Gln Phe Ile Ser
115 120 125

<210> 38
<211> 229
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 38

Glu Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe
1 5 10 15
Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
20 25 30
Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val

35 40 45

Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val
50 55 60

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser
65 70 75 80

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu
85 90 95

Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser
100 105 110

Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro
115 120 125

Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln
130 135 140

Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
145 150 155 160

Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
165 170 175

Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu
180 185 190

Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser
195 200 205

Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
210 215 220

Leu Ser Leu Gly Lys
225

<210> 39
<211> 1254
<212> DNA
<213> 人工
<220>
<223> 合成
<400> 39

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tccgtgttcc tgttcccccc aaagcccaag gacaccctga tgatctcccg gacccccgaa 120
gtgacctgcg tggtggtgga tgtgtcccag gaagatcccg aggtgcagtt caattggtac 180
gtggacggcg tggaagtgca caacgccaag accaagccca gagaggaaca gttcaactcc 240
acctaccggg tggtgtccgt gctgaccgtg ctgcaccagg attggctgaa cggcaaagag 300
tacaagtgca aggtgtccaa caagggcctg ccctccagca tcgaaaagac catctccaag 360
gccaagggcc agccccggga accccaggtg tacacactgc ctccaagcca ggaagagatg 420

accaagaacc aggtgtccct gacctgtctc gtgaagggct tctacccctc cgatatcgcc 480
gtggaatggg agtccaacgg ccagcctgag aacaactaca agaccacccc ccctgtgctg 540
gactccgacg gctccttctt cctgtactcc cgcctgaccg tggacaagtc cagatggcag 600
gaaggcaacg tgttctcctg ctccgtgatg cacgaggccc tgcacaacca ctacacccag 660
aagtccctgt ccctgtctct gggcaagggc ggcggaggat ctggcggagg cggttctggt 720
ggtggtggat ctggtggcgg aggaagtggg ggaggggat ctgtgtctcg gctggaagag 780
gaaatgcgga agctgcaggc caccgtgcag gaactgcaga agcggctgga cagactggaa 840
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gccaagttcg gccccctgcc ttccaagtgg cagatggcct cttccgagcc cccctgcgtg 960
aacaaagtgt ccgactggaa gctggaaatc ctgcagaacg gcctgtacct gatctacggc 1020
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aacaaggaca tgatccagac cctgaccaac aagagcaaga tccagaacgt gggcggcacc 1140
tacgagctgc acgtgggcga taccatcgac ctgatcttca actccgagca ccaggtgctg 1200
aaggacaaca cctactgggg catcatcctg ctggccaacc cccagttcat ctcc 1254

<210> 40
<211> 418
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 40

Glu Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe
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Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
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Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val
35 40 45
Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val
50 55 60
Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser
65 70 75 80
Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu
85 90 95
Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser
100 105 110
Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro
115 120 125
Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln
130 135 140

Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
145 150 155 160
Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
165 170 175
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu
180 185 190
Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser
195 200 205
Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
210 215 220
Leu Ser Leu Gly Lys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
225 230 235 240
Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Val Ser
245 250 255
Arg Leu Glu Glu Glu Met Arg Lys Leu Gln Ala Thr Val Gln Glu Leu
260 265 270
Gln Lys Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys Gly Gly
275 280 285
Gly Gly Gln Leu Glu Thr Ala Lys Glu Pro Cys Met Ala Lys Phe Gly
290 295 300
Pro Leu Pro Ser Lys Trp Gln Met Ala Ser Ser Glu Pro Pro Cys Val
305 310 315 320
Asn Lys Val Ser Asp Trp Lys Leu Glu Ile Leu Gln Asn Gly Leu Tyr
325 330 335
Leu Ile Tyr Gly Gln Val Ala Pro Asn Ala Asn Tyr Asn Asp Val Ala
340 345 350
Pro Phe Glu Val Arg Leu Tyr Lys Asn Lys Asp Met Ile Gln Thr Leu
355 360 365
Thr Asn Lys Ser Lys Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His
370 375 380
Val Gly Asp Thr Ile Asp Leu Ile Phe Asn Ser Glu His Gln Val Leu
385 390 395 400
Lys Asp Asn Thr Tyr Trp Gly Ile Ile Leu Leu Ala Asn Pro Gln Phe
405 410 415
Ile Ser

<210> 41
<211> 1299
<212> DNA
<213> 人工

<220>
<223> 合成
<400> 41

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cctccttgtc ctccttgccc tgcccctgag tttctgggcg gaccttccgt gttcctgttc 120
cccccaaagc ccaaggacac cctgatgatc tcccggaccc ccgaagtgac ctgcgtggtg 180
gtggatgtgt cccaggaaga tcccgaggtg cagttcaatt ggtacgtgga cggcgtggaa 240
gtgcacaacg ccaagaccaa gcccagagag gaacagttca actccaccta ccgggtggtg 300
tccgtgctga ccgtgctgca ccaggattgg ctgaacggca aagagtacaa gtgcaaggtg 360
tccaacaagg gcctgccctc cagcatcgaa aagaccatct ccaaggccaa gggccagccc 420
cgggaacccc aggtgtacac actgcctcca agccaggaag agatgaccaa gaaccaggtg 480
tccctgacct gtctcgtgaa gggcttctac ccctccgata tcgccgtgga atgggagtcc 540
aacggccagc ctgagaacaa ctacaagacc acccccccctg tgctggactc cgacggctcc 600
ttcttcctgt actcccgcct gaccgtggac aagtccagat ggcaggaagg caacgtgttc 660
tcctgctccg tgatgcacga ggccctgcac aaccactaca cccagaagtc cctgtccctg 720
tctctgggca agggcggcgg aggatctggc ggaggcggtt ctggtggtgg tggatctggt 780
ggcggaggaa gtgggggagg gggatctgtg tctcggctgg aagaggaaat gcggaagctg 840
caggccaccg tgcaggaact gcagaagcgg ctggacagac tggaagagac agtgcaggct 900
aagggcggtg gcggacagct cgagacagcc aaagaaccct gcatggccaa gttcggcccc 960
ctgccttcca agtggcagat ggcctcttcc gagcccccct gcgtgaacaa agtgtccgac 1020
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gccaactaca acgatgtggc ccccttcgaa gtgcggctgt acaagaacaa ggacatgatc 1140
cagaccctga ccaacaagag caagatccag aacgtgggcg gcacctacga gctgcacgtg 1200
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<210> 42
<211> 433
<212> PRT
<213> 人工
<220>
<223> 合成
<400> 42

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Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu
20 25 30
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
35 40 45
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser

50 55 60

Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu
65 70 75 80

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr
85 90 95

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
100 105 110

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser
115 120 125

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
130 135 140

Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val
145 150 155 160

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
165 170 175

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
180 185 190

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr
195 200 205

Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val
210 215 220

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
225 230 235 240

Ser Leu Gly Lys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
245 250 255

Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Val Ser Arg
260 265 270

Leu Glu Glu Glu Met Arg Lys Leu Gln Ala Thr Val Gln Glu Leu Gln
275 280 285

Lys Arg Leu Asp Arg Leu Glu Glu Thr Val Gln Ala Lys Gly Gly Gly
290 295 300

Gly Gln Leu Glu Thr Ala Lys Glu Pro Cys Met Ala Lys Phe Gly Pro
305 310 315 320

Leu Pro Ser Lys Trp Gln Met Ala Ser Ser Glu Pro Pro Cys Val Asn
325 330 335

Lys Val Ser Asp Trp Lys Leu Glu Ile Leu Gln Asn Gly Leu Tyr Leu
340 345 350

Ile Tyr Gly Gln Val Ala Pro Asn Ala Asn Tyr Asn Asp Val Ala Pro
355 360 365

Phe Glu Val Arg Leu Tyr Lys Asn Lys Asp Met Ile Gln Thr Leu Thr
370 375 380
Asn Lys Ser Lys Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His Val
385 390 395 400
Gly Asp Thr Ile Asp Leu Ile Phe Asn Ser Glu His Gln Val Leu Lys
405 410 415
Asp Asn Thr Tyr Trp Gly Ile Ile Leu Leu Ala Asn Pro Gln Phe Ile
420 425 430
Ser

<210> 43
<211> 9
<212> PRT
<213> 智人
<400> 43
Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
1 5

<210> 44
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<213> 智人
<400> 44
Asp Met Ile Gln Thr Leu Thr Asn Lys
1 5

<210> 45
<211> 45
<212> PRT
<213> 智人
<400> 45
Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His Val Gly Asp Thr Ile
1 5 10 15
Asp Leu Ile Phe Asn Ser Glu His Gln Val Leu Lys Asn Asn Thr Tyr
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Trp Gly Ile Ile Leu Leu Ala Asn Pro Gln Phe Ile Ser
35 40 45

<210> 46
<211> 17
<212> PRT
<213> 人工
<220>

<223> 合成
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Ser
<210> 47
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<212> PRT
<213> 智人
<400> 47
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Ala Leu Leu Cys Ala Leu Ser Leu Gly Gln Arg Pro Thr Gly Gly Pro
20 25 30
Gly Cys Gly Pro Gly Arg Leu Leu Leu Gly Thr Gly Thr Asp Ala Arg
35 40 45
Cys Cys Arg Val His Thr Thr Arg Cys Cys Arg Asp Tyr Pro Gly Glu
50 55 60
Glu Cys Cys Ser Glu Trp Asp Cys Met Cys Val Gln Pro Glu Phe His
65 70 75 80
Cys Gly Asp Pro Cys Cys Thr Thr Cys Arg His His Pro Cys Pro Pro
85 90 95
Gly Gln Gly Val Gln Ser Gln Gly Lys Phe Ser Phe Gly Phe Gln Cys
100 105 110
Ile Asp Cys Ala Ser Gly Thr Phe Ser Gly Gly His Glu Gly His Cys
115 120 125
Lys Pro Trp Thr Asp Cys Thr Gln Phe Gly Phe Leu Thr Val Phe Pro
130 135 140
Gly Asn Lys Thr His Asn Ala Val Cys Val Pro Gly Ser Pro Pro Ala
145 150 155 160
Glu Pro Leu Gly Trp Leu Thr Val Val Leu Leu Ala Val Ala Ala Cys
165 170 175
Val Leu Leu Leu Thr Ser Ala Gln Leu Gly Leu His Ile Trp Gln Leu
180 185 190
Arg Ser Gln Cys Met Trp Pro Arg Glu Thr Gln Leu Leu Leu Glu Val
195 200 205
Pro Pro Ser Thr Glu Asp Ala Arg Ser Cys Gln Phe Pro Glu Glu Glu
210 215 220
Arg Gly Glu Arg Ser Ala Glu Glu Lys Gly Arg Leu Gly Asp Leu Trp

225 230 235 240
Val

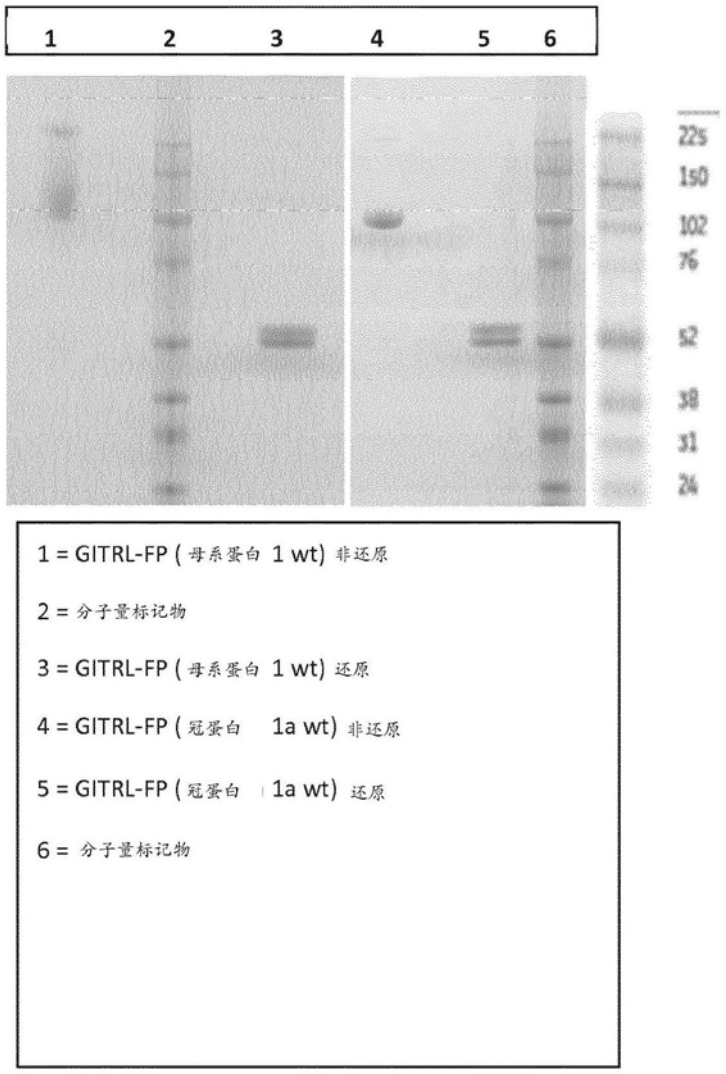

1
2
3
4
5
6
225
150
102
76
52
38
31
24
1 = GITRL-FP (母系蛋白 1 wt) 非还原
2 = 分子量标记物
3 = GITRL-FP (母系蛋白 1 wt) 还原
4 = GITRL-FP (冠蛋白 1a wt) 非还原
5 = GITRL-FP (冠蛋白 1a wt) 还原
6 = 分子量标记物

图1

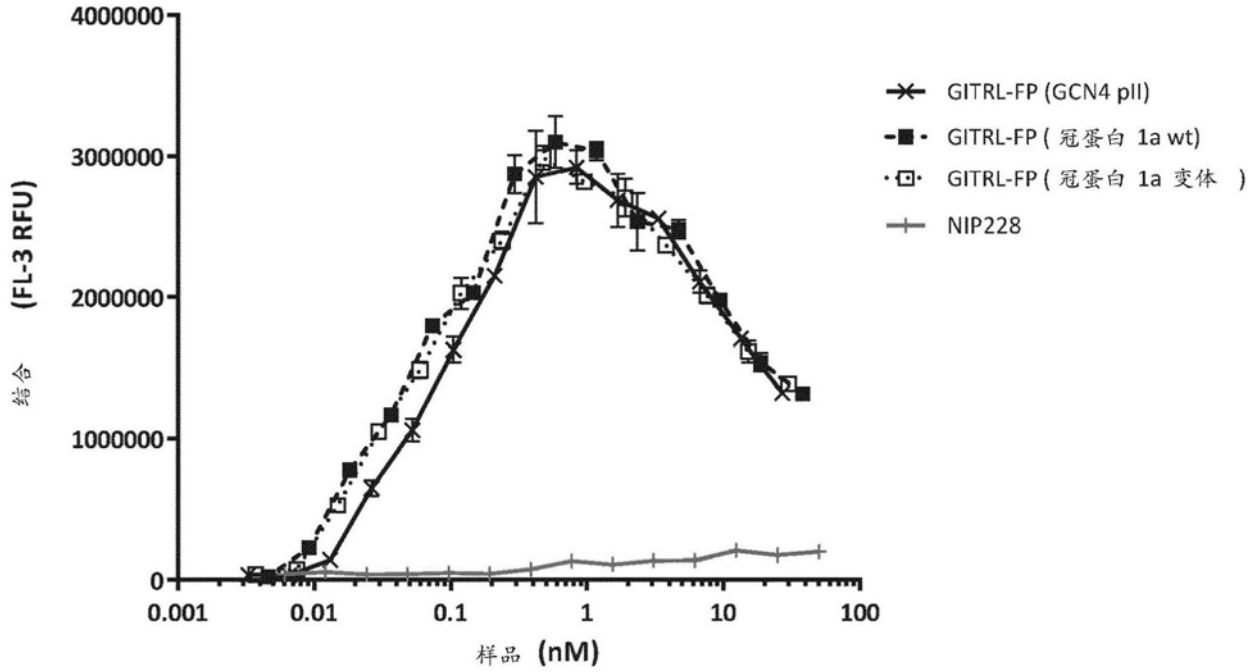

4000000
3000000
2000000
1000000
0
结合 (FL-3 RFU)
0.001
0.01
0.1
1
10
100
样品 (nM)
GITRL-FP (GCN4 pII)
GITRL-FP (冠蛋白 1a wt)
GITRL-FP (冠蛋白 1a 变体)
NIP228

图2A

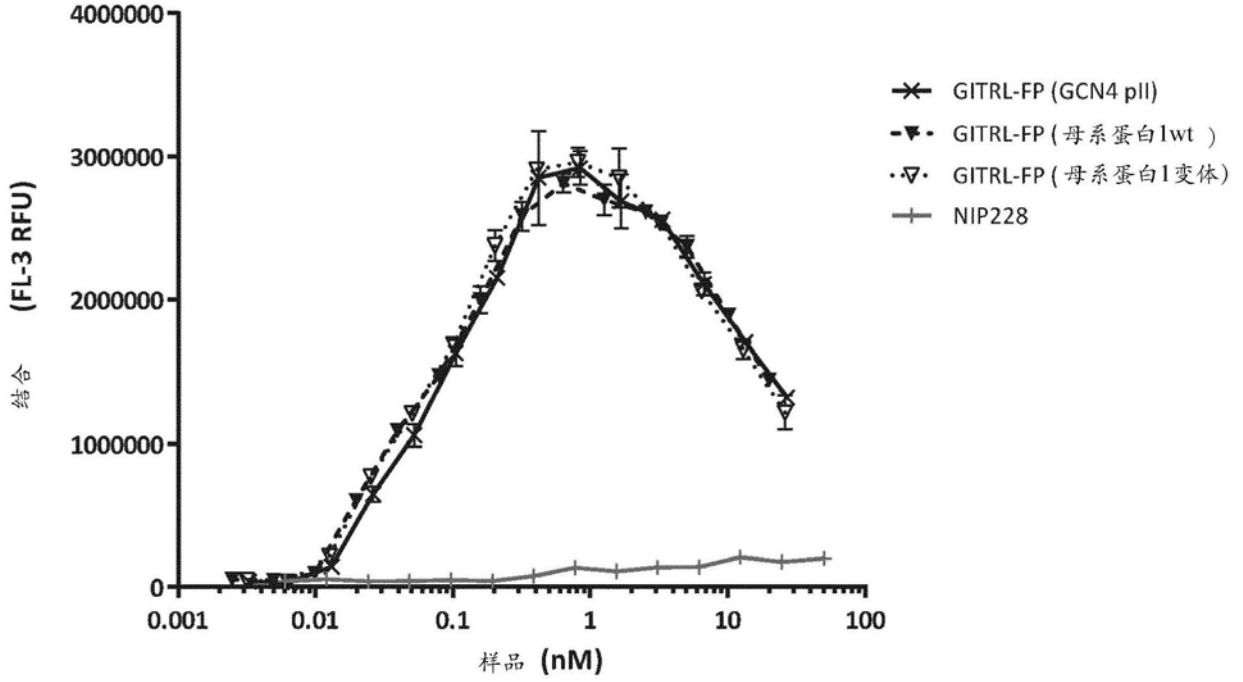

4000000
3000000
2000000
1000000
0
结合 (FL-3 RFU)
0.001
0.01
0.1
1
10
100
样品 (nM)
GITRL-FP (GCN4 pII)
GITRL-FP (母系蛋白1wt)
GITRL-FP (母系蛋白1变体)
NIP228

图2B

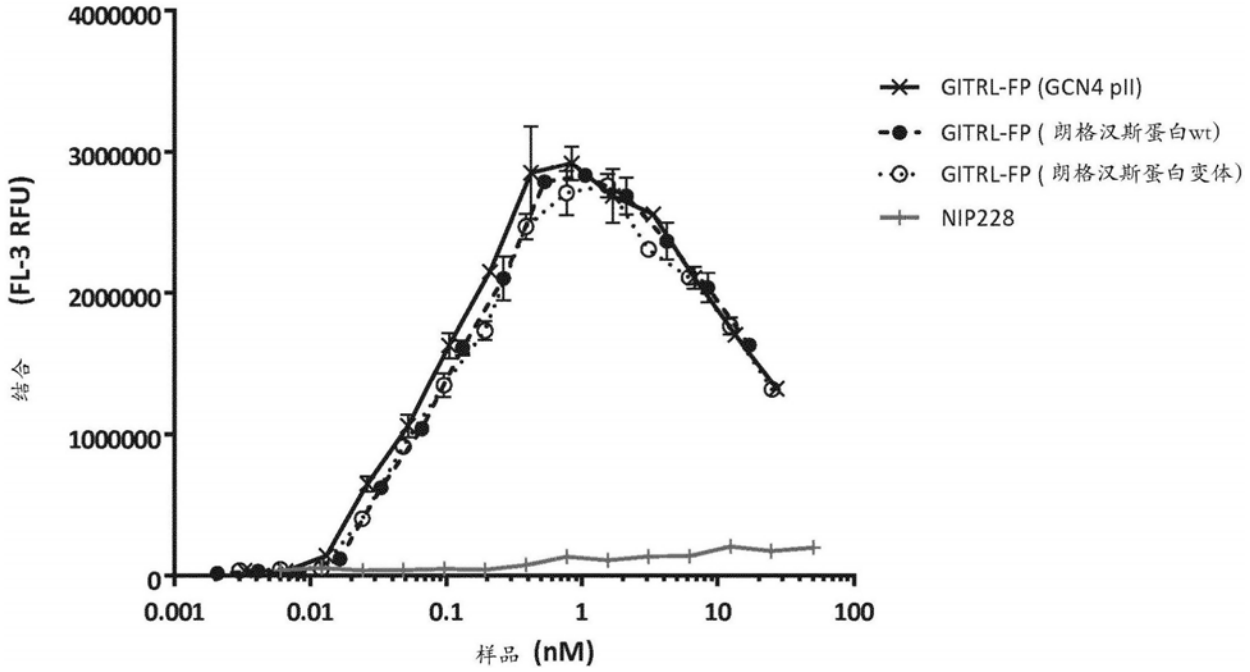

4000000
3000000
2000000
1000000
0
结合 (FL-3 RFU)
0.001
0.01
0.1
1
10
100
样品 (nM)
GITRL-FP (GCN4 pII)
GITRL-FP (朗格汉斯蛋白wt)
GITRL-FP (朗格汉斯蛋白变体)
NIP228

图2C

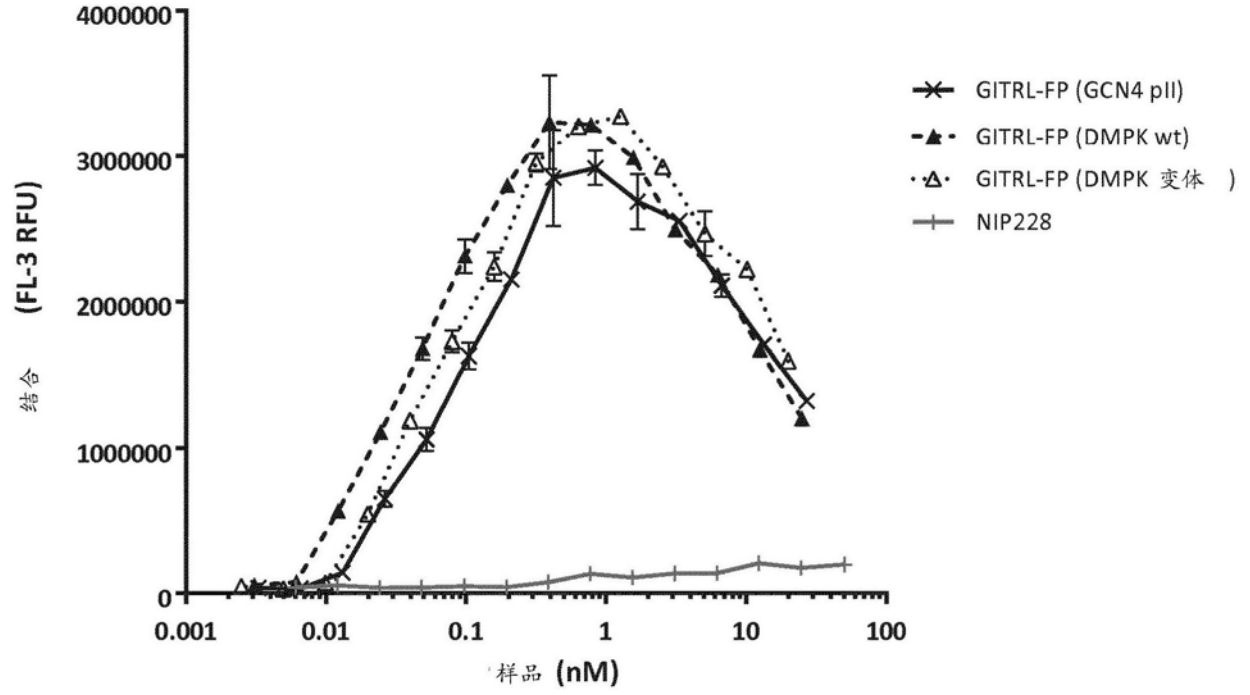

4000000
3000000
2000000
1000000
0
结合 (FL-3 RFU)
0.001
0.01
0.1
1
10
100
样品 (nM)
GITRL-FP (GCN4 pII)
GITRL-FP (DMPK wt)
GITRL-FP (DMPK 变体)
NIP228

图2D

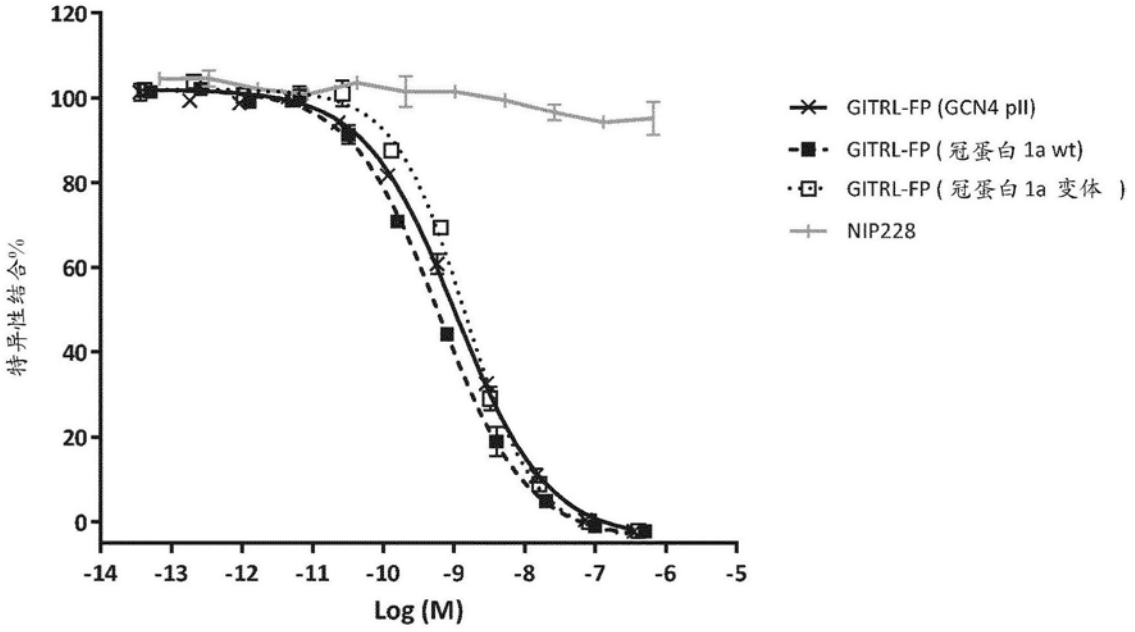

120
100
80
60
40
20
0
特异性结合%
-14
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log (M)
GITRL-FP (GCN4 pII)
GITRL-FP (冠蛋白 1a wt)
GITRL-FP (冠蛋白 1a 变体)
NIP228

图3A

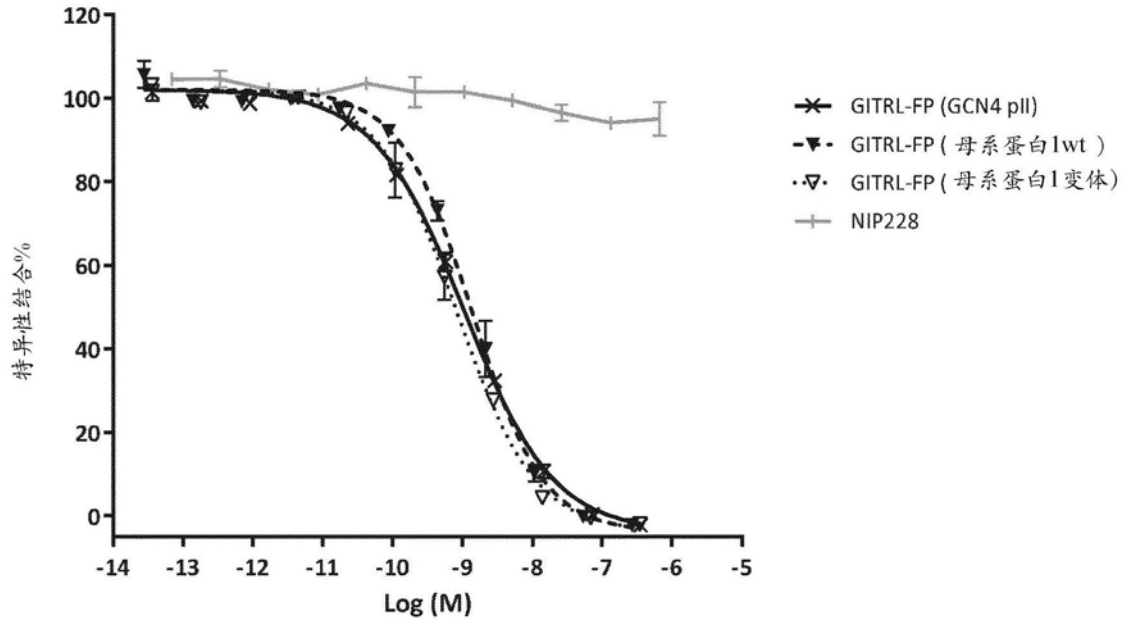

120
100
80
60
40
20
0
特异性结合%
-14
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log (M)
GITRL-FP (GCN4 pII)
GITRL-FP (母系蛋白1wt)
GITRL-FP (母系蛋白1变体)
NIP228

图3B

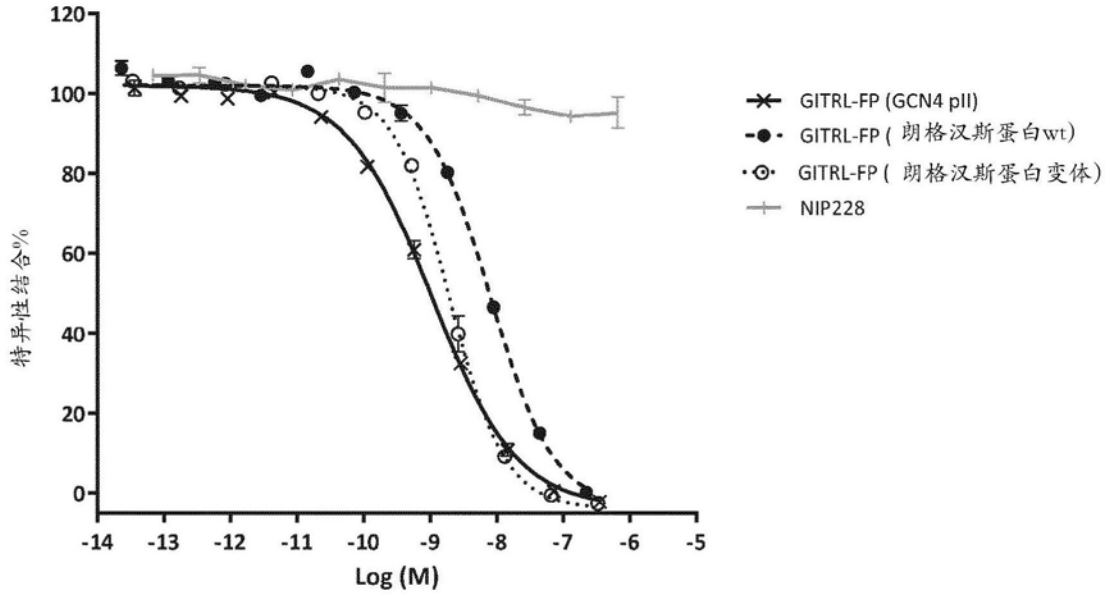

120
100
80
60
40
20
0
特异性结合%
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
Log (M)
GITRL-FP (GCN4 pII)
GITRL-FP (朗格汉斯蛋白wt)
GITRL-FP (朗格汉斯蛋白变体)
NIP228

图3C

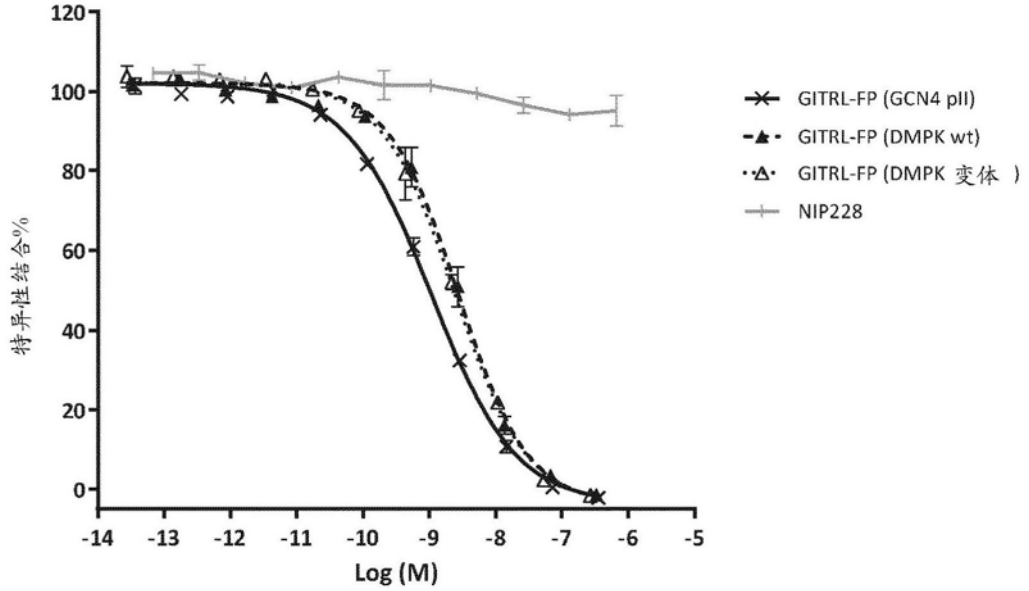

120
100
80
60
40
20
0
特异性结合%
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
Log (M)
GITRL-FP (GCN4 pII)
GITRL-FP (DMPK wt)
GITRL-FP (DMPK 变体)
NIP228

图3D

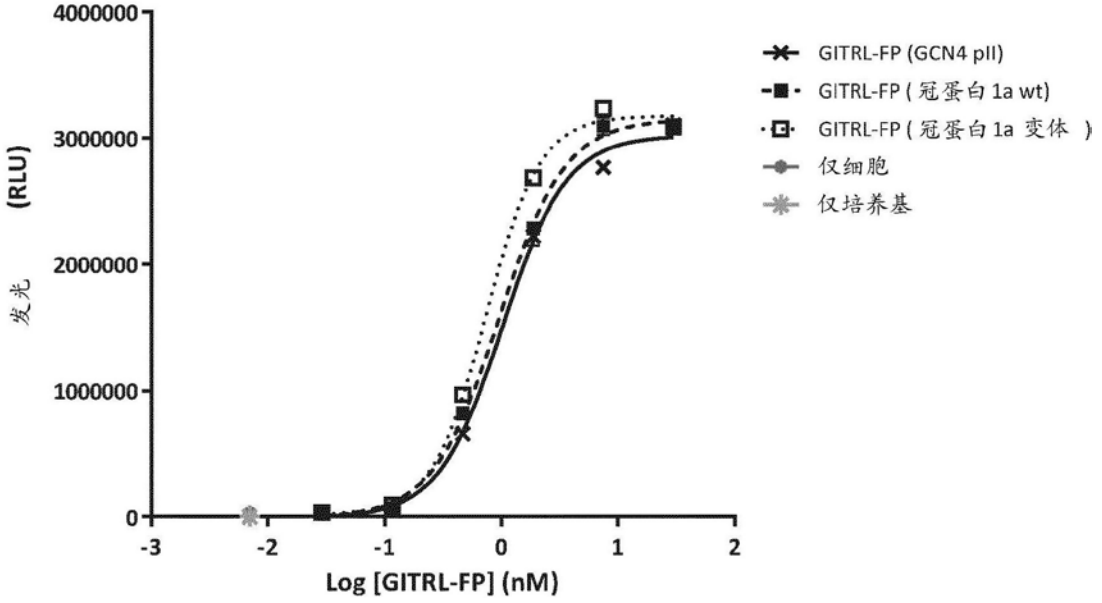

4000000
3000000
2000000
1000000
0
发光 (RLU)
-3
-2
-1
0
1
2
Log [GITRL-FP] (nM)
GITRL-FP (GCN4 pII)
GITRL-FP (冠蛋白 1a wt)
GITRL-FP (冠蛋白 1a 变体)
仅细胞
仅培养基

图4A

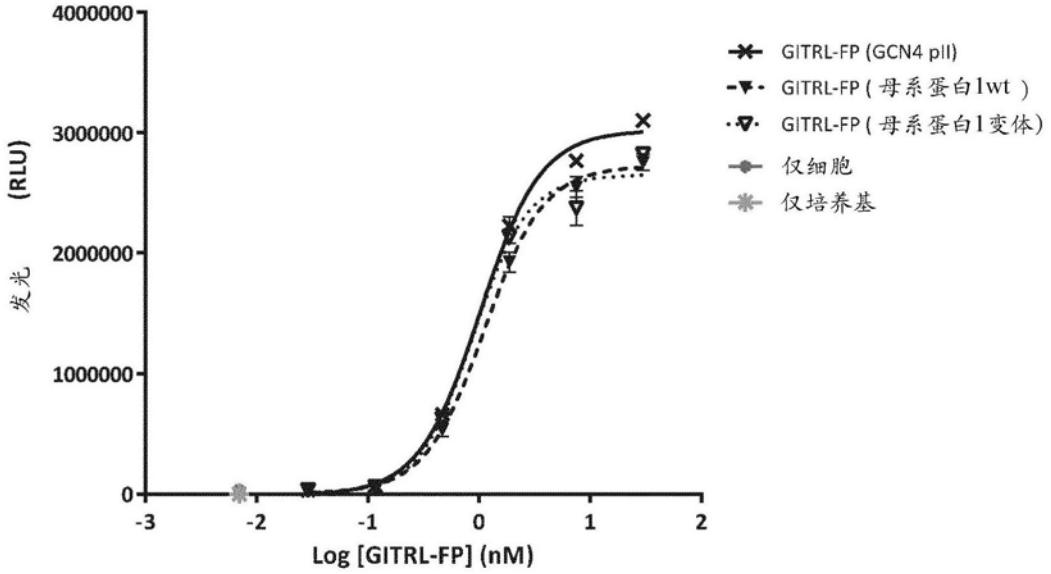

4000000
3000000
2000000
1000000
0
发光 (RLU)
-3
-2
-1
0
1
2
Log [GITRL-FP] (nM)
GITRL-FP (GCN4 pII)
GITRL-FP (母系蛋白1wt)
GITRL-FP (母系蛋白1变体)
仅细胞
仅培养基

图4B

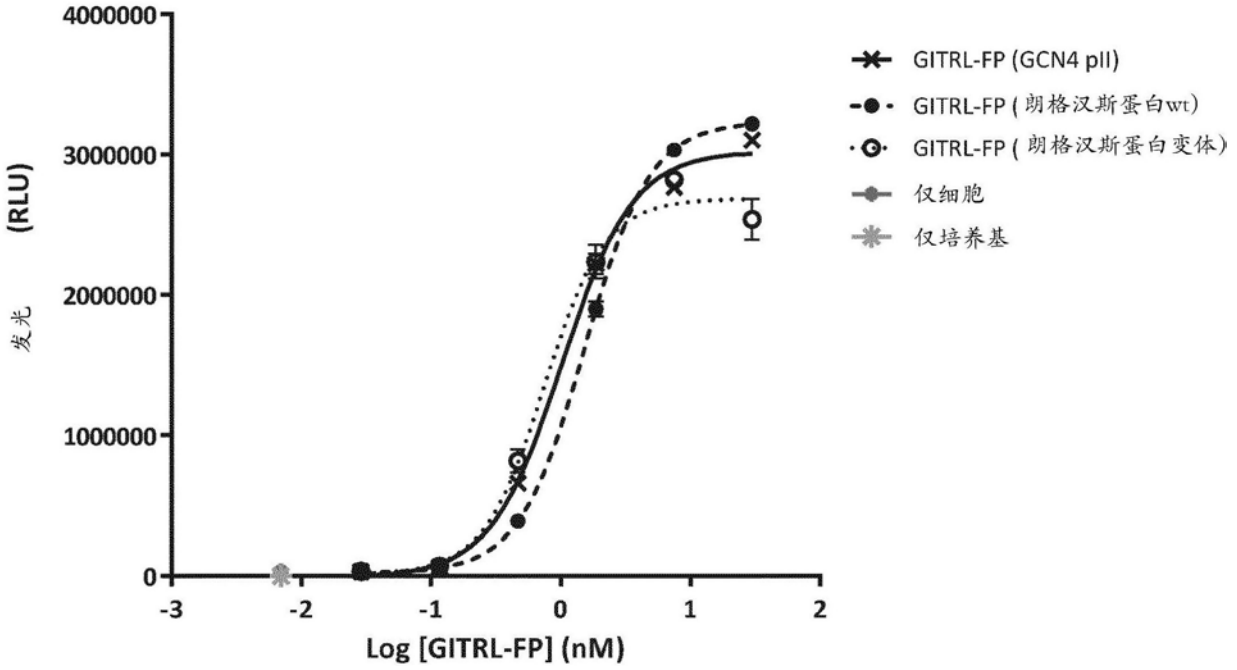

4000000
3000000
2000000
1000000
0
发光 (RLU)
-3
-2
-1
0
1
2
Log [GITRL-FP] (nM)
GITRL-FP (GCN4 pII)
GITRL-FP (朗格汉斯蛋白wt)
GITRL-FP (朗格汉斯蛋白变体)
仅细胞
仅培养基

图4C

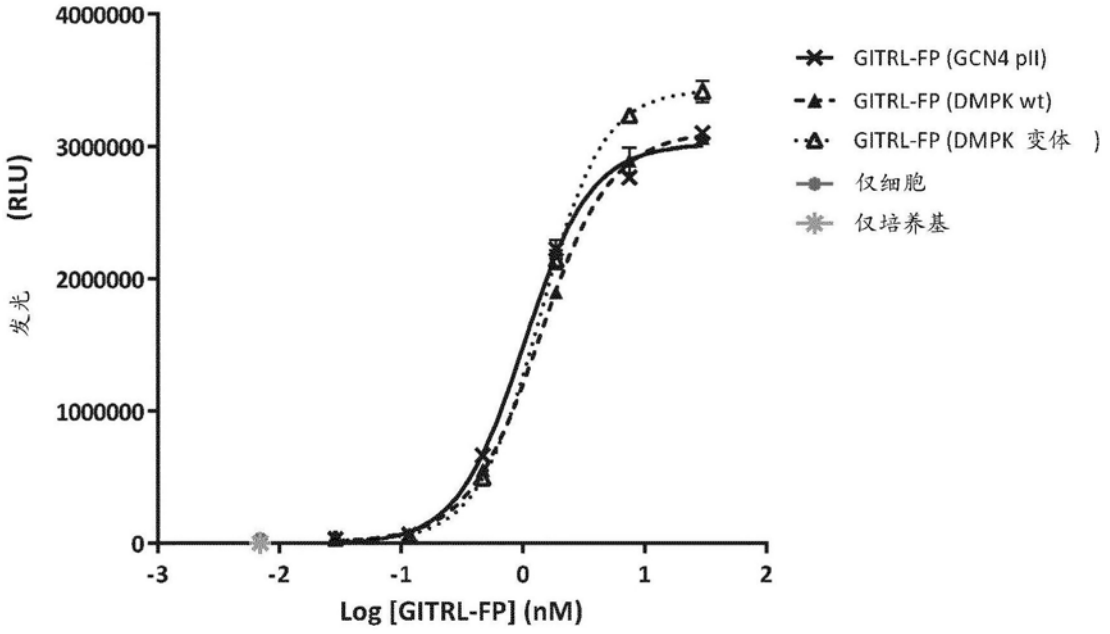

4000000
3000000
2000000
1000000
0
发光 (RLU)
-3
-2
-1
0
1
2
Log [GITRL-FP] (nM)
GITRL-FP (GCN4 pII)
GITRL-FP (DMPK wt)
GITRL-FP (DMPK 变体)
仅细胞
仅培养基

图4D

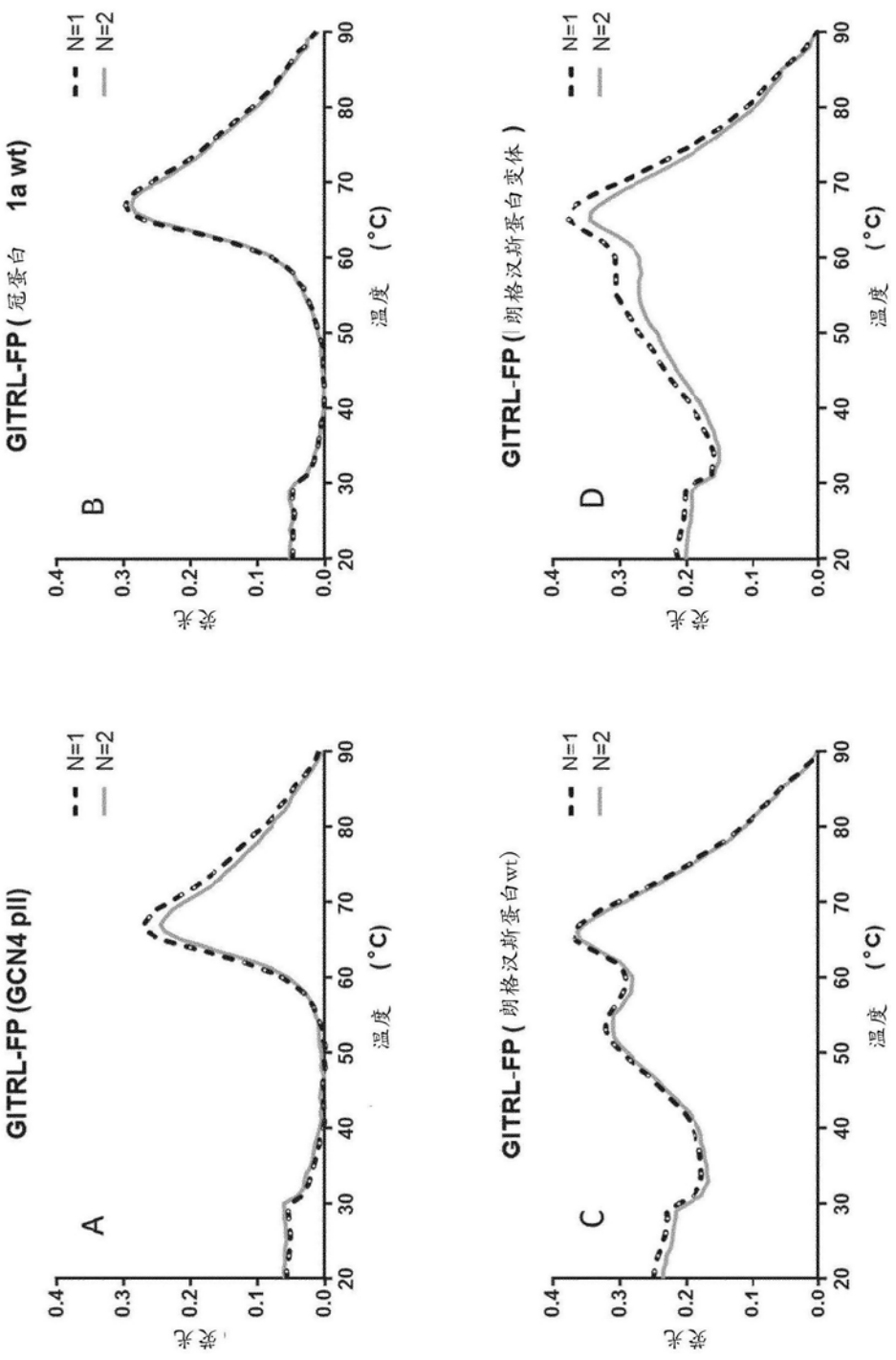

GITRL-FP (GCN4 pII)
A
GITRL-FP (冠蛋白 1a wt)
B
GITRL-FP (朗格汉斯蛋白wt)
C
GITRL-FP (朗格汉斯蛋白变体)
D
N=1
N=2
荧光
温度 (°C)
0.0
0.1
0.2
0.3
0.4
20
30
40
50
60
70
80
90

图5

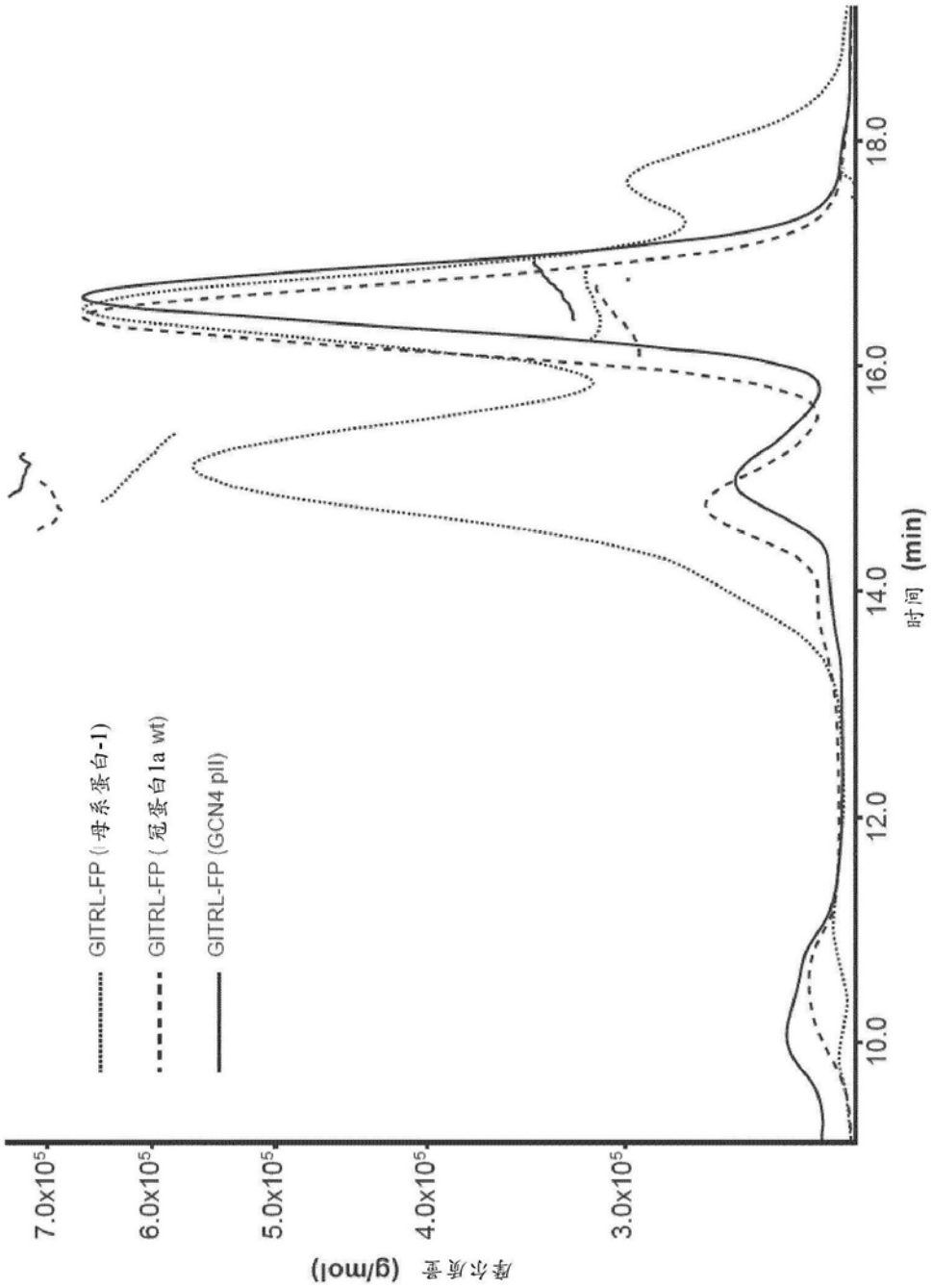

GITRL-FP (母系蛋白-1)
GITRL-FP (冠蛋白1a wt)
GITRL-FP (GCN4 pII)
7.0x10^5
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
摩尔质量 (g/mol)
10.0
12.0
14.0
16.0
18.0
时间 (min)

图6

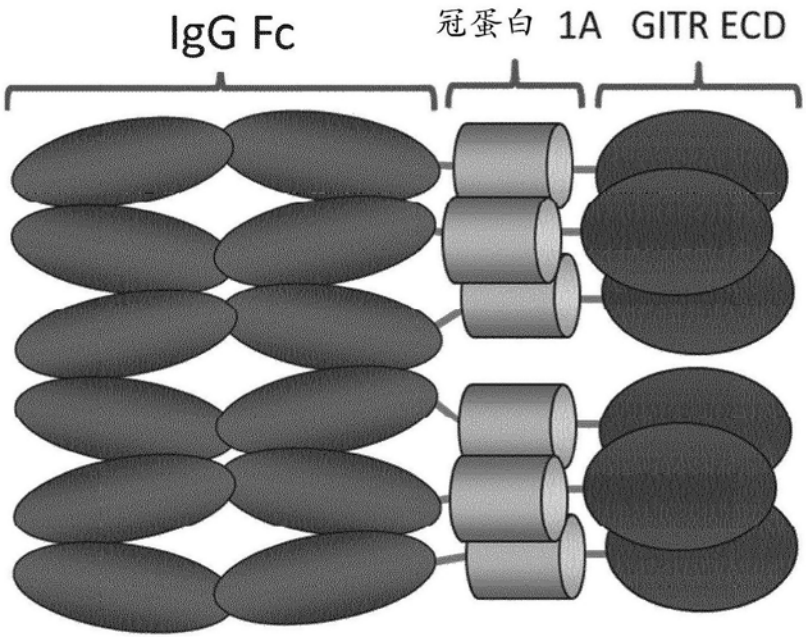


图7

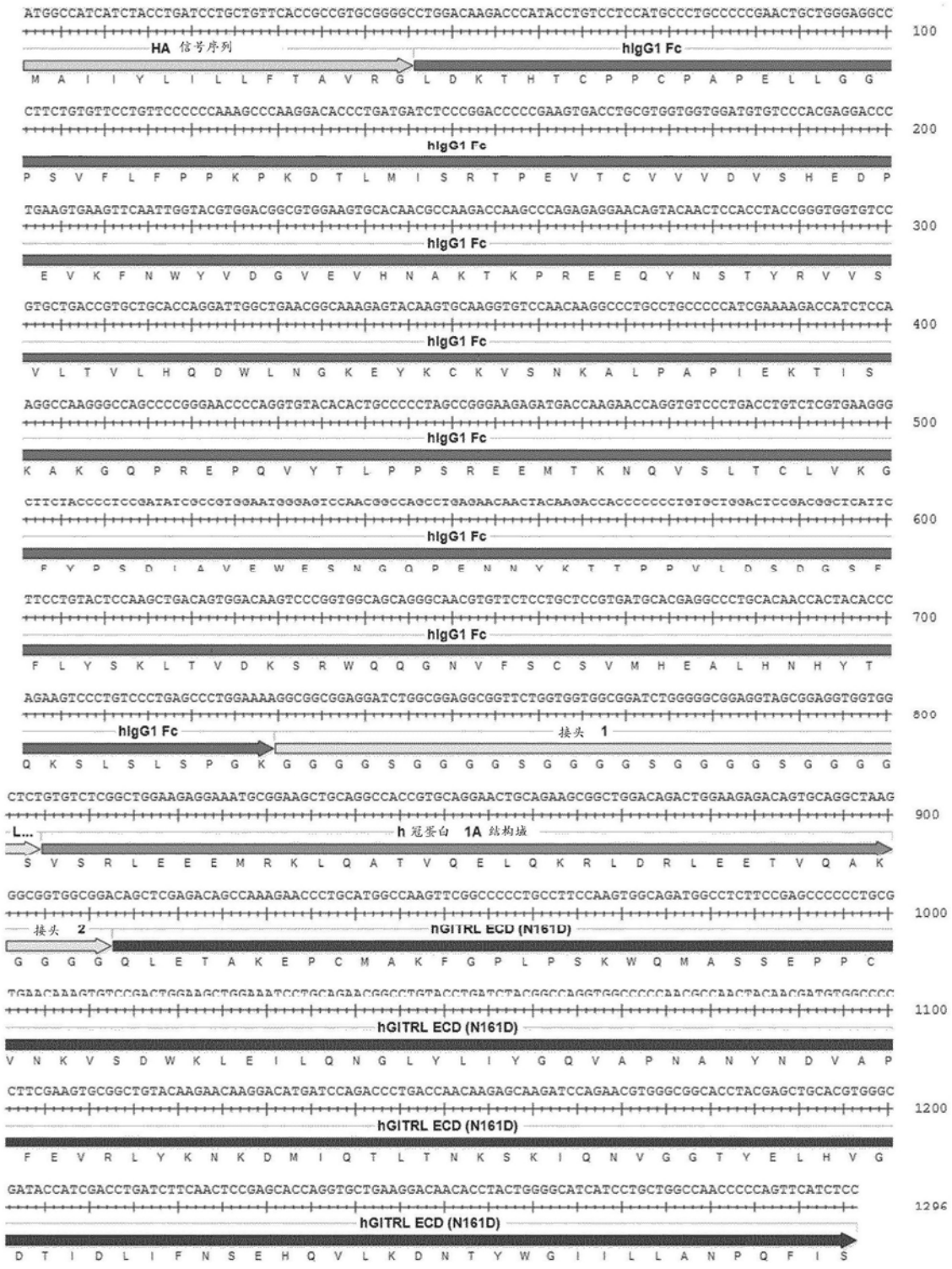

ATGGCCATCATCTACCTGATCCTGCTGTTCACCGCCGTGCGGGGCCTGGACAAGACCCATACCTGTCCTCCATGCCCTGCCCCCGAACTGCTGGGAGGCC
100
HA 信号序列
hIgG1 Fc
M A I I Y L I L L F T A V R G L D K T H T C P P C P A P E L L G G
CTTCTGTGTTCCTGTTCCCCCCAAAGCCCAAGGACACCCTGATGATCTCCCGGACCCCCGAAGTGACCTGCGTGGTGGTGGATGTGTCCCACGAGGACCC
200
hIgG1 Fc
P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P
TGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAAGTGCACAACGCCAAGACCAAGCCCAGAGAGGAACAGTACAACTCCACCTACCGGGTGGTGTCC
300
hIgG1 Fc
E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S
GTGCTGACCGTGCTGCACCAGGATTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAGGCCCTGCCTGCCCCCATCGAAAAGACCATCTCCA
400
hIgG1 Fc
V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S
AGGCCAAGGGCCAGCCCCGGGAACCCCAGGTGTACACACTGCCCCCTAGCCGGGAAGAGATGACCAAGAACCAGGTGTCCCTGACCTGTCTCGTGAAGGG
500
hIgG1 Fc
K A K G Q P R E P Q V Y T L P P S R E E M T K N Q V S L T C L V K G
CTTCTACCCCTCCGATATCGCCGTGGAATGGGAGTCCAACGGCCAGCCTGAGAACAACTACAAGACCACCCCCCCTGTGCTGGACTCCGACGGCTCATTC
600
hIgG1 Fc
F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F
TTCCTGTACTCCAAGCTGACAGTGGACAAGTCCCGGTGGCAGCAGGGCAACGTGTTCTCCTGCTCCGTGATGCACGAGGCCCTGCACAACCACTACACCC
700
hIgG1 Fc
F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T
AGAAGTCCCTGTCCCTGAGCCCTGGAAAAGGCGGCGGAGGATCTGGCGGAGGCGGTTCTGGTGGTGGCGGATCTGGGGGCGGAGGTAGCGGAGGTGGTGG
800
hIgG1 Fc
接头 1
Q K S L S L S P G K G G G G S G G G G S G G G G S G G G G S G G G G
CTCTGTGTCTCGGCTGGAAGAGGAAATGCGGAAGCTGCAGGCCACCGTGCAGGAACTGCAGAAGCGGCTGGACAGACTGGAAGAGACAGTGCAGGCTAAG
900
L...
h 冠蛋白 1A 结构域
S V S R L E E E M R K L Q A T V Q E L Q K R L D R L E E T V Q A K
GGCGGTGGCGGACAGCTCGAGACAGCCAAAGAACCCTGCATGGCCAAGTTCGGCCCCCTGCCTTCCAAGTGGCAGATGGCCTCTTCCGAGCCCCCCTGCG
1000
接头 2
hGITRL ECD (N161D)
G G G G Q L E T A K E P C M A K F G P L P S K W Q M A S S E P P C
TGAACAAAGTGTCCGACTGGAAGCTGGAAATCCTGCAGAACGGCCCTGTACCTGATCTACGGCCAGGTGGCCCCCAACGCCAACTACAACGATGTGGCCCC
1100
hGITRL ECD (N161D)
V N K V S D W K L E I L Q N G L Y L I Y G Q V A P N A N Y N D V A P
CTTCGAAGTGCGGCTGTACAAGAACAAGGACATGATCCAGACCCTGACCAACAAGAGCAAGATCCAGAACGTGGGCGGCACCTACGAGCTGCACGTGGGC
1200
hGITRL ECD (N161D)
F E V R L Y K N K D M I Q T L T N K S K I Q N V G G T Y E L H V G
GATACCATCGACCTGATCTTCAACTCCGAGCACCAGGTGCTGAAGGACAACACCTACTGGGGCATCATCCTGCTGGCCAACCCCCAGTTCATCTCC
1296
hGITRL ECD (N161D)
D T I D L I F N S E H Q V L K D N T Y W G I I L L A N P Q F I S

图8

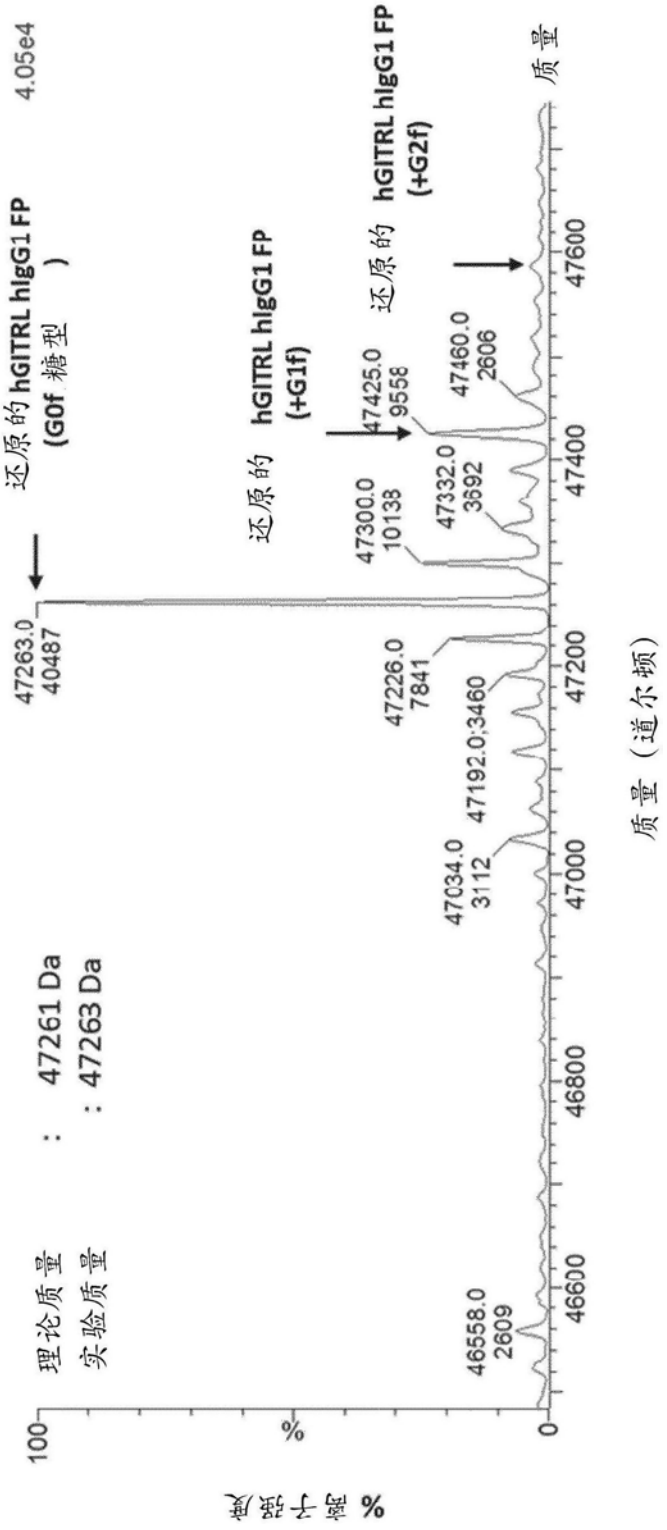

还原的 hGITRL hIgG1 FP
(G0f 糖型)
4.05e4
还原的 hGITRL hIgG1 FP
(+G1f)
还原的 hGITRL hIgG1 FP
(+G2f)
47263.0
40487
47300.0
10138
47425.0
9558
47460.0
2606
47332.0
3692
47226.0
7841
47192.0;3460
47034.0
3112
46558.0
2609
理论质量 : 47261 Da
实验质量 : 47263 Da
100
%
0
46600
46800
47000
47200
47400
47600
质量
质量（道尔顿）
% 颗粒强度

图9

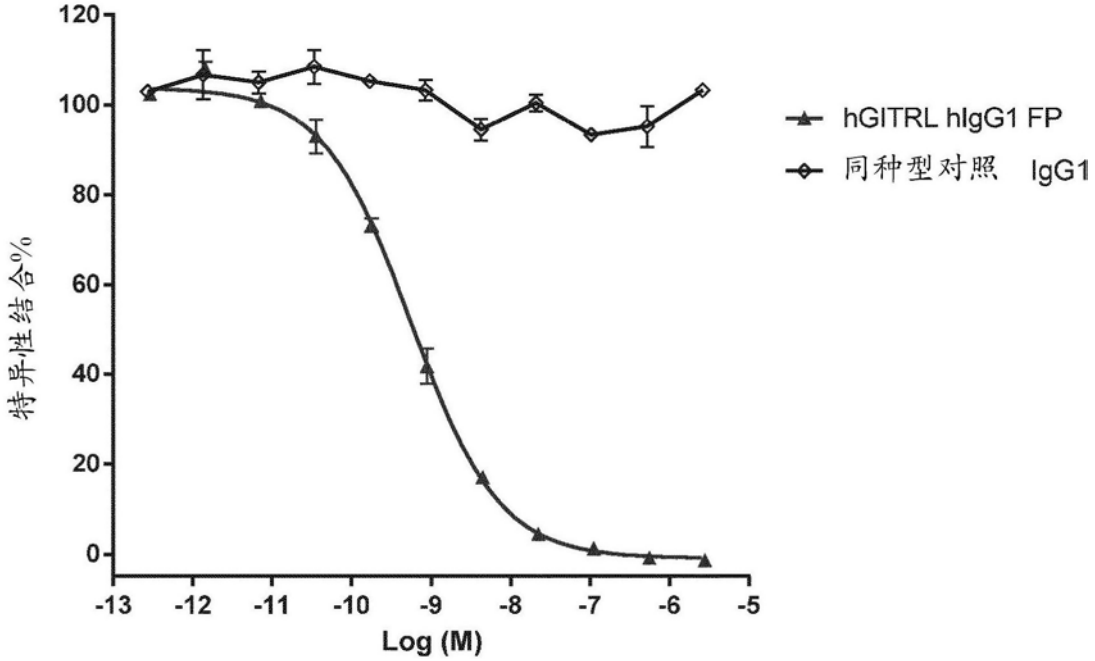

120
100
80
60
40
20
0
特异性结合%
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log (M)
hGITRL hIgG1 FP
同种型对照 IgG1

图10

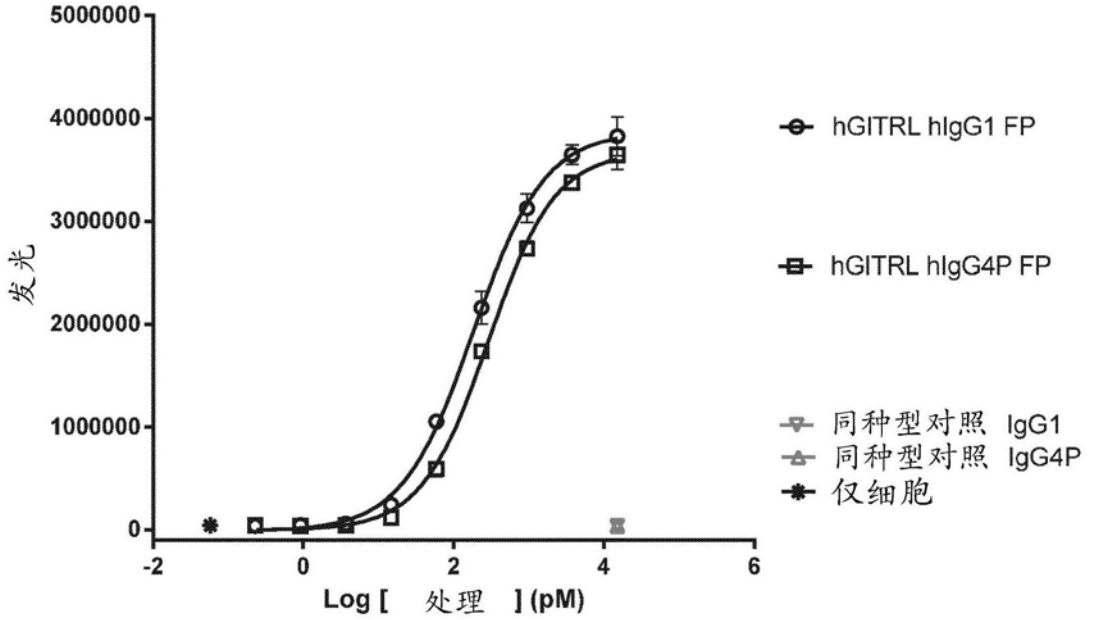

5000000
4000000
3000000
2000000
1000000
0
发光
-2
0
2
4
6
Log [处理] (pM)
hGITRL hIgG1 FP
hGITRL hIgG4P FP
同种型对照 IgG1
同种型对照 IgG4P
仅细胞

图11

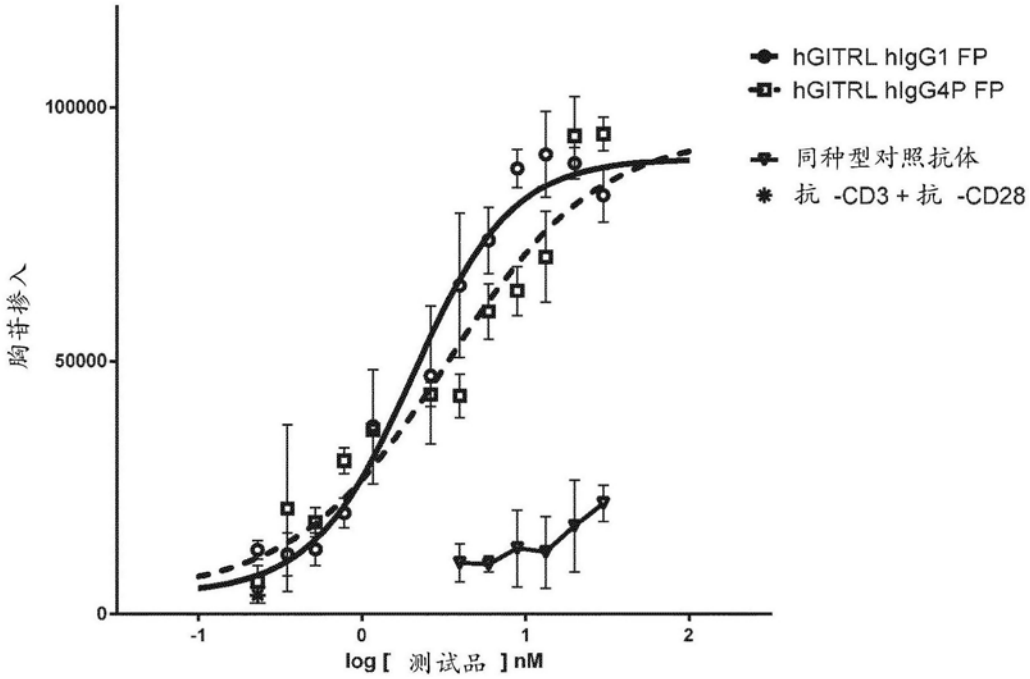

hGITRL hIgG1 FP
hGITRL hIgG4P FP
同种型对照抗体
抗 -CD3 + 抗 -CD28
胸苷掺入
100000
50000
0
-1
0
1
2
log [测试品] nM

图12

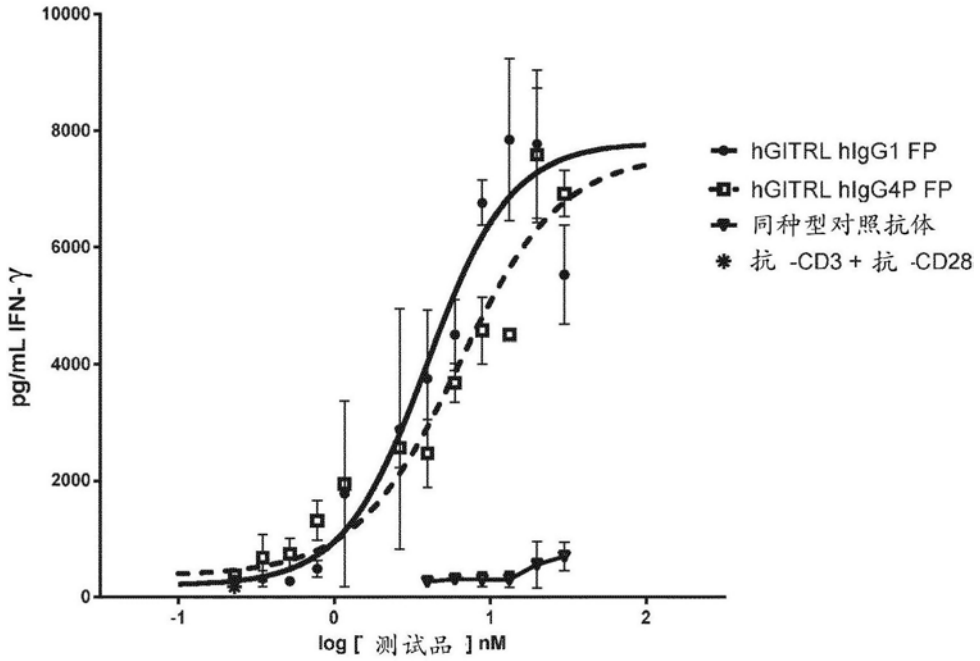

hGITRL hIgG1 FP
hGITRL hIgG4P FP
同种型对照抗体
抗 -CD3 + 抗 -CD28
pg/mL IFN-γ
10000
8000
6000
4000
2000
0
-1
0
1
2
log [测试品] nM

图13

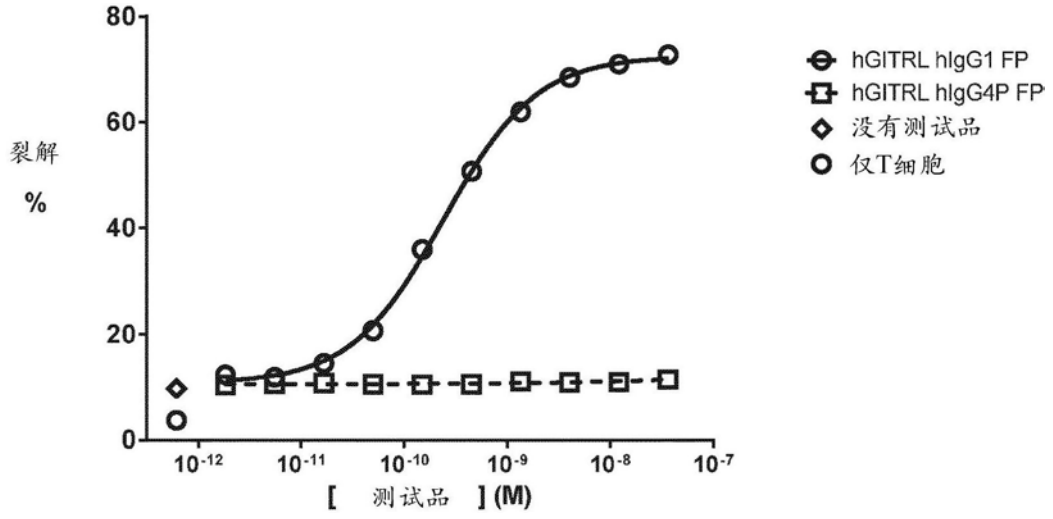

80
60
40
20
0
裂解
%
10-12
10-11
10-10
10-9
10-8
10-7
[测试品] (M)
hGITRL hIgG1 FP
hGITRL hIgG4P FP
没有测试品
仅T细胞

图14

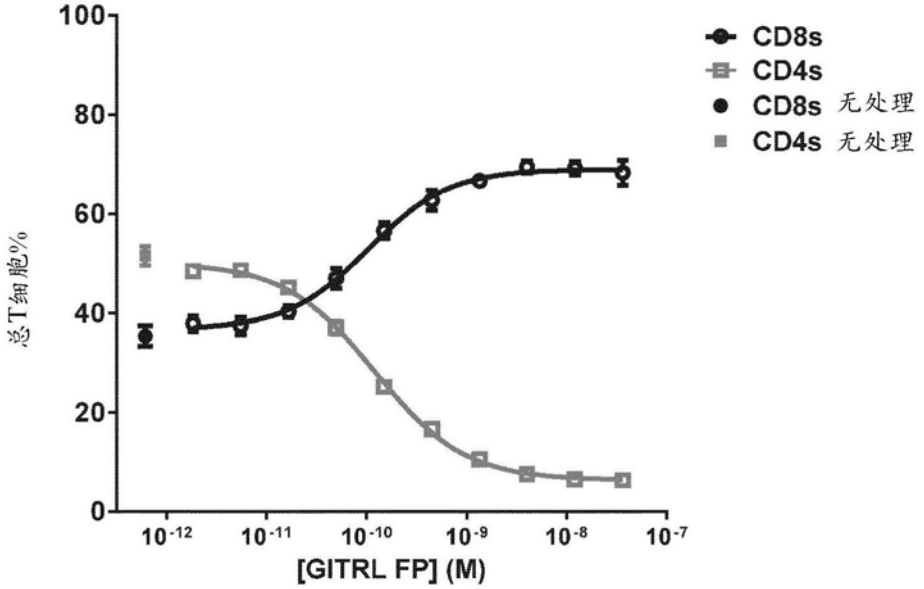

100
80
60
40
20
0
总T细胞%
10-12
10-11
10-10
10-9
10-8
10-7
[GITRL FP] (M)
CD8s
CD4s
CD8s 无处理
CD4s 无处理

图15

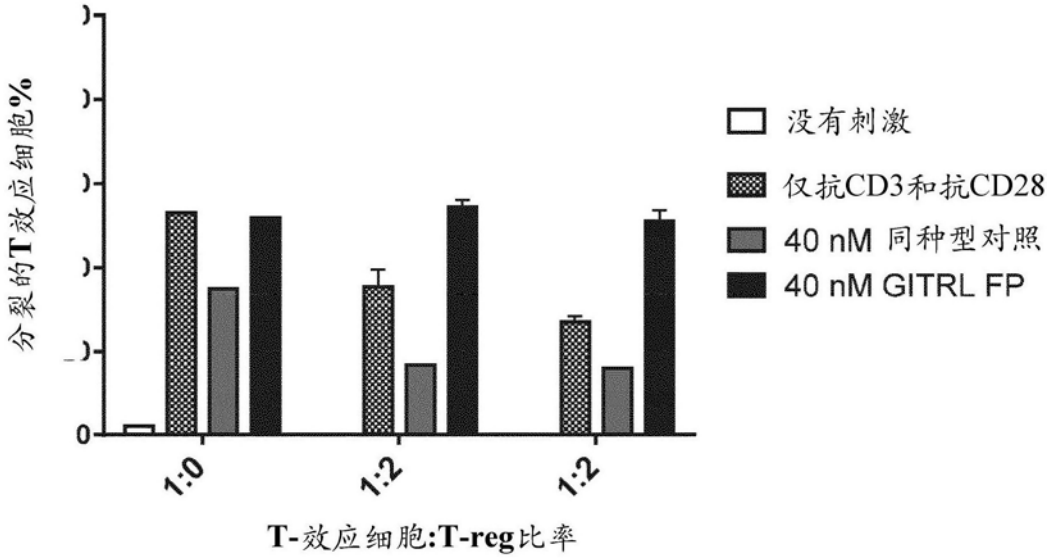

分裂的T效应细胞%
0
1:0
1:2
1:2
T-效应细胞:T-reg比率
没有刺激
仅抗CD3和抗CD28
40 nM 同种型对照
40 nM GITRL FP

图16

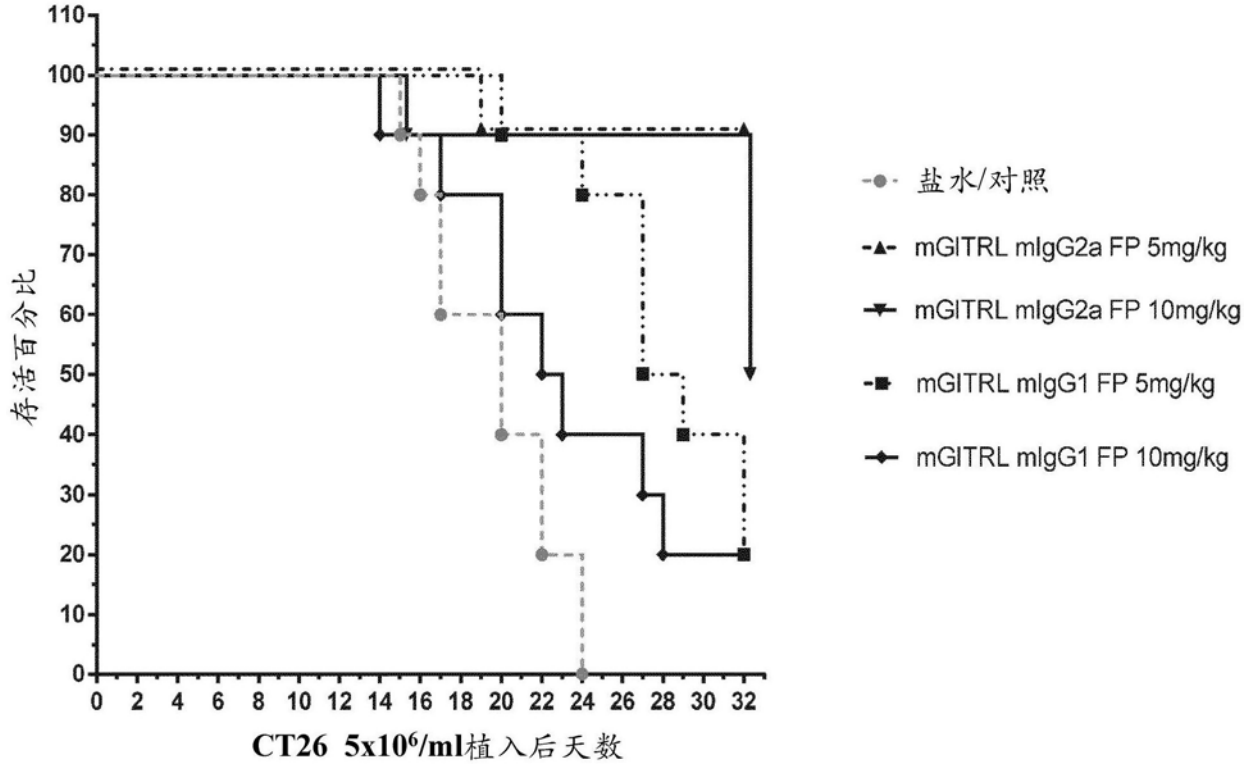

存活百分比
0
10
20
30
40
50
60
70
80
90
100
110
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
CT26 5x10⁶/ml植入后天数
盐水/对照
mGITRL mIgG2a FP 5mg/kg
mGITRL mIgG2a FP 10mg/kg
mGITRL mIgG1 FP 5mg/kg
mGITRL mIgG1 FP 10mg/kg

图17

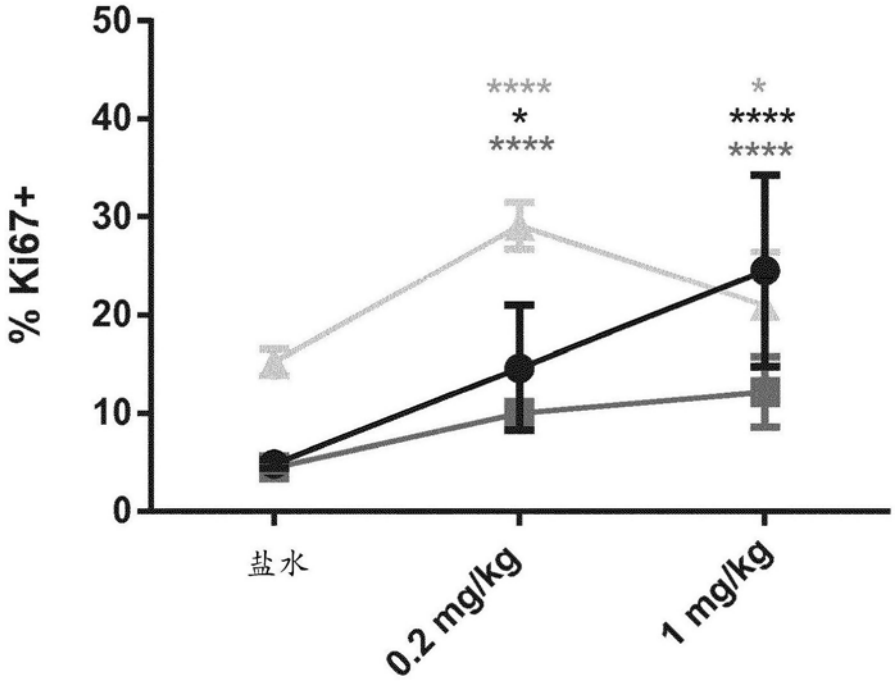

50
40
30
20
10
0
% Ki67+
****
*
****
*
****
****
盐水
0.2 mg/kg
1 mg/kg

图18

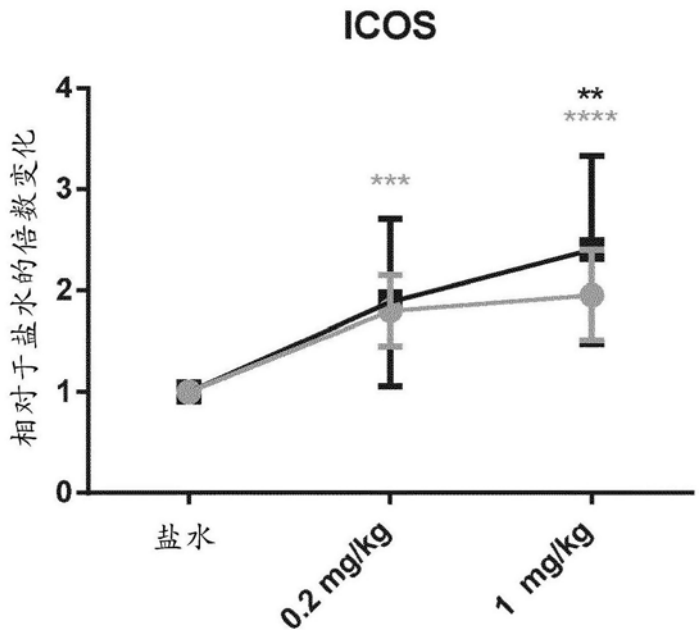

ICOS
相对于盐水的倍数变化
4
3
2
1
0
***
**
****
盐水
0.2 mg/kg
1 mg/kg

图19

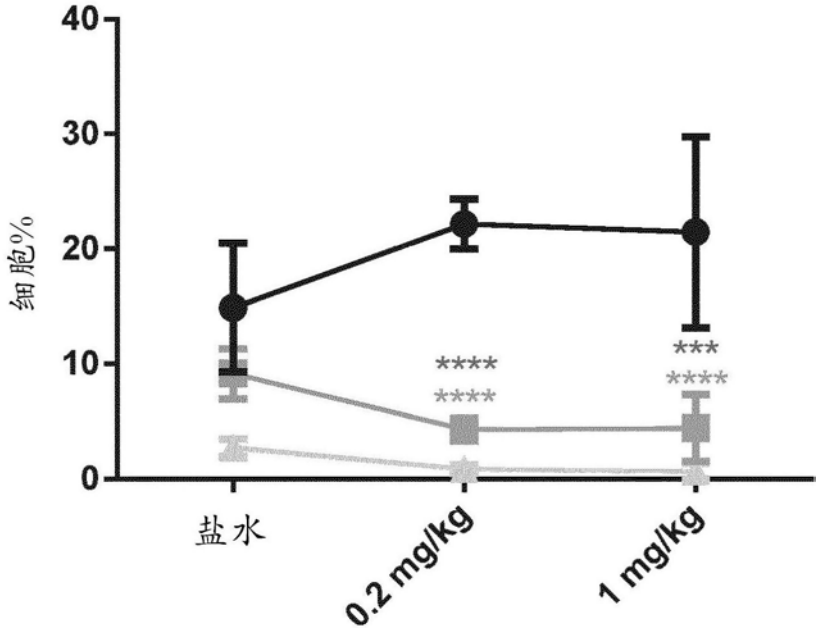

40
30
20
10
0
细胞%
****
****
***
****
盐水
0.2 mg/kg
1 mg/kg

图20

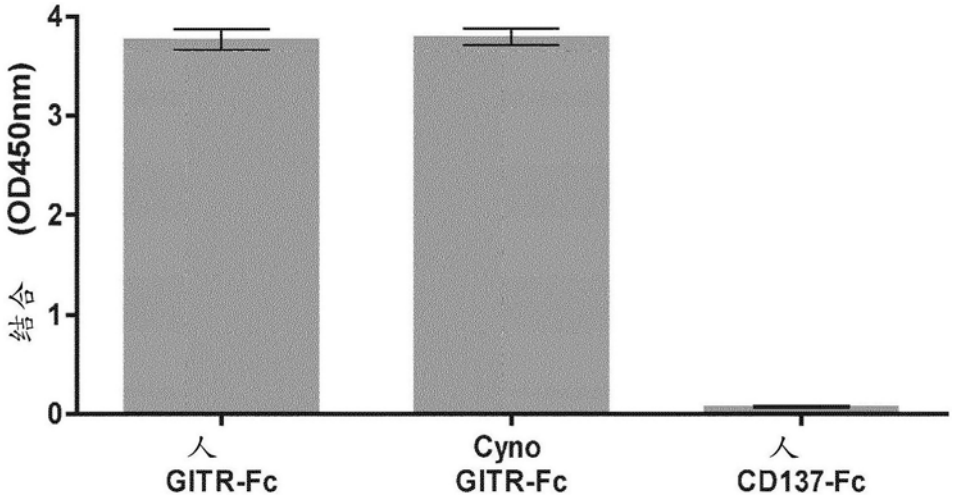

4
3
2
1
0
结合 (OD450nm)
人
GITR-Fc
Cyno
GITR-Fc
人
CD137-Fc

图21

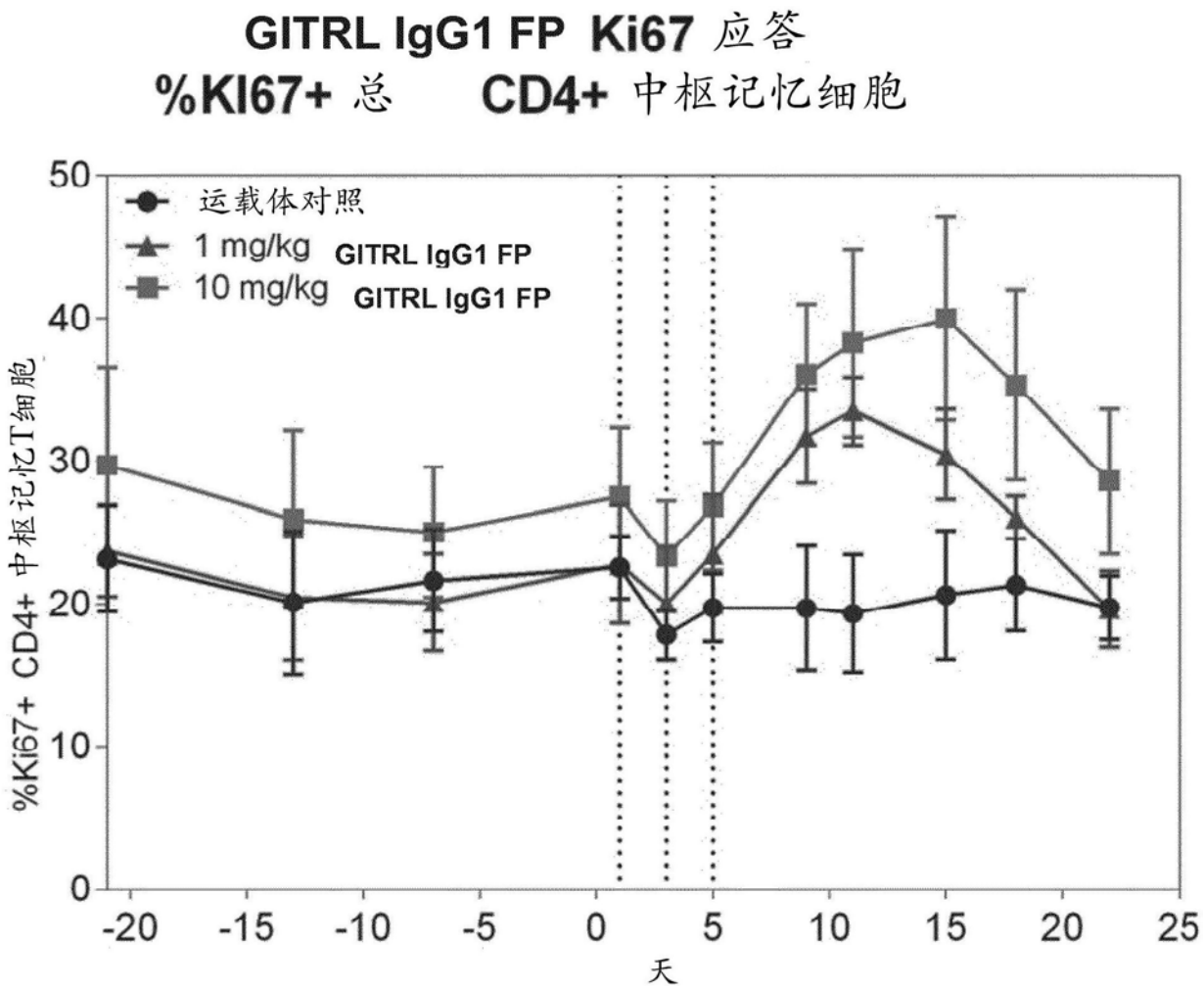

GITRL IgG1 FP Ki67 应答
%KI67+ 总 CD4+ 中枢记忆细胞
运载体对照
1 mg/kg GITRL IgG1 FP
10 mg/kg GITRL IgG1 FP
%Ki67+ CD4+ 中枢记忆T细胞
50
40
30
20
10
0
-20
-15
-10
-5
0
5
10
15
20
25
天

图22

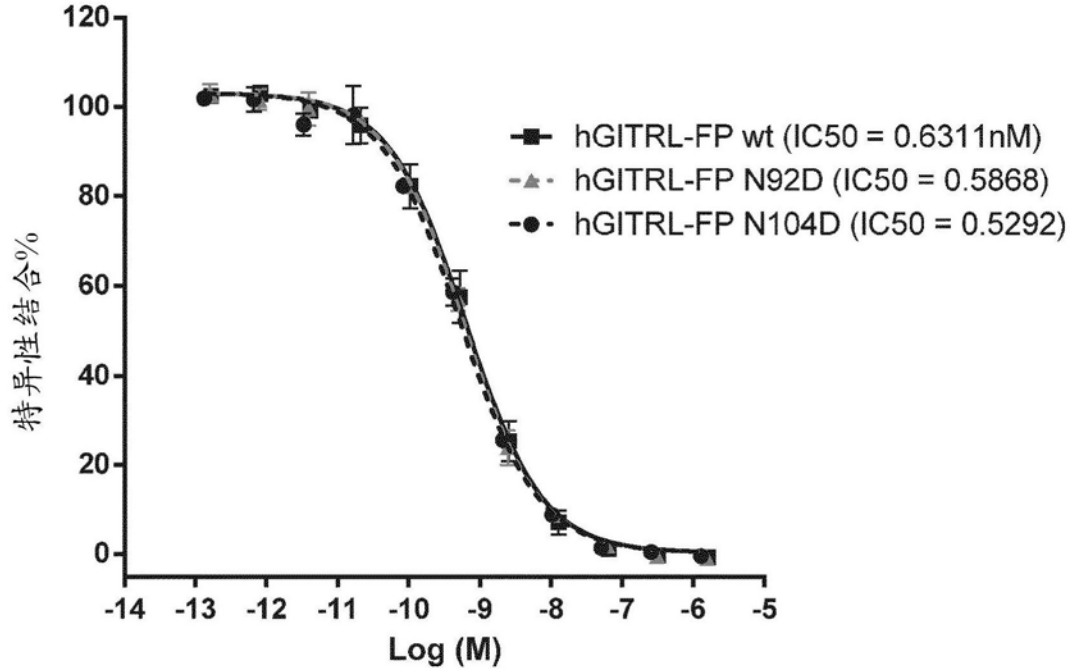

120
100
80
60
40
20
0
特异性结合%
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
Log (M)
hGITRL-FP wt (IC50 = 0.6311nM)
hGITRL-FP N92D (IC50 = 0.5868)
hGITRL-FP N104D (IC50 = 0.5292)

图23A

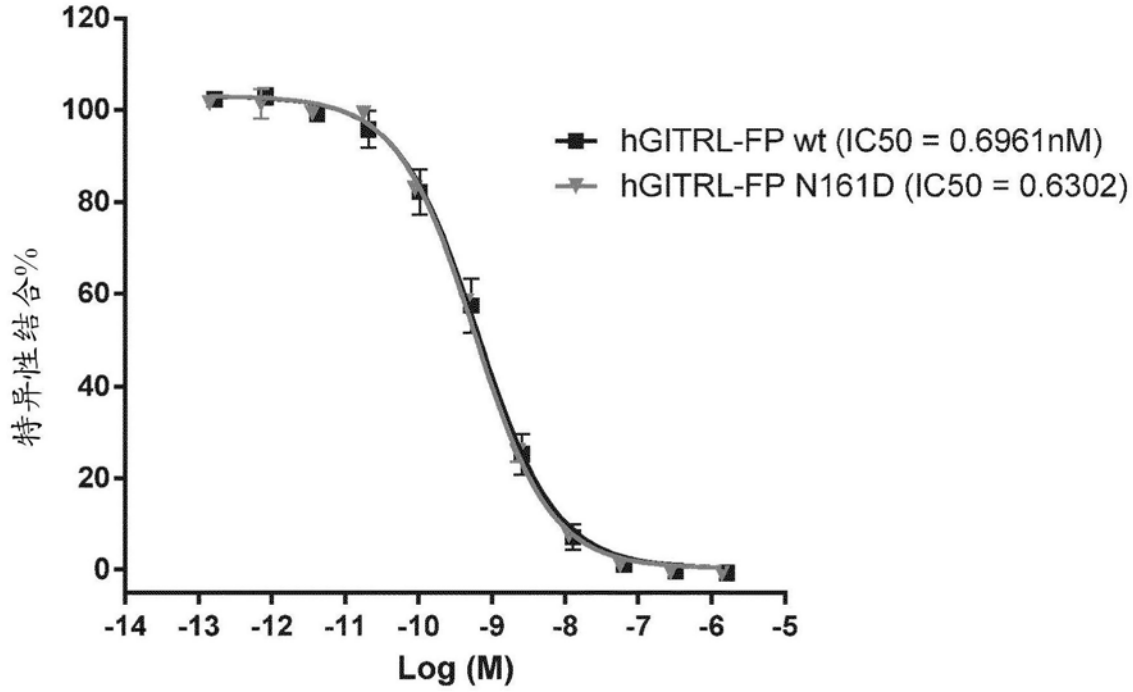

120
100
80
60
40
20
0
特异性结合%
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
Log (M)
hGITRL-FP wt (IC50 = 0.6961nM)
hGITRL-FP N161D (IC50 = 0.6302)

图23B

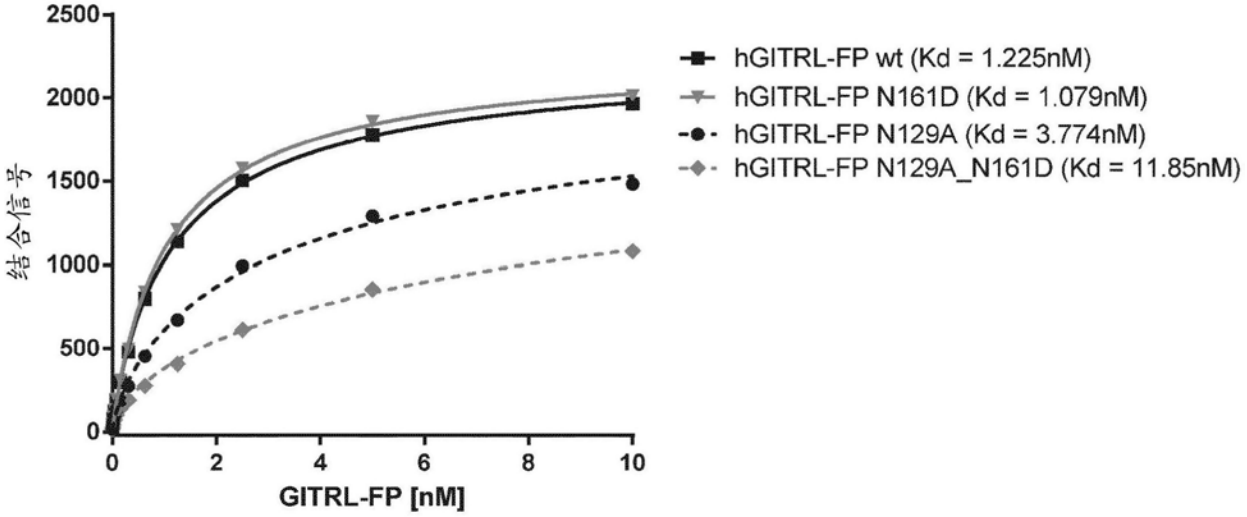

2500
2000
1500
1000
500
0
结合信号
0
2
4
6
8
10
GITRL-FP [nM]
hGITRL-FP wt (Kd = 1.225nM)
hGITRL-FP N161D (Kd = 1.079nM)
hGITRL-FP N129A (Kd = 3.774nM)
hGITRL-FP N129A_N161D (Kd = 11.85nM)

图24

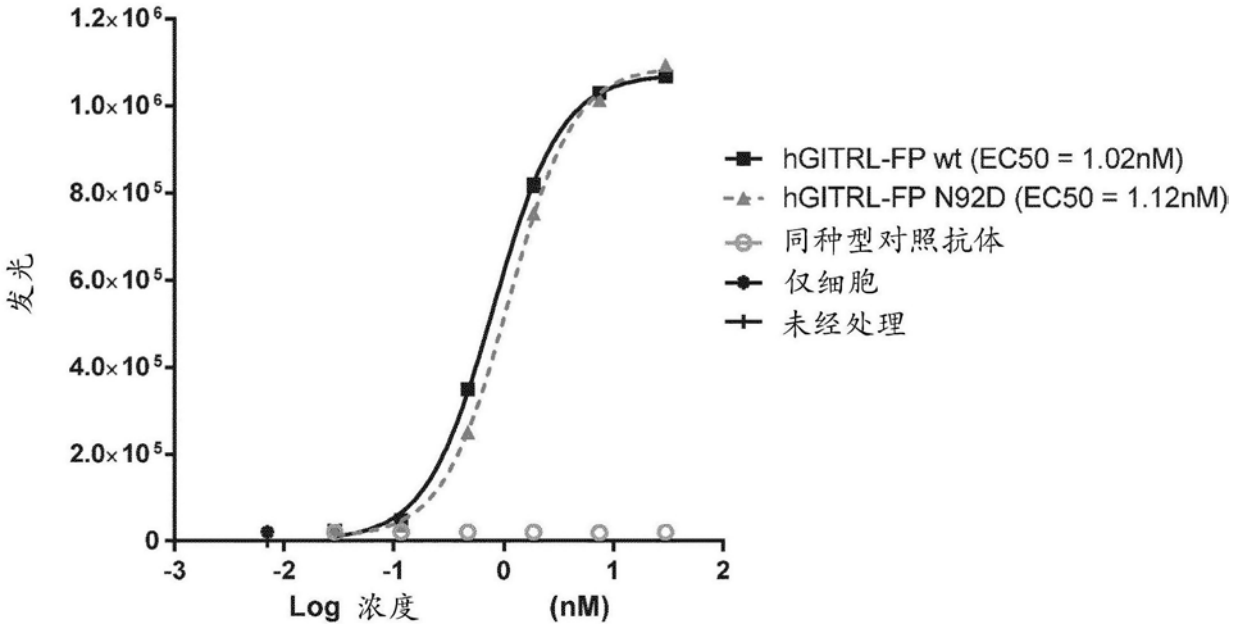

1.2×10⁶
1.0×10⁶
8.0×10⁵
6.0×10⁵
4.0×10⁵
2.0×10⁵
0
发光
-3
-2
-1
0
1
2
Log 浓度 (nM)
hGITRL-FP wt (EC50 = 1.02nM)
hGITRL-FP N92D (EC50 = 1.12nM)
同种型对照抗体
仅细胞
未经处理

图25A

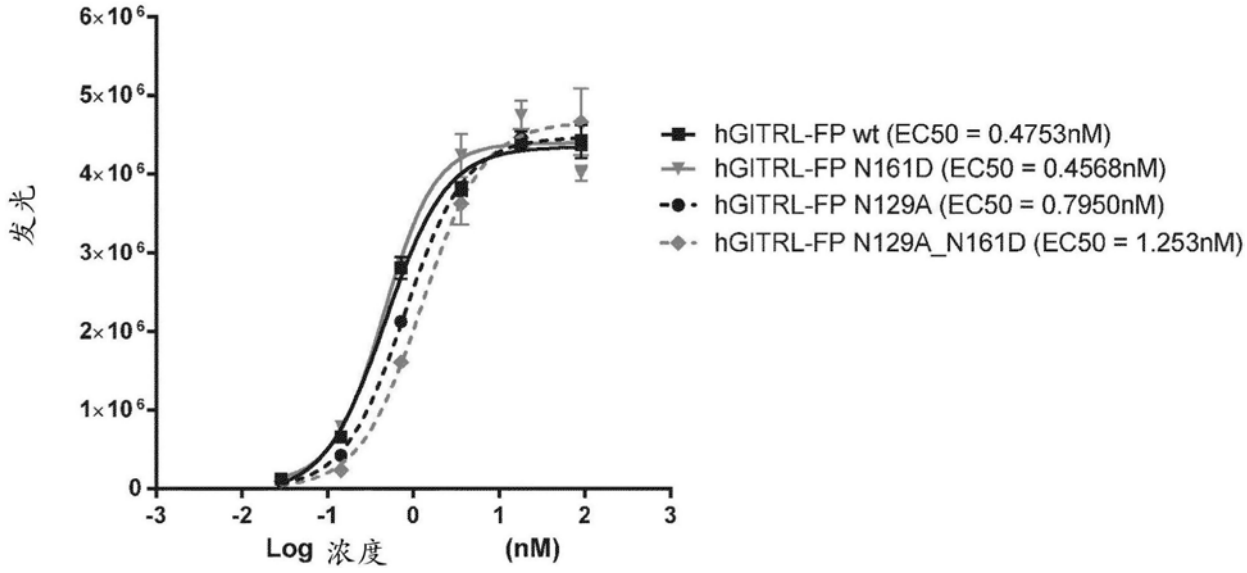

6×10⁶
5×10⁶
4×10⁶
3×10⁶
2×10⁶
1×10⁶
0
发光
-3
-2
-1
0
1
2
3
Log 浓度 (nM)
hGITRL-FP wt (EC50 = 0.4753nM)
hGITRL-FP N161D (EC50 = 0.4568nM)
hGITRL-FP N129A (EC50 = 0.7950nM)
hGITRL-FP N129A_N161D (EC50 = 1.253nM)

图25B

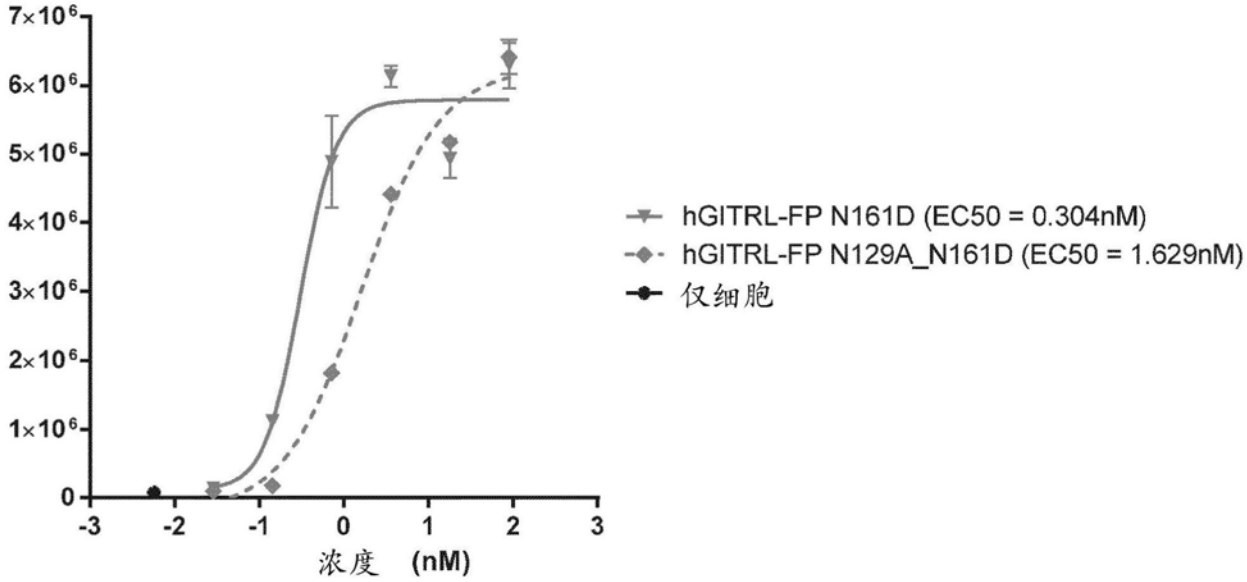

7×10⁶
6×10⁶
5×10⁶
4×10⁶
3×10⁶
2×10⁶
1×10⁶
0
-3
-2
-1
0
1
2
3
浓度 (nM)
hGITRL-FP N161D (EC50 = 0.304nM)
hGITRL-FP N129A_N161D (EC50 = 1.629nM)
仅细胞

图25C

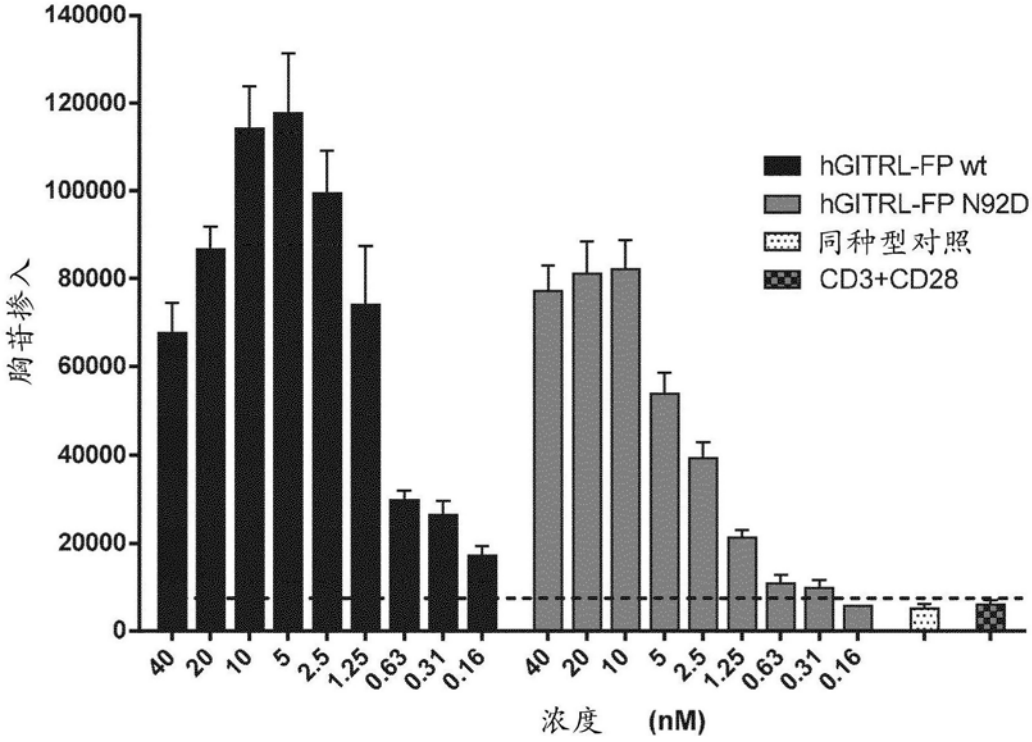

140000
120000
100000
80000
60000
40000
20000
0
胸苷掺入
hGITRL-FP wt
hGITRL-FP N92D
同种型对照
CD3+CD28
40 20 10 5 2.5 1.25 0.63 0.31 0.16
40 20 10 5 2.5 1.25 0.63 0.31 0.16
浓度 (nM)

图26A

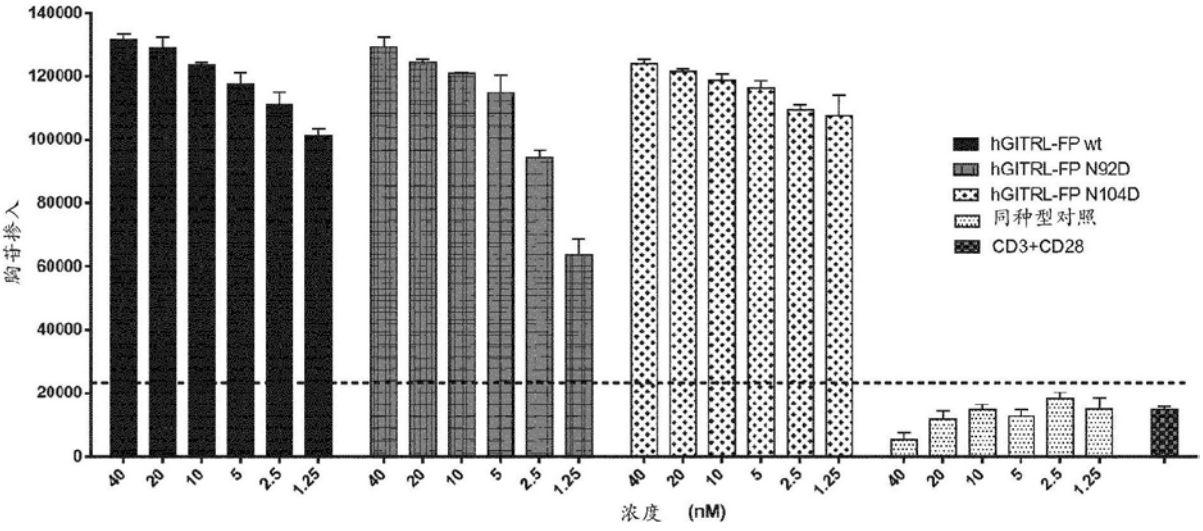

140000
120000
100000
80000
60000
40000
20000
0
胸苷掺入
hGITRL-FP wt
hGITRL-FP N92D
hGITRL-FP N104D
同种型对照
CD3+CD28
40 20 10 5 2.5 1.25
40 20 10 5 2.5 1.25
40 20 10 5 2.5 1.25
40 20 10 5 2.5 1.25
浓度 (nM)

图26B

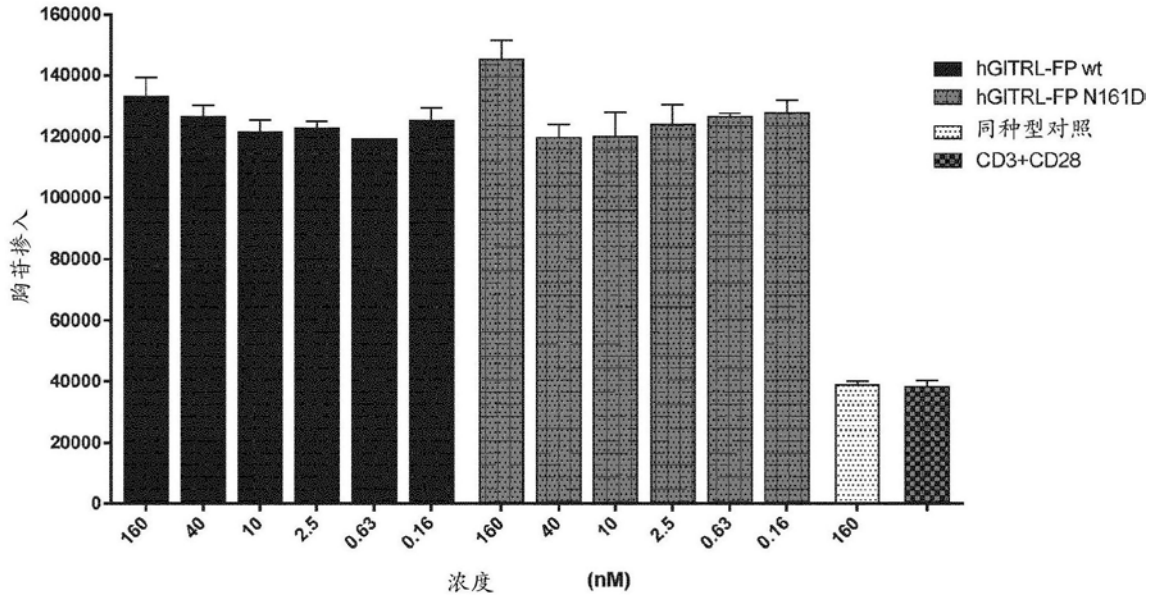

160000
140000
120000
100000
80000
60000
40000
20000
0
胸苷掺入
hGITRL-FP wt
hGITRL-FP N161D
同种型对照
CD3+CD28
160
40
10
2.5
0.63
0.16
160
40
10
2.5
0.63
0.16
160
浓度
(nM)

图26C

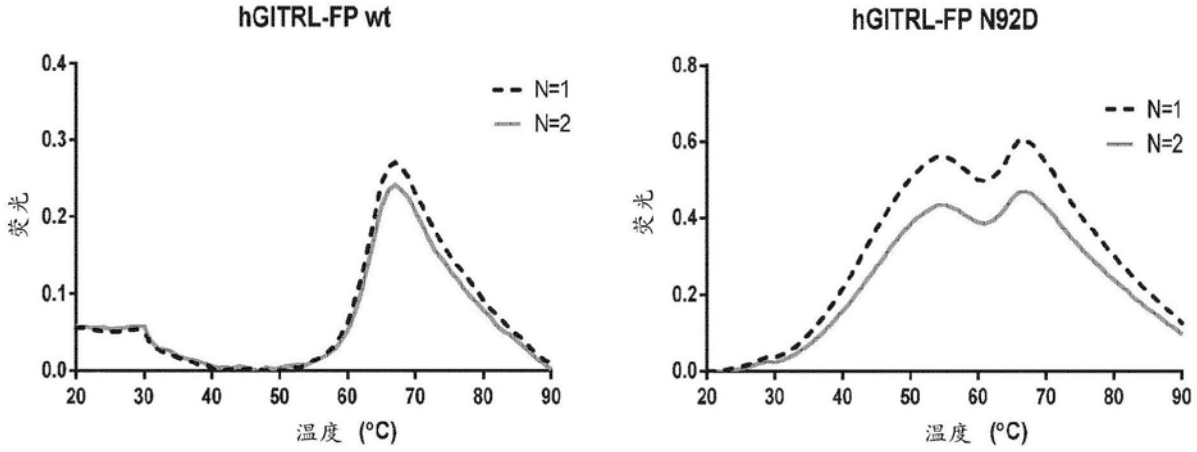

hGITRL-FP wt
0.4
0.3
0.2
0.1
0.0
荧光
N=1
N=2
20
30
40
50
60
70
80
90
温度 (°C)
hGITRL-FP N92D
0.8
0.6
0.4
0.2
0.0
荧光
N=1
N=2
20
30
40
50
60
70
80
90
温度 (°C)

图27

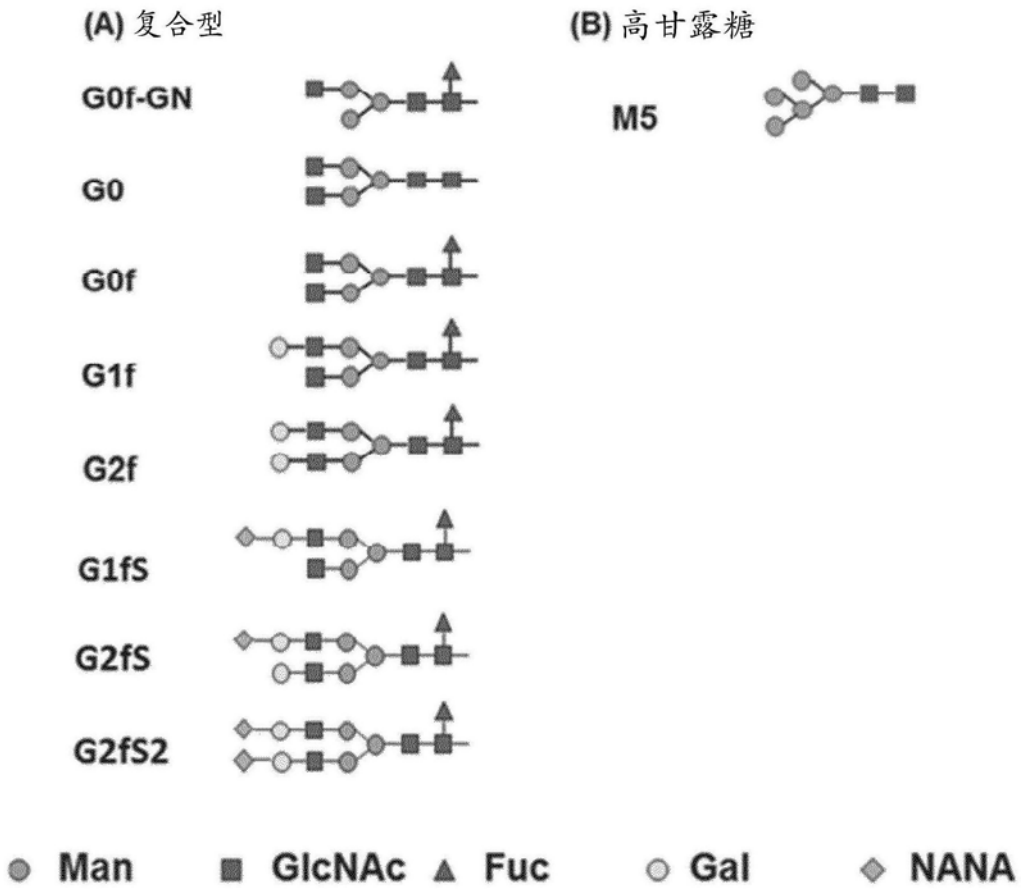

(A) 复合型
G0f-GN
G0
G0f
G1f
G2f
G1fS
G2fS
G2fS2
(B) 高甘露糖
M5
Man
GlcNAc
Fuc
Gal
NANA

图28

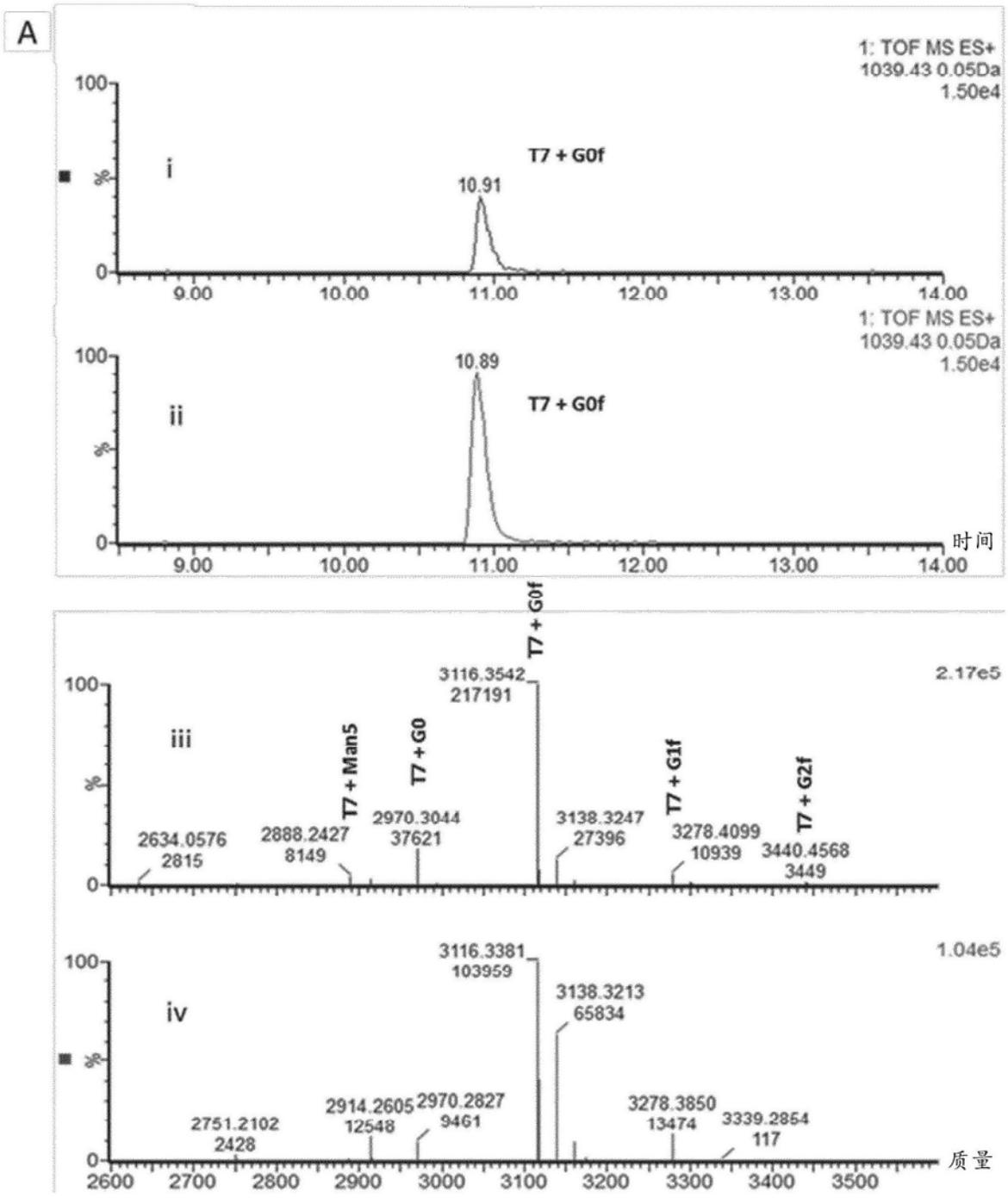

A
1: TOF MS ES+
1039.43 0.05Da
1.50e4
i
T7 + G0f
10.91
ii
10.89
T7 + G0f
时间
iii
T7 + G0f
3116.3542
217191
2.17e5
T7 + Man5
T7 + G0
T7 + G1f
T7 + G2f
2634.0576
2815
2888.2427
8149
2970.3044
37621
3138.3247
27396
3278.4099
10939
3440.4568
3449
iv
3116.3381
103959
1.04e5
3138.3213
65834
2751.2102
2428
2914.2605
12548
2970.2827
9461
3278.3850
13474
3339.2854
117
质量

图29A

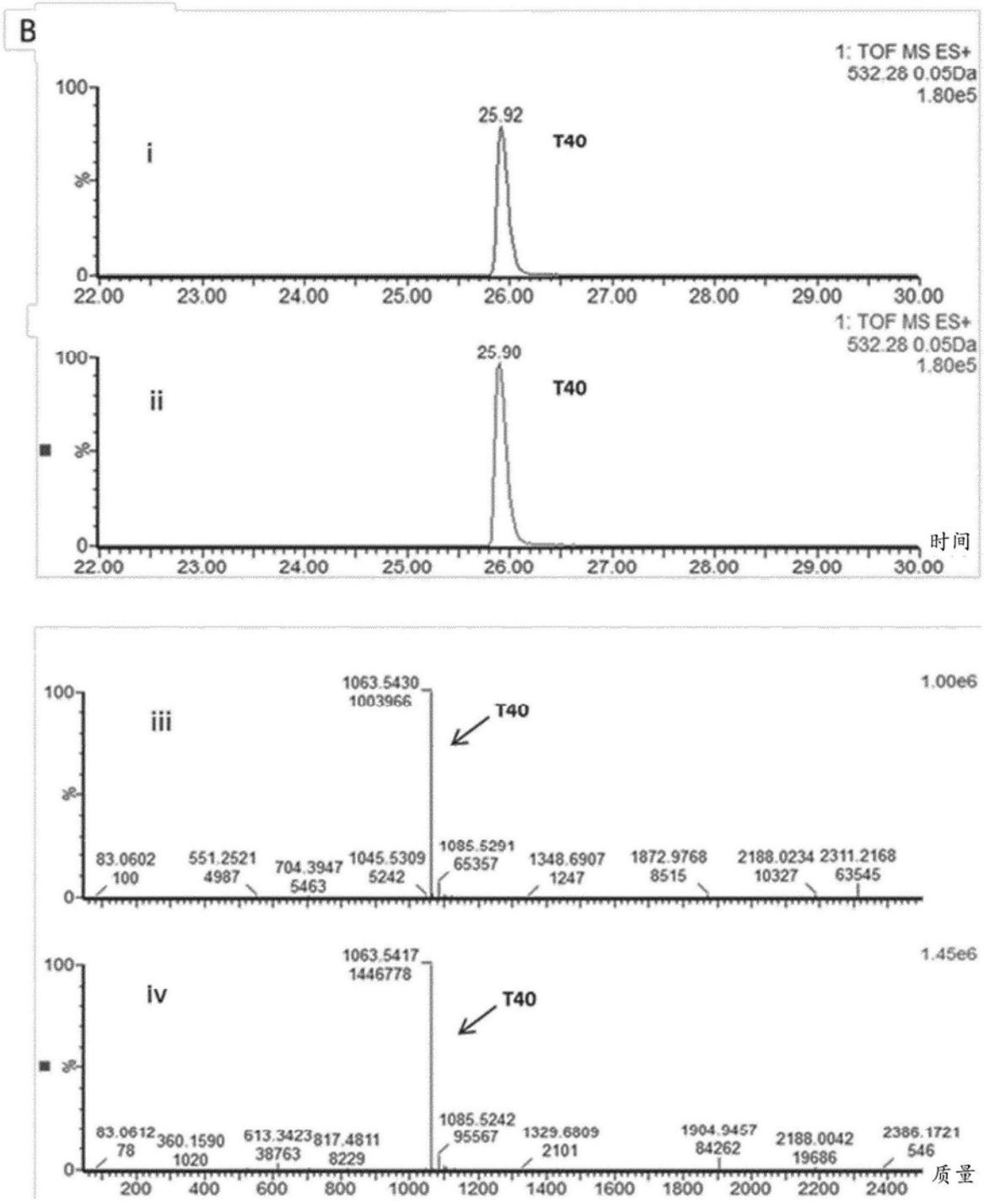

B
1: TOF MS ES+
532.28 0.05Da
1.80e5
i
25.92
T40
ii
25.90
T40
时间
iii
1063.5430
1003966
1.00e6
T40
83.0602
100
551.2521
4987
704.3947
5463
1045.5309
5242
1085.5291
65357
1348.6907
1247
1872.9768
8515
2188.0234
10327
2311.2168
63545
iv
1063.5417
1446778
1.45e6
T40
83.0612
78
360.1590
1020
613.3423
38763
817.4811
8229
1085.5242
95567
1329.6809
2101
1904.9457
84262
2188.0042
19686
2386.1721
546
质量

图29B

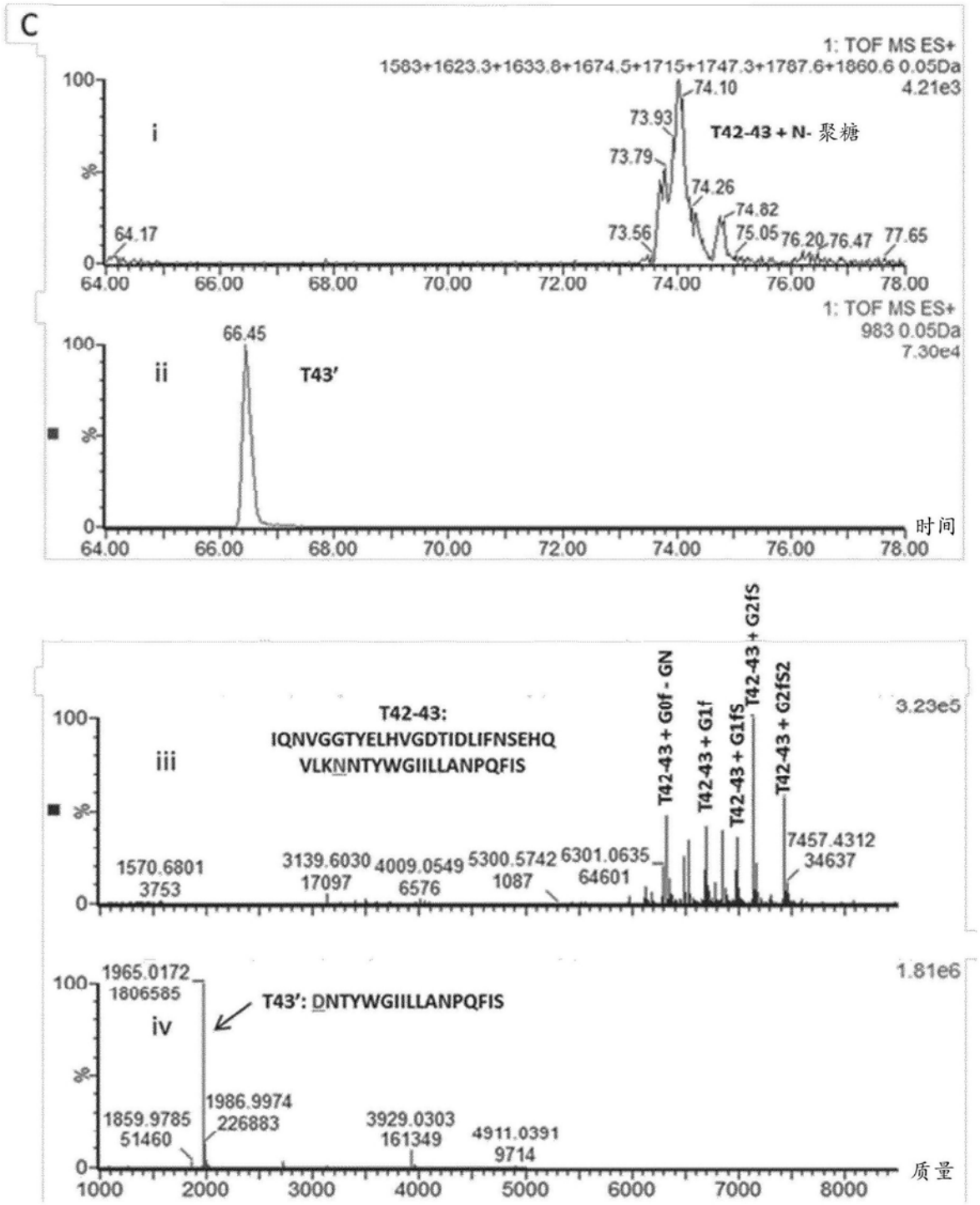

C
1: TOF MS ES+
1583+1623.3+1633.8+1674.5+1715+1747.3+1787.6+1860.6 0.05Da
4.21e3
i
T42-43 + N- 聚糖
74.10
73.93
73.79
74.26
74.82
75.05
76.20
76.47
77.65
73.56
64.17
1: TOF MS ES+
983 0.05Da
7.30e4
ii
66.45
T43'
时间
T42-43:
IQNVGGTYELHVGDTIDLIFNSEHQ
VLKNNTYWGIILLANPQFIS
3.23e5
iii
T42-43 + G0f - GN
T42-43 + G1f
T42-43 + G1fS
T42-43 + G2fS
T42-43 + G2fS2
1570.6801
3753
3139.6030
17097
4009.0549
6576
5300.5742
1087
6301.0635
64601
7457.4312
34637
1.81e6
iv
1965.0172
1806585
T43': DNTYWGIILLANPQFIS
1986.9974
226883
1859.9785
51460
3929.0303
161349
4911.0391
9714
质量

图29C

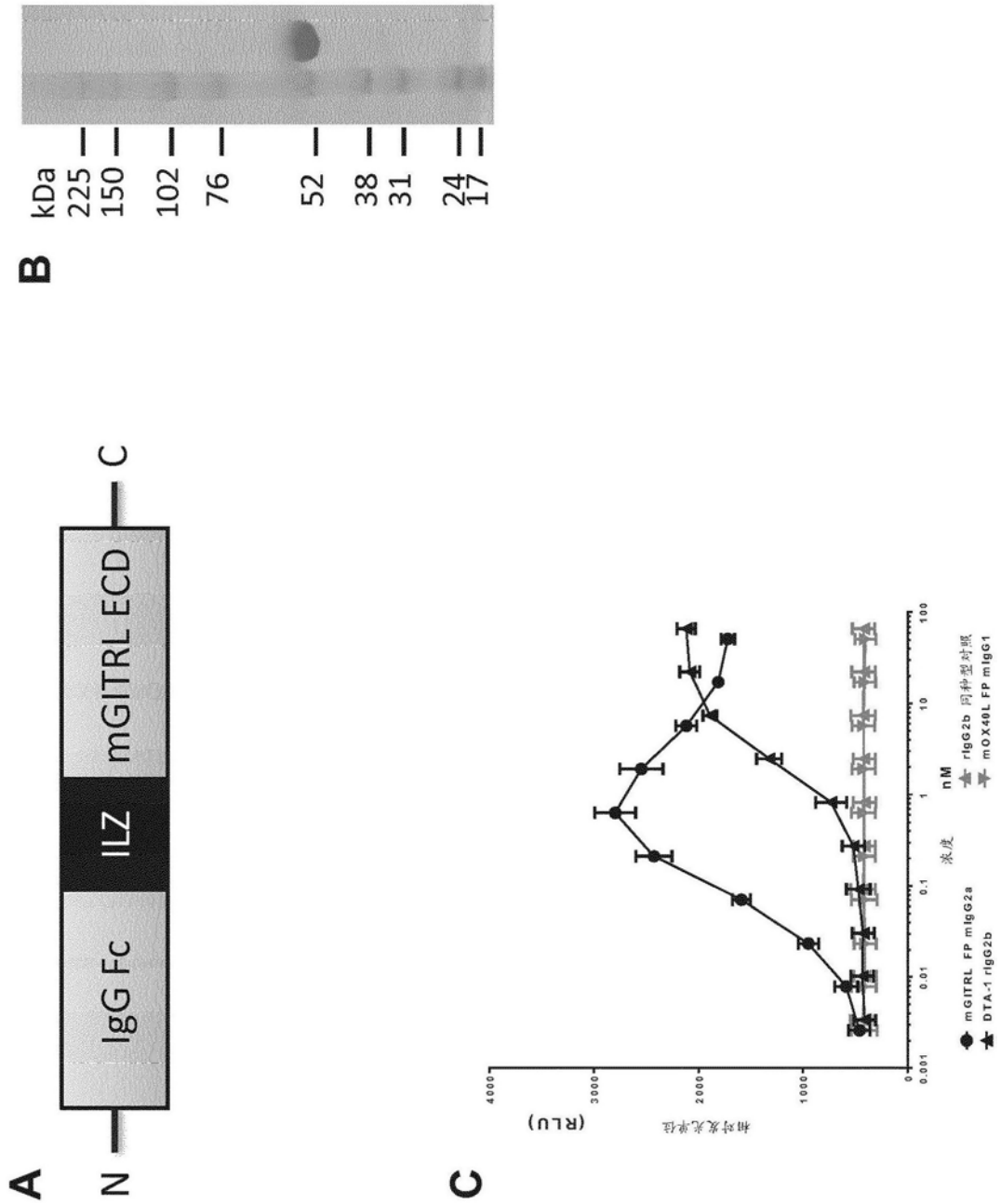

A
N
IgG Fc
ILZ
mGITRL ECD
C
B
kDa
225
150
102
76
52
38
31
24
17
C
相对发光单位
(RLU)
4000
3000
2000
1000
0
浓度
nM
0.001
0.01
0.1
1
10
100
mGITRL FP mIgG2a
DTA-1 rIgG2b
rIgG2b 同种型对照
mOX40L FP mIgG1

图30

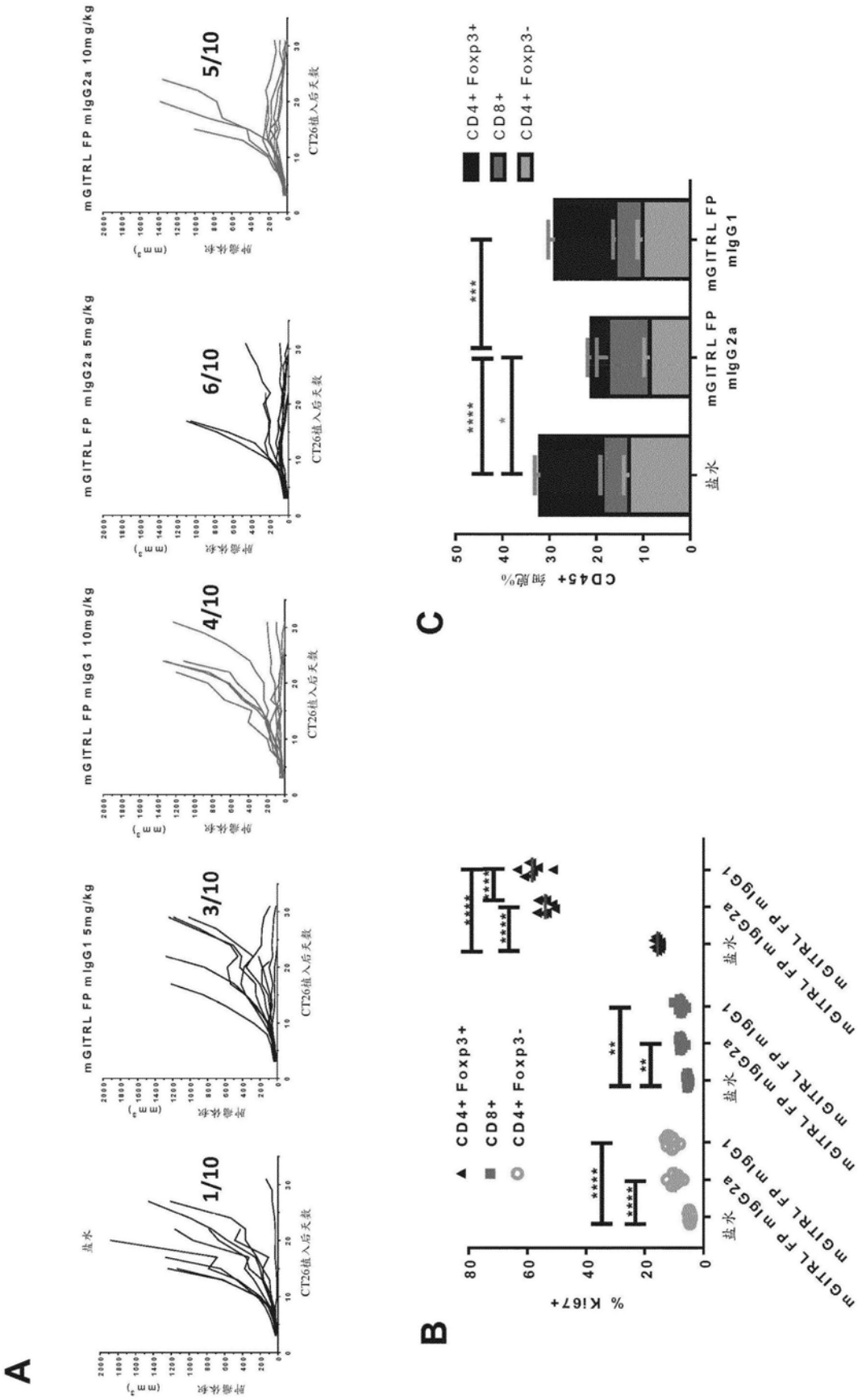
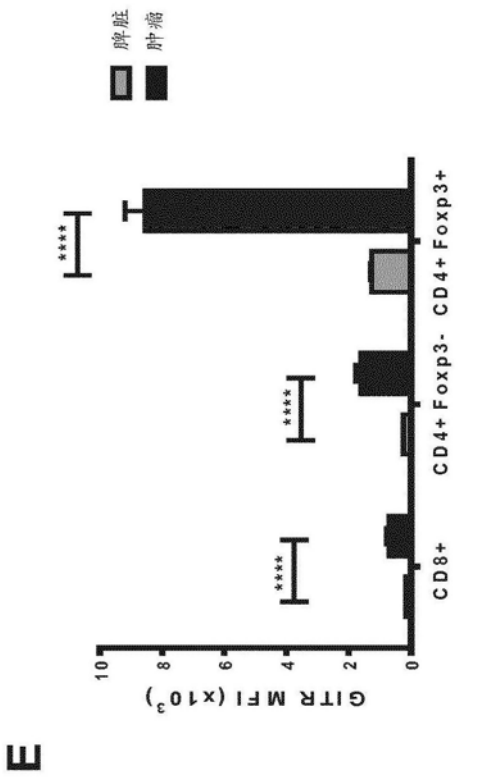

A
盐水
1/10
mGITRL FP mIgG1 5mg/kg
3/10
mGITRL FP mIgG1 10mg/kg
4/10
mGITRL FP mIgG2a 5mg/kg
6/10
mGITRL FP mIgG2a 10mg/kg
5/10
肿瘤体积 (mm³)
CT26植入后天数
B
CD4+ Foxp3+
CD8+
CD4+ Foxp3-
% Ki67+
盐水
mGITRL FP mIgG2a
mGITRL FP mIgG1
C
CD45+ 细胞%
盐水
mGITRL FP mIgG2a
mGITRL FP mIgG1

图31


E
脾脏
肿瘤
GITR MFI (x10³)
0
2
4
6
8
10
****
****
****
CD8+
CD4+ Foxp3-
CD4+ Foxp3+

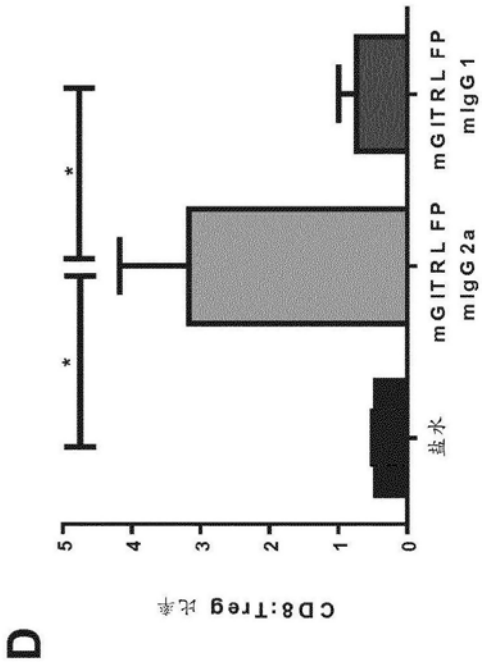

D
CD8:Treg 比率
0
1
2
3
4
5
*
*
盐水
mGITRL FP mIgG2a
mGITRL FP mIgG1

图31续

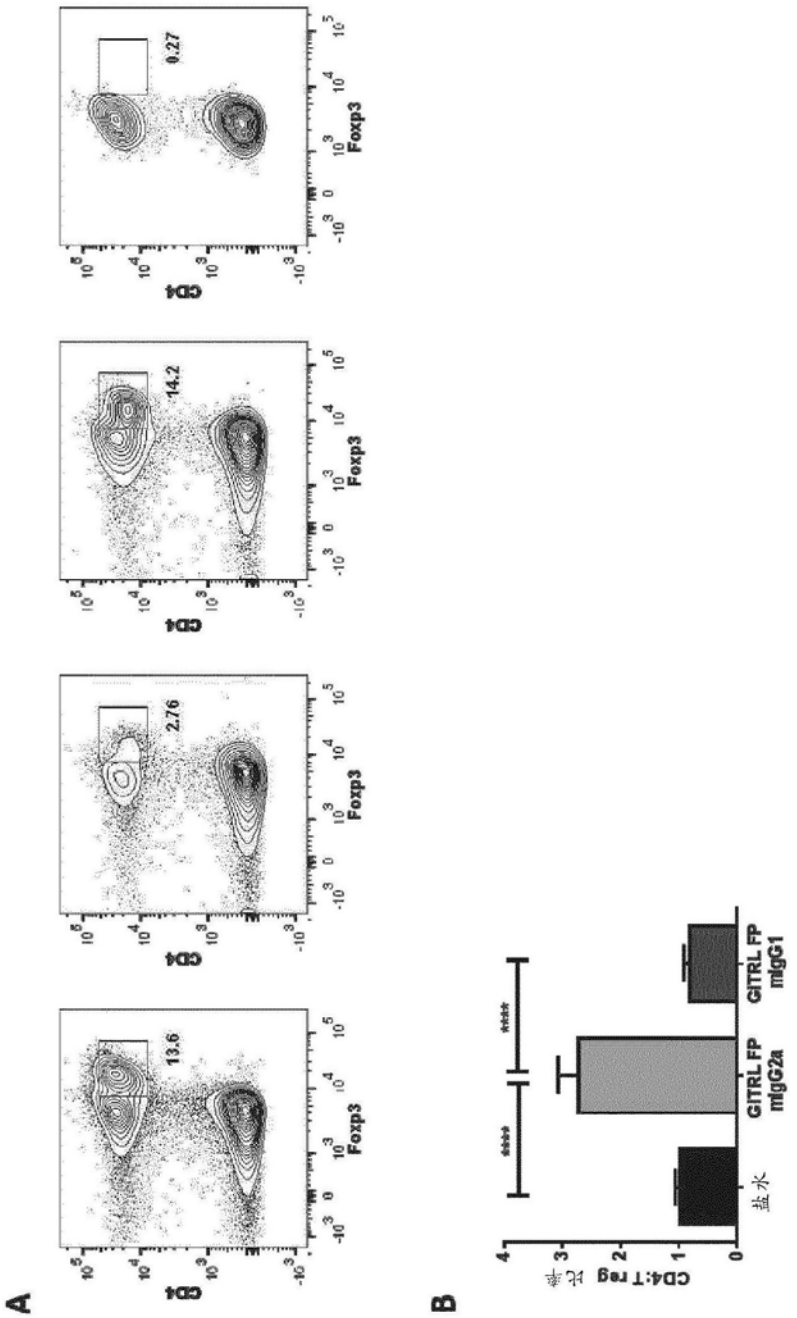

A
B
13.6
2.76
14.2
0.27
CD3
Foxp3
CD4:Treg 比率
盐水
GITRL FP mIgG2a
GITRL FP mIgG1
****

图32

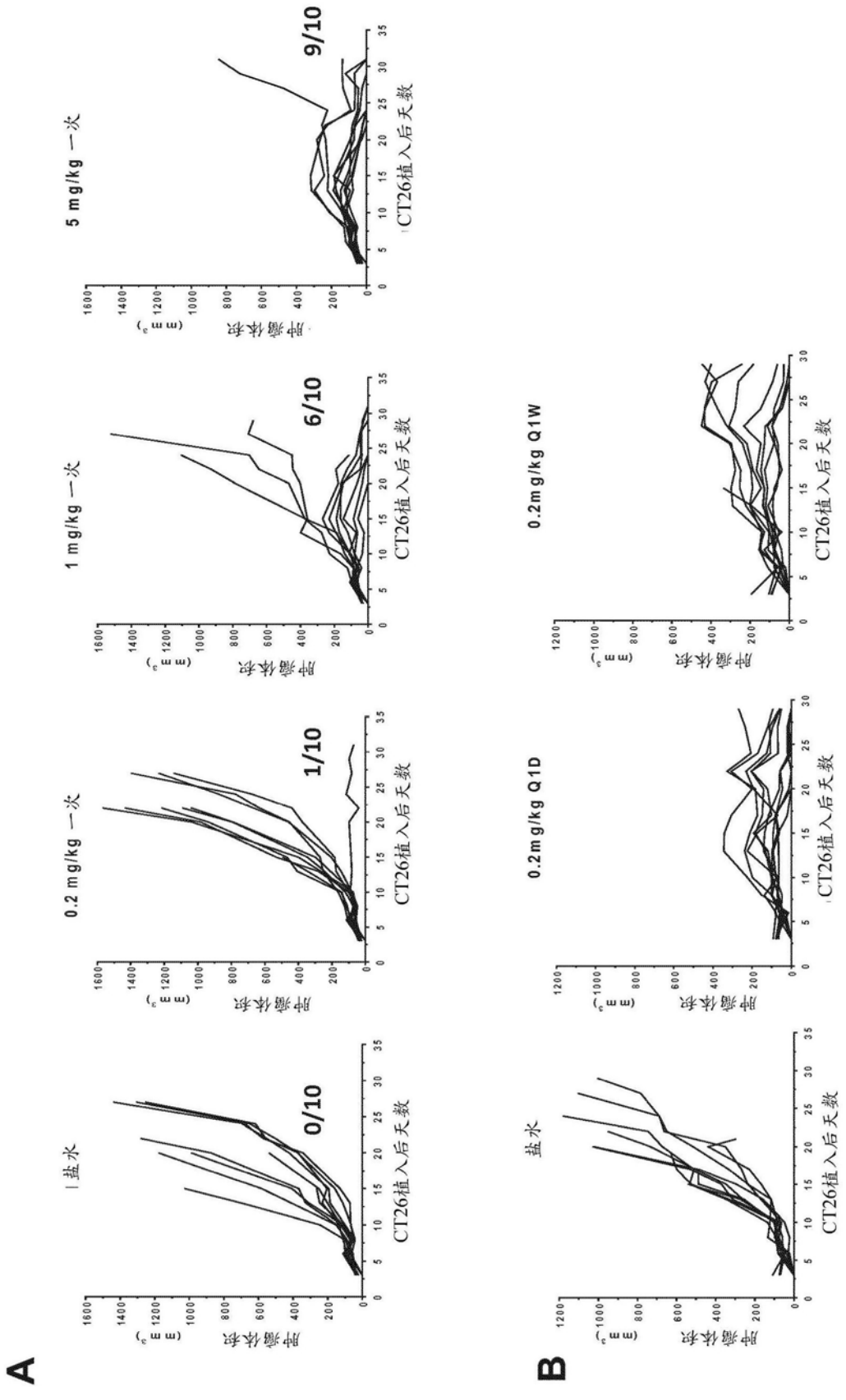
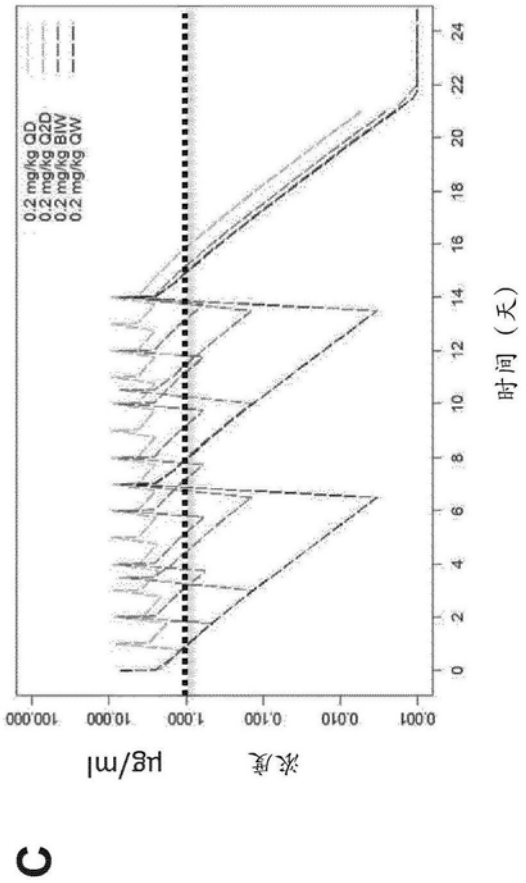

A
盐水
0/10
0.2 mg/kg 一次
1/10
1 mg/kg 一次
6/10
5 mg/kg 一次
9/10
B
盐水
0.2mg/kg Q1D
0.2mg/kg Q1W
肿瘤体积
CT26植入后天数

图33


0.2 mg/kg QD
0.2 mg/kg Q2D
0.2 mg/kg BIW
0.2 mg/kg QW
浓度
μg/ml
0.001
0.010
0.100
1.000
10.000
100.000
0
2
4
6
8
10
12
14
16
18
20
22
24
时间（天）

C

图33续

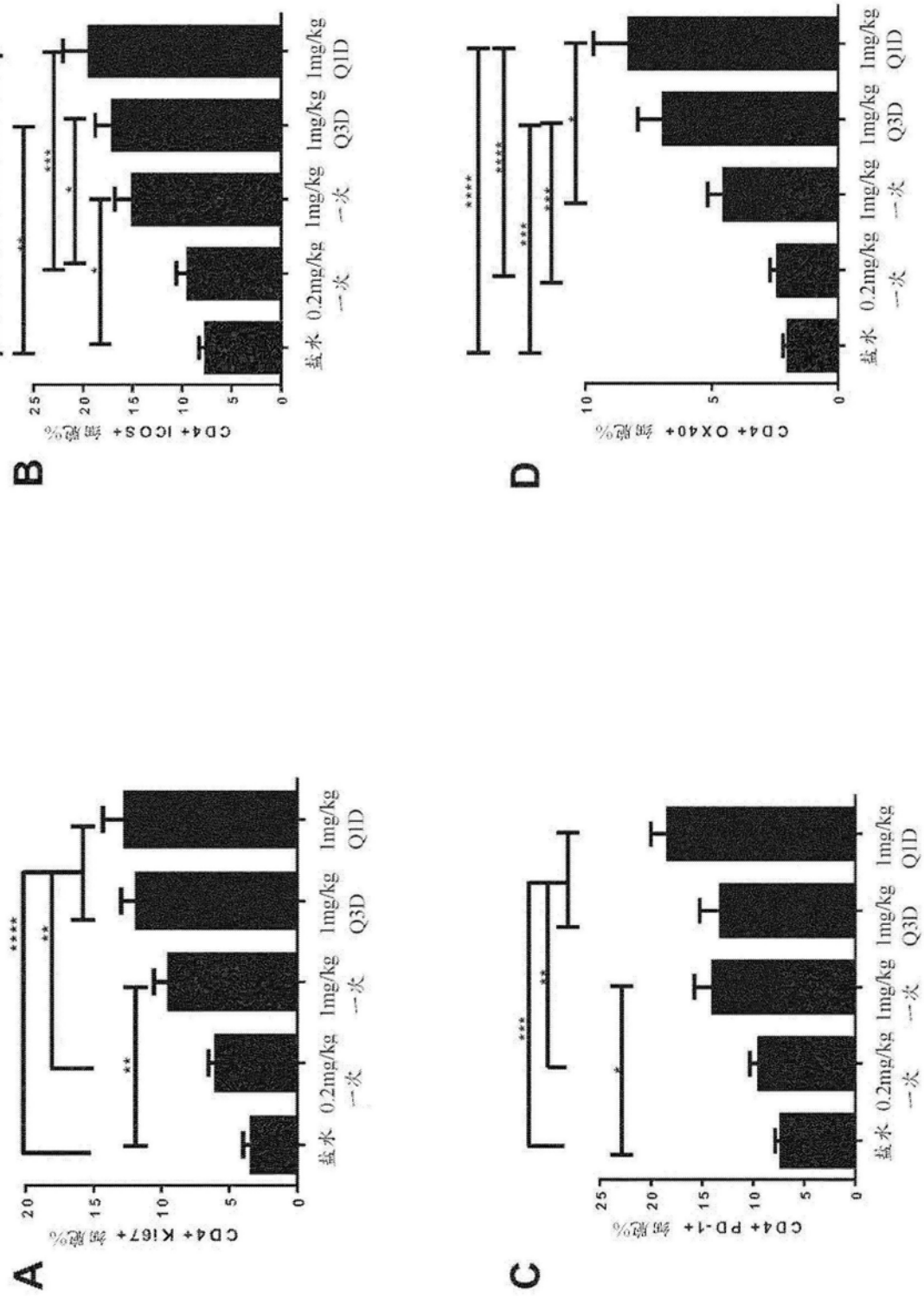

A
CD4+ Ki67+ 细胞%
盐水
0.2mg/kg 一次
1mg/kg 一次
1mg/kg Q3D
1mg/kg Q1D
B
CD4+ ICOS+ 细胞%
C
CD4+ PD-1+ 细胞%
D
CD4+ OX40+ 细胞%

图34

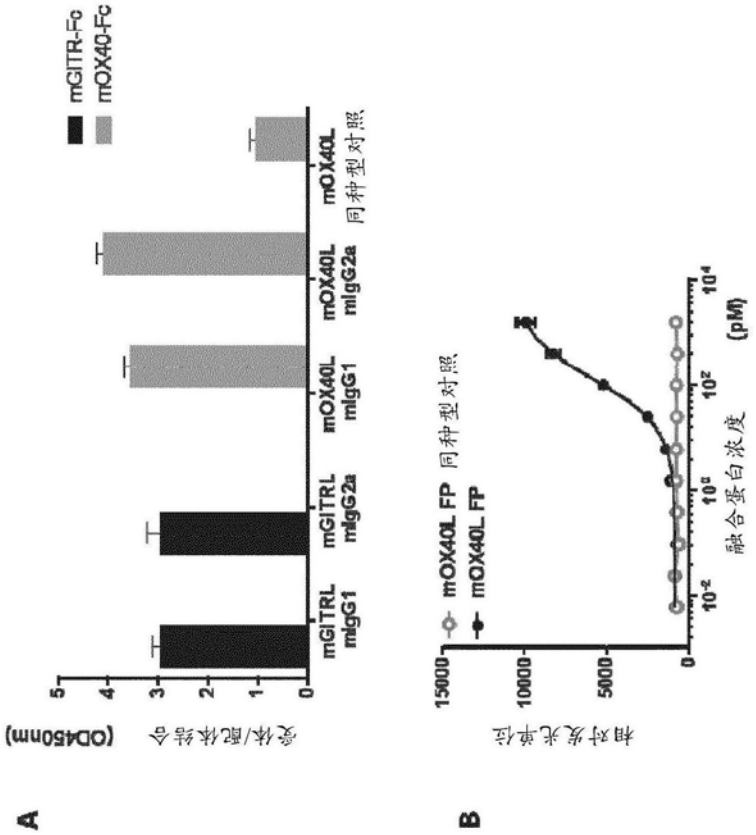

A
mGITR-Fc
mOX40-Fc
mGITRL mIgG1
mGITRL mIgG2a
mOX40L mIgG1
mOX40L mIgG2a
mOX40L 同种型对照
抗体/配体结合 (OD450nm)
0
1
2
3
4
5
B
mOX40L FP 同种型对照
mOX40L FP
相对发光单位
融合蛋白浓度 (pM)
0
5000
10000
15000
10⁻²
10⁻¹
10⁰
10¹
10²
10³
10⁴

图35

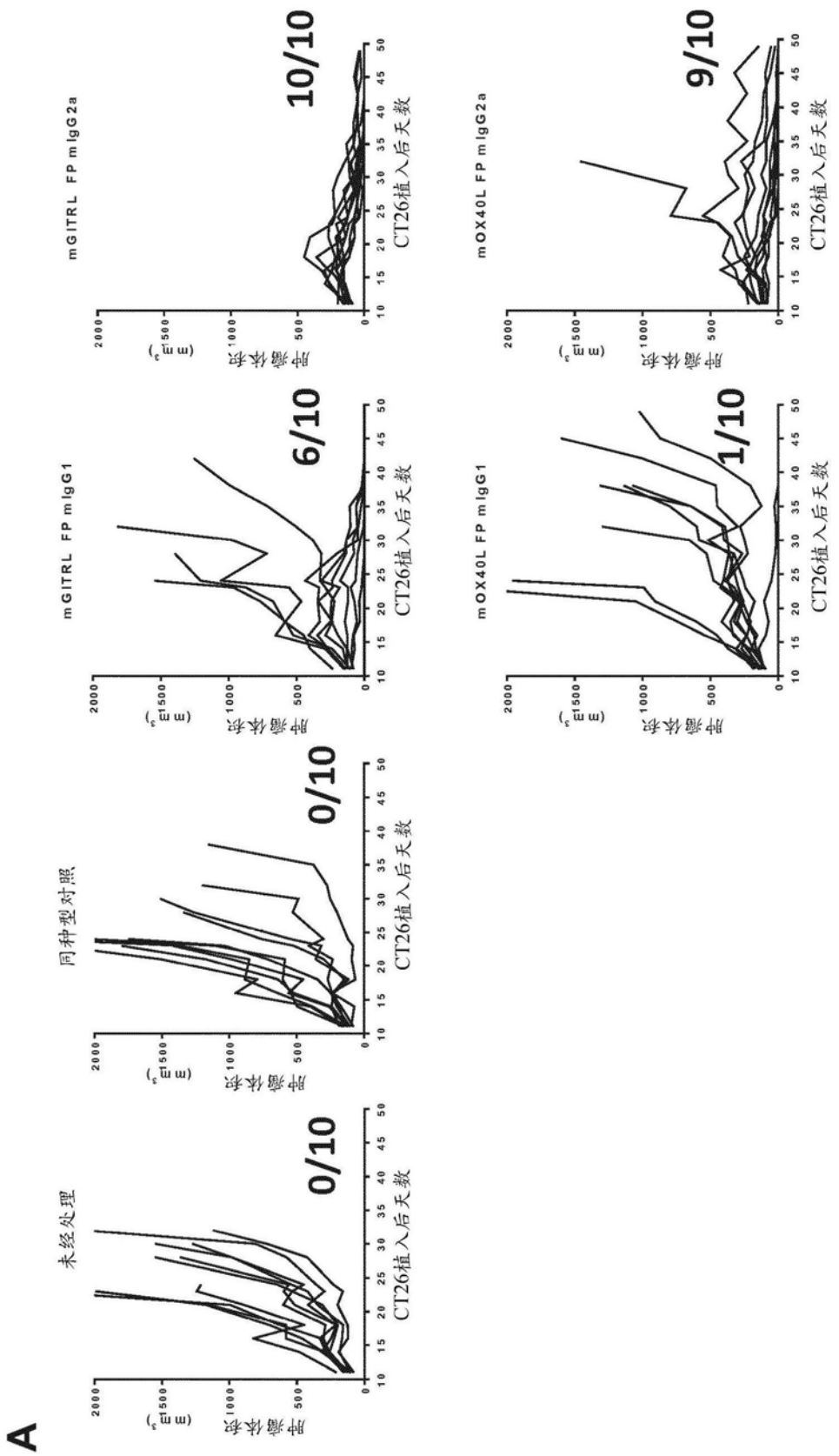

A
未经处理
同种型对照
mGITRL FP mIgG1
mGITRL FP mIgG2a
mOX40L FP mIgG1
mOX40L FP mIgG2a
0/10
0/10
6/10
10/10
1/10
9/10
平均肿瘤体积
CT26植入后天数

图36

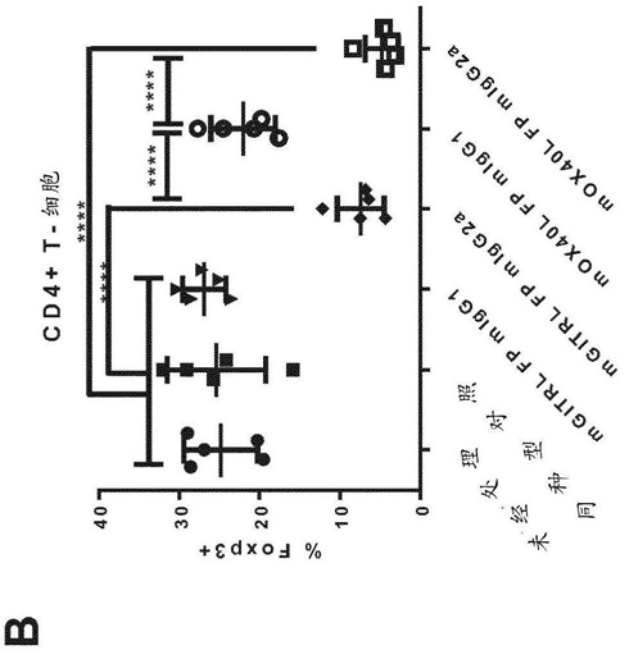

B
CD4+ T-细胞
% Foxp3+
40
30
20
10
0
****
****
****
****
mGITRL FP mIgG1
mGITRL FP mIgG2a
mOX40L FP mIgG1
mOX40L FP mIgG2a
照
对
处
理
型
种
经
同
未

图36续

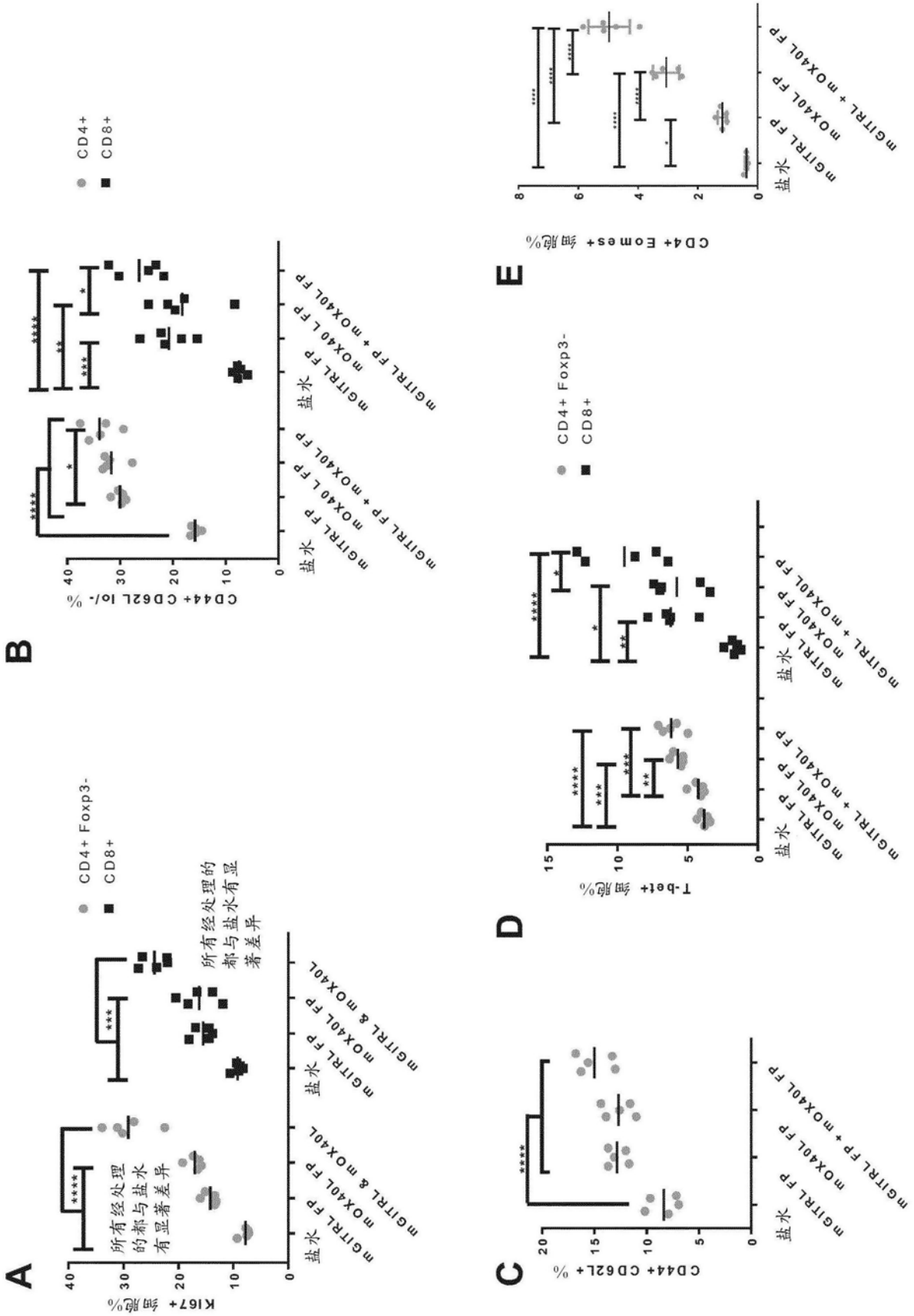

A
B
C
D
E
CD4+ Foxp3-
CD8+
CD4+
CD8+
Ki67+ 细胞%
CD44+ CD62L lo/- %
CD44+ CD62L+ %
T-bet+ 细胞%
CD4+ Eomes+ 细胞%
所有经处理的都与盐水有显著差异
所有经处理的都与盐水有显著差异
盐水
mGITRL FP
mOX40L FP
mGITRL FP + mOX40L FP
mGITRL & mOX40L

图37

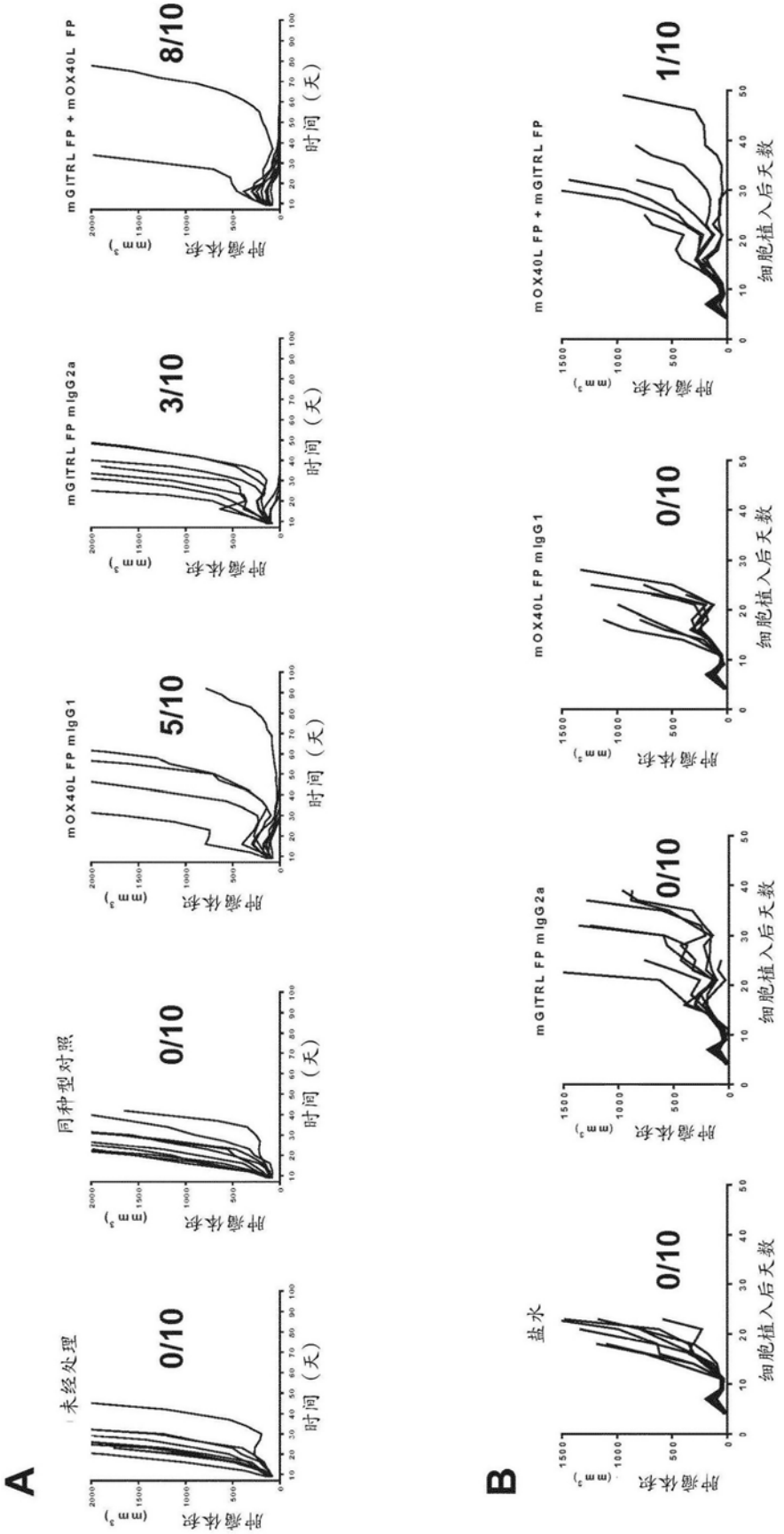

A
未经处理
同种型对照
mOX40L FP mIgG1
mGITRL FP mIgG2a
mGITRL FP + mOX40L FP
0/10
0/10
5/10
3/10
8/10
肿瘤体积
时间（天）
B
盐水
mGITRL FP mIgG2a
mOX40L FP mIgG1
mOX40L FP + mGITRL FP
0/10
0/10
0/10
1/10
细胞植入后天数

图38

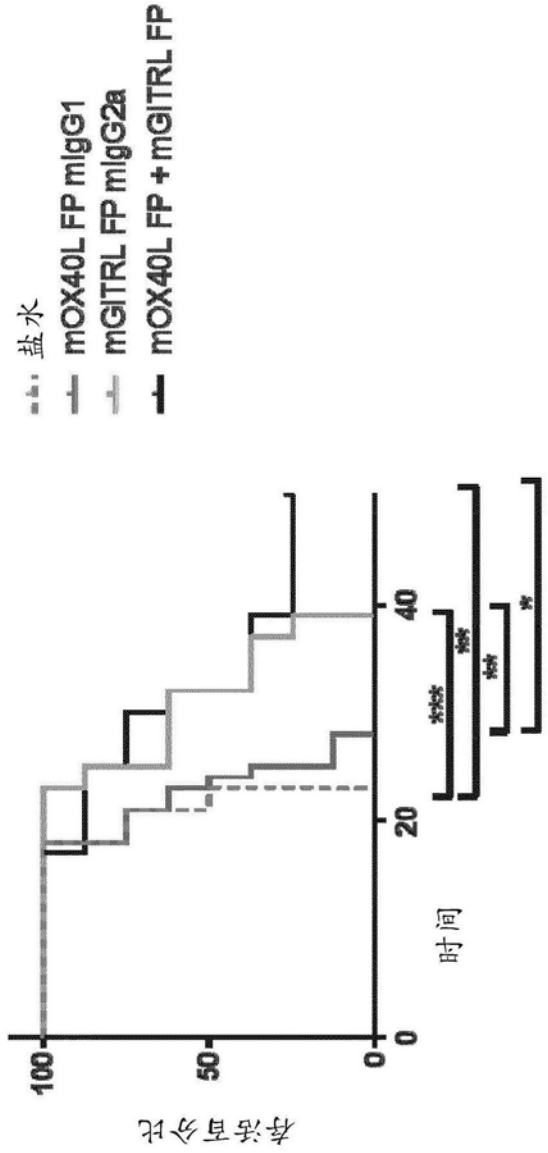

盐水
mOX40L FP mIgG1
mGITRL FP mIgG2a
mOX40L FP + mGITRL FP
存活百分比
100
50
0
0
20
40
时间
***
***
**
*

图39

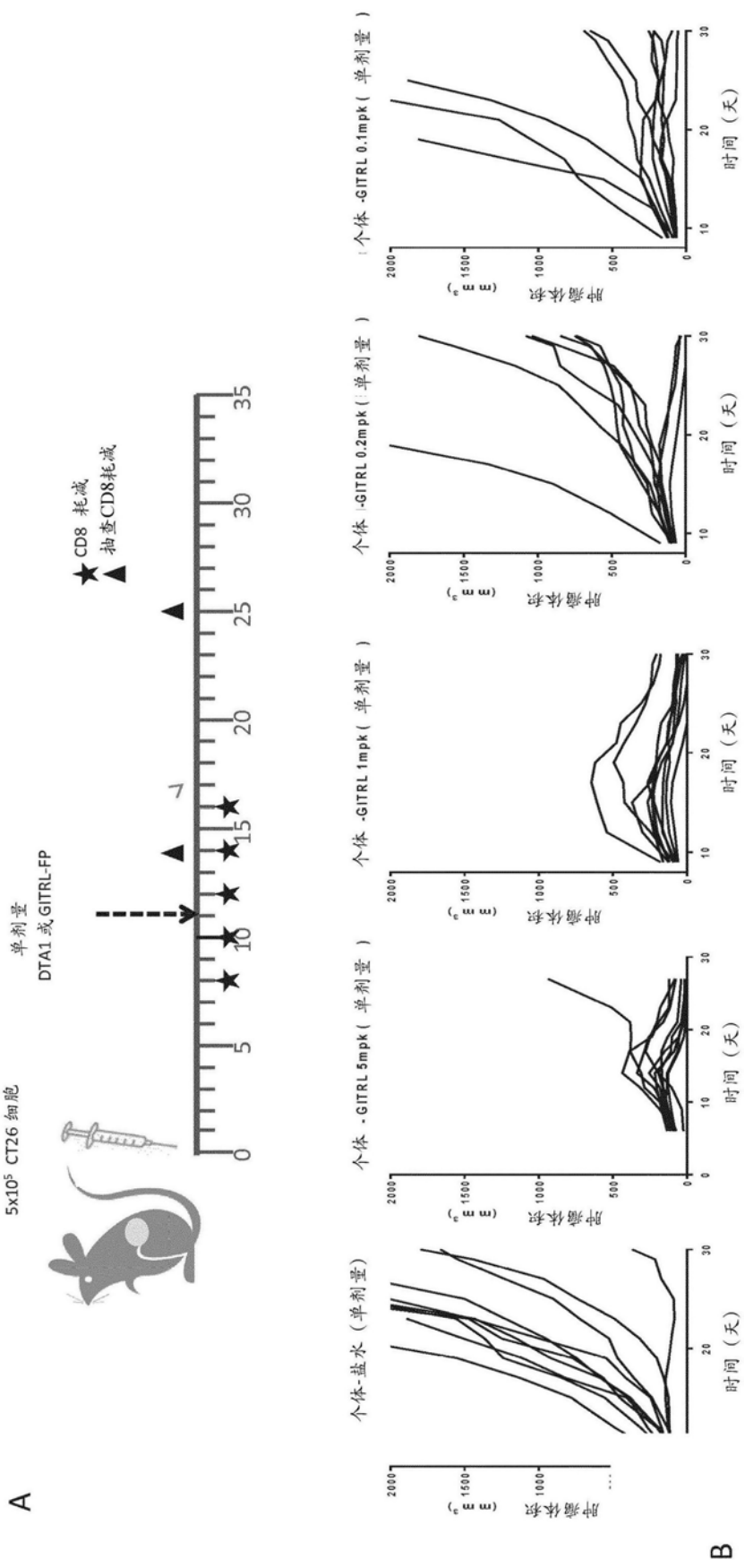

A
5x10^5 CT26 细胞
单剂量
DTA1 或 GITRL-FP
CD8 耗减
抽查CD8耗减
0
5
10
7
15
20
25
30
35
B
个体-盐水（单剂量）
个体 - GITRL 5mpk（单剂量）
个体 -GITRL 1mpk（单剂量）
个体 -GITRL 0.2mpk（单剂量）
个体 -GITRL 0.1mpk（单剂量）
肿瘤体积
时间（天）

图40

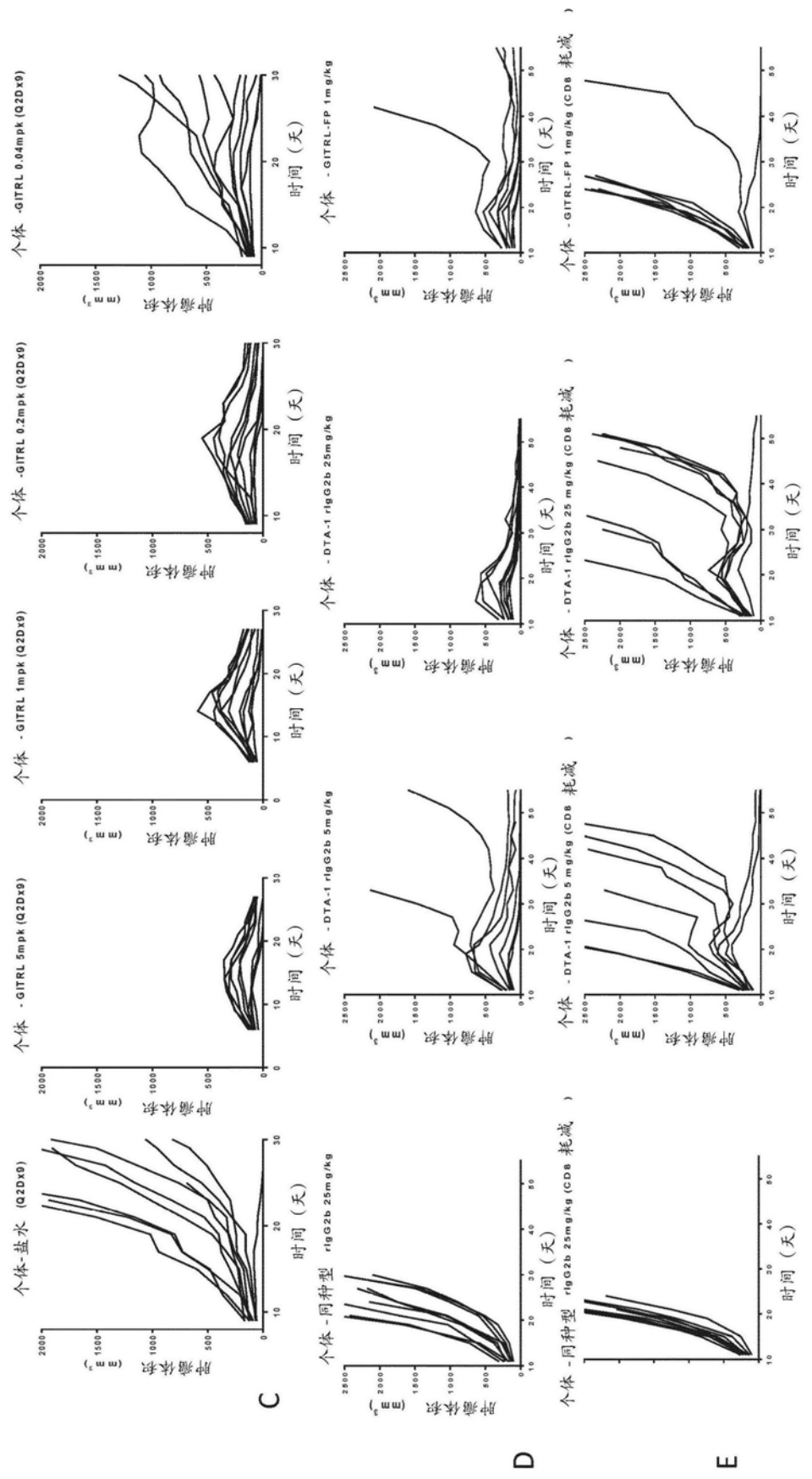
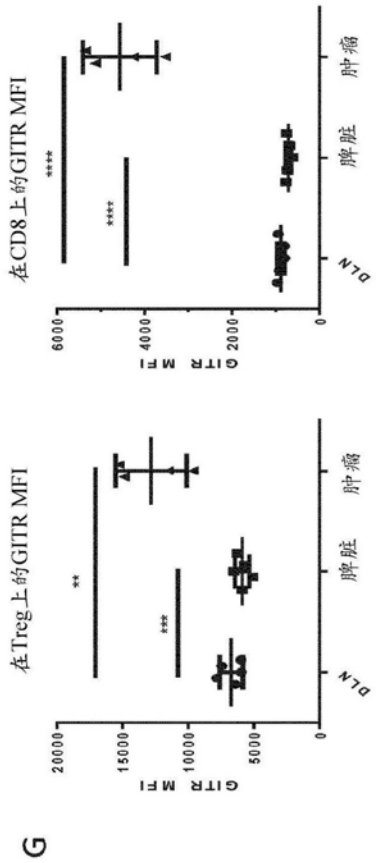

C
D
E
个体-盐水 (Q2Dx9)
个体 - GITRL 5mpk (Q2Dx9)
个体 - GITRL 1mpk (Q2Dx9)
个体 -GITRL 0.2mpk (Q2Dx9)
个体 -GITRL 0.04mpk (Q2Dx9)
个体 - 同种型 rIgG2b 25mg/kg (CD8 耗减)
个体 - DTA-1 rIgG2b 5 mg/kg (CD8 耗减)
个体 - DTA-1 rIgG2b 25mg/kg
个体 - DTA-1 rIgG2b 25 mg/kg (CD8 耗减)
个体 - GITRL-FP 1mg/kg
个体 - GITRL-FP 1mg/kg (CD8 耗减)
肿瘤体积 (mm³)
时间（天）

图40续



G

F

CD8耗减组	剂量 (mg/kg)	存活期中值（天）
同种型	25	24
DTA-1	5	42
DTA-1	25	48
GITRL-FP	1	27

图40续

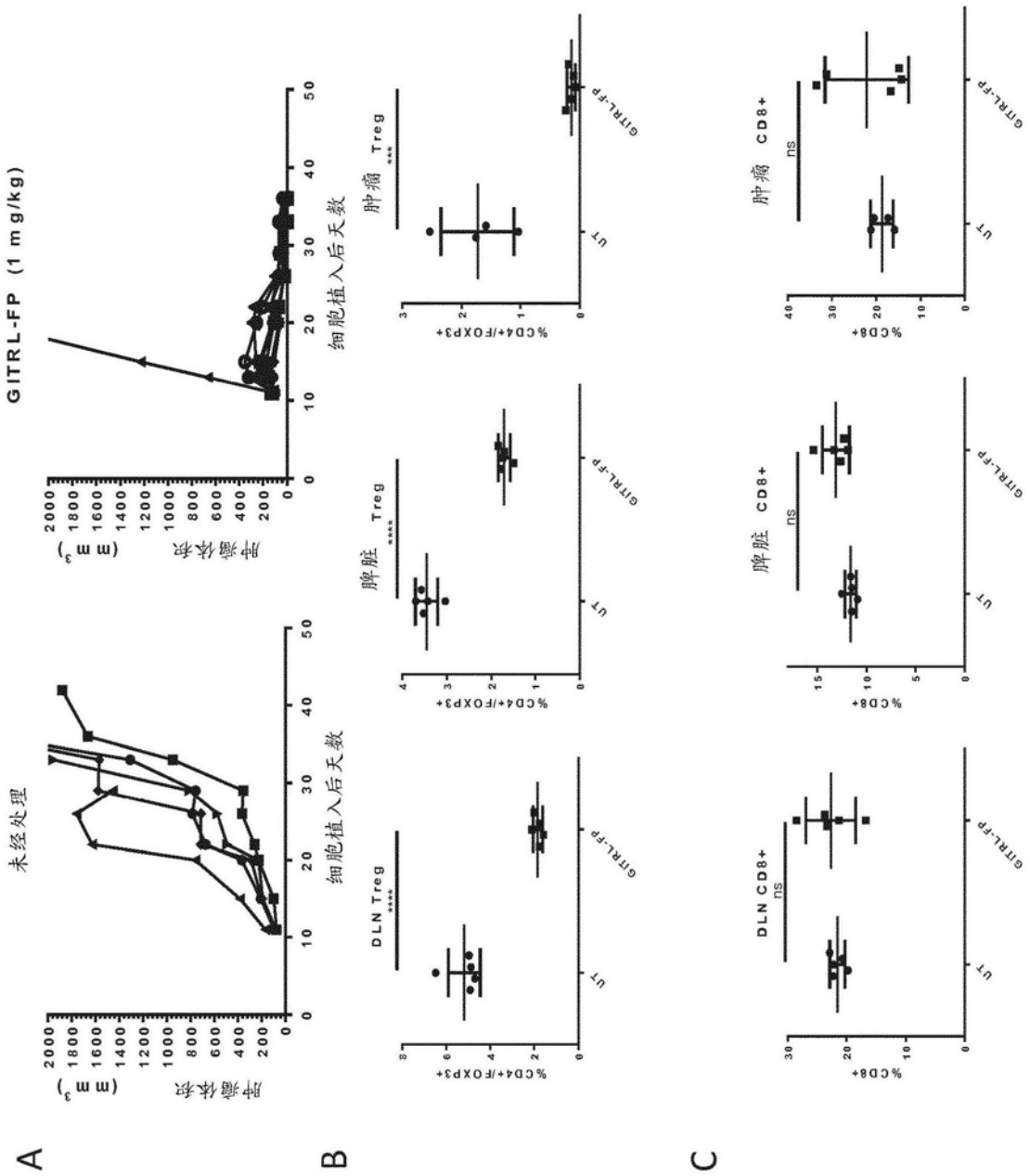

A
未经处理
GITRL-FP (1 mg/kg)
肿瘤体积 (mm³)
细胞植入后天数
B
DLN Treg
脾脏 Treg
肿瘤 Treg
%CD4+/FOXP3+
UT
GITRL-FP
C
DLN CD8+
脾脏 CD8+
肿瘤 CD8+
%CD8+
ns

图41

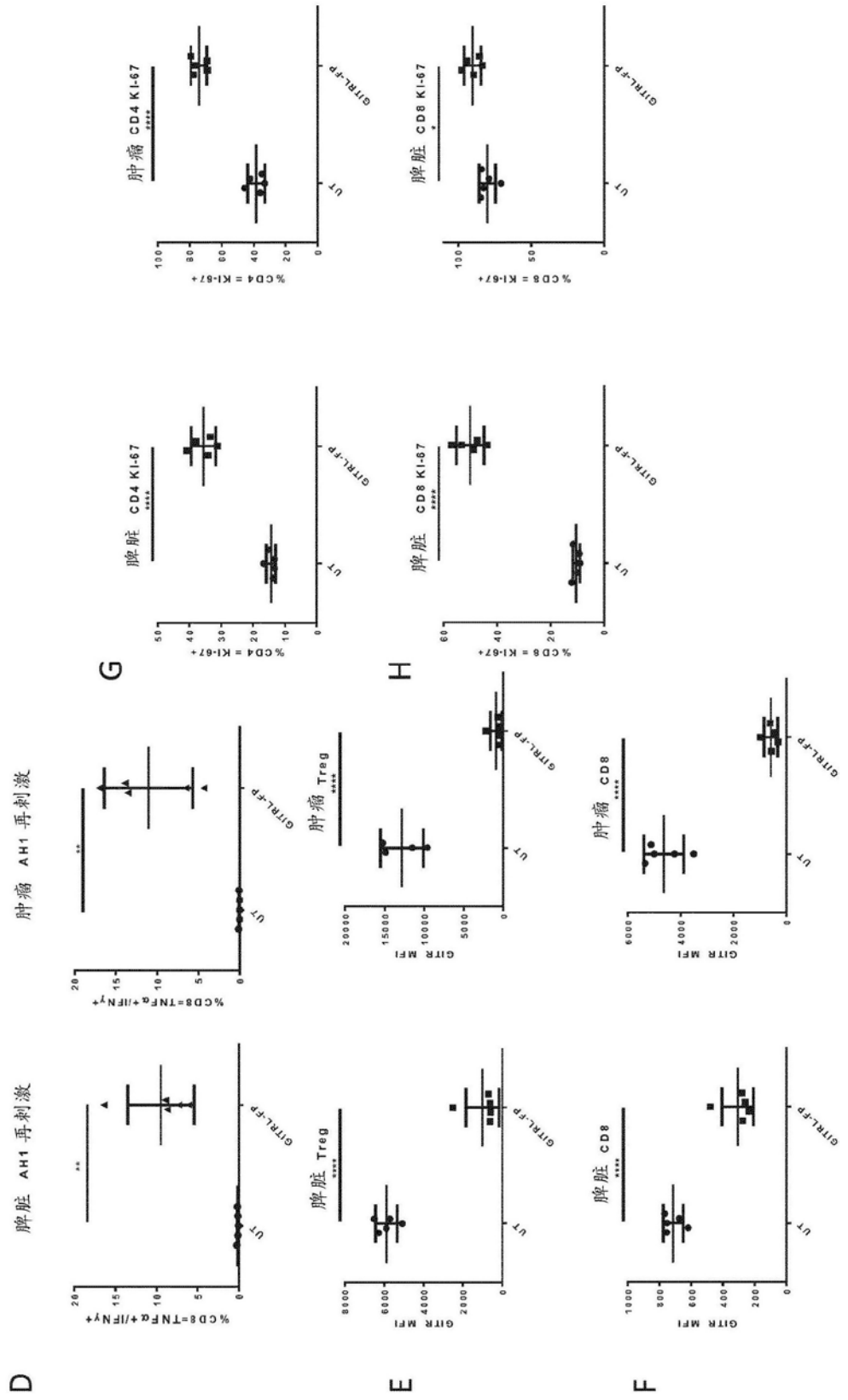

D
脾脏 AH1 再刺激
%CD8=TNFα+/IFNγ+
GITRL-FP
UT
肿瘤 AH1 再刺激
%CD8=TNFα+/IFNγ+
GITRL-FP
UT
E
脾脏 Treg
GITR MFI
GITRL-FP
UT
肿瘤 Treg
GITR MFI
GITRL-FP
UT
F
脾脏 CD8
GITR MFI
GITRL-FP
UT
肿瘤 CD8
GITR MFI
GITRL-FP
UT
G
脾脏 CD4 KI-67
%CD4 = KI-67+
GITRL-FP
UT
肿瘤 CD4 KI-67
%CD4 = KI-67+
GITRL-FP
UT
H
脾脏 CD8 KI-67
%CD8 = KI-67+
GITRL-FP
UT
肿瘤 CD8 KI-67
%CD8 = KI-67+
GITRL-FP
UT

图41续

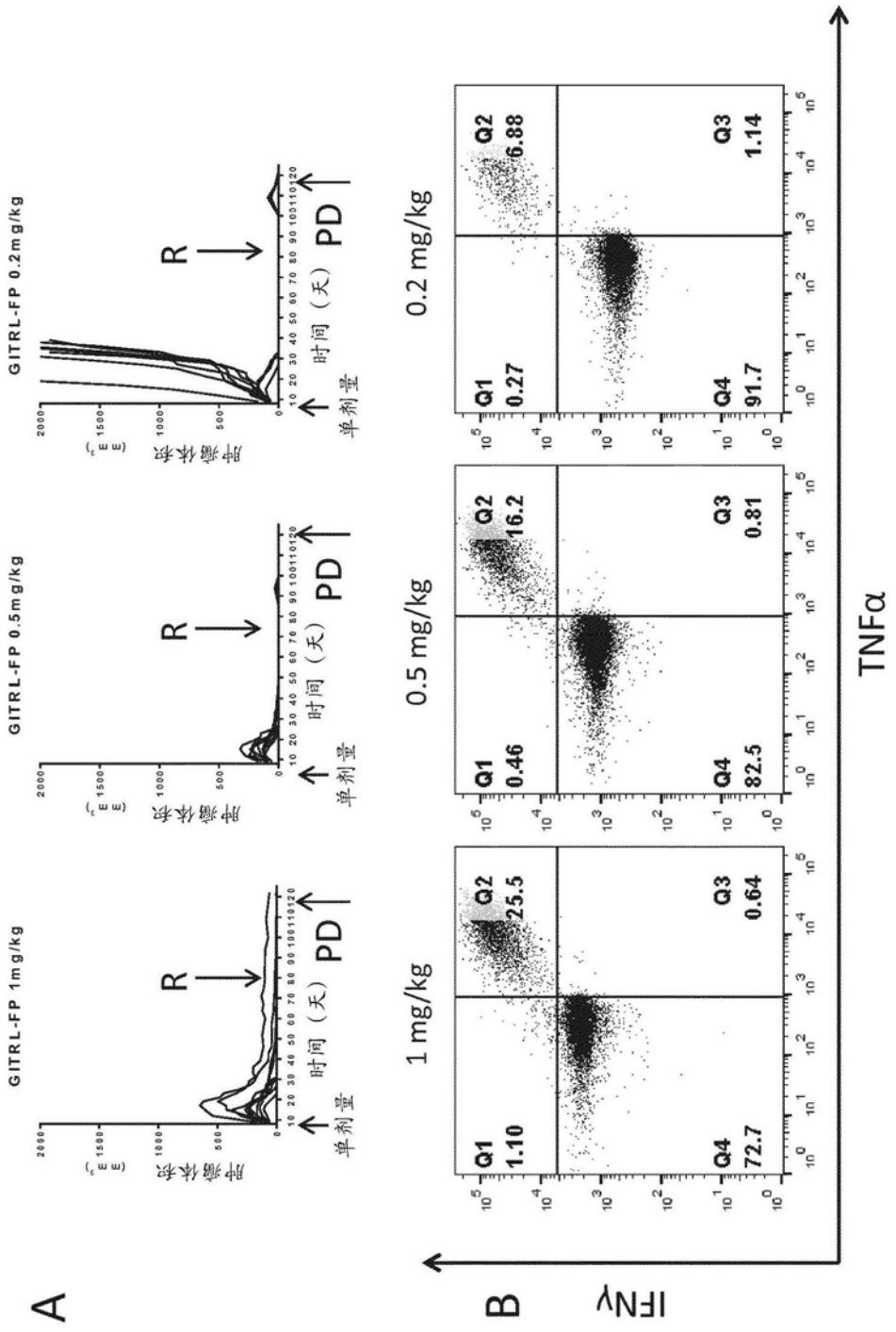

A
GITRL-FP 1mg/kg
GITRL-FP 0.5mg/kg
GITRL-FP 0.2mg/kg
肿瘤体积
时间（天）
单剂量
R
PD
B
1 mg/kg
0.5 mg/kg
0.2 mg/kg
IFNγ
TNFα
Q1 1.10
Q2 25.5
Q4 72.7
Q3 0.64
Q1 0.46
Q2 16.2
Q4 82.5
Q3 0.81
Q1 0.27
Q2 6.88
Q4 91.7
Q3 1.14

图42

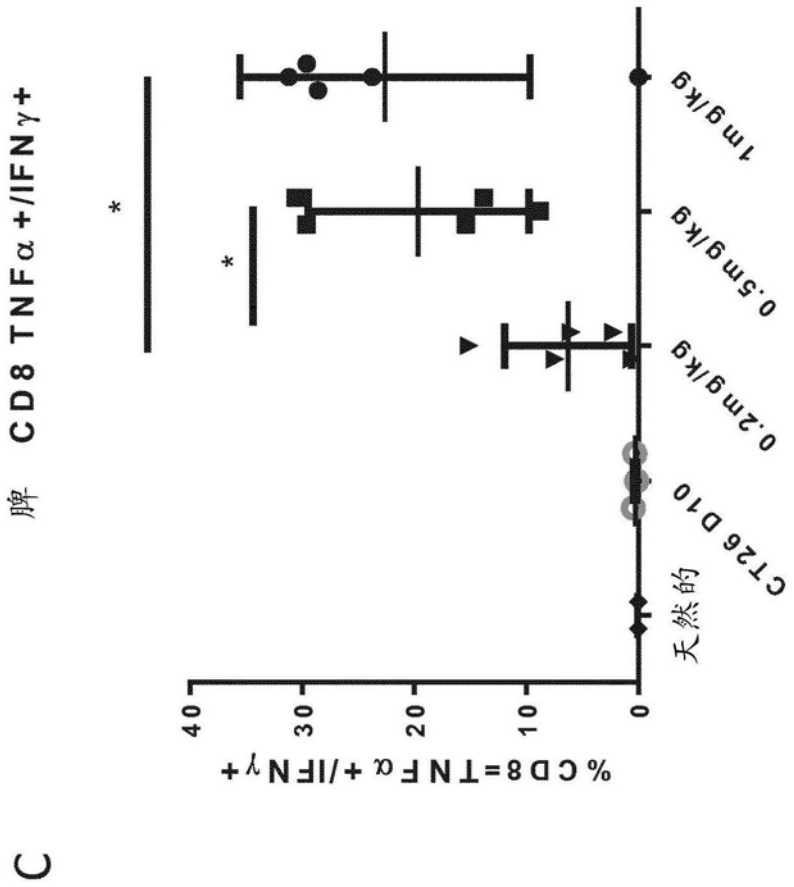

C
脾 CD8 TNFα+/IFNγ+
% CD8=TNFα+/IFNγ+
40
30
20
10
0
*
*
1mg/kg
0.5mg/kg
0.2mg/kg
CT26 D10
天然的

图42续

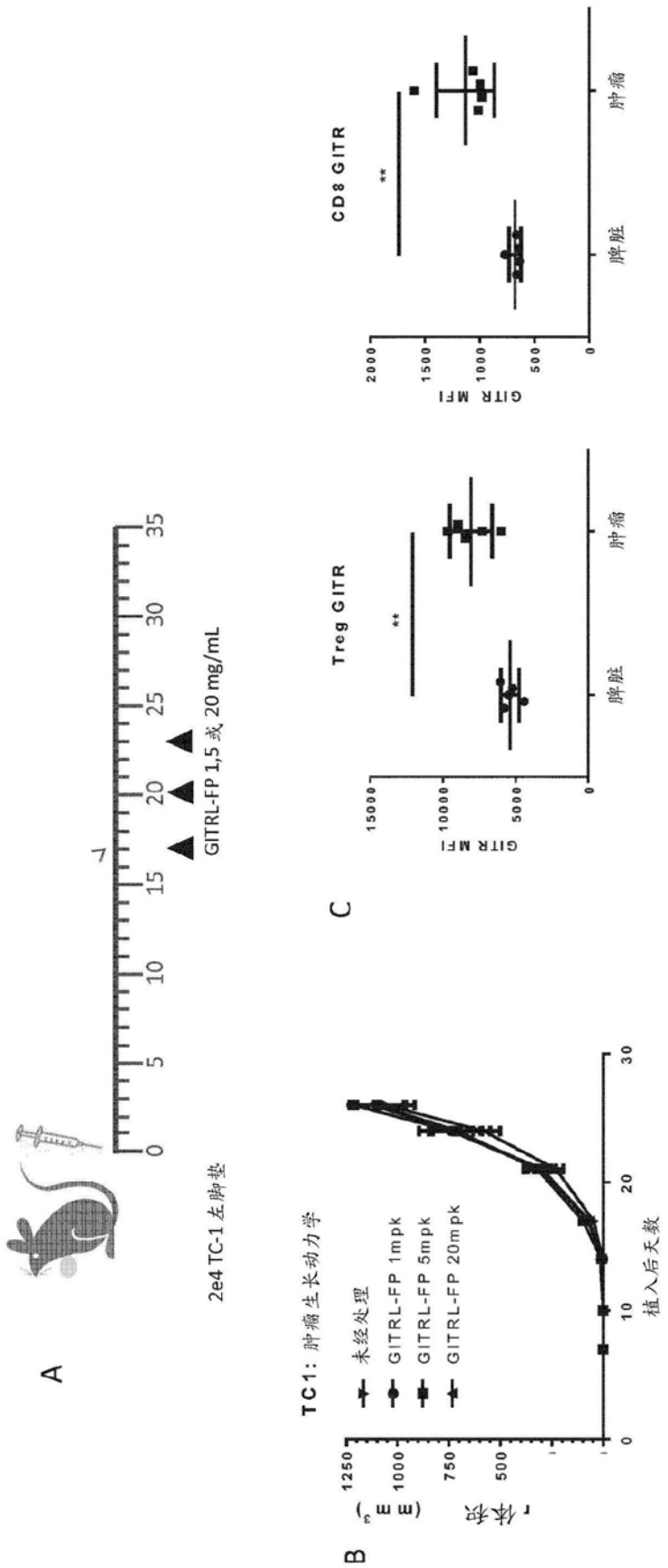

A
0
5
10
15
20
25
30
35
2e4 TC-1 左脚垫
GITRL-FP 1,5 或 20 mg/mL
B
TC1：肿瘤生长动力学
未经处理
GITRL-FP 1mpk
GITRL-FP 5mpk
GITRL-FP 20mpk
肿瘤体积 (mm³)
植入后天数
C
Treg GITR
GITR MFI
脾脏
肿瘤
CD8 GITR
GITR MFI
脾脏
肿瘤

图43

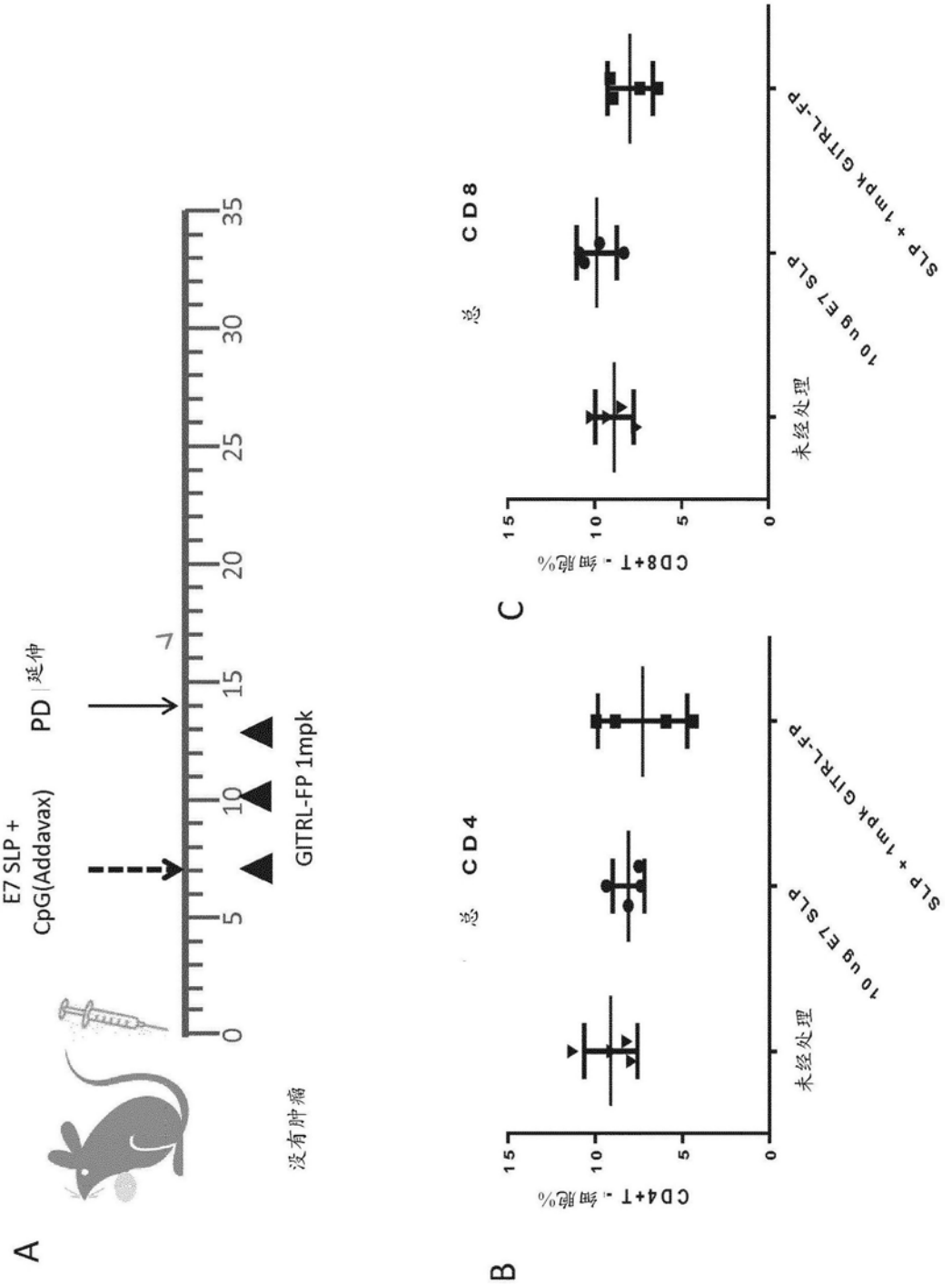

A
E7 SLP +
CpG(Addavax)
PD
延伸
0
5
7
10
15
20
25
30
35
GITRL-FP 1mpk
没有肿瘤
B
总 CD4
CD4+T - 细胞%
15
10
5
0
未经处理
10 u9 E7 SLP
SLP + 1mpk GITRL-FP
C
总 CD8
CD8+T - 细胞%
15
10
5
0
未经处理
10 u9 E7 SLP
SLP + 1mpk GITRL-FP

图44

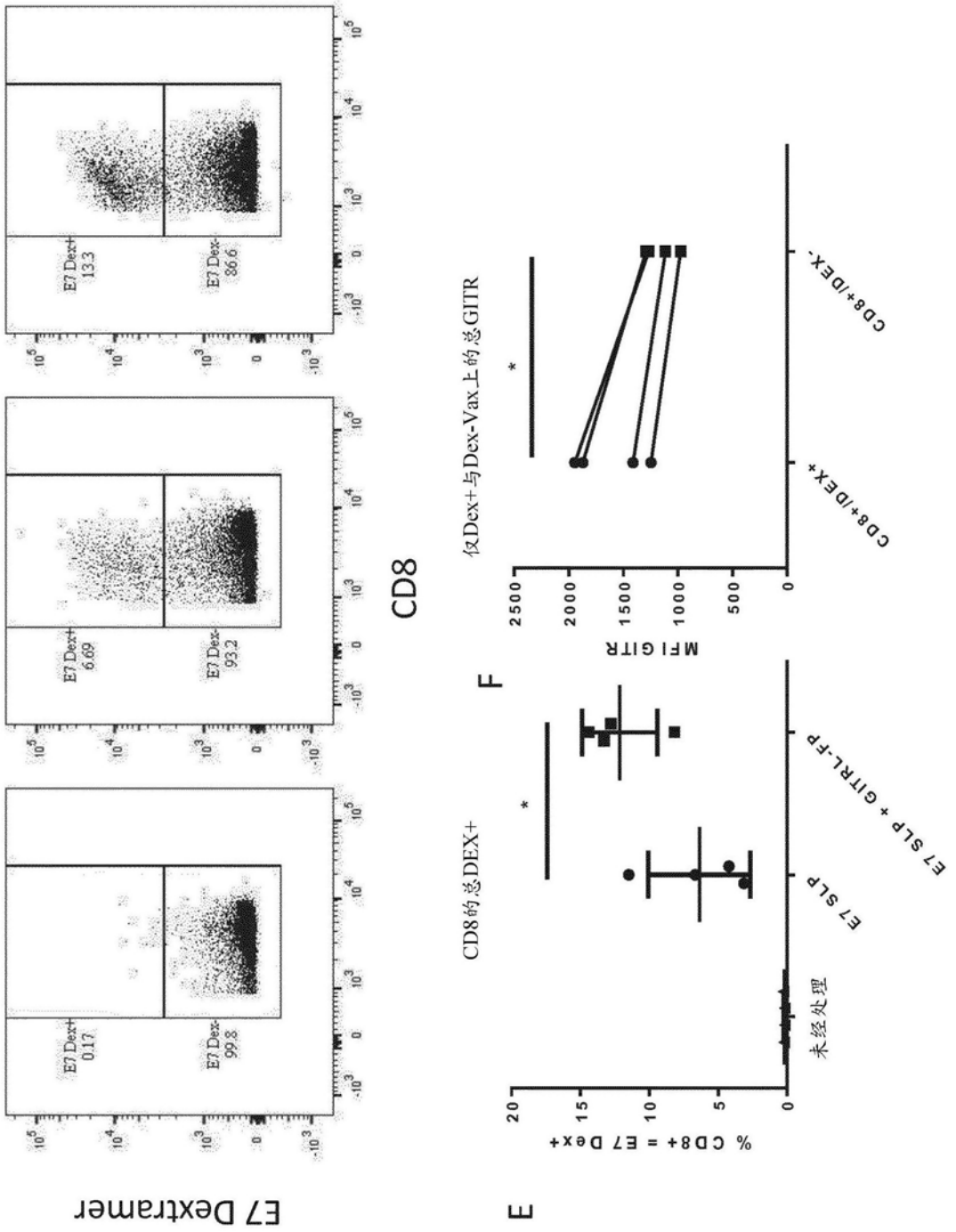

D
E7 Dextramer
CD8
E7 Dex+ 0.17
E7 Dex- 99.8
E7 Dex+ 6.69
E7 Dex- 93.2
E7 Dex+ 13.3
E7 Dex- 86.6
E
CD8的总DEX+
% CD8+ = E7 Dex+
未经处理
E7 SLP
E7 SLP + GITRL-FP
F
仅Dex+与Dex-Vax上的总GITR
MFI GITR
CD8+/DEX+
CD8+/DEX-

图44续

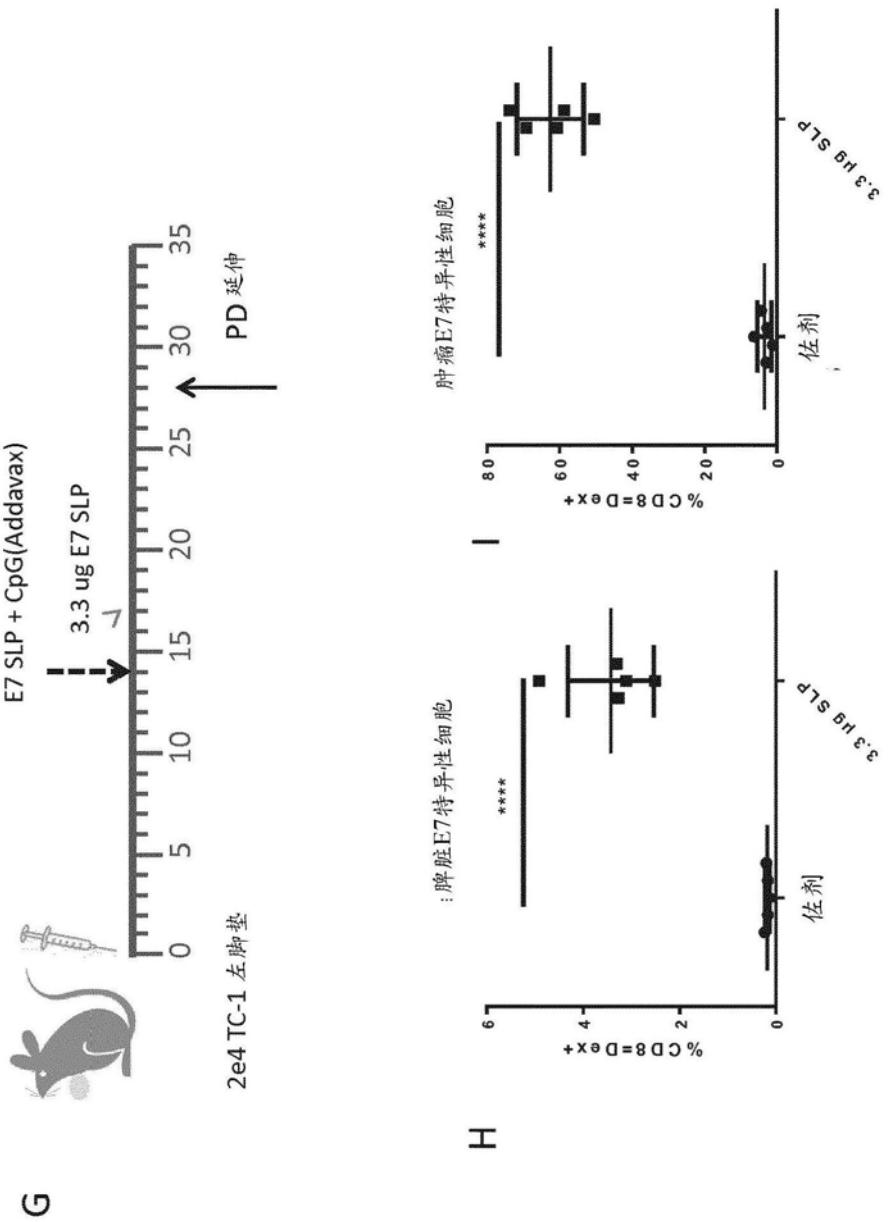

G
E7 SLP + CpG(Addavax)
3.3 ug E7 SLP
7
0
5
10
15
20
25
30
35
PD 延伸
2e4 TC-1 左脚垫
H
脾脏E7特异性细胞
****
% CD8+Dex+
6
4
2
0
佐剂
3.3 μg SLP
I
肿瘤E7特异性细胞
****
% CD8+Dex+
80
60
40
20
0
佐剂
3.3 μg SLP

图44续

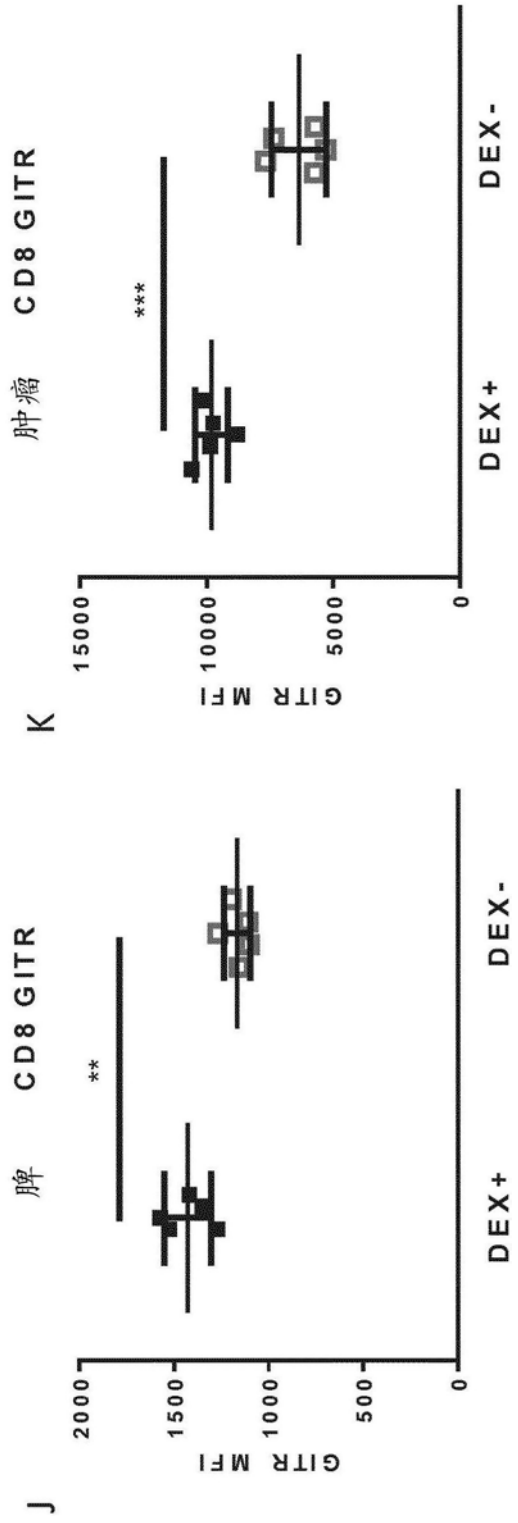

K
肿瘤 CD8 GITR
GITR MFI
15000
10000
5000
0
***
DEX+
DEX-
J
脾 CD8 GITR
GITR MFI
2000
1500
1000
500
0
**
DEX+
DEX-

图44续

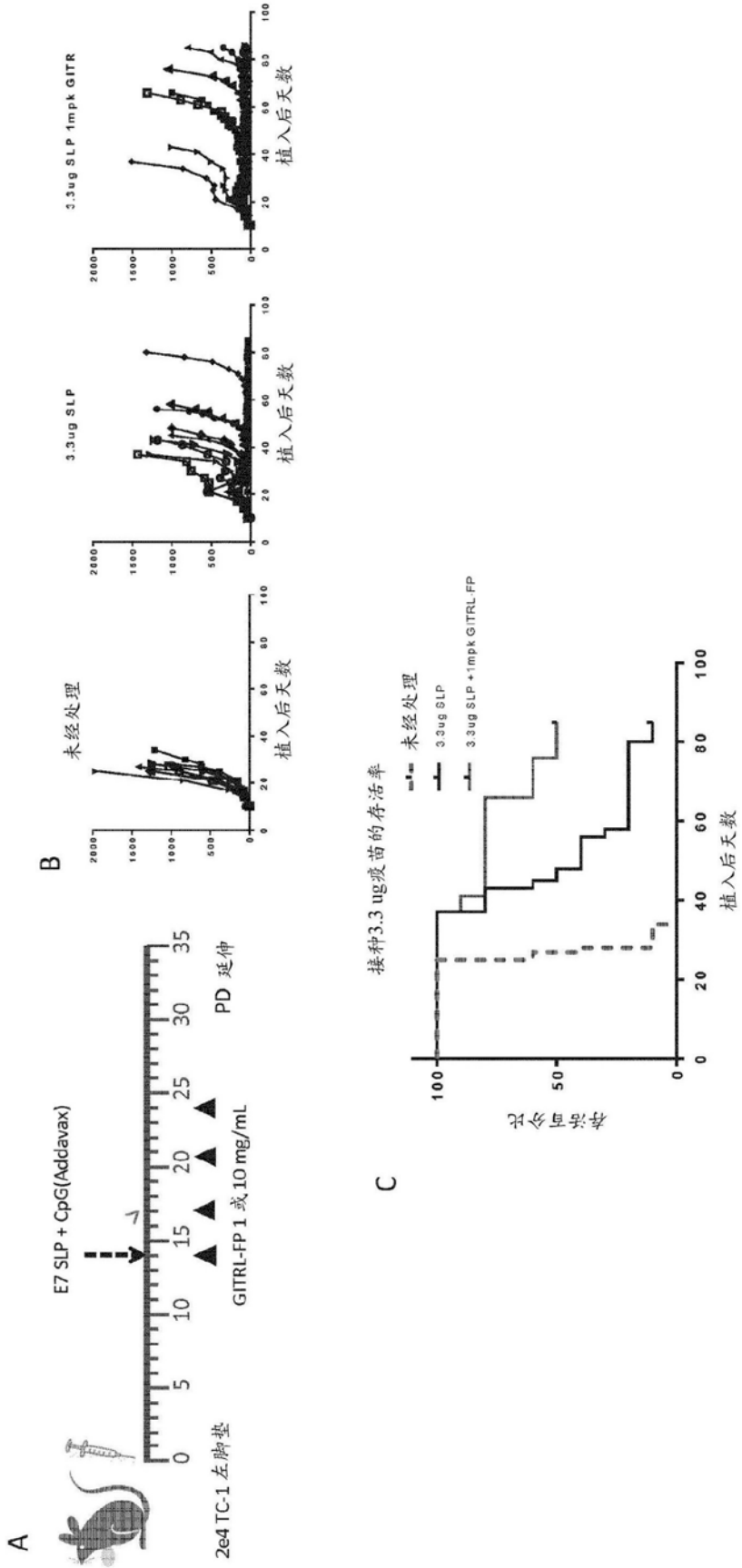
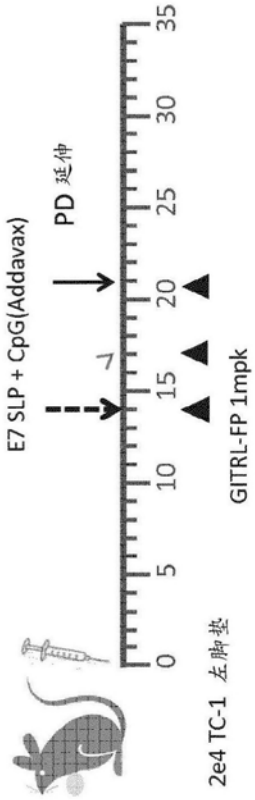

A
2e4 TC-1 左胁垫
E7 SLP + CpG(Addavax)
GITRL-FP 1 或10 mg/mL
PD 延伸
0
5
10
15
20
25
30
35
B
未经处理
3.3ug SLP
3.3ug SLP 1mpk GITR
植入后天数
C
接种3.3 ug疫苗的存活率
未经处理
3.3ug SLP
3.3ug SLP +1mpk GITRL-FP
存活百分比
植入后天数

图45

D

组	存活期中值
未经处理	27
仅 E7 SLP	46.5
E7 SLP + GITRL-FP(1)	80.5
E7 SLP + GITRL-FP(10)	75.5



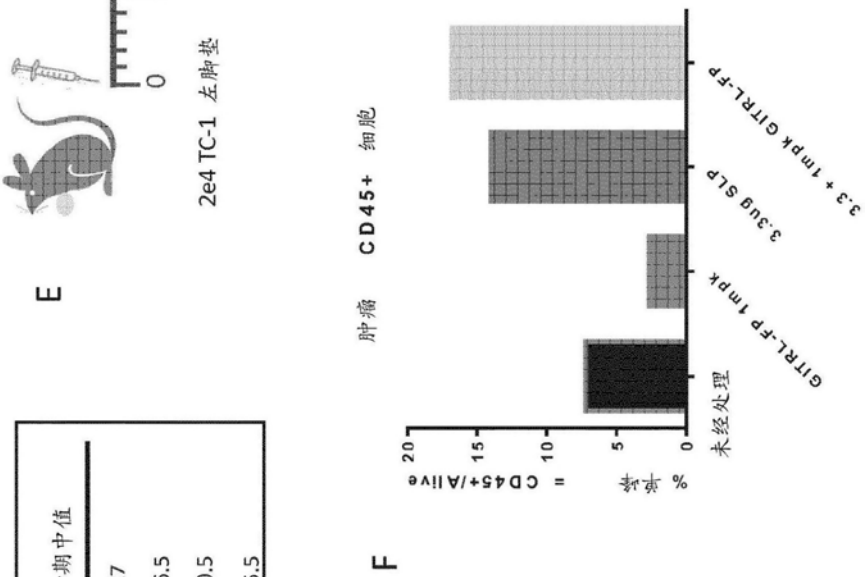


图45续

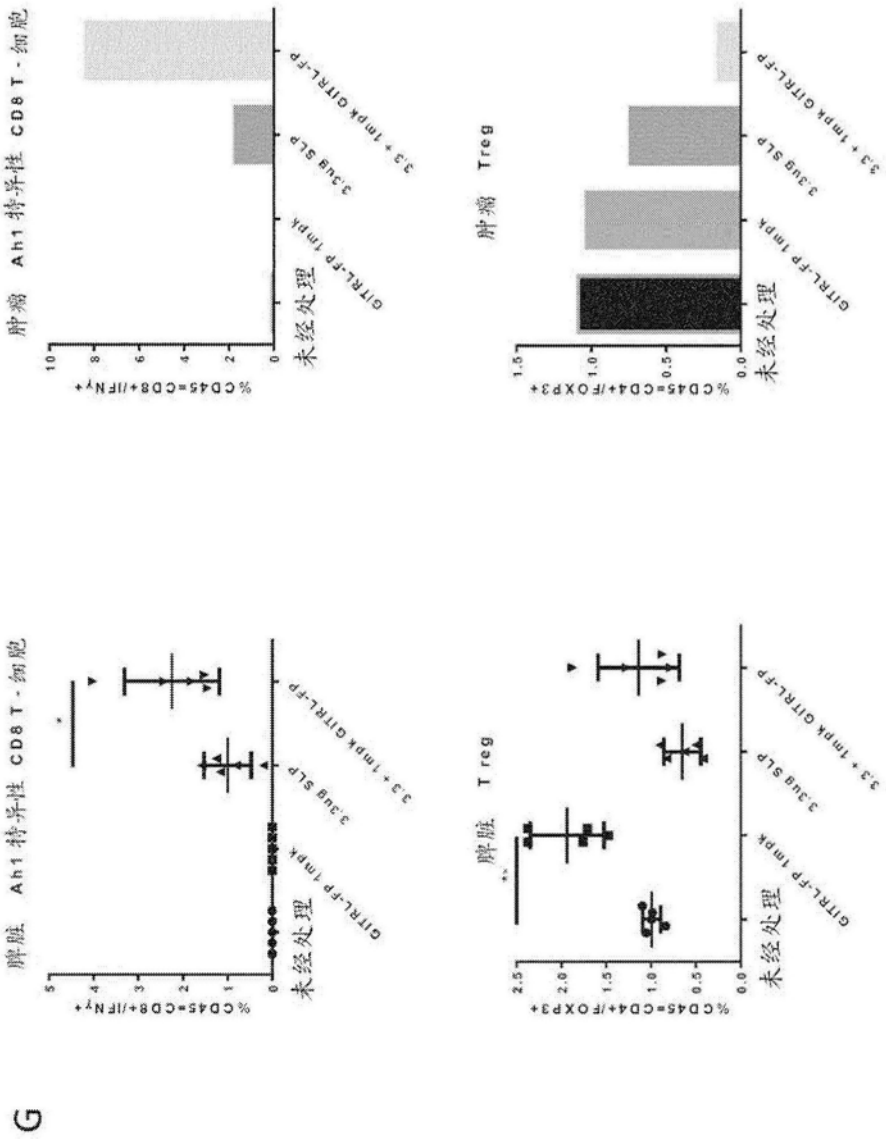

G
脾脏 Ah1 特异性 CD8 T - 细胞
% CD45+CD8+IFNγ+
未经处理
GITRL-FP 1mpk
3.3ug SLP
3.3 + 1mpk GITRL-FP
脾脏 Treg
% CD45+CD4+FOXP3+
肿瘤 Ah1 特异性 CD8 T - 细胞
肿瘤 Treg

图45续