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(54) Title: METHODS AND COMPOSITIONS FOR MODULATION OF PIEZO1 IN THE TREATMENT OF CANCER

(57) Abstract: Provided herein are methods of treating cancer in a subject, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO 1.



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METHODS AND COMPOSITIONS FOR MODULATION OF PIEZO1 IN THE TREATMENT OF CANCER

RELATED APPLICATIONS

This application claims priority to U.S. provisional application 63/406,506, filed
5 September 14, 2022, hereby incorporated by reference in its entirety.

GOVERNMENT SUPPORT

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10 **BACKGROUND**

The coinhibitory receptor Programmed Cell Death-1 (PD-1, CD279) plays a critical role in T cell exhaustion during chronic infection and cancer. PD-1 pathway inhibitors are FDA-approved for treating over 20 cancer types and have shown tremendous success in a subset of patients. However, most cancer patients do not exhibit durable responses and
15 patients with specific cancer types such as glioblastoma experience little to no benefit from PD-1 pathway inhibitors. Consequently, efforts are currently focused on identifying potential combination treatments that can synergize with PD-1 blockade to increase the breadth and durability of response.

Despite its widespread use in the clinic, there is a limited mechanistic understanding
20 of PD-1 signaling. Ligation of PD-1 on CD8⁺ T cells by its ligands Programmed Death Ligand (PD-L)1 and PD-L2 results in the attenuation of multiple cellular processes driven by engagement of the T cell receptor (TCR) and costimulatory receptor CD28. PD-1 plays a pivotal role in regulating the dephosphorylation of TCR-related proteins and in modulating T cell functions such as cytokine production, Ca²⁺ flux, cytolysis, cytoskeletal
25 rearrangements, migration and metabolism. PD-1 exerts its inhibitory functions, at least in part, through the recruitment of SH2-containing tyrosine phosphatases SHP2 (PTPN11) and to a lesser extent, SHP1 (PTPN6), which bind phosphotyrosine residues within the immunoreceptor tyrosine-based switch motif (ITSM) and inhibition motif (ITIM) on the PD-1 cytoplasmic tail. However, the precise mechanism by which PD-1 and SHP2/SHP1
30 counter T cell activation remain unclear. Recent studies have shown that T cell-specific

SHP2 knockout (KO) mice respond to PD-1 blockade and T cells from these mice can become exhausted, showing that PD-1 inhibition can occur in the absence of SHP2. These data suggest that the consequences of phosphatase association with PD-1 are more complex than previously thought and that unidentified proteins and signaling pathways may play a role in mediating PD-1 function. Thus, it was hypothesized that additional unidentified proteins contribute to PD-1-mediated CD8⁺ T cell inhibition and among these proteins potential targets for therapeutics may be identified that can augment PD-1-based immunotherapy.

SUMMARY

10 In some aspects, provided herein are methods of treating cancer in a subject, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1. The agent may be a small molecule agonist of PIEZO1, such as Yoda1, Jedi1, Jedi2, or a modulator of PIEZO1, such as Docosahexaenoic acid. The method may further comprise administering an immune checkpoint inhibitor to the subject.

15 In some aspects, provided herein are methods of treating cancer in a subject, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1 and an immune checkpoint inhibitor. In some embodiments, the immune checkpoint inhibitor is an inhibitor of an immune checkpoint protein selected from CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, 20 ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, BTLA, SIRPalpha (CD47), CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, A2aR, and combinations thereof.

In some embodiments, the agent that increases or stabilizes the activity or expression of PIEZO1 and the immune checkpoint inhibitor are administered conjointly. In 25 some embodiments, the that agent increases or stabilizes the activity or expression of PIEZO1 and the immune checkpoint inhibitor act synergistically when administered.

In some embodiments, the agent is a gRNA fused to a transcription activator, such as a gRNA that comprises a region that is complementary to a portion of a gene that encodes a PIEZO1 protein. In some embodiments, the agent is a vector encoding a PIEZO1 30 protein, such as a viral vector encoding a PIEZO1 protein.

The agent may be an agent is administered systemically. intravenously, subcutaneously, or intramuscularly. The agent may be administered to the subject in a

pharmaceutically acceptable formulation. The method may further comprise administering to the subject an additional agent, such as a chemotherapeutic agent or a cancer vaccine. The method may further comprise administering to the subject a cancer therapy, such as radiation. The subject may be refractory for immune checkpoint inhibitory therapy.

5 In some aspects, provided herein are methods of treating cancer in a subject unresponsive to immune checkpoint inhibitor therapy, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1 (e.g., any agent that increases or stabilizes the activity or expression of PIEZO1 disclosed herein) and an immune checkpoint inhibitor. The agent may be a small molecule agonist of
10 PIEZO1, such as Yoda1, Jedi1, Jedi2, or a modulator of PIEZO1, such as Docosahexaenoic acid. The method may further comprise administering an immune checkpoint inhibitor to the subject, such an inhibitor of PD-1 or PD-L1, or another immune checkpoint inhibitor disclosed herein.

In some aspects, provided herein are methods of treating cancer in a subject, the
15 method comprising administering to the subject T-cells that have been treated *ex vivo* with an agent that increases or stabilizes the activity or expression of PIEZO1 (e.g., any agent that increases or stabilizes the activity or expression of PIEZO1 disclosed herein). The T-cells may be tumor infiltrating lymphocytes. In some embodiments, T-cells are autologous. In some embodiments, T-cells are allogeneic.

20 In some aspects, the subject is a human.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-1J show that quantitative proximity proteomics identifies PD-1 and PIEZO1 association following PD-L1 ligation. Figure 1 A shows a diagram of PD-1-APEX2 proximity labeling dynamics in Jurkat cells treated with TCR-PD-L1 and TCR-control beads. Figure 1B shows an experimental schematic of PD-1-APEX2 proximity labeling time course with bead treatment. TMT ratios (TMT RA) over time (min) of (Fig. 1C) SHP2, (Fig. 1D) PIEZO1, (Fig. 1E) SHP1, (Fig 1F) CD3 ζ and (Fig. 1G) CD28 following stimulation of PD-1-APEX2-expressing Jurkat cells with TCR-PD-L1 (PD-L1) or TCR-control (mIgG1) beads. Data are presented as means from three independent
25 experiments $\pm$ SD. Statistical significance was assessed using two-way ANOVA analysis comparing TCR-PD-L1 and TCR-control bead-treated groups. Only significant differences are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Figure 1H shows
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hierarchical one-way clustering of the averaged TMT ratios from the top 25 proteins calculated from three independent experiments. Figure 1I shows that log-adjusted p-values of significantly enriched GO Terms in TCR-PD-L1 or TCR-control conditions identified from three independent experiments and calculated from fold changes comparing TCR-PD-L1 and TCR-control mean slope of each gene over time. The top 50 genes were selected for analysis using gProfiler g:Ost. Figure 1J shows rank list of the top ten genes identified in TCR-PD-L1 or TCR-control conditions calculated from the mean slope of each gene in three independent experiments. Schematics created using BioRender.

Fig. 2A-Fig 2K shows that PD-1 inhibits TCR-induced PIEZO1 activity around F-actin rings. Figure 2A shows a schematic of the working principle of GenEPi reporter in which GCaMP fluorescence is increased by PIEZO1-specific Ca^{2+} influx. Representative TIRF microscopy images of GenEPi Jurkat (AF488) cells stained with PD-1 (AF647) and F-actin dye (SPY555) and passed through flow cell chambers coated with anti-CD3/CD28 crosslinking antibodies and either Fig. 2b shows control mIgG₁ ligand or Fig. 2C shows PD-L1 to assess PIEZO1 activity over 300 seconds of stimulation. Yellow arrows indicate F-actin rings and merged images contain GenEPi and PD-1 signal only. Normalized fluorescence intensity for Fig. 2D shows that PD-1 expression and Fig. 2E shows PIEZO1 activity calculated from the sum of pixel values within each region of interest (ROI) at 200 seconds of stimulation. ROIs comprise single cells. Figure 2F shows normalized PIEZO1 activity over 200 seconds measured at intervals of 20 seconds. Data are presented as means of n=37-50 ROIs $\pm$ SEM. Statistical significance for a) and b) was measured using Student's unpaired t-test and statistical significance for Figure 2C was measure using two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Representative kymographs for Fig. 2G shows TCR-control and Fig. 2H shows TCR-PD-L1 conditions. Quantification of kymographs for local Fig. 2I shows PD-1 expression and Fig. 2J shows PIEZO1 activity during stimulation. Fig. 2K shows normalized fluorescence intensity for local PIEZO1 activity over 200 seconds calculated from the sum of pixel values within each ROI for TCR-control and TCR-PD-L1 conditions. ROIs comprise PD-1 clustering sites. Data are presented as means of n=5-11 ROIs $\pm$ SEM. Statistical significance for Fig. 2I and Fig. 2J was measured using Student's unpaired t-test and statistical significance for k) was measured using two-way ANOVA Schematic created using BioRender.

Fig. 3A-3N shows that PIEZO1 activity in CD8^+ T cells regulates antitumor immunity. Fig. 3A shows a schematic of E8i-Cre-ER^{T2} *Piezo1*^{flx/flx} tumor growth experiment

and TIL analysis. Tumor volumes (mm^3) were measured over time (days) for Fig. 3B shows MC38 and Fig. 3C shows B16-OVA tumors in Cre⁺ and Cre- E8i-Cre-ER^{T2} *Piezo1*^{flx/flx} mice. Data are presented as means of n=10-11 for MC38 and n=12-18 for B16-OVA $\pm$ SD from two independent experiments for each tumor type including male and female mice.

5 Frequencies of Fig. 3D and Fig. 3E shows CD8⁺ TILs and Fig. 3F and Fig. 3G shows frequencies of CD62L-expressing CD8⁺ TILs isolated from MC38 and B16-OVA tumors on day 15 post-implantation. Ratio of Fig. 3H and Fig. 3I shows frequencies of TIM-3 and Slamf6-expressing CD8⁺ TILs. Data are presented as means of n=18-26 from three independent experiments $\pm$ SD. Statistical significance was measured using Student's

10 unpaired t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Non-parametric t tests were used for graphs that did not exhibit Gaussian distribution. Fig. 3J shows a schematic of tumor growth experiment with PD-1 blockade. Tumor volumes (mm^3) were measured over time (days) for Fig. 3K shows MC38 and Fig. 3L shows B16-OVA tumors in Cre⁺ and Cre- E8i-Cre-ER^{T2} *Piezo1*^{flx/flx} mice with corresponding Fig. 3M shows MC38 and Fig. 3N shows

15 B16-OVA survival analysis. Data are presented as means of n=5-8 $\pm$ SD from one experiment for each tumor type. Statistical significance for tumor growth curves was calculated using two-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Statistical significance of survival curves was measured using Log-rank Mantel Cox test. * p<0.0332, ** p<0.0021, *** p<0.002.

20 **Fig. 4A-4K** shows combined PD-1 blockade and PIEZO1 agonism promote tumor control. Fig. 4A shows a schematic of Yoda1 treatment in MC38 tumor bearing WT mice. Frequencies of b) CD8⁺ T cells, (Fig. 4C) CD62L-expressing, (Fig. 4D) granzyme B-expressing, (Fig. 4E) PD-1-expressing and (Fig. 4F) perforin-expressing CD8⁺ T cells and (Fig. 4G) ratio of TIM-3/Slamf6 expressing CD8⁺ T cells in tumors assessed by flow

25 cytometry. Data are presented as means of n=6-12 $\pm$ SD from one or two independent experiments. Statistical significance was measured using Student's unpaired t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Fig. 4H shows a schematic of combined PD-1 blockade and PIEZO1 agonist Yoda1 administration to B16.F10 tumor-bearing mice. Fig. 4I shows tumor volumes (mm^3) were measured over time (days) and Figure 4J shows

30 individual tumor growth curves from data depicted in Fig. 4I. Fig. 4K shows corresponding survival analysis. Data are combined from two independent experiments and are presented as means of n=13-15. Statistical significance of tumor growth was measured using 3-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Statistical significance of

survival curves was measured using Log-rank Mantel Cox test. * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.002$.

Fig. 5A-5C shows PD-1-APEX2 construct design and cell line optimization. Fig. 5A. Design of human and murine PD-1-APEX2 constructs. hPGK denotes the human phosphoglycerate kinase promoter. Fig. 5B. PD-1-APEX2 expression in Jurkat cells was measured by flow cytometry and compared to unstimulated, non-lentivirally transduced Jurkat cells. Fig. 5C. Labelling efficiency of PD-1-APEX2 probe shown via Western blot stained with Ponceau to assess protein loading and probed with streptactin-HRP.

Fig. 6A-6P shows Dynabead optimization. Tosyl-activated Dynabeads coated with various ratios of crosslinking-CD3/CD28 antibodies and recombinant murine PD-L1 or human IgG₁κ (hIgG₁κ) control ligand (x-axis) were incubated with murine CD8⁺ T cells for 48 h to assess percentages of cells expressing Fig. 6A) CD8β, Fig. 6B) CD44, Fig. 6C) PD-1, Fig. 6D) granzyme B, and percentage of Fig. 6E) live cells via flow cytometry. Cell culture supernatants were collected to assess concentrations of excreted cytokines Fig. 6F) IL-2, Fig. 6G) IFNγ and Fig. 6H) TNFα using CBA assay. The x-axis denotes specified protein ratios where “T” represents the combined percentage of TCR components CD3 and CD28 while the latter number represents percentage of murine PD-L1 or hIgG₁κ on the beads. Increasing concentrations of beads coated with 60% PD-L1 and 40% TCR components selected from the previous assay were incubated with primary murine CD8⁺ T cells for 48 h to assess percentages of cells expressing Fig. 6I) CD8β, Fig. 6J) CD44, Fig. 6K) PD-1, l) granzyme B and percentage of Fig. 6M) live cells via flow cytometry. Cell culture supernatants were collected to assess levels of excreted cytokines Fig. 6N) IL-2, Fig. 6O) IFNγ, Fig. 6P) TNFα using CBA assay. Data are presented as means of $n = 5$ biological replicates $\pm$ SD. Statistical significance was assessed between control and PD-L1 groups using two-way ANOVA analysis. ns, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 7A-7I shows APEX2 proximity labelling identifies PIEZO1 as target of murine PD-1. TMT ratios over time (min) of Fig. 7A) SHP2, Fig. 7B) PIEZO1, Fig. 7C) SHP1, Fig. 7D) CD3ζ, Fig. 7E) CD28, Fig. 7F) ZAP70, Fig. 7G) CD3δ, Fig. 7H) CD3ε and Fig. 7I) CD3γ following stimulation of murine PD-1-APEX2 with murine TCR-PD-L1 (mPD-L1) or TCR-control hIgG₁κ beads at indicated timepoints. Data are represented as means $\pm$ SD from two independent experiments using two-way ANOVA analysis comparing PD-L1

and control hIgG₁κ bead-treated groups. Only significant differences are indicated. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Fig. 8A-8E shows additional PD-1-APEX2 findings. TMT ratios over time (min) of Fig. 8A) CD3δ, Fig. 8B) CD3ε, Fig. 8C) CD3γ and Fig. 8D) ZAP70 following stimulation of PD-1-APEX2 with TCR-PD-L1 or TCR-control mIgG₁ beads. Data are presented as means from three independent experiments ± SD. Statistical significance was assessed using two-way ANOVA analysis comparing TCR-PD-L1 and TCR-control mIgG₁ bead-treated groups. Only significant differences are indicated. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Fig. 8E) Hierarchical one-way clustering of the averaged TMT ratios of all identified proteins calculated from three independent experiments.

Fig. 9A-9G shows PD-L1 inhibits PIEZO1 activity in PD-1-expressing GenEPi Jurkat cells *in vitro* Fig. 9A) Electroporated and blasticidin-selected GenEPi Jurkat cells were incubated with a dose titration of doxycycline (25-200ng/mL) for 24 h and treated with 10μM Yoda1 immediately prior to flow cytometric analysis. Numbers on plots indicate frequencies of FITC+ cells. Fig. 9B) Schematic of TIRF time lapse imaging experimental design. Raw integrated density reported as intensity for Fig. 9C) PD-1 expression and Fig. 9D) PIEZO1 activity calculated from the sum of pixel values within each region of interest (ROI) at 200 seconds of stimulation presented as normalized values in Fig. 2. ROIs comprise single cells. Data are presented as means of n=37-50 ROIs ± SEM. Statistical significance was measured using Student's unpaired t-test. ns, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Flow cytometric analysis of PIEZO1 reporter activity in PD-1-expressing GenEPi Jurkat cells treated with RPMI media, Yoda1 (5μM), TCR-control or TCR-PDL1 beads for Fig. 9E) 10 s or Fig. 9F) 30 s acquisition. Fig. 9G) MFI of XLGenEPi reporter in all conditions following 30 s acquisition. Cells were treated with beads 20 min prior to acquisition and with Yoda1 (5μM) immediately prior to acquisition. Data are presented as means of n=7-12 technical replicates ± SD. Statistical significance was assessed using one-way ANOVA analysis. ns, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Fig. 10A-10G shows E8i-Cre-ER^{T2} PIEZO1 KO is specific to CD8⁺ T cells. Fig. 10A) Schematic of E8i-Cre-ER^{T2} *Piezo1*^{flx/flx} mouse model. Fig. 10B) Relative fold change of PIEZO1 expression in Cre⁺ and Cre⁻ CD8⁺ T cells following 8 doses of 10mg/mL tamoxifen daily using qPCR and TaqMan probes recognizing specific Piezo1 floxed

regions. Data are presented as means of $n=6 \pm \text{SD}$. Flow cytometric analysis comparing Cre expression using an eGFP reporter in Cre⁺ or Cre⁻ Fig. 10C) murine CD8⁺, CD4⁺, CD11b⁺ and CD11c⁺ splenocytes following *in vivo* tamoxifen treatment and Fig. 10D) in murine CD8⁺, CD4⁺ and B220⁺ splenocytes following *in vitro* 4-hydroxy (4-OH) tamoxifen treatment. Numbers on plots indicate frequencies of eGFP-expressing cells. Fig. 10E) Cre expression measured by eGFP and Fig. 10F) Cre activity measured by TD-tomato frequencies in CD8⁺ T cells isolated from the spleens of MC38 and B16-OVA tumor bearing mice at day 15 after tumor implantation. Data are presented as means of $n=22-34 \pm \text{SD}$ from four independent experiments. Fig. 10G) Ratio of bound/unbound Ca²⁺ and fold change of Ca²⁺ influx in non-treated versus Yoda1-treated CD8⁺ T cells from Cre⁺ and Cre⁻ mice stained with Indo-1 following tamoxifen dosing. Data are presented as means of $n=3 \pm \text{SD}$. Statistical significance was measured using Student's unpaired t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Fig. 11A-11K shows E8i-Cre-ER^{T2} *Piezo1*^{flx/flx} CD8⁺ T cells show minimal phenotypic alterations at baseline. CD8⁺ T cells isolated from spleens of Cre⁺ and Cre⁻ E8i-Cre-ER^{T2} *Piezo1*^{flx/flx} mice were stimulated for 24 h with increasing doses of anti-CD3/CD28 crosslinking antibodies (0-10 ug/mL, x-axis) and assessed for frequencies of CD8⁺ Fig. 11A) live cells, Fig. 11B) PD-1, Fig. 11C) CTLA-4, Fig. 11D) CD44, Fig. 11E) CD62L, Fig. 11F) granzyme B, Fig. 11G) Ki-67 and Fig. 11H) IFN γ /TNF α . Cytometric bead array assays were performed on the supernatants of stimulated Cre⁺ and Cre⁻ CD8⁺ T cells to assess concentrations (pg/mL) of Fig. 11I) IFN γ , Fig. 11J) TNF α and Fig. 11K) IL-2. Data are presented as means of $n=3-4 \pm \text{SD}$. Statistical significance was assessed using Student's unpaired t-test. Only significant differences are indicated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Fig. 12A-12S shows flow cytometric analysis of TILs from tumor bearing E8i-Cre-ER^{T2} *Piezo1*^{flx/flx} mice. Fig. 12A) Gating strategy for CD8 TILs isolated from MC38 or B16-OVA tumors. Frequencies of Fig. 12B and E) CD3 ϵ ⁺, Fig. 12C and F) total CD4⁺ T cells and Fig. 12D and G) CD8⁺/CD4⁺ ratio (counts). Frequencies of CD8⁺ cells expressing Fig. 12H and K) PD-1, Fig. 12I and J) CTLA4, Fig. 12J and M) CD69, Fig. 12N and O) granzyme B, Fig. 12P and Q) Slamf6 and Fig. 12R and S) TIM-3 isolated from Cre⁺ and Cre⁻ mice bearing MC38 or B16-OVA tumors on day 15 after tumor implantation. Data are presented as means of $n=18-26 \pm \text{SD}$ from three independent experiments. Statistical

significance was measured using Student's unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 13A-13B shows individual tumor growth curves of E8i-Cre-ER^{T2} mice.

Individual tumor growth curves from data depicted in Figure 2.3k-l of Cre⁺ and Cre⁻ E8i-Cre-ER^{T2} PIEZO1^{flx/flx} mice treated with anti-PD-1 or isotype control antibody and bearing Fig. 13A) MC38 or Fig. 13B) B16-OVA tumors.

Fig. 14A-14D shows PIEZO agonism influences TIL phenotypes in WT mice.

Tumor volume (mm³) measured over time (days) for Fig. 14A) experiment 1 and Fig. 14B) experiment 2 of WT mice bearing MC38 tumors and treated with Yoda1 (7.5 mg/kg). Data are presented as means of $n = 4-8 \pm \text{SD}$. Statistical significance was assessed using 2-way ANOVA analysis. Frequencies of CD8⁺ TILs that are Fig. 14C) Slamf6⁺ TIM-3⁻ and Fig. 14D) Slamf6⁻ TIM-3⁺ assessed by flow cytometry. Data are presented as means of $n = 12 \pm \text{SD}$ from one or two independent experiments. Statistical significance was assessed using Student's unpaired t test. ns, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DETAILED DESCRIPTION

The PD-1 pathway plays a critical role in mediating T cell exhaustion, and blockade of this pathway can promote antitumor immunity. While PD-1 inhibitors are revolutionizing cancer therapy, only a subset of patients respond and show durable remission, highlighting the need to better understand how the PD-1 pathway suppresses T cell functions. Here, PD-1/PD-L1 ligation-induced dynamic changes of the local proteome proximal to PD-1 were quantified using unbiased multiplexed proximity proteomics. The mechanosensitive cation channel PIEZO1 was identified as a primary target of PD-1-mediated inhibition.

Stimulation of CD8⁺ T cells through TCR and CD28 engagement triggered the activation of PIEZO1, while simultaneous PD-1 ligation countered this activation. Mice lacking PIEZO1 selectively on CD8⁺ T cells exhibited increased tumor growth marked by impaired CD8⁺ T cell function, which could not be rescued by PD-1 blockade. Conversely, mice treated with PIEZO1 agonist showed increased numbers and function of CD8⁺ tumor-infiltrating lymphocytes compared to controls. Combined administration of PIEZO1 agonist and anti-PD-1 significantly reduced tumor burden and improved survival in a tumor model unresponsive to PD-1 blockade. These findings identify PIEZO1 inhibition as an important mechanism by which PD-1 signaling regulates CD8⁺ T cell functions and suggest PIEZO1 agonism and/or modulation as a novel approach for augmenting cancer immunotherapy.

Provided herein is unbiased proximity-labeling as applied to the characterization of the dynamic behavior of proteins recruited to the cytoplasmic tail of PD-1 following ligation and identification of the mechanosensitive ion channel PIEZO1 (FAM38a) as a primary target of PD-1-mediated inhibition. PIEZO1 is expressed on a variety of cancers, which include epithelial and immune cells, where its activity can be regulated by external forces such as shear stress, cyclical hydrostatic pressure, and membrane deformation (force-from-lipid), as well as actomyosin contractility and extracellular matrix tethering (force-from-filament). Both external and internal forces contribute to PIEZO1 gating, a process by which an ion channel transitions between its open and closed conformations as a means of regulating the passage of electrical current through the ion-conducting pore; this, in turn, induces downstream signaling. The stimulation of CD8⁺ T cells through TCR and CD28 engagement triggers the activation of PIEZO1. Notably, it is also demonstrated that simultaneous ligation of PD-1 and engagement of TCR and CD28 significantly reduces PIEZO1-mediated Ca²⁺ influx. Moreover, knockout mice (KO) deficient in PIEZO1 only in CD8⁺ T cells exhibited markedly increased tumor growth. CD8⁺ tumor infiltrating lymphocytes (TILs) isolated from these mice were less abundant and less activated. CD8⁺ T cell-specific PIEZO1 KO decreased the efficacy of PD-1 blockade in tumor-bearing mice. In complementary studies, stimulating PIEZO1 activity in wild-type (WT) tumor-bearing mice by systemic treatment with the PIEZO1 agonist Yoda1 resulted in increased activation of effector CD8⁺ T cells in the tumor microenvironment (TME). Importantly, combined administration of anti-PD-1 and Yoda1 decreased tumor burden and improved survival in WT mice bearing tumors unresponsive to PD-1 blockade. These summarized results identify reduction of PIEZO1 activity as a novel mechanism by which PD-1 mediates its inhibitory functions and pharmacological agonism of PIEZO1 as a potentially effective combination therapy to improve PD-1 inhibitors in cancer patients.

Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

The term “*agent*” is used herein to denote a chemical compound, a small molecule, a mixture of chemical compounds and/or a biological macromolecule (such as a nucleic acid, an antibody, an antibody fragment, a protein or a peptide). The activity of such agents may render them suitable as a “therapeutic agent” which is a biologically, physiologically, or pharmacologically active substance (or substances) that acts locally or systemically in a subject.

Unless otherwise specified here within, the terms “*antibody*” and “*antibodies*” broadly encompass naturally-occurring forms of antibodies (e.g. IgG, IgA, IgM, IgE) and recombinant antibodies such as single-chain antibodies, chimeric and humanized antibodies and multi-specific antibodies, as well as fragments and derivatives of all of the foregoing, which fragments and derivatives have at least an antigenic binding site. Antibody derivatives may comprise a protein or chemical moiety conjugated to an antibody.

The term “*antibody*” as used herein also includes an “*antigen-binding portion*” of an antibody (or simply “*antibody portion*”). The term “*antigen-binding portion*”, as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., a biomarker polypeptide or fragment thereof). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term “antigen-binding portion” of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent polypeptides (known as single chain Fv (scFv); see e.g., Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Osbourn *et al.* 1998, *Nature Biotechnology* 16: 778). Such single chain antibodies are also intended to be encompassed within the term “antigen-binding portion” of an antibody. Any VH and VL sequences of specific scFv can be linked to human immunoglobulin constant region cDNA or genomic

sequences, in order to generate expression vectors encoding complete IgG polypeptides or other isotypes. VH and VL can also be used in the generation of Fab, Fv or other fragments of immunoglobulins using either protein chemistry or recombinant DNA technology. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6444-6448; Poljak *et al.* (1994) *Structure* 2:1121-1123).

An antibody for use in the instant invention may be a bispecific antibody. A bispecific antibody has binding sites for two different antigens within a single antibody polypeptide. Antigen binding may be simultaneous or sequential. Triomas and hybrid hybridomas are two examples of cell lines that can secrete bispecific antibodies. Examples of bispecific antibodies produced by a hybrid hybridoma or a trioma are disclosed in U.S. Patent 4,474,893. Bispecific antibodies have been constructed by chemical means (Staerz *et al.* (1985) *Nature* 314:628, and Perez *et al.* (1985) *Nature* 316:354) and hybridoma technology (Staerz and Bevan (1986) *Proc. Natl. Acad. Sci. USA*, 83:1453, and Staerz and Bevan (1986) *Immunol. Today* 7:241). Bispecific antibodies are also described in U.S. Patent 5,959,084. Fragments of bispecific antibodies are described in U.S. Patent 5,798,229.

Bispecific agents can also be generated by making heterohybridomas by fusing hybridomas or other cells making different antibodies, followed by identification of clones producing and co-assembling both antibodies. They can also be generated by chemical or genetic conjugation of complete immunoglobulin chains or portions thereof such as Fab and Fv sequences.

Still further, an antibody or antigen-binding portion thereof may be part of larger immunoadhesion polypeptides, formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion polypeptides include use of the streptavidin core region to make a tetrameric scFv polypeptide (Kipriyanov *et al.* (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, biomarker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv polypeptides (Kipriyanov *et al.* (1994) *Mol.*

Immunol. 31:1047-1058). Antibody portions, such as Fab and F(ab')₂ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion polypeptides can be obtained using standard recombinant DNA

5 techniques, as described herein.

Antibodies may also be “*humanized*,” which is intended to include antibodies made by a non-human cell having variable and constant regions which have been altered to more closely resemble antibodies that would be made by a human cell. For example, by altering the non-human antibody amino acid sequence to incorporate amino acids found in human
10 germline immunoglobulin sequences. The humanized antibodies of the disclosure may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs. The term “humanized antibody”, as used herein, also includes antibodies in which CDR sequences derived from the germline of
15 another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The terms “*cancer*” or “*tumor*” refer to the presence of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation, immortality, metastatic potential, rapid growth and proliferation rate, and certain
20 characteristic morphological features.

Cancer cells are often in the form of a tumor, but such cells may exist alone within an animal, or may be a non-tumorigenic cancer cell, such as a leukemia cell. As used herein, the term “cancer” includes premalignant as well as malignant cancers. Cancers include, but are not limited to, B cell cancer, *e.g.*, myelomas like multiple myeloma,
25 Waldenström's macroglobulinemia, the heavy chain diseases, such as, for example, alpha chain disease, gamma chain disease, and mu chain disease, benign monoclonal gammopathy, and immunocytic amyloidosis, melanomas, breast cancer, lung cancer, bronchus cancer, colorectal cancer, prostate cancer, pancreatic cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral
30 nervous system cancer, esophageal cancer, cervical cancer, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testicular cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, cancer of hematologic tissues, and

the like. Other non-limiting examples of types of cancers applicable to the methods encompassed by the present disclosure include human sarcomas and carcinomas, *e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, colorectal cancer, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, liver cancer, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, bone cancer, brain tumor, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.*, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), myeloma, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease. In some embodiments, cancers are epithelial in nature and include but are not limited to, bladder cancer, breast cancer, cervical cancer, colon cancer, gynecologic cancers, renal cancer, laryngeal cancer, lung cancer, oral cancer, head and neck cancer, ovarian cancer, pancreatic cancer, prostate cancer, or skin cancer. In other embodiments, the cancer is breast cancer, prostate cancer, lung cancer, or colon cancer. In still other embodiments, the epithelial cancer is non-small-cell lung cancer, nonpapillary renal cell carcinoma, cervical carcinoma, ovarian carcinoma, or breast carcinoma. The epithelial cancers may be characterized in various other ways including, but not limited to, serous, endometrioid, mucinous, clear cell, Brenner, or undifferentiated. In some embodiments, the cancer comprises a solid tumor. In some embodiments, the tumor is an adenocarcinoma, an adrenal tumor, an anal tumor, a bile duct tumor, a bladder tumor, a bone tumor, a blood born tumor, a brain/CNS tumor, a breast tumor, a cervical tumor, a colorectal tumor, an endometrial tumor, an esophageal tumor, an Ewing tumor, an eye tumor, a gallbladder tumor, a gastrointestinal, a kidney

tumor, a laryngeal or hypopharyngeal tumor, a liver tumor, a lung tumor, a mesothelioma tumor, a multiple myeloma tumor, a muscle tumor, a nasopharyngeal tumor, a neuroblastoma, an oral tumor, an osteosarcoma, an ovarian tumor, a pancreatic tumor, a penile tumor, a pituitary tumor, a primary tumor, a prostate tumor, a retinoblastoma, a
 5 Rhabdomyosarcoma, a salivary gland tumor, a soft tissue sarcoma, a melanoma, a metastatic tumor, a basal cell carcinoma, a Merkel cell tumor, a testicular tumor, a thymus tumor, a thyroid tumor, a uterine tumor, a vaginal tumor, a vulvar tumor, or a Wilms tumor.

As used herein, the phrase “*conjoint administration*” refers to any form of administration of two or more different therapeutic agents such that the second agent is
 10 administered while the previously administered therapeutic agent is still effective in the body (*e.g.*, the two agents are simultaneously effective in the subject, which may include synergistic effects of the two agents). For example, the different therapeutic agents can be administered either in the same formulation or in separate formulations, either concomitantly or sequentially. In certain embodiments, the different therapeutic agents can
 15 be administered within about one hour, about 12 hours, about 24 hours, about 36 hours, about 48 hours, about 72 hours, or about a week of one another. Thus, a subject who receives such treatment can benefit from a combined effect of different therapeutic agents. As used herein, any two agents and/or additional agents may be conjointly administered according to the methods provided herein.

20 PIEZO1 is a mechanosensitive ion channel protein that in humans is encoded by the gene *PIEZO1*. It is a mechanosensitive non-specific cation channel. It plays a key role in epithelial cell adhesion by maintaining integrin activation through R-Ras recruitment to the ER, likely in its activated state, and subsequent stimulation of calpain signaling. Exemplary nucleotide and amino acid sequences of human PIEZO1, which correspond to GenBank
 25 Accession numbers, are listed below in Table 1. In some embodiments, an agent described herein targets an amino acid sequence disclosed in Table 1 or at least partially encodes an sequence listed in Table 1. In some embodiments, an agent described herein targets a nucleic acid sequence described in Table 1 or at least partially encodes an sequence listed in Table 1. As used herein, PIEZO1. may comprise a sequence listed below in Table 1, or may
 30 comprise at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95% homology to a sequence listed in Table 1.

Table 1

SEQ ID NO: 1: Human PIEZO1 Amino Acid Sequence; NP_001136336.2

MEPHVLGAVLYWLLLPALLAACLLRFSGLSLVYLLFLLLLPWFPGPTRCGLQGHTRGLLRALLGLSLFL
LVAHLALQICLHIVPRLDQLLGPSCSRWETLSRHIGVTRLDLKDIPNAIRLVAPDLGILVVSSVCLGICG
RLARNTRQSPHPRELDDDERDVDASPTAGLQEAATLAPTRRSRLAARFRVTAHWLLVAAGRVLAVTLLAL
AGIAHPSALSSVYLLLFALCTWWACHFPISTRGFSRLCVAVGCFGAGHLICLYCYQMPLAQALLPPAGI
WARVLGLKDFVGPTNCSSPHALVLNTGLDWVPVYASPGVLLLLCYATASLRKLRAYRPSGQRKEAAKGYEA
RELELAELDQWPQERESDQHVVPTAPDTEADNCIVHELTGQSSVLRPVRPKRAEPREASPLHSLGHLIM
DQSYVCALIAMMVWSITYHSWLTFFVLLWACL IWTVRSRHQLAMLCSPCILLYGMTLCLCLRYVWAMDRLR
ELPTTLGPVSLRQLGLEHTRYPCLDLGAMLLYTTLTFWLLLRQFVKEKLLKWAESPAALTEVTVADTEPTR
TQTLLQSLGELVKGVYAKYWIYVCAGMFIVVSFAGRLVVYKIVYMFLFLLCLTLFQVYYSLWRKLLKAFW
WLVVAYTMLVLIAVYTFQFQDFPAYWRNLTGFTDEQLGDLGLEQFSVSELSILVPGFFLLACILQLHY
FHRPFMQLTDMEHVSLPGTRLPRWAHRQDAVSGTPLLREEQQEHQQQQQEEEEEEEDSRDEGLGVATPHQ
ATQVPEGAAGKGLVAERLLELAAGFSDVLSRVQVFLRRLLELHVFKLVALYTVWVALKEVSMNLLLVLV
WAFALPYPRFRPMASCLSTVWTCVIVCKMLYQLKVVNPQEYSSNCTEPPNSTNLLPTEISQSLLYRGP
VDPANWFGVRKGFPNLGYIQNHLQVLLLLLVFEAIVYRRQEHYRRQHQLAPLPAQAVFASGTRQQLDQDLL
GCLKYFINFFFYKFGLEICFLMAVNVIGQRMNFLVTLHGCVLVAILLTRHRQATARLWPNYCLFLALFLL
YQYLLCLGMPPALCIDYPWRWSRAVPMNSALIKWLYLPDFFRAPNSTNLISDFLLLLL CASQQWQVFS
AERTEEWQRMAGVNTDRLEPLRGEPNPVPNF IHCRSYLDMLKVAVFRYLFWLVLVVVFTGATRISIFGLGYL
LACFYLLLFGTALLQRDTRARLVLWDCILLYNVTVIISKNMLSLLACVFVEQMQTGFCWVIQLFSLVCTV
KGYDPKEMMDRDQDCLLPVEEAGI IWDSCFFFLLLQRRVFLSHYYLHVRADLQATALLASRGFALYNA
ANLKSIDFHRRIEEKSLAQLKRQMERIRAKQEKHRQGRVDRSRPQDTLGPKDPGLEPGDPSPGSSPPRR
QWWRPWL DHATVIHSGDYFLFESDSEEEEEAVPEDPRPSAQSAFQLAYQAWVTNAQAVLRRRQQEQEQAR
QEQAQQLPTGGGPSQEVEPAEGPEEAAAAGRSHVVQRVLSTAQFLWMLGQALVDELTRWLQEFTRHHGTMS
DVLRAERYLLTQELLQGGEVHRGVLDQLYTSQAEATLPGPTEAPNAPSTVSSGLGAEPELSSMTDDMGSP
LSTGYHTRSGSEEA VTDPGEREAGASLYQGLMRTASELLLDRLRLIPELEEAELFAEGQGGRALRLLRVY
QCVAHSELLEYFIIILNMHV TASAGSLVLPVLVFLWAMLSIPRPSKRFWMTAIVFTEIAVVVKYLFQFG
FFPWNSHVVLRRYENKPYFPFPRILGLEKTDGYIKYDLVQLMALFFHRSQLLCYGLWDHEEDSPSKEHDKS
GEEEGQGAEEGPGVPAATTEDHIQVEARVGPTDGTPEPQVELRPRDTRRISLRFRRRKKEGPARKGAAAIE
AEDREEEEGEEKEAPTGREKPRSRSGRVRAAGRRLQGFCLSLAQGTYRPLRRFFHDILHTKYRAATDV
YALMFLADVDFIIIIIFGFWAFGKHSAAITITSSLSDDQVPEAFVLMLLIQFSTMVVDRALYLRKTVLGL
LAFQVALVLA IHLWMFFILPAVTERMFNQNVVAQLWYFVKCIYFALSAYQIRC GYPTRILGNFLT KKYNH
LNLFLFQGRFVLVFLVELRAVMDWVWTDTTLSLSSWMCVEDIYANIFI IKSRETEKKYPQPKGQKKKKI
VKYMGGLIILFLIAI IWFPLLFMSLVRSVGVVNGPIDVTYTLKLGGYEPLFTMSAQPSIIPFTAQAY
EELSRQFDPQLAMQFISQSPEDIVTAQIEGSSGALWRISPPSRAQMKRELYNGTADITLRF TWNFQRD
LAKGGTVEYANEKHMALAPNSTARRQLASLLEGTSDQSVVIPNLF PKYIRAPNGPEANPVKQLQPNEEA
DYLGVRIQLRREQGAGATGFLEWWVIELQE CRTDCNLLPMVIFSDKVSPPSLGFLAGY GIMGLYVSIVLV
IGKFVRGFFSEISHS IMFEELPCVDRILKLCQDIFLVRETRELELEEEELYAKLIFLYRSPETMIKW TREK
E

SEQ ID NO: 2: Human NM_001142864.4 Homo sapiens piezo type mechanosensitive ion channel component 1 (PIEZO1), mRNA

45 GGGAGCCGCCGTCCTCGGCCACGCTCGGCCCACTGAGCCGAGCGCTGCGCTCCGCCGAGGGGCAGGGCGGT
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55 AGCCACATCCACGGGAGCTGGATGATGATGAGAGGGATGTGGATGCCAGCCCCGACGGCAGGGCTGCAGG
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GCTGGTGGCGGCTGGGCGGGTCTGGGCGGTAACTGCTTGGCACTGGCAGGCATCGCCCCCACTGGCT

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CTACCAGATGCCCTTGCCACAGGCTCTGCTCCCCGCTGCCGGCATCTGGGCTAGGGTGCTGGGTCTCAAG
5 GACTTCGTGGGTCCCACCAACTGCTCCAGCCCCACGCGCTGGTCCTCAACACCGGCCCTGGACTGGCCTG
TGTATGCCAGCCCCGGCGTCTCTGCTGCTGTGTACGCCACGGCCTCTCTGCGCAAGCTCCGCGCGTA
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GACCAGTGGCCCCAGGAACGGGAGTCTGACCAGCACGTGGTGGCCACAGCACCCGACACCGAGGCTGATA
10 ACTGCATCGTGACAGAGCTGACCGGCCAGAGCTCCGTCTGCGGCGGCCTGTGCGGCCCAAGCGGGCTGA
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15 ACACCCTGACCTTCTGGCTCTGCTGCGCCAGTTTGTGAAAAGAGAAGCTGCTGAAGTGGGCAGAGTCTCC
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55 CCAGGCGGTGCTGAGGCGGGCAGCAGGAGCAGGAGCAGGCAAGGCAGGAACAGGCAGGACAGCTACCC
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 10 ACATCAAGTACGACCTGGTGCAGCTCATGGCCCTTTTCTTCCACCGCTCCCAGCTGCTGTGCTATGGCCT
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 15 CGGCCGGGCGGCGGCTGCAGGGCTTCTGCCTGTCCCTGGCCAGGGCACATATCGGCCGCTACGGCGCTT
 CTTCCACGACATCCTGCACACCAAGTACCGCGCAGCCACCGACGTCTATGCCCTCATGTTCTTGGCTGAT
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 GAGCGCCAGCAGCCGTCCATCATCCCCCTTACGGCCAGGCCATGAGGAGCTGTCCCGGCAGTTTGAC
 CCCCAGCCGCTGGCCATGCAGTTCATCAGCCAGTACAGCCCTGAGGACATCGTCACGGCGCAGATTGAGG
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ACCACTCCCCACGTAAGAAAGTACAACCTAGGGGTTTCTAGTGGATTACAGTTGAGCATCTGCCTTCTC

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 5 TACAAAAAATAAACGTTAGCTGGGTGTGATGGTGCACACTTGTGCTCCCAGCTACTCTGGAGGCTGAGG
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 GGCAACAGAGTGAGACCCCTGTCTCTAAAAAAGTAAAAAGAAGAAACCCAGTGCCCATTTGGCTGTCACTCG
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 10 TATTTTCATGAAAACAGGCTCAGACAGCGTTTGTCTTTTGTAAATCAGCTTTTCTTGCTCAGCATGGTGTG
 TCTGAGGTCCACCCACGTGGCAGTATGGCCTTCTGTCTTATGGGCAAATGATATTCTGTGGCATGGATGG
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 35 CCTGTGAAAGCTGGTGTGGGGAGGCGGCTGCAGTCACTGAGGGTGTCACTTGTACCCAGCTGGCCATGCA
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 ACCTTGGTGTCTGACGGCTGGTGTGAAGACAGAAGGAACGGAGAACAGGCGGTTGTAGAAAGCGACCTCCT
 20 TCAGGGTGTGGTCCCGCATGGGCCGCACACCACCCACCGTCCCCGTGCCGCTCATCCGAGAAGCCCTGCCG
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 AGGCCCCCTGACCCAGCGCCAGGTTGGTTCATGAGCTTGATAGCCAAGCGTGTGCAGCTGTCCCCAGTCAT
 25 GACCTTGGAGTAGCCGTGGGCTC

It will be appreciated that specific sequence identifiers (SEQ ID NOs) have been
 referenced throughout the specification for purposes of illustration and should therefore not
 be construed to be limiting. Any marker encompassed by the present invention, including,
 30 but not limited to, the markers described in the specification and markers described herein,
 are well-known in the art and may be used in the embodiments encompassed by the present
 invention.

The phrase “*pharmaceutically-acceptable carrier*” as used herein means a
 pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid
 35 filler, diluent, excipient, or solvent encapsulating material, involved in carrying or
 transporting the subject compound from one organ, or portion of the body, to another organ,
 or portion of the body.

The terms “*polynucleotide*”, and “*nucleic acid*” are used interchangeably. They
 refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or
 40 ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional
 structure, and may perform any function, known or unknown. The following are non-
 limiting examples of polynucleotides: coding or non-coding regions of a gene or gene
 fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA
 (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides,

branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified, such as by conjugation with a labeling component. The term “recombinant” polynucleotide means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in a non-natural arrangement.

The terms “*prevent*,” “*preventing*,” “*prevention*,” and the like refer to reducing the probability of developing a disease, disorder, or condition in a subject, who does not have, but is at risk of or susceptible to developing a disease, disorder, or condition.

The term “*small molecule*” is a term of the art and includes molecules that are less than about 1000 molecular weight or less than about 500 molecular weight. In one embodiment, small molecules do not exclusively comprise peptide bonds. In another embodiment, small molecules are not oligomeric. Exemplary small molecule compounds which can be screened for activity include, but are not limited to, peptides, peptidomimetics, nucleic acids, carbohydrates, small organic molecules (e.g., polyketides) (Cane *et al.* (1998) *Science* 282:63), and natural product extract libraries. In another embodiment, the compounds are small, organic non-peptidic compounds. In a further embodiment, a small molecule is not biosynthetic.

As used herein, the term “*subject*” means a human or non-human animal selected for treatment or therapy.

The “*tumor microenvironment*” is an art-recognized term and refers to the cellular environment in which the tumor exists, and includes, for example, interstitial fluids surrounding the tumor, surrounding blood vessels, immune cells, other cells, fibroblasts, signaling molecules, and the extracellular matrix.

The phrases “*therapeutically-effective amount*” and “*effective amount*” as used herein means the amount of an agent which is effective for producing the desired therapeutic effect in at least a sub-population of cells in a subject at a reasonable benefit/risk ratio applicable to any medical treatment.

“*Treating*” a disease in a subject or “*treating*” a subject having a disease refers to subjecting the subject to a pharmaceutical treatment, e.g., the administration of a drug, such that at least one symptom of the disease is decreased or prevented from worsening.

In some aspects, provided herein are methods of treating cancer in a subject, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1. The agent may be a small molecule agonist of PIEZO1, such as Yoda1, Jedi1, Jedi2, or a modulator of PIEZO1, such as Docosahexaenoic acid. The method may further comprise administering an immune checkpoint inhibitor to the subject.

In some aspects, provided herein are methods of treating cancer in a subject, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1 and an immune checkpoint inhibitor. In some embodiments, the immune checkpoint inhibitor is an inhibitor of an immune checkpoint protein selected from CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, BTLA, SIRPalpha (CD47), CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, A2aR, and combinations thereof. In some preferred embodiments, the immune checkpoint inhibitor is an inhibitor of PD-1 or PD-L1.

In some embodiments, the agent that increases or stabilizes the activity or expression of PIEZO1 and the immune checkpoint inhibitor are administered conjointly. In some embodiments, the agent increases or stabilizes the activity or expression of PIEZO1 and the immune checkpoint inhibitor act synergistically when administered. In some embodiments, the agent is a gRNA fused to a transcription activator, such as a gRNA that comprises a region that is complementary to a portion of a gene that encodes a PIEZO1 protein. In some embodiments, the agent is a vector encoding a PIEZO1 protein, such as a viral vector encoding a PIEZO1 protein. For example, without being bound by theory or methodology, the agent that increases or stabilizes the activity or expression of PIEZO1 may be a small molecule agonist of PIEZO1, such as Yoda1, Jedi1, Jedi2, a modulator of PIEZO1, such as Docosahexaenoic acid, a gRNA disclosed herein, or any combination thereof.

The agent may be administered systemically, intravenously, subcutaneously, or intramuscularly. The agent may be administered to the subject in a pharmaceutically acceptable formulation. The method may further comprise administering to the subject an additional agent, such as a chemotherapeutic agent or a cancer vaccine. The method may

further comprise administering to the subject a cancer therapy, such as radiation. The subject may be refractory for immune checkpoint inhibitory therapy.

In some aspects, provided herein are methods of treating cancer in a subject unresponsive to immune checkpoint inhibitor therapy, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1 (e.g., any agent that increases or stabilizes the activity or expression of PIEZO1 disclosed herein) and an immune checkpoint inhibitor. The agent may be a small molecule agonist of PIEZO1, such as Yoda1, Jedi1, Jedi2, a modulator of PIEZO1, such as Docosahexaenoic acid, or any combination thereof. The method may further comprise administering an immune checkpoint inhibitor to the subject, such an inhibitor of PD-1 or PD-L1, or another immune checkpoint inhibitor disclosed herein.

In some aspects, provided herein are methods of treating cancer in a subject, the method comprising administering to the subject T-cells that have been treated *ex vivo* with an agent that increases or stabilizes the activity or expression of PIEZO1 (e.g., any agent that increases or stabilizes the activity or expression of PIEZO1 disclosed herein). The T-cells may be tumor infiltrating lymphocytes. In some embodiments, T-cells are autologous (*i.e.*, derived from the subject). In some embodiments, T-cells are allogeneic (*i.e.*, derived from a donor).

In some aspects, the subject is a human.

Modulators of PIEZO1

Provided herein are methods and compositions for preventing or treating cancer in a subject, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1.

5 The agent may increase the activity of PIEZO1 by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100%. The agent may increase the expression of PIEZO1 by
10 at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100%.

An agent disclosed herein may increase PIEZO1 mRNA by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at

least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100%. Measurement of PIEZO1 can be done in a biological sample or multiple biological samples taken from the subject over a period of time.

5 Polypeptide Agents

In some embodiments, the agent provided herein is a polypeptide agent (e.g., a polypeptide that binds to a PIEZO1 protein). A polypeptide agent disclosed herein may increase or stabilize the expression or activity of PIEZO1 by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%,
10 at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100%. In some embodiments, the agent may be a chimeric or fusion polypeptide. In some embodiments, the agent may be a ligand or binding partner of PIEZO1. A fusion or chimeric polypeptide can be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different
15 polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional
20 techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and re-amplified to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons: 1992). Moreover, many
25 expression vectors are commercially available that already encode a fusion moiety.

The polypeptides described herein can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding a polypeptide(s). Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous polypeptides in recombinant hosts, chemical synthesis of polypeptides, and *in vitro*
30 translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N. Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques

(1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) J. Am. Chem. Soc. 91:501; Chaiken I. M. (1981) CRC Crit. Rev. Biochem. 11:255; Kaiser et al. (1989) Science 243:187; Merrifield, B. (1986) Science 232:342; Kent, S. B. H. (1988) Annu. Rev. Biochem. 57:957; and Offord, R. E. (1980) Semisynthetic Proteins, Wiley Publishing, which are incorporated herein by reference.

Nucleic Acids

In certain embodiments, provided herein are agents that are vectors that contain the isolated nucleic acid molecules described herein, such as those that encode a PIEZO1 peptide. As used herein, the term “vector,” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a “plasmid”, which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby be replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes. Such vectors are referred to herein as “recombinant expression vectors” (or simply, “expression vectors”). In certain embodiments, provided herein are cells that contain a nucleic acid described herein (*e.g.*, a nucleic acid encoding an antibody, antigen binding fragment thereof, antibody-like molecule, or polypeptide described herein). The cell can be, for example, prokaryotic, eukaryotic, mammalian, avian, murine and/or human.

The interfering nucleic acids described herein may be contacted with a cell or administered to an organism (*e.g.*, a human). Alternatively, constructs and/or vectors encoding the interfering RNA molecules may be contacted with or introduced into a cell or organism. In certain embodiments, a viral, retroviral or lentiviral vector is used.

In some embodiments, agents for increasing the expression or activity of PIEZO1, are delivered to subjects by use of viral vectors. Viral vector systems which can be utilized with the methods and compositions described herein include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors, including but not limited to lentiviral vectors, moloney murine leukemia virus, *etc.*; (c) adeno-associated virus vectors; (d) herpes simplex

virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; (i) pox virus vectors such as an orthopox, *e.g.*, vaccinia virus vectors or avipox, *e.g.* canary pox or fowl pox; and (j) a helper-dependent or gutless adenovirus.

5 In some embodiments, adenoviruses can be used to deliver nucleic acid agents for increasing the expression or activity of PIEZO1. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, *Current Opinion in Genetics and Development* 3:499-503 (1993) present a review of adenovirus-based gene therapy.

10 In some embodiments, nucleic acid agents for increasing the expression or activity of PIEZO1 can be delivered by adeno-associated virus (AAV) vectors. In some embodiments, a AAV vector that expresses a nucleic acid agent for increasing the expression or activity of PIEZO1 is a recombinant AAV vector having, for example, either an U6 or H1 RNA promoter, or a cytomegalovirus (CMV) promoter. Suitable AAV vectors for use in agents, compositions, and methods described include, but are not limited to AAVs described in Passini *et al.*, *Methods Mol. Biol.* 246: 225-36 (2004).

15 In another embodiments, the agent comprises a CRISPR activation agent and/or a sgRNA. In some embodiments, the agent is an sgRNA. An sgRNA combines tracrRNA and crRNA, which are separate molecules in the native CRISPR/Cas9 system, into a single RNA construct, simplifying the components needed to use CRISPR activation system. In some embodiments, the crRNA of the sgRNA has complementarity to *PIEZO1* DNA.

20 Antibody Agents

Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof (*e.g.* humanized, chimeric, etc.). Antibodies may also be fully human. The terms “monoclonal antibodies” and “monoclonal antibody composition”, as used herein, refer to a population of antibody polypeptides that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term “polyclonal antibodies” and “polyclonal antibody composition” refer to a population of antibody polypeptides that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody composition typically displays a single binding affinity for a particular antigen with which it immunoreacts.

30 In certain embodiments, the methods and compositions provided herein relate to antibodies and antigen binding fragments thereof that bind specifically to a PIEZO1

protein. Such antibodies can be polyclonal or monoclonal and can be, for example, murine, chimeric, humanized or fully human. In some embodiments, the agent may be a recombinant antibodies specific for a PIEZO1 protein, such as chimeric or humanized monoclonal antibodies, can be made using standard recombinant DNA techniques. Such

5 chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in US Pat No. 4,816,567; US Pat. No. 5,565,332; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci.* 84:214-218; Nishimura *et al.* (1987) *Cancer Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *Biotechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

15 Human monoclonal antibodies specific for a PIEZO1 protein can be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system. For example, "HuMAb mice" which contain a human immunoglobulin gene miniloci that encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the

20 endogenous μ and κ chain loci (Lonberg, N. *et al.* (1994) *Nature* 368(6474): 856 859). Accordingly, the mice exhibit reduced expression of mouse IgM or κ , and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgG κ monoclonal antibodies (Lonberg, N. *et al.* (1994), *supra*; reviewed in Lonberg, N. (1994) *Handbook of*

25 *Experimental Pharmacology* 113:49 101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* Vol. 13: 65 93, and Harding, F. and Lonberg, N. (1995) *Ann. N. Y Acad. Sci* 764:536 546). The preparation of HuMAb mice is described in Taylor, L. *et al.* (1992) *Nucleic Acids Research* 20:6287 6295; Chen, J. *et al.* (1993) *International Immunology* 5: 647 656; Tuaillon *et al.* (1993) *Proc. Natl. Acad. Sci USA* 90:3720 3724; Choi *et al.* (1993)

30 *Nature Genetics* 4:117 123; Chen, J. *et al.* (1993) *EMBO J.* 12: 821 830; Tuaillon *et al.* (1994) *J. Immunol.* 152:2912 2920; Lonberg *et al.*, (1994) *Nature* 368(6474): 856 859; Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49 101; Taylor, L. *et al.* (1994) *International Immunology* 6: 579 591; Lonberg, N. and Huszar, D. (1995) *Intern.*

Rev. Immunol. Vol. 13: 65-93; Harding, F. and Lonberg, N. (1995) Ann. N.Y. Acad. Sci. 764:536-546; Fishwild, D. *et al.* (1996) Nature Biotechnology 14: 845-851. See further, U.S. Pat. Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; 5,770,429; and 5,545,807.

5 Small Molecule Agents

Certain embodiments of the methods and compositions disclosed herein relate to the use of small molecule agents e.g., small molecule agents that increase or stabilize the expression or activity of a PIEZO1 protein. Such agents include those known in the art and those identified using the screening assays described herein. A small molecule provided
10 herein may have at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% specificity for a PIEZO1 protein.

In some embodiments, assays used to identify agents (e.g., anti-cancer agents) in the
15 methods described herein include obtaining a population of cells and a small molecule agent, wherein the cells are incubated with a small molecule agent and the level of a PIEZO1 protein is measured. Agents identified *via* such assays, may be useful, for example, for increasing the expression or the activity of a PIEZO1 protein in a subject.

Agents useful in the methods disclosed herein may be obtained from any available
20 source, including systematic libraries of natural and/or synthetic compounds. Agents may also be obtained by any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, e.g.,
25 Zuckermann *et al.*, 1994, *J. Med. Chem.* 37:2678-85); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide
30 oligomer or small molecule libraries of compounds (Lam, 1997, *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of agents may be presented in solution (e.g., Houghten, 1992, *Biotechniques* 13:412-421), or on beads (Lam, 1991, *Nature* 354:82-84), chips (Fodor, 1993, *Nature* 364:555-556), bacteria and/or spores, (Ladner, USP 5,223,409), plasmids (Cull *et al.*, 1992, *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith, 1990, *Science* 249:386-390; Devlin, 1990, *Science* 249:404-406; Cwirla *et al.*, 1990, *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici, 1991, *J. Mol. Biol.* 222:301-310; Ladner, *supra.*).

Pharmaceutical Compositions

In certain embodiments, provided herein is a composition, e.g., a pharmaceutical composition, containing at least one agent described herein together with a pharmaceutically acceptable carrier. In one embodiment, the composition includes a combination of multiple (e.g., two or more) agents described herein.

In some embodiments, the pharmaceutical composition is delivered locally or systemically. In some embodiments, the pharmaceutical composition may be administered to a tumor present in the subject. In some embodiments, the agent or pharmaceutical composition is administered with an additional cancer therapeutic agent. In some embodiments, the additional cancer therapeutic agent is a chemotherapeutic agent. In some embodiments, the pharmaceutical composition further comprises an additional agent for treatment of cancer. In some embodiments, the additional agent is a tumor vaccine. In certain embodiments, the additional therapeutic agent is a chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CytosanTM); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; emylerumines and memylamelamines including alfretamine, triethylenemelamine, triethylenephosphoramidate, triethylenethiophosphoramidate, and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins

(articularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, foremustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin phili); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromomophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, carrinomyacin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (Adramycin™) (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as demopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptapurine, thiamiprine, thioguanine; pyrimidine analogues such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replinisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; hestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziqune; elformthine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK™; razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-tricUorotriemylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethane; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiopeta; taxoids, e.g.,

paclitaxel (Taxol™, Bristol Meyers Squibb Oncology, Princeton, N.J.) and docetaxel (Taxoteret™, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine (Gemzar™); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; 5 mitroxantrone; vancristine; vinorelbine (Navelbine™); novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeoloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in the definition of “chemotherapeutic agent” are anti-hormonal agents that act to regulate 10 or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including Nolvadex™), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston™); inhibitors of the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, 15 aminoglutethimide, megestrol acetate (Megace™), exemestane, formestane, fadrozole, vorozole (Rivisor™), letrozole (Femara™), and anastrozole (Arimidex™); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprohde, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above. In some embodiments, the additional cancer therapeutic agent is an immune checkpoint inhibitor. 20 Immune Checkpoint inhibition broadly refers to inhibiting the checkpoints that cancer cells can produce to prevent or downregulate an immune response. Examples of immune checkpoint proteins are CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, BTLA, SIRPalpha (CD47), CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, 25 TIGIT, HHLA2, butyrophilins, A2aR, and combinations thereof.

The pharmaceutical compositions and/or agents disclosed herein may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, e.g., those targeted for buccal, sublingual, and systemic 30 absorption, boluses, powders, granules, pastes for application to the tongue; or (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous, intrathecal, intracerebral or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation. Methods of preparing pharmaceutical

formulations or compositions include the step of bringing into association an agent described herein with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association an agent described herein with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Pharmaceutical compositions suitable for parenteral administration comprise one or more agents described herein in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain sugars, alcohols, antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions include water, ethanol, dimethyl sulfoxide (DMSO), polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

Therapeutic Methods

In some aspects, provided herein are methods of treating a cancer by administering to a subject (e.g., to a tumor present in a subject) an agent and/or a pharmaceutical composition described herein. In some aspects, the subject is a human.

In some embodiments, the methods described herein may be used to treat any cancerous or pre-cancerous tumor. In some embodiments, the cancer includes a solid tumor. Cancers that may be treated by methods and compositions provided herein include, but are not limited to, cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma;

basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometrioid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; mammary paget's disease; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; malignant thymoma; malignant ovarian stromal tumor; malignant thecoma; malignant granulosa cell tumor; and malignant robblastoma; sertoli cell carcinoma; malignant leydig cell tumor; malignant lipid cell tumor; malignant paraganglioma; malignant extra-mammary paraganglioma; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; malignant blue nevus; sarcoma; fibrosarcoma; malignant fibrous histiocytoma; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; malignant mixed tumor; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; malignant mesenchymoma; malignant brenner tumor; malignant phyllodes tumor; synovial sarcoma; malignant mesothelioma; dysgerminoma; embryonal carcinoma; malignant teratoma; malignant struma ovarii; choriocarcinoma; malignant mesonephroma; hemangiosarcoma; malignant hemangioendothelioma; kaposi's sarcoma; malignant hemangiopericytoma; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; malignant chondroblastoma; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; malignant odontogenic tumor; ameloblastic odontosarcoma; malignant ameloblastoma; ameloblastic fibrosarcoma; malignant

pinealoma; chordoma; malignant glioma; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor;

5 malignant meningioma; neurofibrosarcoma; malignant neurilemmoma; malignant granular cell tumor; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; small lymphocytic malignant lymphoma; diffuse large cell malignant lymphoma; follicular malignant lymphoma; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small

10 intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

In some embodiments, the subject has cancer. In some embodiments, the cancer

15 comprises a solid tumor. In some embodiments, the tumor is an adenocarcinoma, an adrenal tumor, an anal tumor, a bile duct tumor, a bladder tumor, a bone tumor, a blood born tumor, a brain/CNS tumor, a breast tumor, a cervical tumor, a colorectal tumor, an endometrial tumor, an esophageal tumor, an Ewing tumor, an eye tumor, a gallbladder tumor, a gastrointestinal, a kidney tumor, a laryngeal or hypopharyngeal tumor, a liver

20 tumor, a lung tumor, a mesothelioma tumor, a multiple myeloma tumor, a muscle tumor, a nasopharyngeal tumor, a neuroblastoma, an oral tumor, an osteosarcoma, an ovarian tumor, a pancreatic tumor, a penile tumor, a pituitary tumor, a primary tumor, a prostate tumor, a retinoblastoma, a Rhabdomyosarcoma, a salivary gland tumor, a soft tissue sarcoma, a melanoma (such as uveal melanoma (UVM), a metastatic tumor, a basal cell carcinoma, a

25 Merkel cell tumor, a testicular tumor, a thymus tumor, a thyroid tumor, a uterine tumor, a vaginal tumor, a vulvar tumor, or a Wilms tumor. Actual dosage levels of the active ingredients in the pharmaceutical compositions or agents to be administered may be varied so as to obtain an amount of the active ingredient (e.g., an agent described herein) which is effective to achieve the desired therapeutic response for a particular patient, composition,

30 and mode of administration, without being toxic to the patient.

The compositions disclosed herein may be administered over any period of time effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The period of time may be at

least 1 day, at least 10 days, at least 20 days, at least 30, days, at least 60 days, at least three months, at least six months, at least a year, at least three years, at least five years, or at least ten years. The dose may be administered when needed, sporadically, or at regular intervals. For example, the dose may be administered monthly, weekly, biweekly, triweekly, once a day, or twice a day. In certain embodiments, a dose of the composition is administered at regular intervals over a period of time. In some embodiments, a dose of the composition is administered at least once a week. In some embodiments, a dose of the composition is administered at least twice a week. In certain embodiments, a dose of the composition is administered at least three times a week. In some embodiments, a dose of the composition is administered at least once a day. In some embodiments, a dose of the composition is administered at least twice a day. In some embodiments, doses of the composition are administered for at least 1 week, for at least 2 weeks, for at least 3 weeks, for at least 4 weeks, for at least 1 month, for at least 2 months, for at least 3 months, for at least 4 months, for at least 5 months, for at least 6 months, for at least 1 year, for at least two years, at least three years, or at least five years.

The selected dosage level will depend upon a variety of factors including the activity of the particular agent employed, the route of administration, the time of administration, the rate of excretion or metabolism of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician could prescribe and/or administer doses of the compounds employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

Exemplification

Example 1: Proximity proteomics identifies PD-1 and PIEZO1 association

Previous proteomic studies involving PD-1 pulldown have identified direct binding partners of PD-1 but have not been successful in tracking the time-resolved behavior of

proteins recruited to PD-1 following PD-L1 ligation. To overcome these issues and to test if important factors in PD-1-mediated CD8⁺ T cell inhibition remain unidentified, proximity-labeling combined with multiplexed quantitative mass spectrometry was utilized. This made possible time-resolved detection of proteins lying within ~10nm radius of the PD-1 cytoplasmic tail in the presence or absence of PD-L1 ligation (Fig. 1A). Jurkat cell lines stably expressing human or murine PD-1 fused to an ascorbate peroxidase 2 (APEX2) labeling probe via the C-terminus of the PD-1 cytoplasmic tail was utilized (Fig. 5A). PD-1-APEX2-expressing Jurkat cells were sorted and assessed for labeling efficiency using anti-streptavidin Western blotting (Fig. 5B,C). Tosyl-activated Dynabeads were covalently coupled to CD3 and CD28 crosslinking antibodies using either control murine IgG₁ ligand (mIgG₁, TCR-control) or recombinant PD-L1-Fc fusion protein (TCR-PD-L1) to provide TCR and co-stimulatory signals for T cell activation while also driving strong inhibitory signals through PD-1, respectively (Fig. 6). Following incubation in labeling media containing biotinyl tyramide, PD-1-APEX2-expressing Jurkat cells were co-cultured with TCR-PD-L1 or TCR-control beads for 2, 5, 10 and 20 min while the 0 min condition contained no beads (Fig. 1B). Biotinylation of proteins proximal to the PD-1 cytoplasmic tail was induced by the addition of H₂O₂ to the cell:bead mixture during the last minute of each timepoint (Fig. 1A, Fig. 5C). Cell pellets were lysed and biotinylated proteins were enriched using streptavidin beads under denaturing conditions. Trypsin digest-derived peptides were labelled with tandem mass tags (TMT) and quantified by triple-stage mass spectrometry (MS³)³¹.

To quantify proteins co-localized with PD-1 over time following PD-L1 ligation relative to TCR/CD28 stimulation alone, scaled TMT ratios (TMT RA) of identified peptides for each condition were calculated. A strong 2-3-fold, time-dependent enrichment of SHP2 following TCR-PD-L1 ligation was observed as compared to TCR-control stimulation. Enrichment was observed using either human or murine PD-1 peroxidase fusion proteins, confirming that SHP2 is recruited to PD-1 following ligation (Fig. 1C, Fig. 7A). A robust 2-3-fold enrichment of the mechanosensitive ion channel PIEZO1 following PD-L1 ligation compared to TCR-control stimulation using both human and murine PD-1 peroxidase fusion proteins was observed (Fig. 1D, Fig. 7B): among 1247 quantified proteins, the peptides corresponding to PIEZO1 fell within a small group that associated with PD-1 in a time-dependent manner following PD-L1 ligation. One-way hierarchical

data clustering confirmed near-identical recruitment kinetics for SHP2 and PIEZO1 to PD-1 (Fig. 1H). Thus, following PD-L1 binding to PD-1, PIEZO1 associates with PD-1.

The recruitment kinetics of previously reported mediators and targets of PD-1 signaling, including SHP1, CD3 subunits, CD28 and Zap70 were also identified. While significant enrichment of SHP1 following 10 and 20 min of PD-1 ligation (as compared to TCR stimulation alone) was detected, the degree of enrichment was markedly lower as compared to SHP2 or PIEZO1 (Fig. 1E, Fig. 7C). Although CD3 subunits, CD28 and ZAP70 were identified as binding to PD-1, significant increases in association of these proteins with PD-1 following PD-L1 ligation were not observed. These data suggest that CD3 subunits, CD28 and ZAP70 remain proximal to PD-1 at the immune synapse in the presence or absence of PD-L1 ligation (Fig. 1F-1G, Fig. 7D-I and Fig. 8A-D). One-way hierarchical clustering confirmed different recruitment kinetics for SHP1, CD3 subunits, CD28 or Zap70 (Fig. 1H, Fig. 8E). The limited changes in PD-1 binding by known mediators as compared to the robust, ligand-dependent association of PIEZO1 and PD-1 implicates an uncharacterized mechanism of PD-1 that involves PIEZO1 function.

To further assess the landscape of protein localization changes proximal to PD-1, gProfiler g:OSt gene ontology (GO) term functional enrichment analysis was used to compare pathways that were enriched in TCR-PD-L1 or TCR-control stimulated conditions. It was found that clathrin-mediated endocytosis and membrane trafficking were among the most significantly enriched pathways in TCR-PD-L1 conditions as compared to TCR-controls, suggesting that PD-1 ligation may alter receptor clustering and turnover in the plasma membrane to prevent stable contact with antigen presenting cells at the immune synapse (Fig. 1I). As anticipated, signaling pathways involved in TCR and immune response activation, as well as actin cytoskeleton organization, were enriched in TCR-control conditions compared to those treated with TCR-PD-L1 beads (Fig. 1I). When the mean slope for each gene across all time points was calculated in both TCR-PD-L1 and TCR-control conditions as a means to rank the most highly enriched proteins, SHP2 and PIEZO1 ranked highest along with sorting nexins involved in intracellular trafficking (SNX12), actin polymerizing subunits (ARPC2, TFG) and membrane transporters (SLC34A1, SLC44A1) (Fig. 1J). In addition, the observed enrichment for PD-1 following PD-L1 ligation signifies an increase in local PD-1 concentration, indicating clustering of PD-1 molecules that undergo enhanced biotinylation (Fig. 1J). As expected, various TCR- and CD28-related genes enriched in the TCR-control-treated samples, including CD28,

CD3 ζ , ZAP70, VAV3 and PI3K-related proteins were identified (Fig. 1J). Collectively, these data highlight the changing membrane dynamics that occur following TCR stimulation and PD-L1 ligation in T cells, which not only confirm previous findings, but also identify the association between PD-1 and PIEZO1.

5 **Example 2: PD-1 ligation inhibits PIEZO1-mediated Ca²⁺ influx**

Since PIEZO1 activation has been shown to promote optimal TCR signaling and PD-1 ligation attenuates T cell function, it was hypothesized that PD-1 association with PIEZO1 would result in PIEZO1 inhibition and thereby impair T cell activation. Upon TCR stimulation, there is a tightly regulated response to Ca²⁺ influx, with Ca²⁺ release-activated channels (CRAC) and voltage gated Ca²⁺ channels being necessary for the TCR signaling cascade. Due to the involvement of multiple Ca²⁺ channels, uncoupling the individual contribution of each ion channel during T cell activation is challenging. The role of PIEZO1 in T cell activation and its regulation by PD-1 using a Jurkat cell line stably expressing an inducible, fluorescent PIEZO1-specific activity reporter (GenEPi) was explored. GenEPi contains a low affinity and high dynamic range GFP, calmodulin and M13 peptide fusion protein (GCaMP) engineered to ensure the specificity of sensing PIEZO1-mediated Ca²⁺ influx. The GenEPi reporter was expressed using a Tet-On system and is comprised of GCaMP fused to the cytoplasmic tail of PIEZO1 using a flexible linker (Fig. 2A). GenEPi Jurkat cells exhibited increasing levels of reporter expression and PIEZO1 activity with increasing doses of doxycycline in the presence of the PIEZO1 agonist Yoda1, verifying the inducibility and functional specificity of GenEPi (Fig. 9A).

To visualize and compare changes in PIEZO1 activity in the presence or absence of PD-1 ligation, PD-1-expressing GenEPi Jurkat cells were passed through a flow-cell chamber coated with crosslinking CD3 and CD28 antibodies and either PD-L1 or control mIgG₁ ligand (Fig. 9B). Using Total Internal Reflection Fluorescence (TIRF) time lapse imaging, the intensity of PIEZO1 activity and PD-1 expression were assessed at the ligand-cell interface at various times after TCR-control and TCR-PD-L1 stimulation (Fig. 2B and 2C and Fig. 9B). It was found that PIEZO1 activation in GenEPi Jurkat cells ligated with PD-L1 was delayed and had lower intensity overall as compared to TCR-stimulated cells, while PD-1 intensity remained the same in both conditions, suggesting that PD-1 inhibits PIEZO1 activation (Fig. 2B and 2E, Fig. 9C). Specifically, GenEPi Jurkat cells exhibited strong PIEZO1 activity following 100 s of TCR stimulation that peaked at 200 s and

persisted for 5 min (300 s), further confirming that TCR stimulation induces PIEZO1 activity (Fig. 2B, and 2E-2F, Fig. 9D, and captured via extended videos, incorporated herein by reference). This activity coincided with F-actin ring formation, where PIEZO1 activity appeared to localize around F-actin ring structures (Fig. 2B). A significant decrease in PIEZO1 activity over time in the presence of PD-L1 was observed; PIEZO1 activity was visible starting at 140 s, but remained less intense than in TCR-stimulated conditions throughout the time course (Fig. 2C, and 2E-2F, and captured via extended videos, incorporated herein by reference). Interestingly, local PIEZO1 activity was significantly decreased upon PD-1 ligation in the presence of PD-L1 at PD-1 clustering sites during the process of immune synapse and F-actin ring formation implying that PD-1 ligation results in the attenuation of PIEZO1 activity through physical association (Fig. 2C, 2G-2K).

In complementary studies flow cytometry was used to assess changes in PIEZO1 activity following PD-1 ligation by quantifying mean fluorescence intensity (MFI) of GCaMP fluorescence and frequency of GFP-positive, PD-1-expressing GenEPi Jurkat cells treated with TCR-control or TCR-PD-L1 beads. PD-1-expressing GenEPi Jurkat cells exhibited a significant decrease in PIEZO1 activity following exposure to TCR-PD-L1 beads compared to GenEPi Jurkat cells treated with TCR-control beads or PIEZO1 agonist Yoda1 (Fig. 9E-G). Together, these data imply that contractile F-actin rings formed at the immune synapse provide sufficient force to gate PIEZO1 in an open conformation, thereby sustaining Ca^{2+} influx for optimal TCR signaling (Fig. 2C). Importantly, it was demonstrated that this activity is inhibited by PD-1 ligation (Fig. 2C).

Example 3: CD8⁺-specific PIEZO1 KO impairs antitumor immunity

To investigate PD-1 and PIEZO1 function specifically in CD8⁺ T cells, a tamoxifen-inducible CD8⁺-specific PIEZO1 KO mouse was developed by crossing *Piezo1*^{flx/flx} mice with E8i-Cre-ER^{T2} Rosa26 tdTomato reporter mice (Extended Data Fig. 6a). Following tamoxifen treatment, the efficacy of PIEZO1 deletion was ~70% in Cre⁺ CD8⁺ T cells as compared to Cre⁻ controls, as assessed by RT-qPCR using probes specific for the loxP regions flanking exons 20-23 of PIEZO1 (Fig. 10B). The frequency of CD8⁺ T cells expressing Cre protein was consistent with decreased PIEZO1 transcript levels assessed by RT-qPCR (Fig. 10B-D). As an additional control, the level of Cre reporter in CD8⁺ splenocytes was assessed on day 15 following tumor implantation and observed sustained Cre activity in Cre⁺ CD8⁺ T cells, confirming the efficacy of our inducible Cre system (Fig.

10E,F). Functionally, a significant decrease in PIEZO-1-specific Ca^{2+} influx induced by Yoda1 treatment (visualized using the cell permeable Ca^{2+} dye Indo-1) was observed in $\text{Cre}^+ \text{CD8}^+$ T cells compared to $\text{Cre}^- \text{CD8}^+$ T cells; Fig. 10G). *In vitro* stimulation of tamoxifen-treated $\text{Cre}^+ \text{E8i-Cre-ER}^{\text{T2}} \text{Piezo1}^{\text{flx/flx}} \text{CD8}^+$ T cells with a titration of crosslinking CD3 and CD28 antibodies revealed slight decreases in viability, CD44, granzyme B and $\text{IFN}\gamma/\text{TNF}\alpha$ co-expression, as well as increased expression of CD62L at higher CD3/CD28 stimulation concentrations compared to controls (Extended Data Fig. 7a,d-f,h), suggesting that PIEZO1 KO impairs CD8^+ T cell activation and effector function. Significant differences were not observed in PD-1, CTLA4 and Ki-67 expression or production of IL-2, $\text{IFN}\gamma$ or $\text{TNF}\alpha$ between the two groups (Fig. 11B-C, G, I-K).

Given the increase in PIEZO1 activity observed following TCR stimulation (Fig. 2D), it was hypothesized that loss of PIEZO1 in CD8^+ T cells would lead to more rapid tumor growth as a consequence of impaired T cell activation. Indeed, when MC38 or B16-OVA tumor cells were implanted in tamoxifen-treated $\text{E8i-Cre-ER}^{\text{T2}} \text{Piezo1}^{\text{flx/flx}}$ mice and tumor growth was monitored, loss of PIEZO1 in CD8^+ T cells alone was sufficient to drive significantly increased tumor burden in Cre^+ mice as compared to Cre^- controls in both tumor models (Fig. 3A-3C). There also was a significant decrease in CD8^+ T cell frequencies (Fig. 3D-3E and Fig. 12A), an increase in total CD4^+ T cell frequencies and lower $\text{CD8}^+/\text{CD4}^+$ T cell ratios in the tumor with no significant changes in CD3e frequencies (Fig. 12A-G). In addition, $\text{Cre}^+ \text{CD8}^+$ TILs from MC38 tumors expressed significantly more CD62L and significantly less PD-1, CTLA4 and CD69 compared to Cre^- controls (Fig. 3F and Fig. 12H-J). $\text{Cre}^+ \text{CD8}^+$ TILs from B16-OVA tumors also expressed significantly more CD62L and significantly less CTLA4 as compared to Cre^- controls but did not show significant differences in the frequencies of PD-1- or CD69-expressing cells although both trended toward decreased frequency (Fig. 3G and Fig. 12K-M). Moreover, $\text{Cre}^+ \text{CD8}^+$ TILs from B16-OVA tumors expressed significantly less granzyme B. CD8^+ TILs from MC38 tumors also expressed less granzyme B, but this trend was not significant (Fig. 12N,O). Interestingly, PIEZO1 KO CD8^+ TILs from MC38 and B16-OVA models consistently retained high expression of Slamf6 and had minimal expression of TIM-3 (Fig. 3H and 3I, and Fig. 12P-S), suggesting that PIEZO1 is involved in regulating the transition of Slamf6 progenitor cells to TIM-3 effector-like CD8^+ TILs, thereby controlling exhausted CD8^+ T cell subpopulations. Together, these findings demonstrate that loss of PIEZO1

activity in CD8⁺ T cells decreases their activation and acquisition of effector functions to impair antitumor immunity and promote tumor growth, similar to PD-1-mediated inhibition of T cell activation.

To further study PD-1 regulation of PIEZO1, E8i-CRE-ER^{T2} *Piezo1*^{flx/flx} Cre⁺ and
 5 Cre⁻ tumor-bearing mice were treated with anti-PD-1 or isotype control antibody (Fig. 3J). Strikingly, administration of PD-1 blocking antibody on days 14 and 17 reduced clearance of MC38 tumors, which are highly sensitive to PD-1 blockade, in E8i-CRE-ER^{T2} *Piezo1*^{flx/flx} Cre⁺ mice compared to anti-PD-1 treated Cre⁻ mice (Fig. 3K and Fig. 13A). Administration of PD-1 blockade on days 10 and 13 following tumor implantation did not significantly
 10 reduce growth of B16-OVA tumors in E8i-CRE-ER^{T2} *Piezo1*^{flx/flx} Cre⁺ mice in contrast to marked tumor control in anti-PD-1 treated Cre⁻ mice (Fig. 3J,L, and Fig. 13B). PIEZO1 KO in CD8⁺ T cells significantly impaired overall survival in both tumor models regardless of PD-1 blockade (Fig. 3M and 3N). Thus, PD-1-mediated inhibition of PIEZO1 in CD8⁺ T cells contributes to immune evasion by tumors, underscoring the importance of PIEZO1
 15 function in shaping T cell responses.

Example 4: PIEZO1 agonism improves CD8⁺ T cell antitumor immunity

The impaired antitumor immunity observed in CD8⁺-specific PIEZO1 KO mice led us to ask whether PIEZO1 agonism would enhance antitumor immunity. To answer this question, MC38 tumors were implanted subcutaneously in WT mice and administered
 20 Yoda1 (7.5mg/kg) intraperitoneally (i.p.) (Fig. 4A). While Yoda1 treatment had variable effects on MC38 tumor growth in WT mice (Fig. 14A-B), phenotypic analysis of the TILs at day 15 following MC38 tumor implantation revealed increased percentages of CD8⁺ TILs in Yoda1-treated mice compared to controls (Fig. 4B). Additionally, Yoda1-treated CD8⁺ TILs expressed more activation and effector proteins, including PD-1, granzyme B and
 25 perforin and lower expression of CD62L (Fig. 4C-F). PIEZO1-agonized CD8⁺ TILs expressed high levels of terminal exhaustion marker TIM-3 and low levels of progenitor stem-like marker Slamf6 compared to controls, further suggesting a role for PIEZO1 in driving an effector-exhausted phenotype (Fig. 4G, Fig. 14C-D).

Next considered was if the increased CD8⁺ T cell effector function observed in
 30 PIEZO1-agonized CD8⁺ TILs would improve the efficacy of PD-1 blockade. To assess the effects of combined anti-PD-1 and PIEZO1 agonist Yoda1 treatment, the B16.F10 tumor model was chosen because it is unresponsive to PD-1 blockade (Fig. 4H). Mice treated with

either PD-1 blockade or Yoda1 alone did not show decreased tumor growth (Fig. 4I and 4J). In marked contrast, tumor growth was significantly attenuated when PD-1 blockade and Yoda1 were administered together (Fig. 4I and 4J). Moreover, combined anti-PD-1 and Yoda1 treatment significantly improved survival in B16.F10 tumor-bearing mice as compared to single and negative controls, such that 40% of mice treated with combination therapy – but not mice treated with monotherapy – survived beyond 30 days (Fig. 4K). These findings point to PIEZO1 agonism as a potential avenue for therapeutic intervention in cancer.

Example 5: Discussion

While PD-1 signaling counters T cell activation, the understanding of the signaling mechanisms by which PD-1 exerts its inhibitory functions is incomplete. Herein a novel function was identified for PD-1 in restraining T cell signals driven by a mechanosensitive ion channel. Specifically, PD-1 ligation induces co-localization of PD-1 with PIEZO1 and this association impairs PIEZO1 activation and subsequent Ca^{2+} influx that drives T cell activation, acquisition of effector functions and key cell fate decisions. Knockout of PIEZO1 in murine CD8^+ T cells alone is sufficient to impair CD8^+ TIL-mediated tumor control and this cannot be overcome by PD-1 blockade. Conversely, PIEZO1-agonism improves CD8^+ T cell activation and function in the TME and has significant therapeutic benefit in mouse models when combined with PD-1 blockade to control tumor growth. These findings are the first to document coinhibitory receptor-mediated negative regulation of a mechanosensor and highlight PIEZO1 agonism in combination with PD-1 blockade as a possible therapeutic approach for targeting immune checkpoint blockade-resistant cancers.

Recent studies have demonstrated that PIEZO1-driven Ca^{2+} influx in T cells leads to calpain activation and reorganization of cortical F-actin scaffolding, linking PIEZO1 to optimal TCR signaling. In contrast, PD-1 ligation has been shown to impair F-actin clearance from the cellular interface, reduce Ca^{2+} signaling and abrogate tumor cell killing through decreased tumor-T cell couplings. These findings confirm these opposing functions of PD-1 and PIEZO1. Based on the data, PD-1 targets PIEZO1 to prevent optimal T cell activation through inhibition of Ca^{2+} influx and destabilization of the immune synapse (Fig. 2). This model is supported by the *in vitro* studies, which demonstrate that PD-1 ligation inhibits PIEZO1 at the immune synapse, and the *in vivo* studies provided herein, which

highlight the necessity of PIEZO1 for promoting optimal CD8⁺ TIL function in tumor models and the inability of PD-1 blockade to reverse accelerated tumor growth resulting from PIEZO1 KO in CD8⁺ T cells (Fig. 2, 3). These findings imply that expression of PIEZO1 is required for PD-1 to fully execute T cell inhibition (Fig. 3K-3N). Although
5 PIEZO1 agonism alone is not sufficient to consistently decrease tumor burden in WT mice (Fig. 14A,B), combining PD-1 blockade with PIEZO1 agonism improved tumor control in PD-1 blockade-unresponsive tumors (Fig. 4H-4K). The effectiveness of this combination may result from 1) prevention of PIEZO1 inactivation as well as inhibitory phosphatase recruitment by PD-1 blockade and 2) PIEZO1 agonism further increasing the number and
10 function of cytotoxic CD8⁺ T cells in the tumor, leading to enhanced tumor control.

PD-1-mediated inhibition of PIEZO1 is demonstrated herein (Fig. 2). PIEZO1 activity may be regulated by TCR-induced cytoskeletal rearrangements, specifically retrograde actin flow, which is supported by the data capturing PIEZO1 activity concentrated around F-actin ring structures. The mechanism by which Ca²⁺ influx is
15 sustained to induce specific gene transcription following CRAC channel activation in T cells remains unclear. Previous studies have proposed a positive feedback loop between F-actin polymerization and Ca²⁺ influx, which persists over hours at the immune synapse to maintain TCR signaling⁷. It is possible that actomyosin contractile forces modulate PIEZO1 to sustain Ca²⁺ influx, resulting in downstream gene transcription. Moreover, the proximity
20 proteomics experiments demonstrate PD-1 co-localization with various proteins involved in actin polymerization, membrane trafficking and endocytosis (Fig. 1H-J). Thus, PD-1 sequestration of actin cytoskeletal regulatory proteins and proteins that mediate membrane turnover may play a role in impairing actin cytoskeletal dynamics and stability at the immune synapse, thereby attenuating actomyosin contractile forces that activate PIEZO1.

25 Although previous proteomic methods have been used to characterize PD-1-mediated T cell inhibition, PD-1 co-localization with PIEZO1 and subsequent inhibition of PIEZO1 have never been documented. However, rather than studying PD-1 in isolation, insight into the spatial and temporal behavior of proteins involved in PD-1 signaling at the membrane was gained by using a multiplexed proximity proteomic approach that assesses
30 changes in membrane dynamics, receptor clustering and protein recruitment immediately proximal to PD-1. Given the dynamic nature of immune cell receptor signaling, high-resolution proximity proteomic methods may be instrumental in studying additional

macromolecular immunoreceptor complexes for which, despite clinical relevance, signaling remains largely unclear.

In summary, a new mechanism by which PD-1 exerts its inhibitory signals through negative regulation of the mechanosensitive ion channel PIEZO1 is described herein. These studies are the first to demonstrate PD-1 co-localization with PIEZO1 following PD-L1 ligation. This association results in the inhibition of PIEZO1-mediated Ca^{2+} influx, while TCR/CD28 stimulation in the absence of PD-L1 ligation promotes PIEZO1 activity around F-actin rings. The selective inhibition of PIEZO1 by PD-1 illuminates the significance of PIEZO1 in regulating CD8^+ T cell function and introduces a novel receptor target class. Moreover, these findings in mice show that modulating PIEZO1 activity influences the anti-tumor activity of CD8^+ T cells, subsequently affecting tumor growth. Strikingly, combined therapy using PIEZO1 agonist and PD-1 blockade improved tumor control in mice harboring tumors unresponsive to PD-1 blockade. Together, these studies expand our understanding of the diverse mechanisms by which PD-1 inhibits T cell function, identify a novel role for PIEZO1 in controlling CD8^+ T cell function and reveal a new combination therapeutic strategy using PD-1 blockade and PIEZO1 agonism. Further understanding of the mechanisms controlling PD-1 and PIEZO1 association, the specific downstream pathways involved and the effects of external mechanical force on this biology should provide additional fundamental and therapeutic insights.

Example 6: Methods

Cell lines

Jurkat E6.1 and human PD-1-expressing Jurkat E6.1 cells (35.8 line transduced with an ORF-T2A/IRES-GFP reporter) (a gift from N. Haining/K. Yates, Dana Farber Cancer Institute) were cultured in RPMI media supplemented with 10% FBS, 1% penicillin/streptomycin, 1% HEPES and 0.1% BME (denoted R10). MC38-OVA (a gift from N. Haining/N. Collins, Dana-Farber Cancer Institute), B16-OVA (generated in collaboration with the N. Haining lab), MC38-WT (a gift from D. Vignali, University of Pittsburgh School of Medicine), B16.F10 (a gift from G. Dranoff, Novartis Institutes for Biomedical Research, Cambridge, MA) and 293x (a gift from C. Kadoch, Dana-Farber Cancer Institute) cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 20 $\mu\text{g}/\text{mL}$ gentamicin. MC38-OVA and B16-OVA cells were

produced by transduction of parental MC38 and B16.F10 cells with the lentiviral vector TRC-pLX305 (Broad Institute) containing OVA protein.

PD-1-APEX2 plasmid design, cloning and stable expression

5 PD-1 sequences were obtained from NCBI and were modified using the codon optimization tool from Integrated DNA Technologies (IDT). APEX2 sequences have been previously described¹⁷. PD-1-APEX2 fusion sequences contain human or murine PD-1 linked to APEX2 on the C-terminal tail by an 8-residue serine-glycine linker (GGSSGGSS) and a FLAG-TAG added to the C-terminal tail of APEX2. All human and murine PD-1-
10 APEX2 double-stranded gene fragments (gBlocks) were generated by IDT. Cloning of gBlock gene fragments into the pXPR_053 plasmid (Addgene #113591) was completed by GenScript.

To generate stable PD-1-APEX2-expressing Jurkat cell lines, 293x cells were transfected with PD-1-APEX2 fusion plasmids along with PsPax2 and MD2G packaging
15 plasmids suspended in Optimem (Life Technologies #31985-062) and polyethylamine “Max” (PEI, Polysciences #24765-2), and supplemented with fresh media after 24 h. Lentivirus was harvested 48 h following media supplementation by ultracentrifugation for 2 h at 71,934.8 RCF at 4°C. Viral titer was calculated using serial dilutions of the viral stock on 293x cells in DMEM complete media containing PEI “Max”. A multiplicity of infection
20 of 30 was used to spin transduce Jurkat cells for 2 h at 652 RCF at 37°C. Jurkat cells were stained with human PD-1 surface antibody (BioLegend, clone EH12.2H7, #329936) and evaluated for PD-1 expression using flow cytometry 24 h and 48 h following transduction. Highly expressing PD-1-APEX2 fusion clones were sorted on the BD Aria II. Stocks were frozen in R10 media containing 10% dimethyl sulfoxide (DMSO) for future experiments.

25 *APEX2 proximity labeling and sample processing*

PD-1-APEX2-expressing Jurkat cells were cultured at a concentration of 1 million cells/mL in labeling media (RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, 1% HEPES, 0.1% BME and 500µM biotinyl tyramide purchased
30 from Toronto Research Chemicals) for 2 h. TCR-PD-L1 or TCR-control Dynabeads (see supplemental methods for PD-L1 bead generation) were added to PD-1-APEX2-expressing Jurkat cells at a ratio of 1:4 cells/beads for the specified timepoints. No beads were added to

the 0 min control to assess protein localization at baseline. Following each timepoint, hydrogen peroxide (H₂O₂, Sigma Aldrich) was added at a final concentration of 1mM for 1 min to each condition not including the no H₂O₂-treated control used to quantify background labelling. The labeling reaction was quenched using 2X quenching buffer (DPBS supplemented with 20mM sodium ascorbate, 10mM trolox and 20mM sodium azide) and cells were spun down immediately at 726 RCF for 2 min. Cells were immediately washed three times with ice-cold 1X quenching buffer and spun down at 726 RCF for 2 min after each wash. Following the third 1X quenching wash, cells were washed with 10 mL ice-cold PBS and 1 million cells were taken for Western blotting analysis. Cells were then spun down a final time at 726 RCF for 2 min. All PBS was removed from cell pellets, which were flash-frozen on dry ice and stored at -80°C until processing and streptavidin pulldown.

PD-1-APEX2-expressing Jurkat cell pellets were processed using previously described methods with slight modification³. Cell pellets were lysed in filtered 8M urea with 1% sodium dodecyl sulfate (SDS) at room temperature for 15 min. 55% ice-cold trichloroacetic acid (TCA) was added to lysates at a 1:1 ratio and left on ice for 15 min to precipitate the protein. Lysates were spun down at 20,817 RCF for 15 min at room temperature to prevent urea from precipitating out of solution. The supernatant was discarded, and protein pellets were washed 4X with ice-cold acetone and spun down at 20,817 RCF for 10 min following each wash. Protein pellets were then air-dried and resuspended in 8M urea lysis buffer supplemented with 1% SDS and 10mM tris(2-carboxyethyl)phosphine (TCEP, Sigma) and 100 mM NH₄CO₃. Pellets were water-bath sonicated for 3 cycles of 30 s and vortexed mixed at 37°C for 2 h until the pellets were completely resuspended. Redissolved pellets were checked for an alkaline pH and then spun down at 20,817 RCF for 15 min at room temperature. Clear supernatants were transferred to new microcentrifuge tubes and freshly prepared 50mM ammonium bicarbonate (NH₄CO₃) containing 400mM iodoacetamide was added to each sample at a final concentration of 20mM iodoacetamide. Samples were immediately vortexed and incubated in the dark at room temperature for 25 min. During the incubation period, streptavidin beads (Pierce) were washed twice with 4M urea and 0.5% SDS. Alkylation of cysteine residues was quenched using a solution of 100mM dithiothreitol (DTT) containing streptavidin beads such that each sample received 100μL beads and reached a final

concentration of 50mM DTT, 4M urea and 0.5% SDS (1:1). Samples containing streptavidin beads were rotated overnight at 4°C and the following day were applied to the Dyna-Mag-2 to remove the supernatant. Beads were washed with 4M urea and 0.5% SDS and transferred to new Eppendorf tubes to decrease background signal. Beads were washed
5 3X more with 4M urea and 0.5% SDS followed by 3 washes with 4M urea to remove all detergent.

Mass spectrometry sample preparation

Streptavidin beads were digested with lysyl endopeptidase (LysC, 2mg/mL, Wako) in 50µL 200mM 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (EPPS) pH 8.5 and 2% acetonitrile at 37°C. Trypsin (50µL stock, Promega #V5111, in EPPS buffer and at a final dilution of 1:100) was added for additional digest overnight at 37°C. Beads were removed with a magnetic rack and clear supernatants were transferred to new tubes. Peptide digest reactions were directly labelled with TMT11plex (Thermo Fisher Scientific
15 #A34808) reagents in 200mM EPPS pH 8.5, 30% acetonitrile for 1 h at room temperature. Labeling efficiency was measured by MS of mixed small aliquots of the labeling reactions and was >95%. Frozen and thawed TMT labeling reactions were quenched with 0.3% hydroxylamine for 15 min at room temperature. Reactions were then mixed, dried in a speed vac centrifuge to near completion and subjected to alkaline reversed phase
20 fractionation (Thermo Fisher Scientific #84868) with 12 elution subsequent fractions of 10%, 12.5%, 15%, 17.5%, 20%, 25%, 30%, 35%, 40%, 50%, 65% and 80% acetonitrile. Fraction pairs 1+7, 2+8, 3+9, 4+10, 5+11, 6+12 were mixed, dried down to completion, desalted with Stage tips and run on Orbitrap Lumos mass spectrometers (Thermo Fisher Scientific).

25

Mass spectrometry analysis

Data collection followed a synchronous precursor selection (SPS) MS³ TMT method⁴⁹. Peptides were separated prior to the electrospray ionization with a Proxeon EASY-nLC1200 system over a ~35cm capillary column of 100µm inner diameter packed
30 with Accucore C18 beads (2.6 µm, 150Å, Thermo Fisher Scientific). Service MS¹ scans were performed in the Orbitrap at a resolution of 120,000, mass range 400-1400 thomson (Th). After collision induced dissociation (CID, CE=35) and using an isolation window of

0.4 m/z , MS² was performed in the ion-trap with maximum injection times of 150-400 ms. For MS³ quantification, precursors were selected following a Top10 method followed by high-energy collision-induced dissociation (HCD, CE=65). Orbitrap MS³ analysis was done at a resolution of 60,000 at 200 Th with varying injection times of up to 650 ms and charge-state dependent variable isolation windows from 0.7 to 1.2 Da as described previously⁵⁰. Peptide-spectrum matches (PSM) were obtained by a SEQUEST (v.28, rev.12) based software, searching a database with respective UniProt mouse and human reference proteomes with added common contaminants and reverse peptide sequences as decoy. After mzXML conversion of spectra, searches used a mass tolerance of 20 p.p.m. for precursors and a fragment-ion tolerance of 0.9 Da. Searches allowed for up to two missed trypsin cleavage sites with dynamic modification of oxidized methionines (+15.9949 Da) and static peptide N-terminal and lysine modifications with TMT11 (+229.1629 Da). PSM were filtered by linear discriminant analysis with a false discovery rate (FDR) of 1% and a following FDR of 1% for collapsed proteins. MS¹ data were calibrated post search and searched again. TMT signal to noise quantification for peptides was filtered for an MS² isolation specificity of 70% or greater and a summed signal to noise of 200 or greater for all TMT channels for each peptide. Details of TMT intensity quantification applied were described previously⁵⁰. Scaled quantification data were analyzed by one-way clustering (Ward's method) using the JMP Pro statistical software package. Proteomics raw data and search results were deposited in the PRIDE archive⁵¹ and can be accessed under ProteomeXchange⁵² accession numbers PXD036136, PXD036207 and PXD036147 for human PD-1-APEX2 experiments and PXD036218 and PXD036216 for murine PD-1-APEX2 experiments.

Summary statistics and GO Term analysis

Raw data from three independent proximity labeling time course experiments were normalized to ACACA. Proteins that were not identified in all three experiments were excluded from the analysis. Each protein was then normalized to its respective 0 min control condition and the mean slope over time for TCR-control and TCR-PD-L1 conditions was calculated for each protein, where larger slope values indicate a stronger association with PD-1. The top 10 mean slope values for TCR-control and TCR-PD-L1 conditions were reported in Fig. 1j. The mean slope ratios between TCR-PD-L1/TCR-control and TCR-control/TCR-PD-L1 conditions were then calculated for all proteins to

assess protein enrichment per condition. The top 50 proteins were selected for GO Term functional enrichment analysis using g-Profiler g:GOST functional profiling³³. Adjusted p-values of significantly enriched functions for TCR-control and TCR-PD-L1 conditions were reported in Fig. 1i.

5

Jurkat GenEPi nucleofection and stable clone selection

The GenEPi reporter plasmid (XLGenEPi) was generated by the Pantazis lab. GenEPi reporter expression was driven by a doxycycline-inducible Tet-On system. Jurkat cells were co-nucleofected with the XLGenEPi plasmid and pCMV_pBase at a molecular ratio of 1:1 using Lonza's SE Cell line kit (cat. V4XC-2024) and corresponding CL-120 program on Lonza's Amaxa-4D Nucleofector. The pCMV_pBase plasmid is a non-viral vector that expresses the piggyBac transposase (pBSe) which, when co-transfected, allows for the integration of the GenEPi transgene into the genome using two terminal repeat domains flanking the GenEPi reporter⁵³. This integration into the genome allows for the generation of stable GenEPi-expressing clones that can be selected for using blasticidin (BSD). 2µg of XLGenEPi and equimolecular amounts of pCMV_pBase plasmids were used to nucleofect 1 million Jurkat cells in cuvettes. Cells were rested in complete RPMI media (RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, 1% HEPES, 0.1% BME, 1mM sodium pyruvate and 4.5g/L of glucose) for 24 h following nucleofection. To generate stable clones, GenEPi nucleofected cells were selected with 8µg/mL BSD for 7 days. Cells were then rested in complete RPMI media for 24 h and treated with 200 ng/mL doxycycline for 24 h to induce GenEPi reporter expression. Selected Jurkat cells were then treated with 5µM Yoda1 and GFP-positive clones were immediately sorted on the BD FACS Aria and cultured to generate stable cell lines.

25

Flow cell chamber preparation and TIRF imaging

6-channel µ-Slide VI Glass Bottom slides (Ibidi #80607) were coated with Poly-D-Lysine for 1 h at room temperature. Excess Poly-D-Lysine was removed from the channels and the channels were washed 4X with PBS. Chambers were then coated with 2µg/mL CD3 and CD28 crosslinking antibodies and 4µg/mL PD-L1 or mIgG₁ overnight at 4°C. Slides were then washed 4X with PBS and 80µL of PBS was added to the flow cell chamber to prevent drying out prior to use.

30

Stable GenEPi Jurkat cells were treated with ImmunoCult Human CD3/CD28 T cell activator (STEMCELL Technologies #10971) for 48 h prior to imaging to induce PD-1 expression. GenEPi Jurkat cells were treated with doxycycline (200ng/mL) for 24 h prior to imaging to temporally control and induce GenEPi expression. GenEPi Jurkat cells were then stained with F-actin SPY550-FastAct (1:500, Cytoskeleton #CY-SC205) and anti-human PD-1 AF647 (1:50, Biolegend #329910 clone EH12.2H7) for 1 h at 37°C in PBS supplemented with 10% FBS (PBS-F). Cells were washed twice with PBS-F and resuspended at a concentration of 1 million/mL in complete RPMI media.

TIRF imaging was performed using a fully motorized Nikon Ti inverted microscope equipped with a Nikon Ti-TIRF-EM Motorized Illuminator and a Nikon LUN-F Laser Launch with single fiber output (488nm, 90mW; 561 nm, 70mW; 640nm, 65mW). To immobilize the sample, 80 µL of stained XLGenEPi Jurkat cells were flowed into one of the 6 coated µ-Slide channels (Ibidi #80607). Flow was established by capillarity using a Kimwipe. Imaging was performed under static conditions (e.g., not flow). Images were collected using an Apo TIRF 100x/1.49 DIC oil immersion objective lens with Nikon NF immersion oil, adjusting the correction collar to minimize spherical aberration. Images were captured with an Andor Zyla 4.2 Plus sCMOS monochrome camera using the 16-bit dual gain digitizer mode, 540 MHz readout rate and 2x2 pixel binning (resulting pixel size 0.1 µm/px) and Nikon Elements Acquisition Software AR 5.02. The TIR angle for each channel was adjusted using fluorescence beads in the same flow chambers as used in the experiments and verified with a control sample. Signals from the different channels were acquired sequentially using a Chroma ZT 405/488/561/640 multi-band pass dichroic mirror mounted on a Nikon TIRF filter cube located in the filter cube turret, and band pass emission filters for GenEPi AF488 (Chroma ET525/50m), SPY5550-FastAct (Chroma ET 595/50m) and PD-1 AF647 (Chroma ET 655lp) channels, respectively, located on a Sutter emission filter wheel within the infinity space of the stand. Time-lapse imaging was performed with a time interval of 20 s and total acquisition time of 5 min. An ND16 filter was introduced in the light-path to reduce irradiation. Imaging conditions were optimized to reduced photobleaching and phototoxicity.

Fiji analysis for TIRF imaging

TIRF image analysis was completed using a custom workflow built in Fiji/ImageJ⁵⁴. The TIRF images were opened in Fiji and split into the different fluorescent channels. A 500 pixel rolling ball background subtraction was applied to reduce background intensities close to 0. The individual PD-1 and PIEZO1 channels were processed by performing a Tophat/difference of Gaussian filter to emphasize the small puncta. The processed images were segmented by intensity-based thresholding. A close filter followed by a size filter was applied to the thresholded masks to generate the final masks for PD-1 and PIEZO1. Regions of interest (ROI) were then drawn to demarcate cell boundaries to calculate the parameters on a per cell basis. The mask areas, the intensities of the signal and all ROIs were measured. Normalized fluorescence intensity for PIEZO1 activity was calculated from the sum of pixel values within each ROI for TCR-control and TCR-PD-L1 conditions. Only cells expressing both PD-1 and the PIEZO1 GenEPi reporter were analyzed. Representative images were processed using Nikon Imaging Software (NIS) and Fiji/ImageJ. AI-denoise was applied to F-actin channels using NIS. A 25 pixel rolling ball background subtraction was then applied to F-actin channels in Fiji to reduce background. A 50 pixel rolling ball background subtraction was applied to GenEPi reporter and PD-1 channels in Fiji to reduce background. A median filter of 1 pixel was applied to GenEPi reporter and PD-1 channels in Fiji. Gamma adjustments of 0.8 for PD-1 and GenEPi channels and 1.0 for the F-actin channel were also applied to supplemental videos. Coloring for supplemental videos was adjusted such that PD-1 is displayed in magenta, GenEPi in yellow and F-actin in cyan to clearly convey changes in each channel when merged.

Mice

Seven- to ten-week-old age-matched female or male mice were used for all *in vivo* experiments and seven- to fourteen-week-old mice were used for CD8⁺ T cell isolation and *in vitro* experimentation. Wild type C57BL/6J mice were purchased from Jackson Laboratories. E8I-Cre-ER^{T2} mice were a generous gift from the Vignali lab. Homozygous E8I-Cre-ER^{T2} mice were crossed with homozygous Piezo1^{flx/flx} mice purchased from Jackson Laboratories (Stock #029213). Mice were crossed until they were fixed for the Piezo1^{flx/flx} mutant allele as assessed by the Transnetyx genotyping service. The Piezo1-2 WT probe was used to detect the WT *Piezo1* allele (forward primer: CTGTCCCCTTCCCCATCAAG; reverse primer: GGGTCCAGGGTAGACAACAG). The

L1L2-Bact-P MD probe (forward primer: GCTGGCGCCGGAAC; reverse primer: GCGACTATAGAGATATCAACCACTTTGT) was used to detect the L1L2_Bact_P cassette, composed of an FRT site followed by a lacZ sequence and a loxP site, confirming the integration of the floxed *Piezo1* mutant allele. eGFP E8I-Cre-ER^{T2} and Rosa26-LSL TD

5 tomato knock-in activity reporters were also quantified by Transnetyx using the eGFP (forward primer: CGTCGTCCTTGAAGAAGATGGT; reverse primer: CACATGAAGCAGCACGACTT) and tdRFP (forward primer: AGATCCACCAGGCCCTGAA; reverse primer: GTCTTGAACCTCCACCAGGTAGTG) probes, respectively. Heterozygous Cre mice were bred to generate both Cre⁺ and Cre-

10 littermates. To induce *Piezo1* deletion, Cre⁺ and Cre- mice were intraperitoneally injected with 8 doses of 10mg/mL tamoxifen daily. Deletion efficiency was assessed using RT-qPCR with probes Mm01241547_g1 spanning exons 21-22 (ThermoFisher #4351372) and Mm01241549_m1 spanning exons 23-24 (ThermoFisher #4331182) to specifically assess the region containing loxP sites in exons 20 and 23. Results indicated that Cre⁺ mice

15 required two copies of the Cre⁺ allele for ~70% PIEZO1 deletion. Cre⁺ and Cre- mice *Piezo1*^{flx/flx} mice were then bred separately to ensure the homozygous expression of Cre alleles for efficient gene deletion. All mice were housed in specific pathogen-free conditions and all animal experimentation was performed in accordance with regulations and animal care guidelines from the Harvard Medical School Standing Committee on

20 Animals (IACUC) and the National Institute of Health.

Tumor implantation

Mice were anesthetized with 2.5% 2,2,2-tribromoethanol (Avertin, Sigma-Aldrich catalog no. T48402-25G) and injected in the flank subcutaneously with 2.5×10^5 MC38,

25 B16.F10 or B16-OVA tumor cells. Once palpable tumors were observed, tumors were measured every other day to calculate tumor volume over time. Tumor volume was calculated using the equation $(L \times W^2)/2$ where L denotes tumor length and W denotes tumor width. Mice were monitored for body condition and weight loss. Mice with tumors that exceeded 2000mm³, that were severely ulcerated or that infiltrated the i.p. cavity were

30 sacrificed.

Tumor infiltrating lymphocyte isolation

Tumors were harvested at day 15 following implantation and processed for analysis. Extracted tumors were mechanically chopped and treated with collagenase type 1 (Worthington Biochemical, #LS004194) and mixed for 25 min at 37°C. Lymphocytes were enriched using a 40/70% Percoll gradient that was centrifuged at 805 RCF for 20 min (no brake). Immune cells were harvested from the interface between 40% and 70% Percoll and resuspended in MACS buffer (PBS with 1 % FBS and 2 mM EDTA) for staining for flow cytometry analyses.

10 *Flow cytometry staining*

Cells were surface stained with antibodies listed below at a 1:100 dilution (unless otherwise noted) in 96-well V-bottom plates for 45 min in the dark on ice. Samples were washed twice with MACS buffer and fixed for 20 min in the dark at room temperature and permeabilized with FoxP3/Transcription Factor Staining Buffer Set Kit according to the manufacturer's protocol (eBioscience #00-5523-00). Cells were stained with intracellular antibodies listed below at a 1:100 dilution (unless otherwise noted) for 1 h in the dark on ice and washed twice with eBioscience Permeabilization Buffer. All washes used 200µL of the specified buffers and spins were performed at 726 RCF for 2 min at 4°C. Fixed and stained samples were acquired the following day on the BD FACSymphony and were analyzed using FlowJo software.

Antibodies for flow cytometry and sorting

Flow cytometry analyses were performed on a BD FACSymphony and cell sorting was performed on a BD Aria II. The following fluorescent antibodies were purchased from Biolegend for flow cytometry and cell sorting: human PD-1 (clone EH12.2H7, #329936) and murine CD45.2 (APC-Cy7, clone 104, #109824), CD8b (Alexa Fluor700, clone 53-6.7, #00730), CD44 (APC, clone IM7, #103012), PD-1 (PE-Cy7, RMP1-30, #109110), CD69 (BV421, H1.2F3, #104528), CD62L (BV605, PerCPCy5.5, clone MEL-14), CD3e (BUV395, PerCpCy55, FITC, clone 145-2C11), perforin (PE, clone S16009A), granzyme B (BV421, clone GB11, #515408), CTLA4 (BV605, clone UC10-4B9, #106323), TIM-3 (BV711, BV421, clone RMT3-23, #119727), Slamf6 (PE, APC, clone 330-AJ), IFNγ (APC, clone XMG1.2, #505810) and TNFα (PerCP/Cy5.5, clone MP6-XT22, #506322).

The following fluorescent antibodies were purchased from BD Biosciences: CD4 (BUV496, BUV737, clone GK1.5), LFA1 (BV786, clone M17/4) and Ki67 (PerCpCy55, clone B56). LIVE/DEAD Fixable Aqua dead cell stain (1:600, L34957) and LIVE/DEAD Fixable Near-IR dead cell stain (1:600, L34976) were purchased from Thermo Fisher Scientific.

In vivo PD-1 blockade and Piezo1 agonist treatment

Two 100µg doses of anti-PD-1 (Freeman lab or BioXcell, clone 29F.1A12, #BE0273) or rat IgG2a isotype control (BioXcell, clone 2A3, #BP0089) were administered interperitoneally to mice on days 10 and 13 (B16-OVA model) or 14 and 17 (MC38 model) following tumor implantation. Yoda1 (Tocris, #5586) was reconstituted in DMSO to reach a final stock concentration of 17 mg/mL. Reconstituted Yoda1 was freshly formulated with PBS and sonicated prior to each experiment to obtain a dose of 7.5mg/kg per mouse (250µg/200µL) administered interperitoneally 3-5 times between days 5 and 13.

Western blotting

Jurkat cells or tumor cells were lysed in Pierce RIPA Buffer supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (100X) for 15 min on ice. Whole cell lysates were spun down at 20,817 RCF at 4°C for 15 min. Supernatants were collected and transferred to new Eppendorf tubes and 10µL of each lysate was taken for protein estimation using the Pierce BCA Protein Assay Kit to normalize for protein loading. Lysates were denatured with 4X Laemmli Sample Buffer (BioRad) or 4X NuPAGE LDS (Invitrogen) containing beta mercaptoethanol (BME) and boiled for 5 min at 95°C. 15-40µg of protein per lysate was loaded and run on a NuPAGE 4-12% Bis-Tris protein gel and then transferred onto a nitrocellulose membrane. Ponceau staining was performed to check transfer efficiency and protein loading. Membranes were then blocked for 1 h in TBS supplemented with 1% Tween (TBS-T) and 5% milk supplemented with 0.2% Tween at room temperature. Membranes were then incubated with primary antibody Streptactin-HRP (Bio-Rad, 1:50,000, #1610381) and rocked overnight in blocking buffer at 4°C. Membranes were washed 3X for 5 min in TBS-T buffer prior to imaging. Membranes were then treated with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher #34580) and imaged using the Amersham Imager 600.

PD-L1 bead generation

M-450 Tosyl-activated magnetic Dynabeads (Invitrogen #14013) were washed using the Dyna-Mag-2 in 0.1M sodium phosphate buffer, pH 7.4-8.0. Magnetic beads were then treated with a mixture of human or mouse CD3 crosslinking antibody (BioXcell, human clone OKT3 #BE0001-2, murine clone 145-2C11 #BE0001-1), CD28 (BioXcell, human clone 9.3 #BE0248, murine clone 37.51 #BE0015-1) crosslinking antibody and either recombinant human (for use in Jurkat cells, R&D Systems #156-B7-100) or murine (for use in primary murine CD8⁺ T cells, R&D Systems #1019-B7-100) PD-L1 fusion protein or recombinant human IgG₁κ (for use in primary murine CD8⁺ T cells, SouthernBiotech #0151k-01) or mouse IgG₁ (for use in Jurkat cells, SouthernBiotech #0102-01) control protein depending on the PD-1 construct and origin of cells used. Multiple titrations of protein concentrations were tested to determine which ratio generated the most significant inhibition of T cells: 10/90%, 20/80%, 40/60%, 60/40% and 80/20% of TCR/CD28 antibodies and recombinant PD-L1 or control respectively. Antibodies and recombinant protein mixtures were crosslinked to beads using a total of 200μg of protein per 1mL of beads. Beads were incubated and rotated for 16-24 h at room temperature. Beads were then applied to the Dyna-Mag-2 to remove supernatant and washed in PBS supplemented with 0.1% BSA and 2mM EDTA pH 7.4. To deactivate the remaining free tosyl groups on the beads, beads were rotated overnight at room temperature in 0.2M Tris-HCL supplemented with 0.1% BSA, pH 8.5. Beads were washed 3X more with PBS buffer supplemented with 0.1% BSA and 2mM EDTA pH 7.4 and reconstituted in this buffer at 100 million beads per 1mL and stored at 4°C prior to use.

On-bead protein ratios and cell:bead ratios

Naïve murine CD8⁺ T cells were isolated from spleens of wild type mice using negative selection magnetic-activated cell sorting (MACS) isolation (Miltenyi #130-096-543). Naïve CD8⁺ T cells were stimulated for 24 h on 96-well U-bottom plates coated with anti-CD3 and anti-CD28 crosslinking antibodies. After 24 h, cells were moved to a new U-bottom plate. Murine TCR-PD-L1 or TCR-control hIgG₁κ beads containing various protein ratios outlined above were added to CD8⁺ T cells at various cell:bead ratios (1:2, 1:4, 1:6) for 48 h. Supernatant was collected 48 h following the addition of beads for cytometric bead array (CBA) analysis (BD Biosciences). After 48 h, CD8⁺ T cells were stained and

acquired the following day on the BD FACSymphony for flow cytometric analysis as described in Flow Cytometry Staining in Main Methods.

Indo-1 Ca^{2+} flux assay

5 Naïve CD8⁺ T cells were isolated from the spleens of WT mice or E8i-Cre-ER^{T2} Piezo1^{flx/flx} Cre⁺ or Cre⁻ mice treated with 10mg/mL tamoxifen for 8 days using naïve CD8⁺ T cell MACS. Purified CD8⁺ T cells were stimulated on 96-well U-bottom dishes coated with 4μg/mL of crosslinking murine CD3 and CD28 antibodies for 48 h. Stimulated CD8⁺ T cells were then rested for 4-12 h in R10 media and moved to a fresh, uncoated 96-
10 well U-bottom plate. WT or E8i-Cre-ER^{T2} Piezo1^{flx/flx} CD8⁺ T cells were then stained in PBS containing 1% FBS for 1 h at room temperature with near IR fixable LIVE/DEAD stain and 1μM of Indo-1 (Thermo Fisher Scientific #I1226). Cells were then washed twice with 1% FBS PBS and resuspended in R10 media in 5mL round-bottom polystyrene tubes. TCR-PD-L1 or TCR-control beads were added at a ratio of 1:5 cell:bead and Yoda1 was
15 added to samples at a final concentration of 5μM. Following addition of PIEZO1 agonist or beads for specified timepoints, cells were immediately acquired for 1 min using tube mode on the BD FACSymphony or BD LSRII and analyzed using FlowJo software. Ca²⁺ influx was quantified by calculating the ratio of bound (BUV395) to unbound (BUV496) Ca²⁺ using Indo-1.

20

RT-qPCR

Murine CD8⁺ T cells were isolated from the spleens and inguinal lymph nodes of Cre⁺ and Cre⁻ naïve mice treated with 10mg/mL tamoxifen for 8 days using CD8⁺ T cell positive selection MACS (Miltenyi #130-117-044) or cell sorting. Isolated CD8⁺ T cells
25 were washed with PBS and spun down at 453 RCF for 5 min. PBS was removed from the cell pellets and cell pellets were frozen at -80°C or immediately processed for RNA. RNA was isolated from purified CD8⁺ T cell pellets using Qiagen's RNeasy Mini Kit (#74104). RNA was quantified using the Qubit RNA HS Assay Kit (#Q32852) and RNA concentration was normalized across samples. Reverse transcription was performed using the
30 Superscript VILO cDNA Synthesis Kit (Thermo Fisher Scientific #11-754-050) to generate cDNA. cDNA was diluted and mixed with *Piezo1* specific fluorescein amidites (FAM) Taqman probes (Thermo Fisher Scientific, Assay ID # Mm01241547_g1 and

#Mm01241549_m1) and control eukaryotic 18s rRNA FAM probe (Thermo Fisher Scientific, Assay ID #Hs99999901_s1) according to the Fast Advanced Mastermix real time PCR protocol. PCR mixtures were plated in Roche LightCycler 480 96-well plates (#04729692001) and sealed. Plates were run on the Roche LightCycler 480 with a 20 s
5 polymerase activation step at 95°C followed by 40 cycles of 1 s of denaturing at 95°C and 20 s of annealing and extending at 60°C.

PIEZO1 KO CD8⁺ T cell stimulation and flow cytometry

E8I-Cre-ER^{T2} Cre⁺ and Cre⁻ mice were treated with 8 doses of 100mg/mL
10 tamoxifen. Naïve CD8⁺ T cells were isolated from spleens of tamoxifen-treated mice using negative selection MACS isolation (Miltenyi). Naïve CD8⁺ T cells were stimulated for 48 h on 96-well U-bottom plates coated with a titration of anti-CD3 and anti-CD28 crosslinking antibodies (0, 0.1, 1.0, 5.0 and 10µg/mL). Supernatant from each sample was collected 48 h following the addition of beads for CBA analysis (BD Biosciences). After 48 h, CD8⁺ T
15 cells were stained as described in Flow Cytometry Staining in Main Methods and analyzed on the BD FACSymphony or CytoFLEX (Beckman Coulter).

Incorporation by Reference

All publications, patents, and patent applications mentioned herein are hereby
20 incorporated by reference in their entirety as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Equivalents

25 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

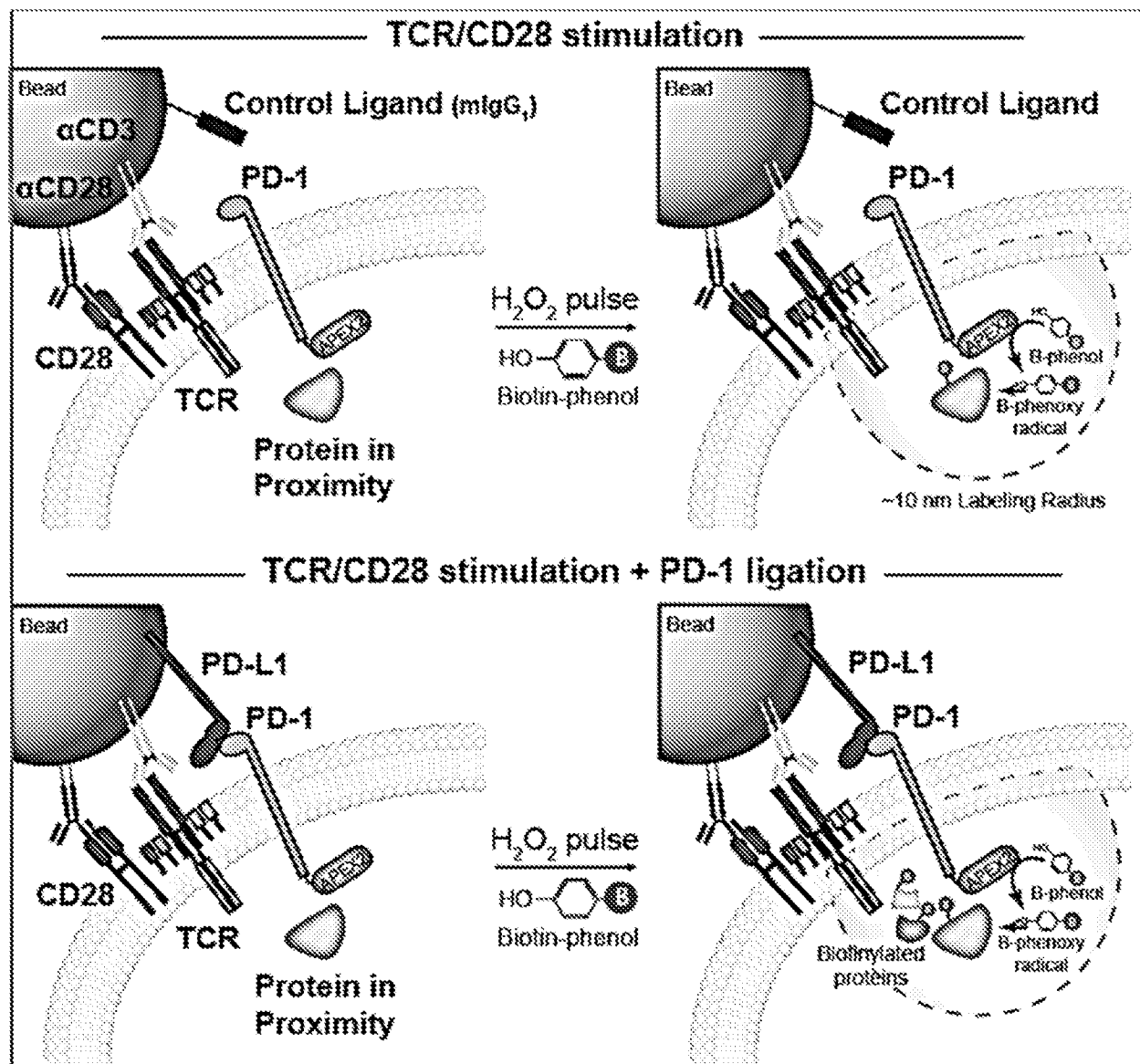
What is claimed is:

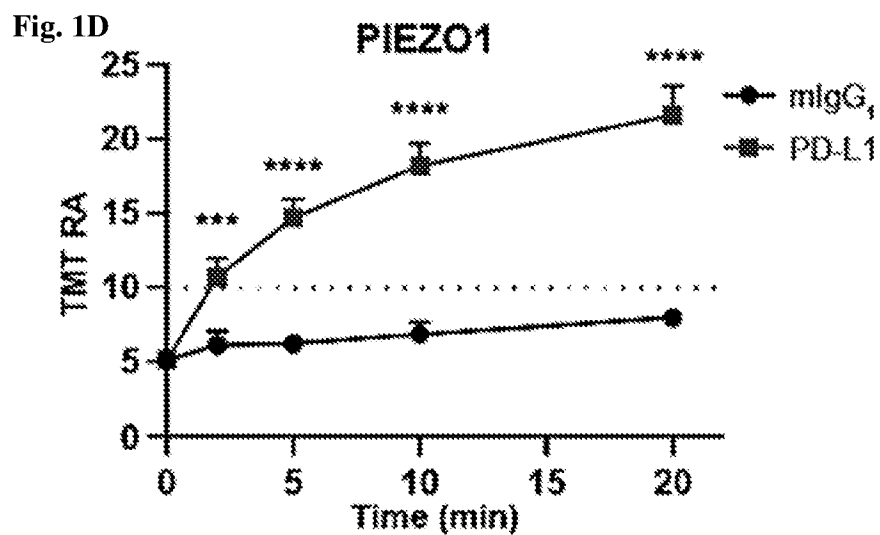
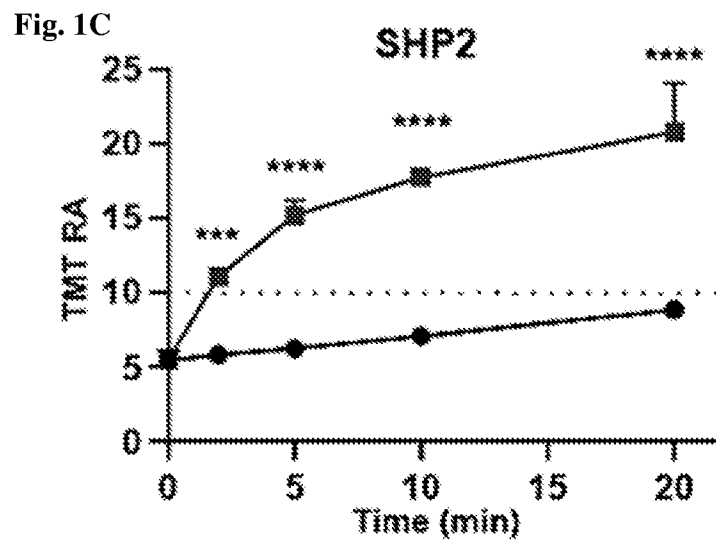
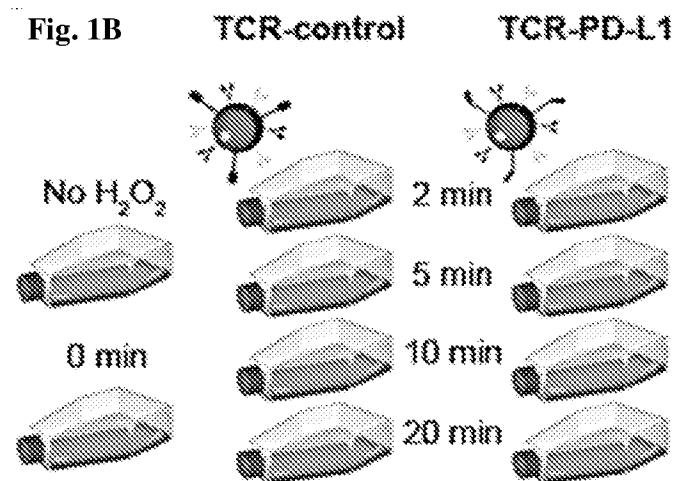
1. A method of treating cancer in a subject, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1.
2. The method of claim 1, wherein the agent is a small molecule agonist of PIEZO1.
3. The method of claim 2, wherein the small molecule agonist is Yoda1, Jedi1 or Jedi2.
4. The method of any one of claim 1 to 3, wherein the method further comprises administering an immune checkpoint inhibitor to the subject.
5. A method of treating cancer in a subject, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1 and an immune checkpoint inhibitor.
6. The method of claim 4 or 5, wherein the immune checkpoint inhibitor is an inhibitor of an immune checkpoint protein selected from CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, BTLA, SIRPalpha (CD47), CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, A2aR, and combinations thereof.
7. The method of claim 6, wherein the immune checkpoint inhibitor is an inhibitor of PD-1 or PD-L1.
8. The method of any one of claims 4 to 7, wherein the agent that increases or stabilizes the activity or expression of PIEZO1 and the immune checkpoint inhibitor are administered conjointly.
9. The method of claim 8, wherein the administration of the agent that increases or stabilizes the activity or expression of PIEZO1 and the immune checkpoint inhibitor act synergistically.

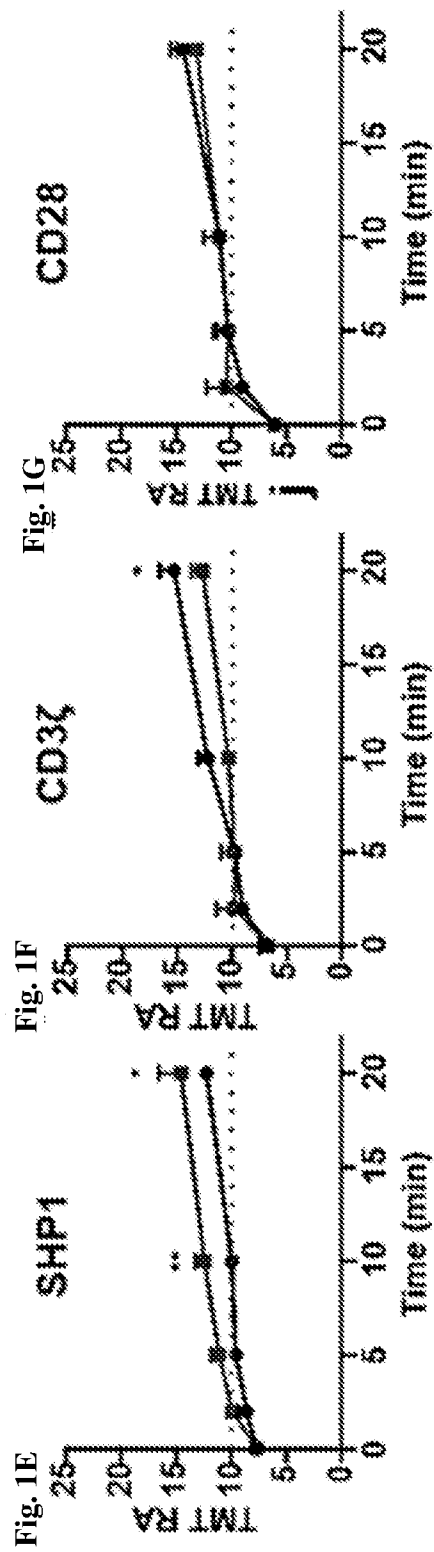
10. The method of claim 1, wherein the agent is a gRNA fused to a transcription activator.
11. The method of claim 10, wherein the gRNA comprises a region that is complementary to a portion of a gene that encodes a PIEZO1 protein.
12. The method of claim 1, wherein the agent is a vector encoding a PIEZO1 protein, such as a viral vector encoding a PIEZO1 protein.
13. The method any one of claims 1 to 12, wherein the agent is administered systemically, intravenously, subcutaneously, or intramuscularly.
14. The method of any one of the previous claims, wherein the agent is administered to the subject in a pharmaceutically acceptable formulation.
15. The method of any one of claims 1 to 14, wherein the method further comprises administering an additional agent or cancer therapy.
16. The method of claim 15, wherein
 - i) the additional agent is a chemotherapeutic agent or a cancer vaccine; and/or
 - ii) the cancer therapy is radiation.
17. The method of any one of claims 1 to 16, wherein the subject is refractory for immune checkpoint inhibitory therapy.
18. A method of treating cancer in a subject unresponsive to immune checkpoint inhibitor therapy, the method comprising administering to the subject an agent that increases or stabilizes the activity or expression of PIEZO1 and an immune checkpoint inhibitor.
19. The method of claim 18, wherein the agent is a small molecule agonist of PIEZO1.
20. The method of claim 19, wherein the small molecule agonist is Yoda1, Jedi 1 or Jedi2.

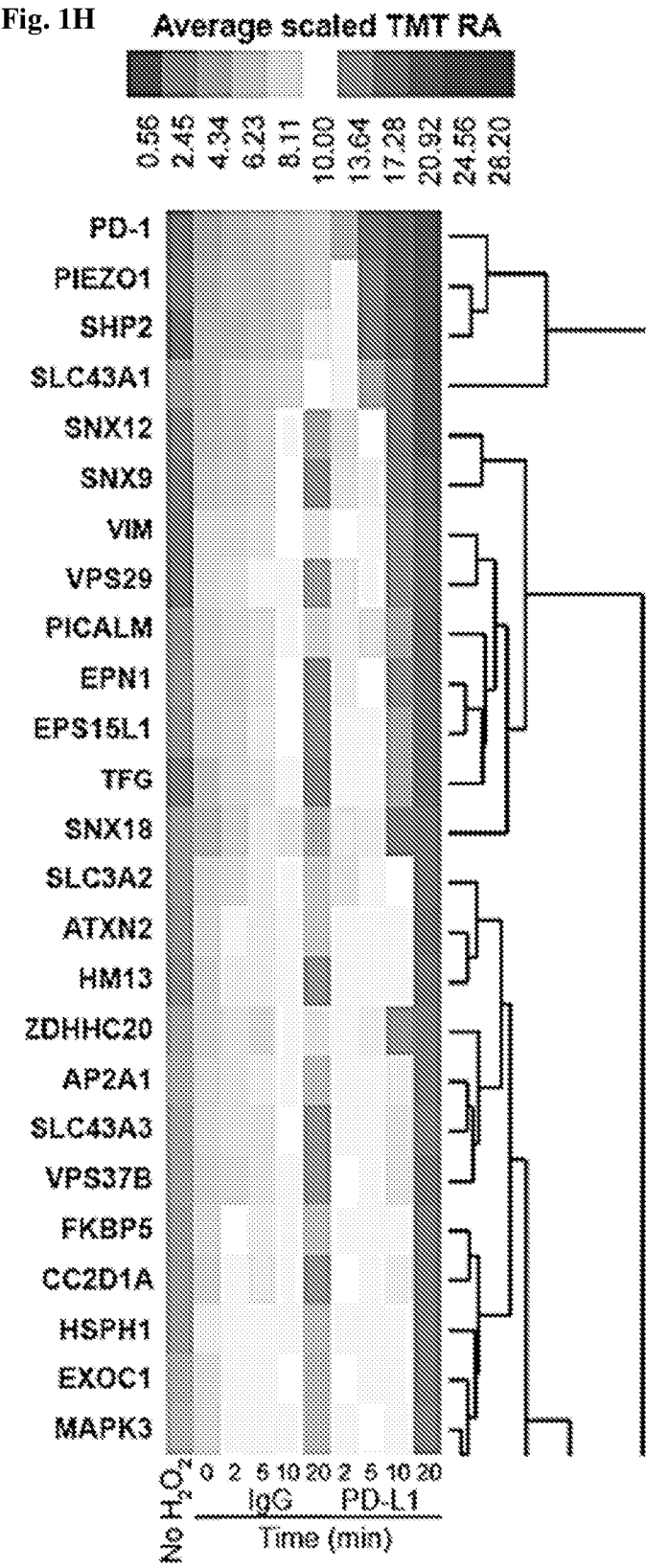
21. The method of any one of claim 18 to 20, wherein the immune checkpoint inhibitor therapy comprises an inhibitor of PD-1 or PD-L1.
22. A method of treating cancer in a subject, the method comprising administering to the subject T-cells that have been treated *ex vivo* with an agent that increases or stabilizes the activity or expression of PIEZO1.
23. The method of claim 22, wherein the T-cells are tumor infiltrating lymphocytes.
24. The method of claim 22 or claim 23, wherein the T-cells are autologous.
25. The method of claim 22 or claim 23, wherein the T-cells are allogeneic.
26. The method of any one of claims 1 to 25, wherein the subject is a human.

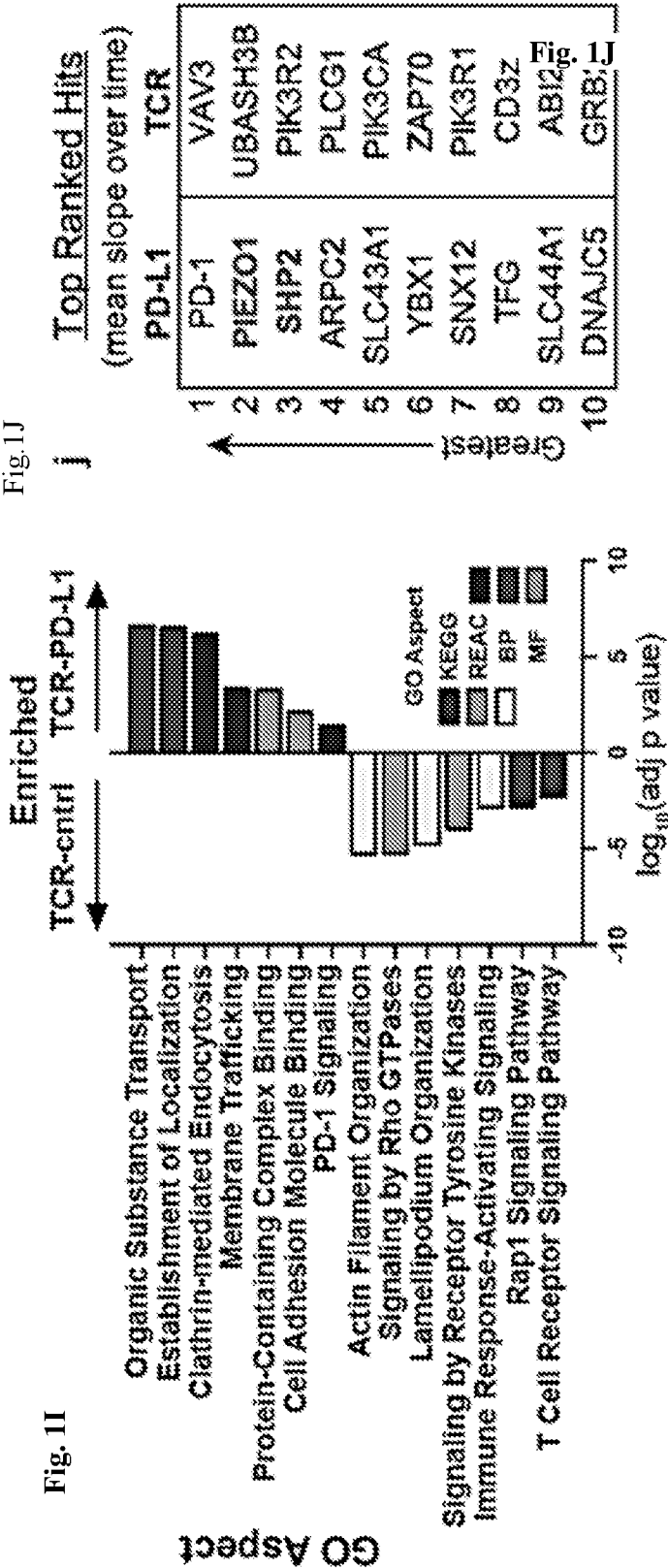
Fig. 1A

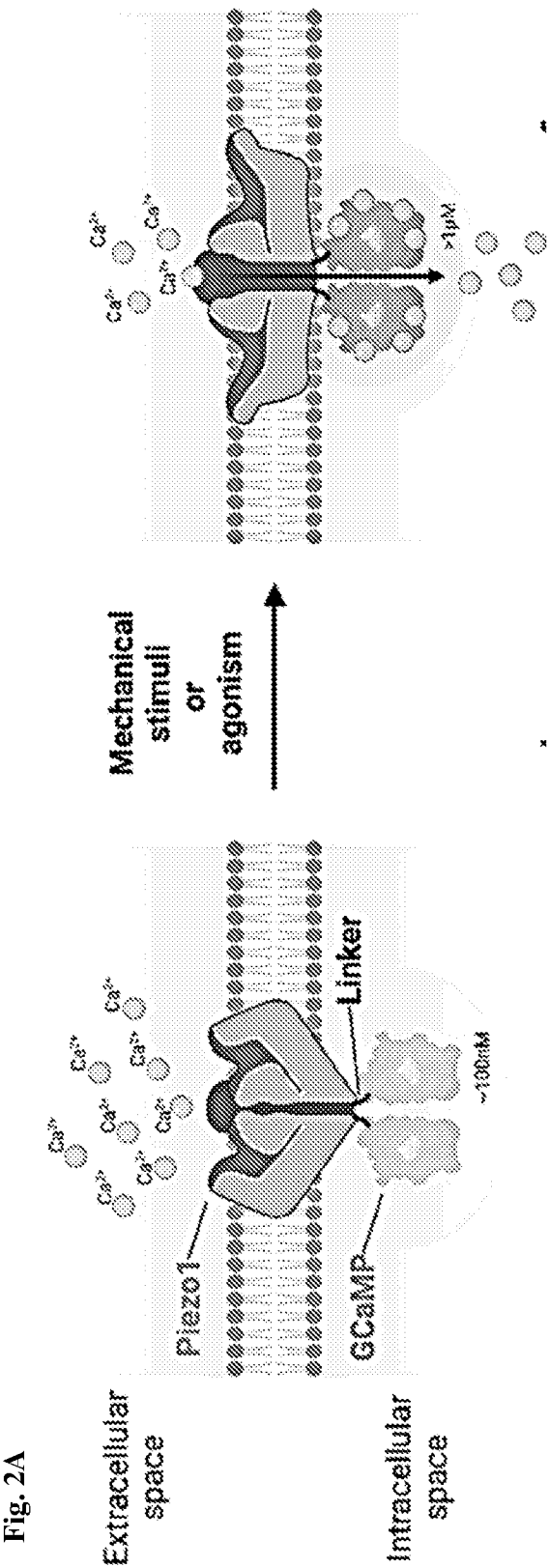


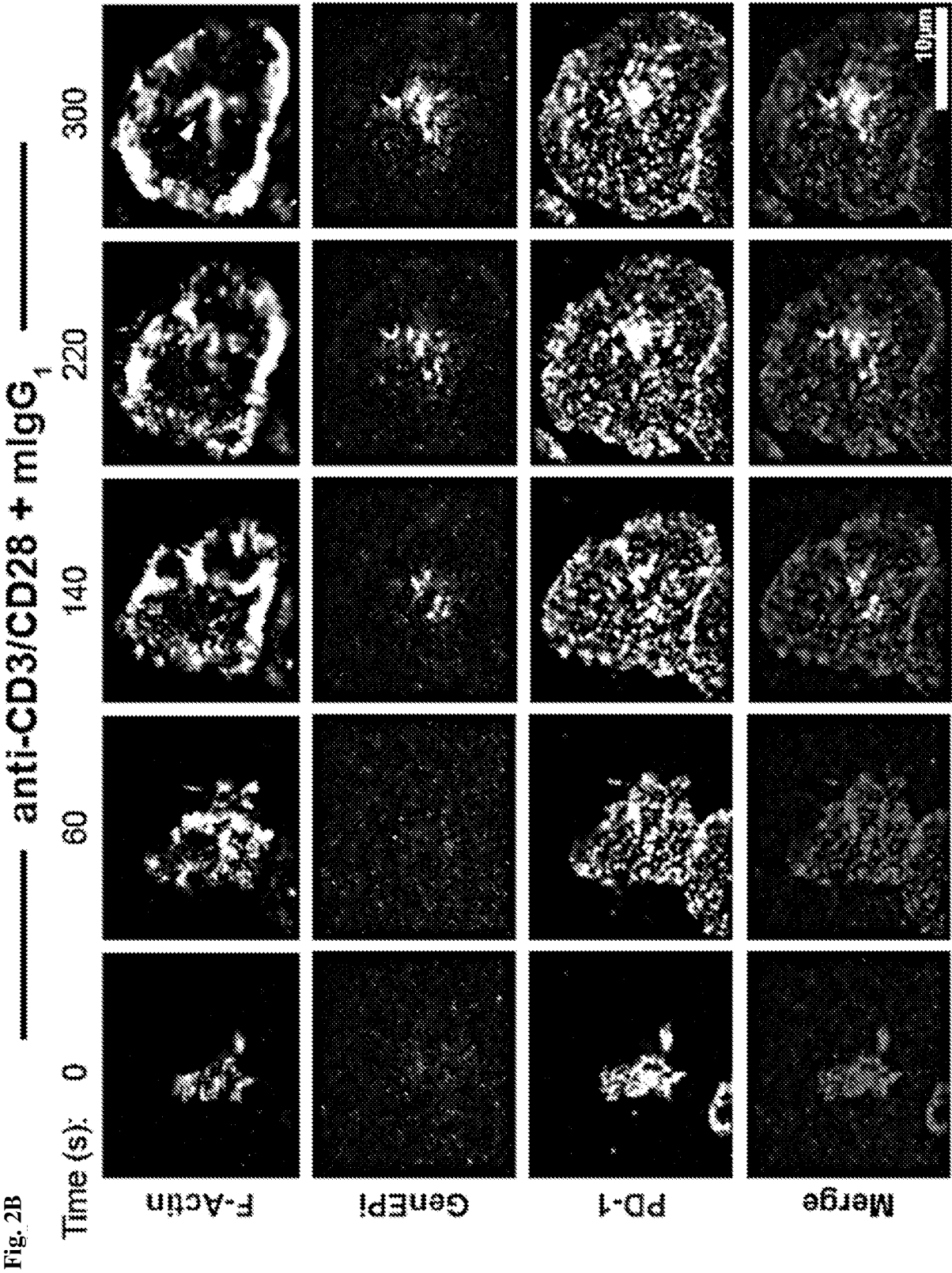












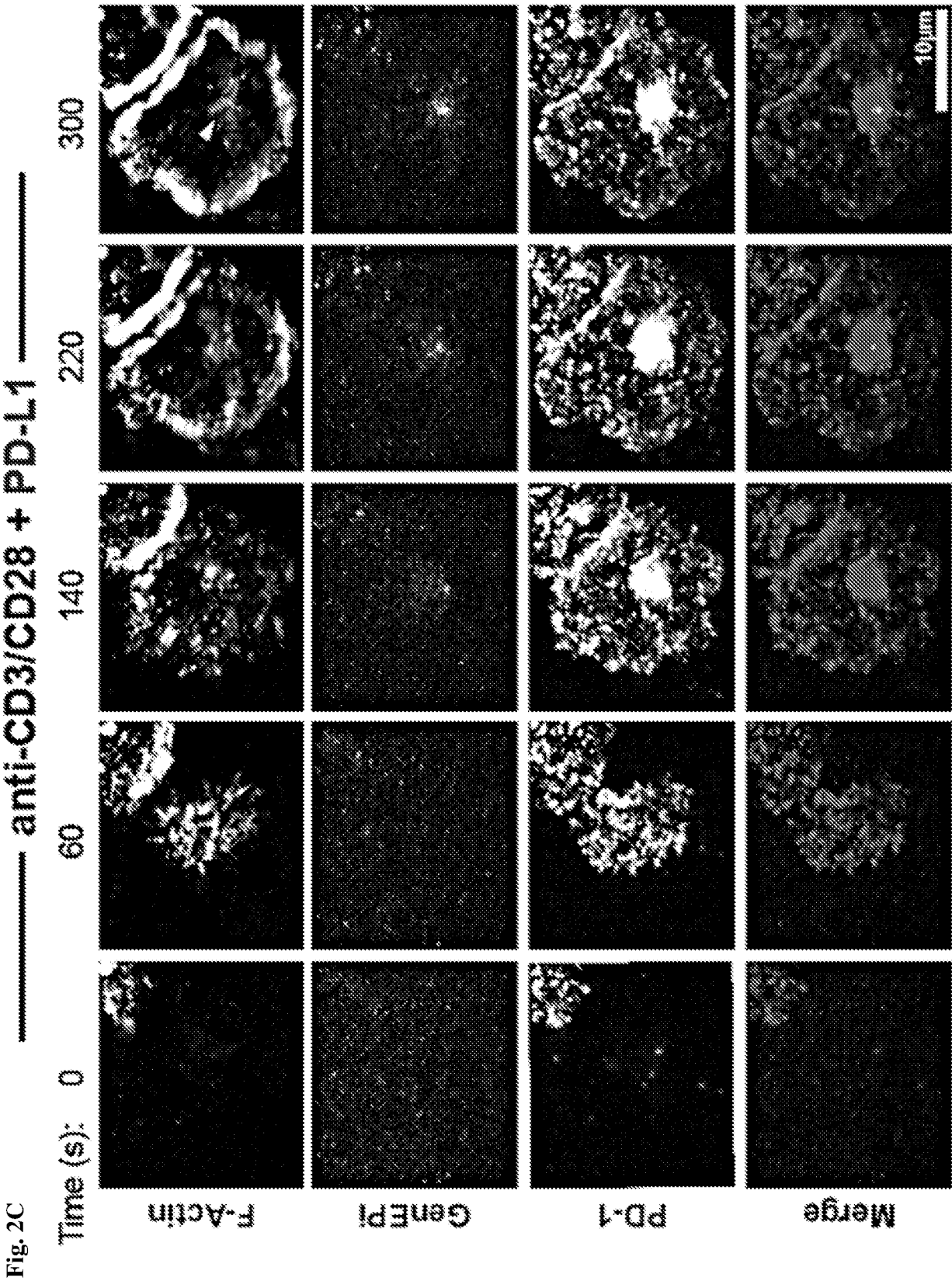


Fig. 2D

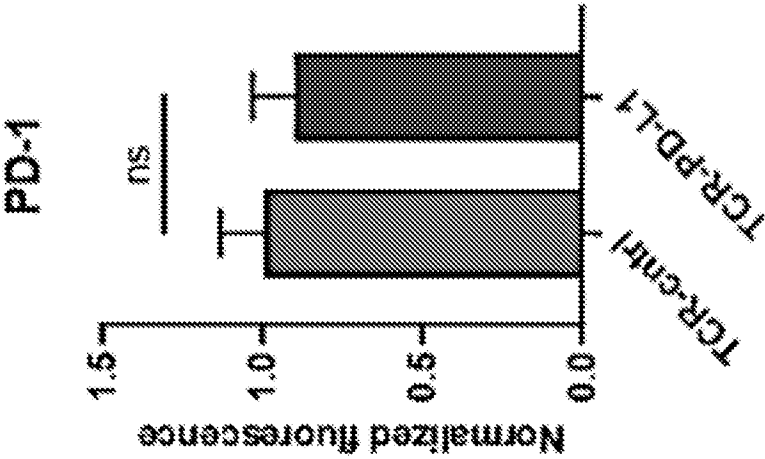


Fig. 2E

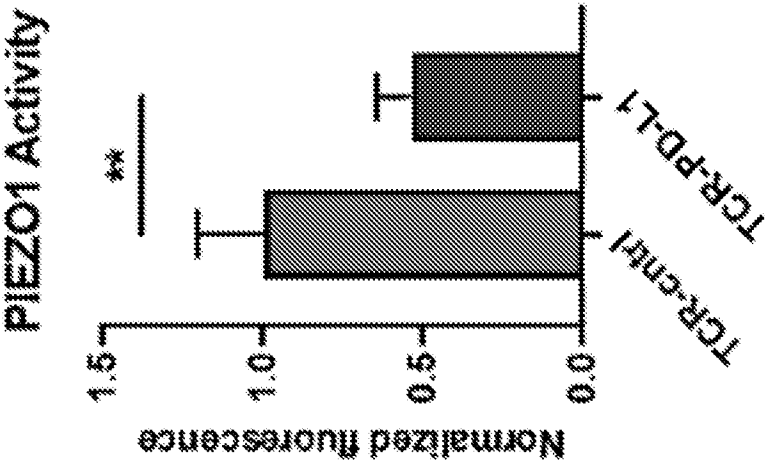
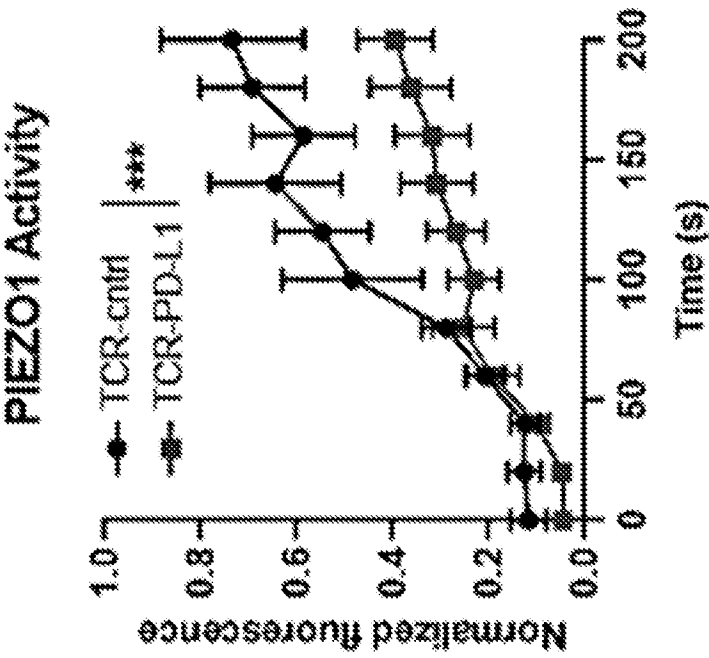


Fig. 2F



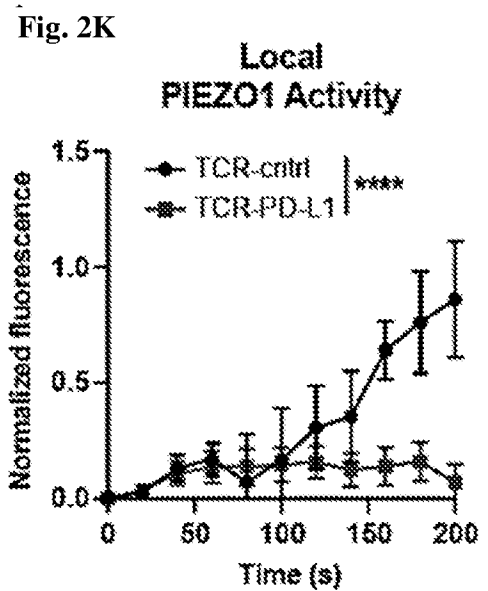
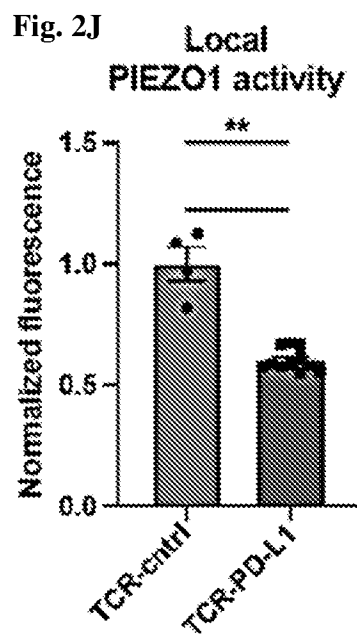
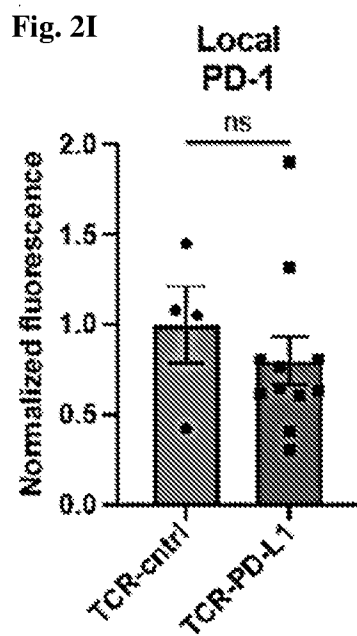
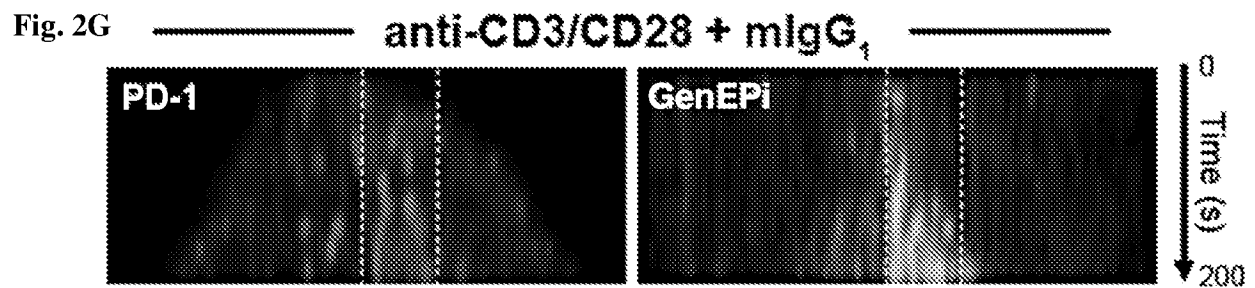


Fig. 3A

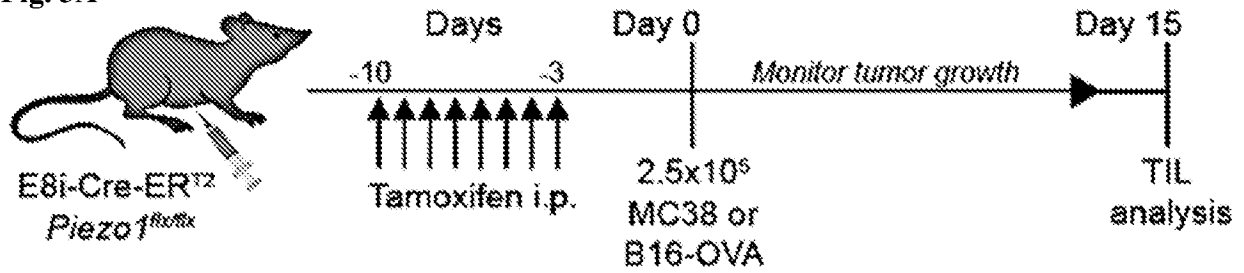


Fig. 3B

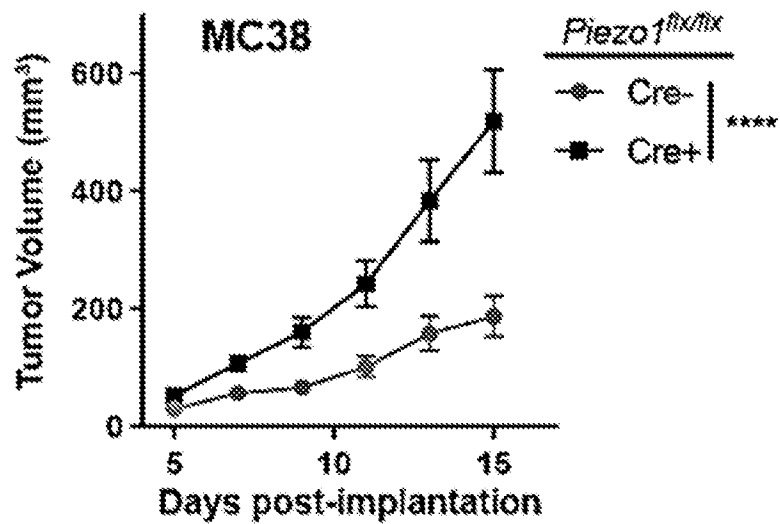


Fig. 3C

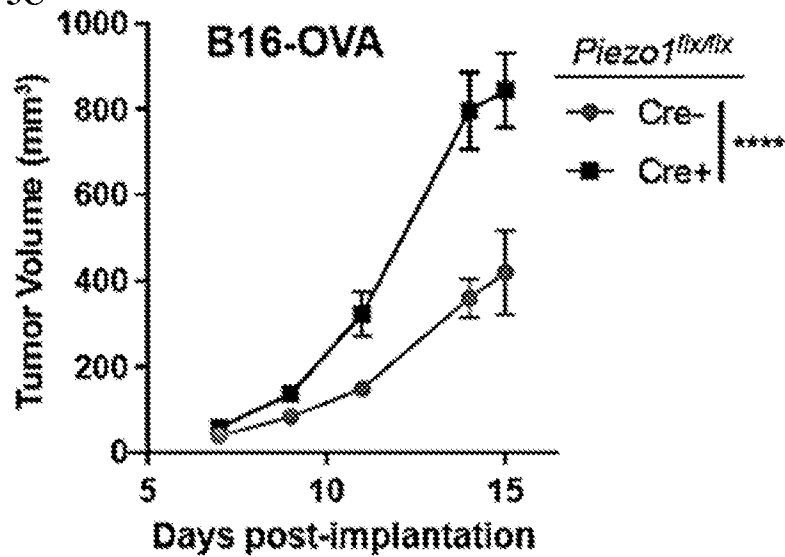


Fig. 3D

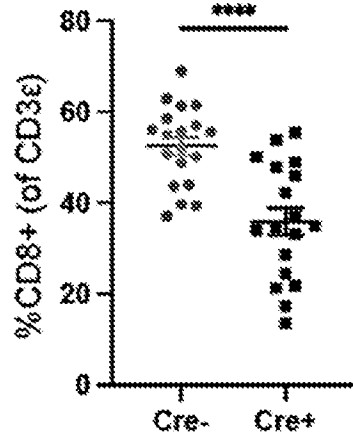


Fig. 3F

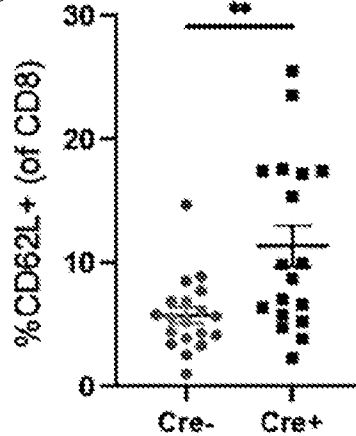


Fig. 3H

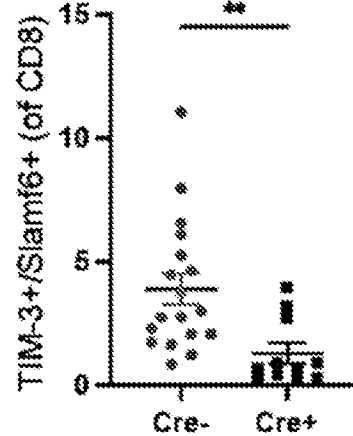


Fig. 3E

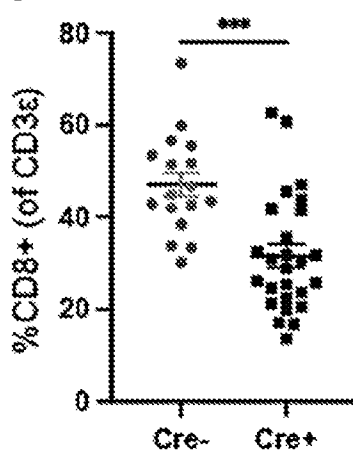


Fig. 3G

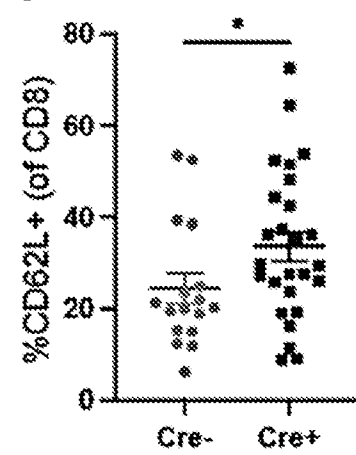


Fig. 3I

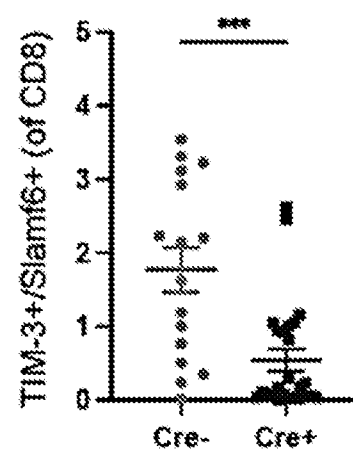


Fig. 3J

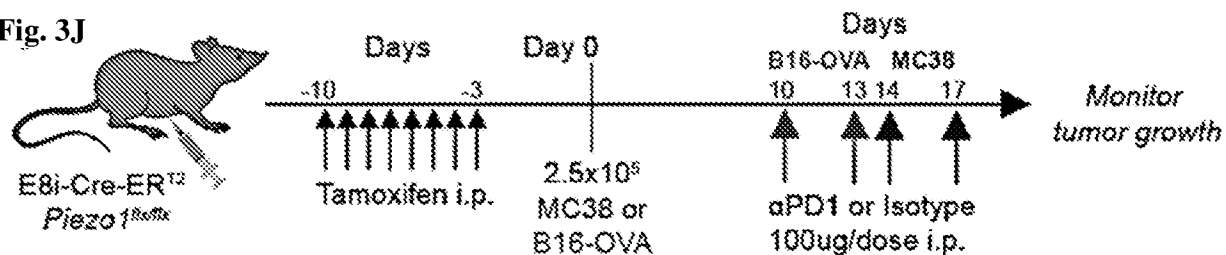


Fig. 3K

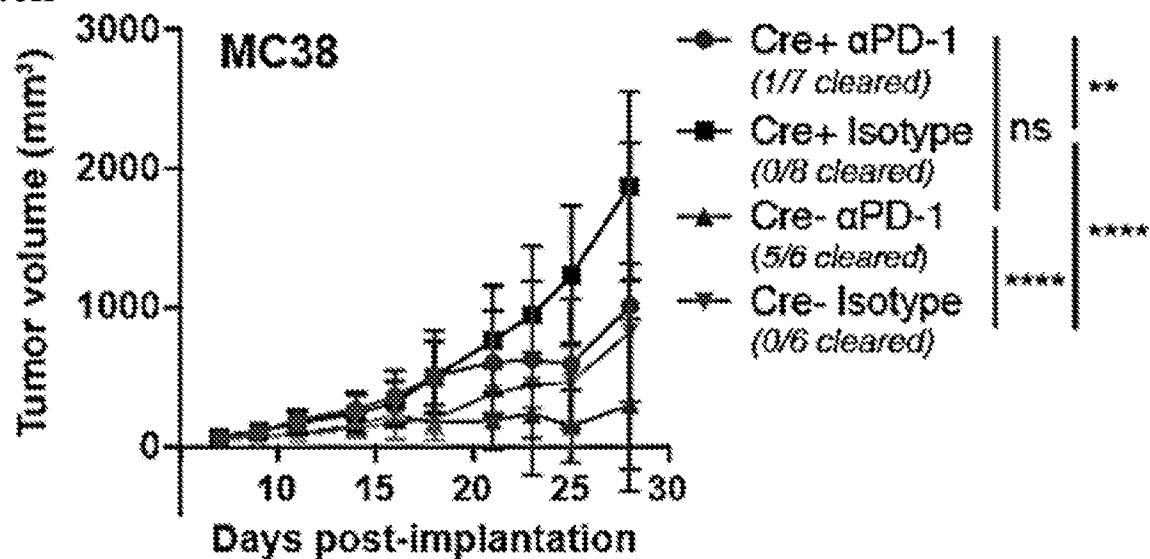
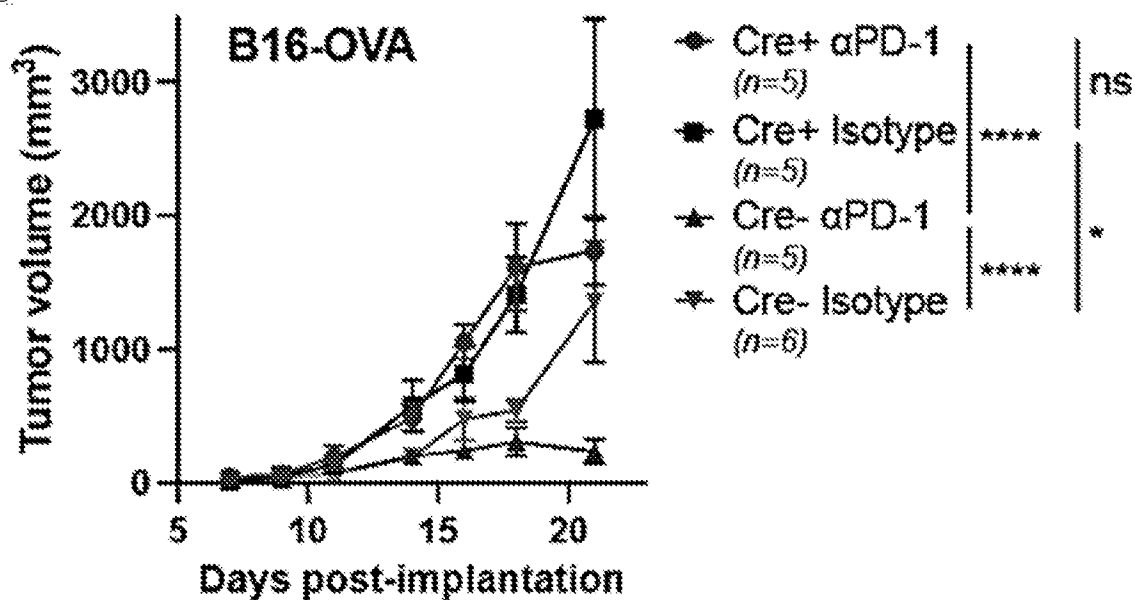


Fig. 3L



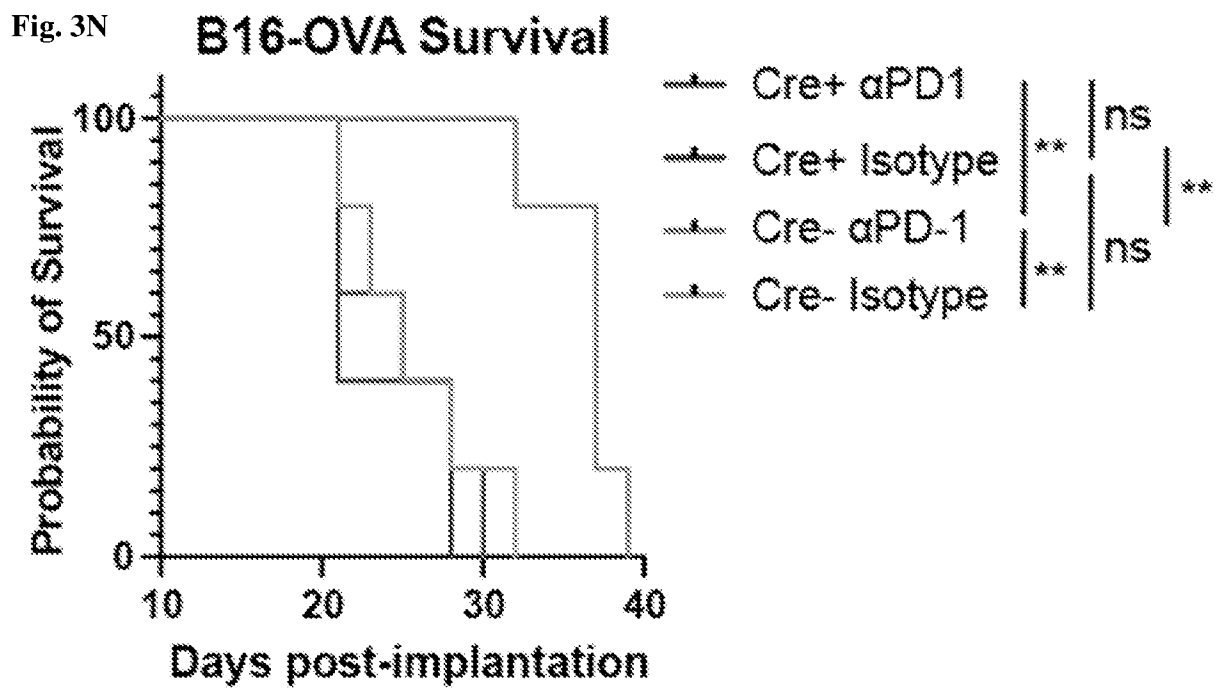
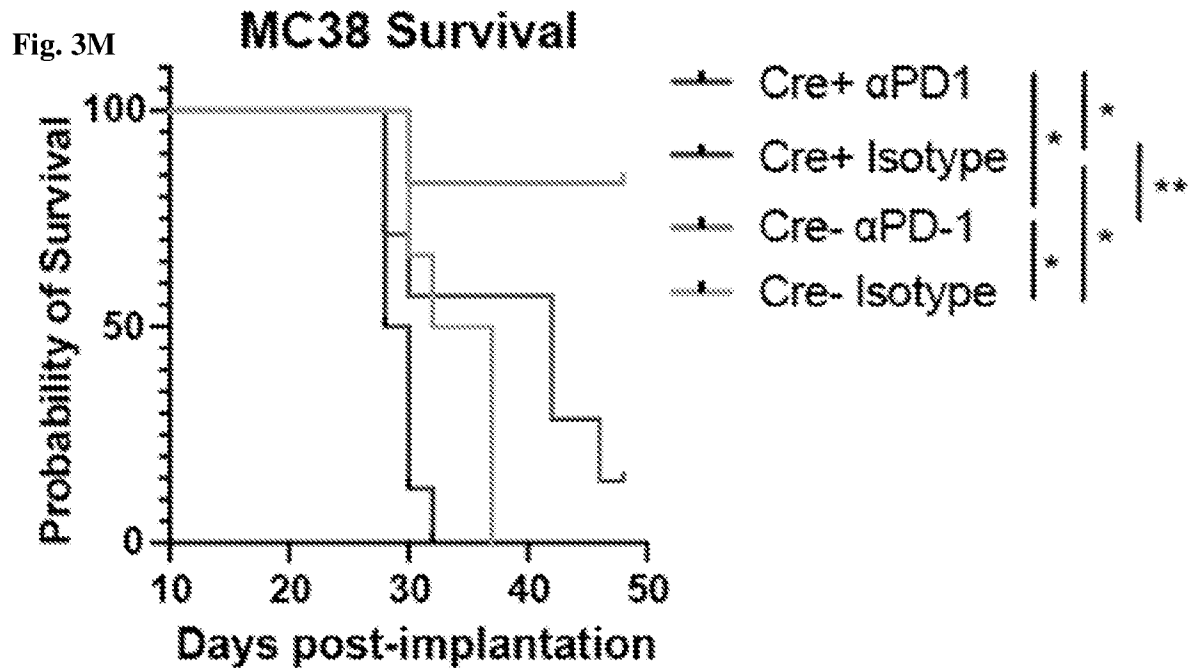


Fig. 4A

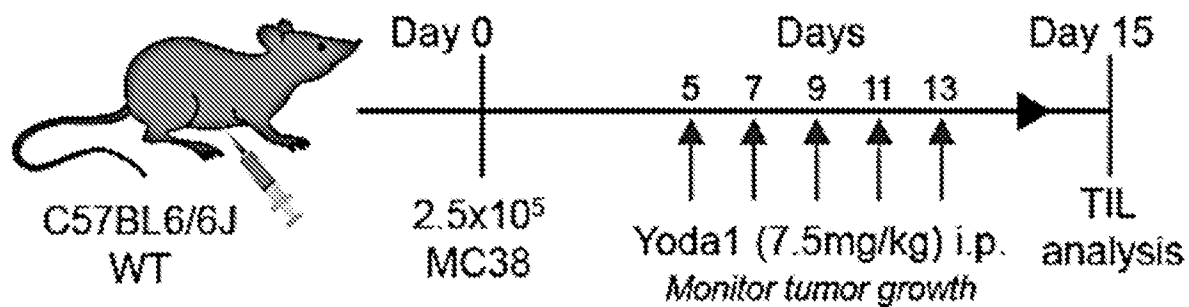


Fig. 4B

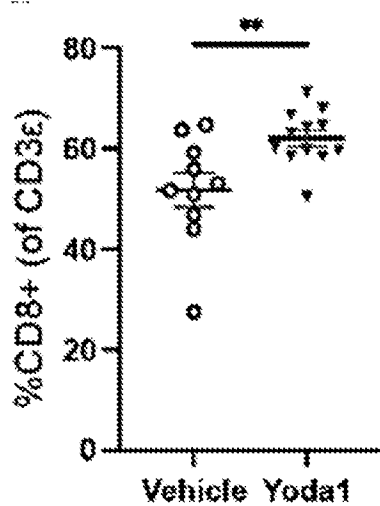


Fig. 4C

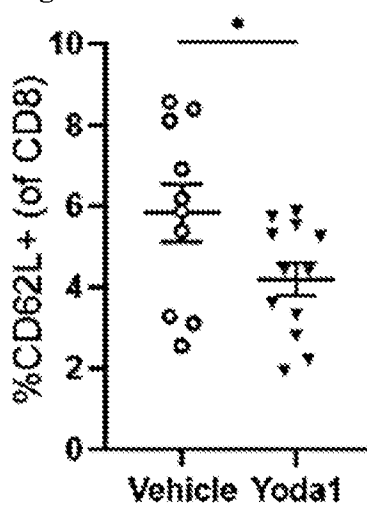


Fig. 4D

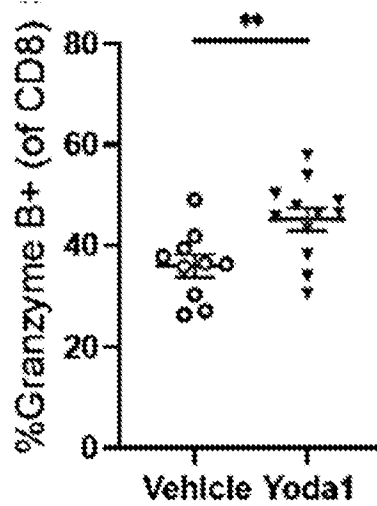


Fig. 4E

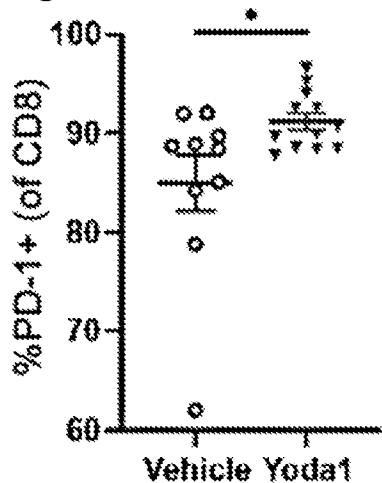


Fig. 4F

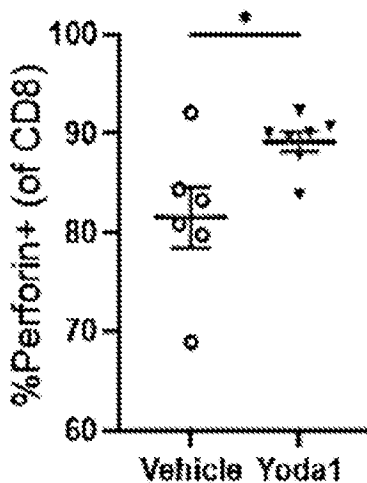


Fig. 4G

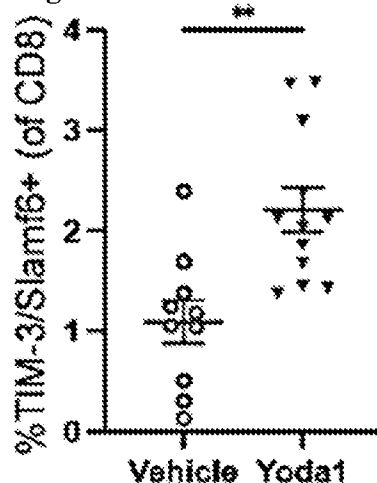


Fig. 4H

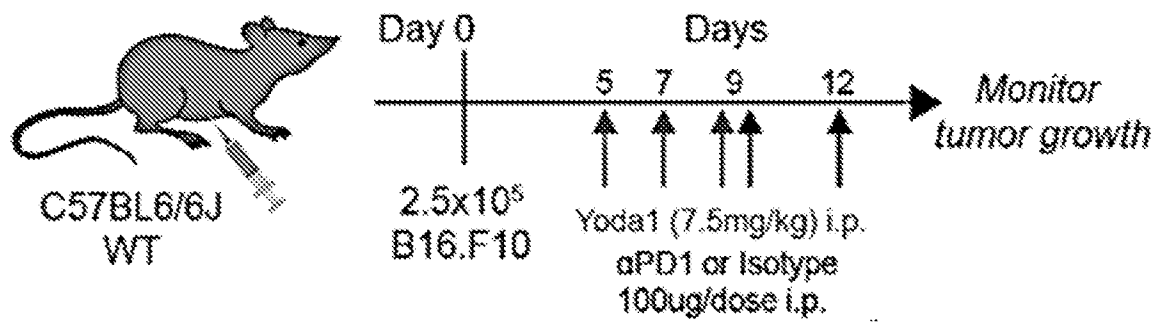


Fig. 4I

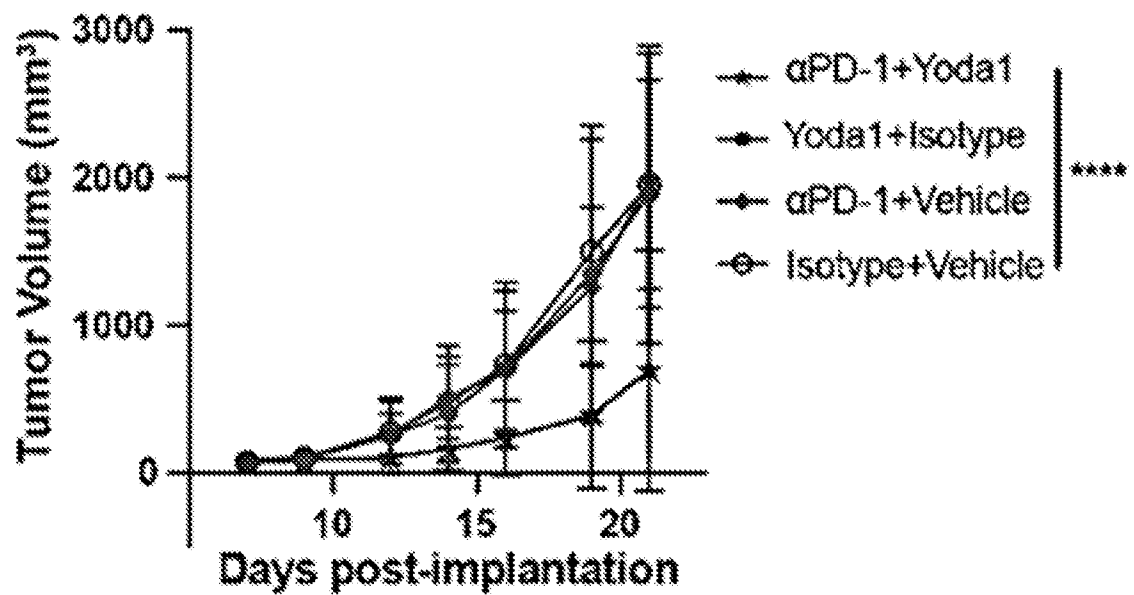


Fig. 4J

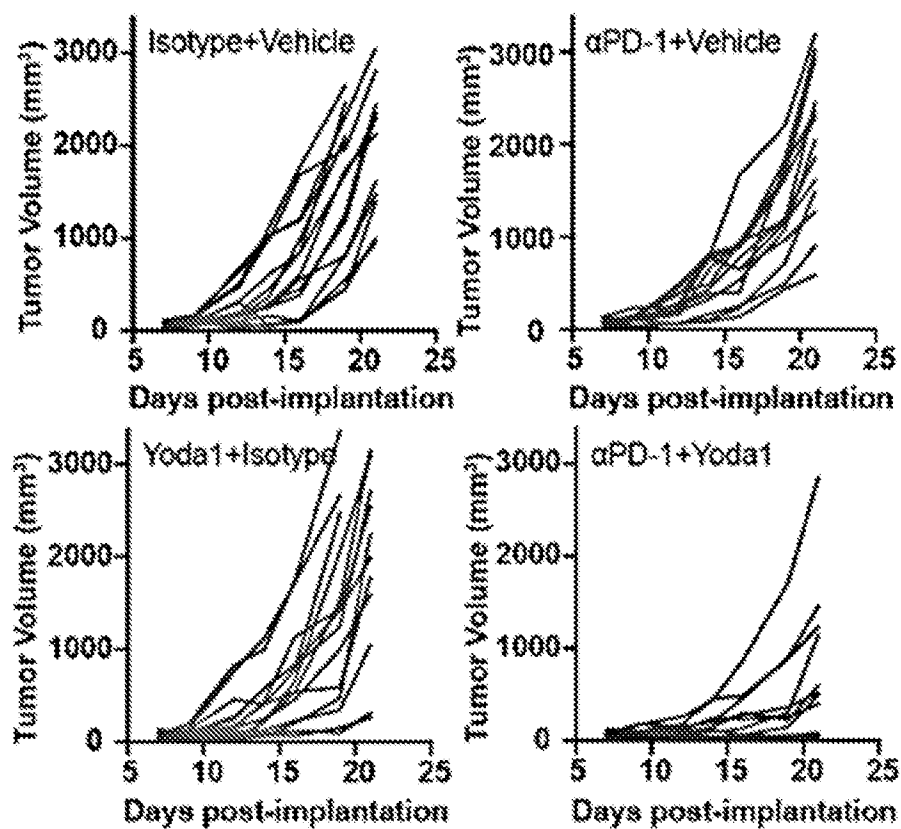


Fig. 4K

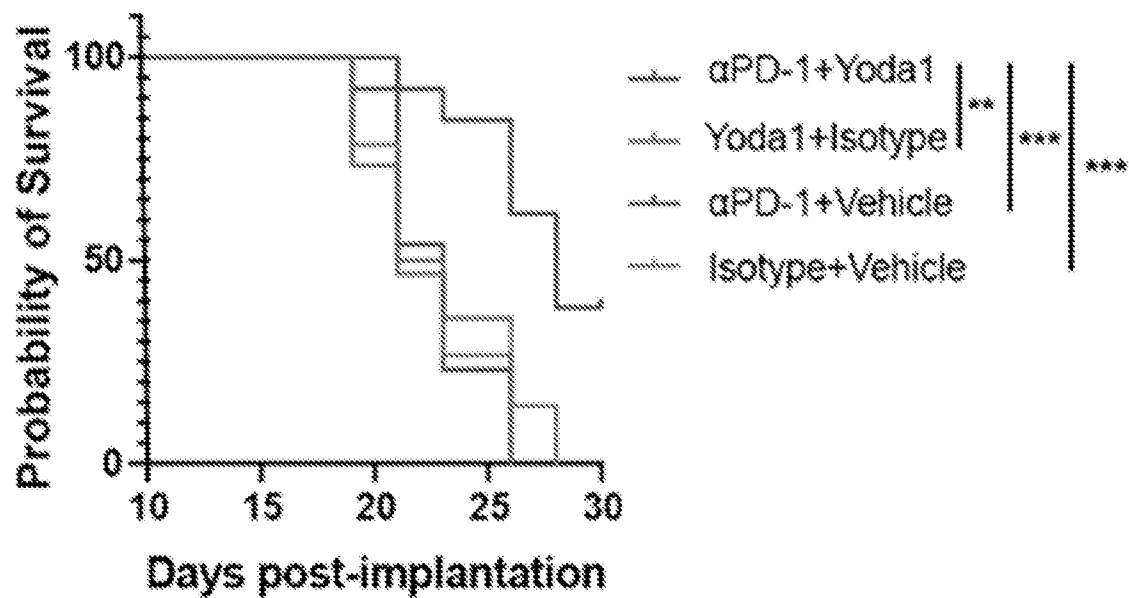


Fig. 5A

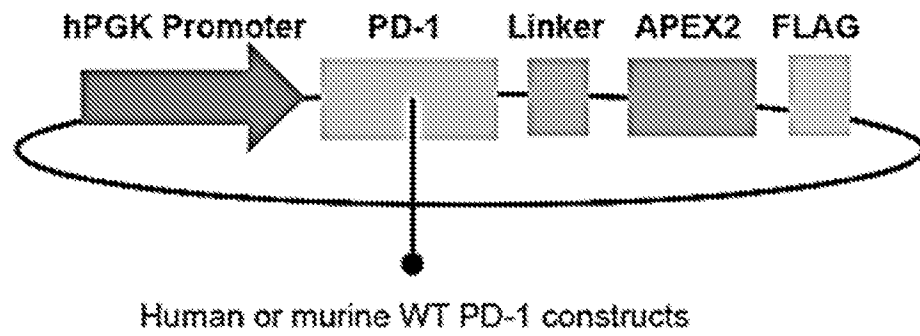
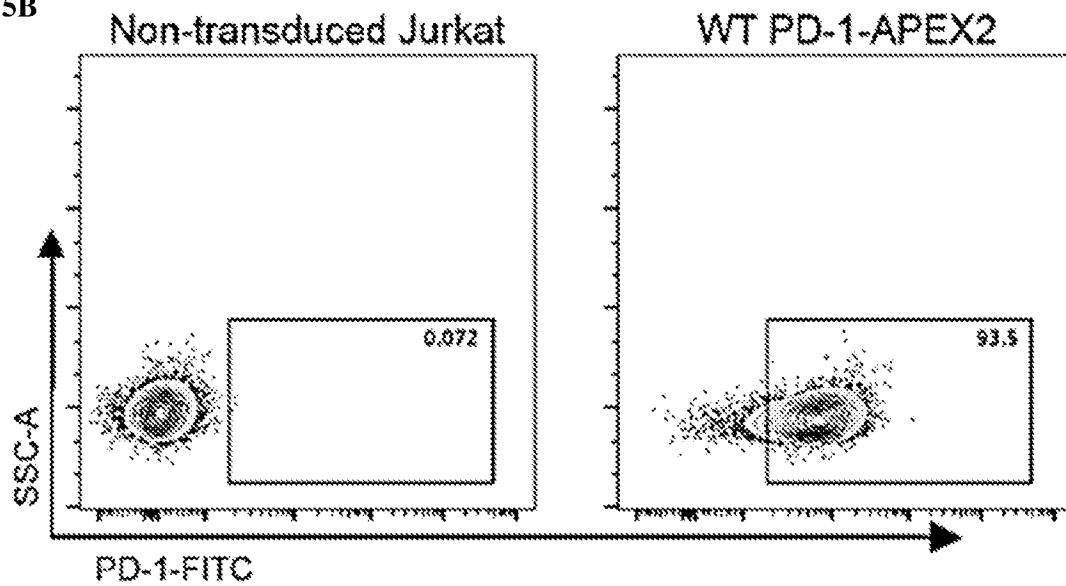


Fig. 5B



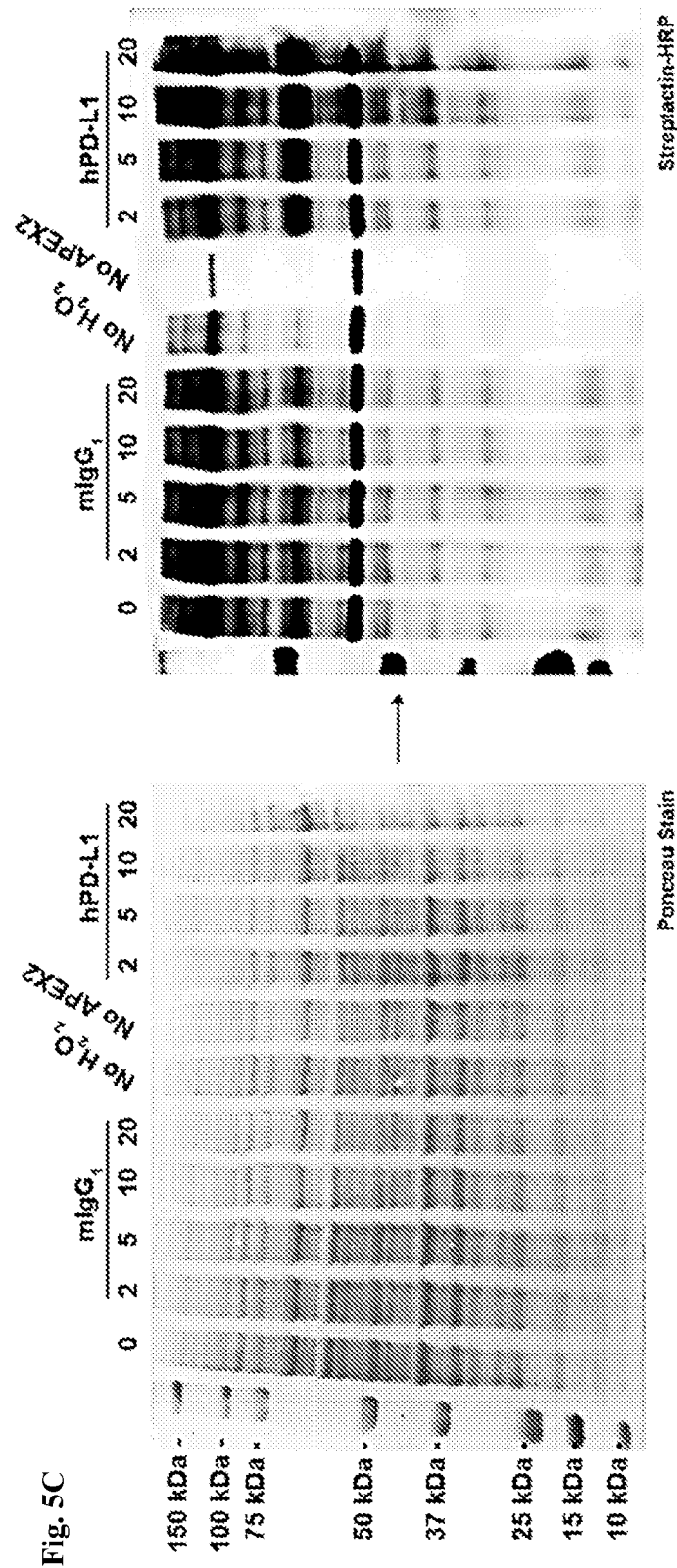


Fig. 6A

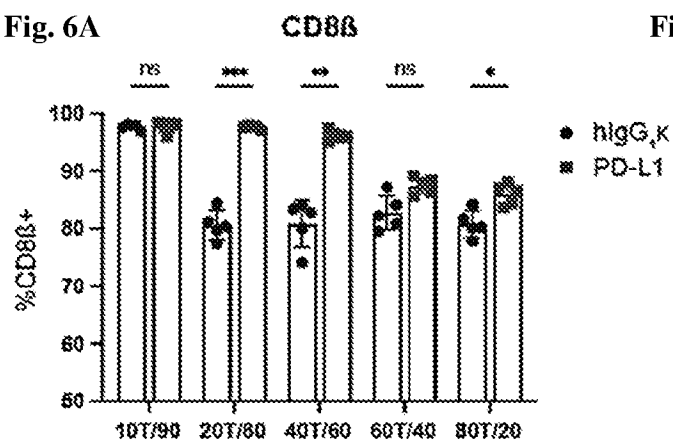


Fig. 6B

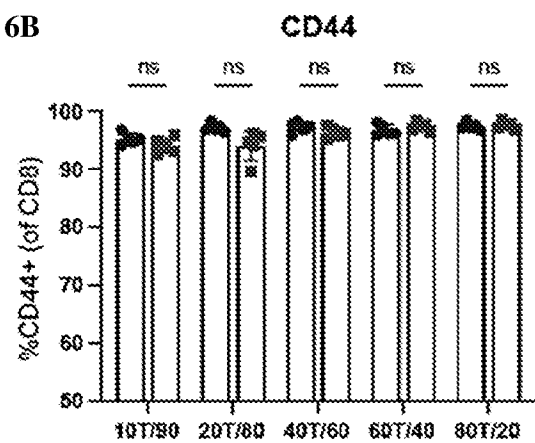


Fig. 6C

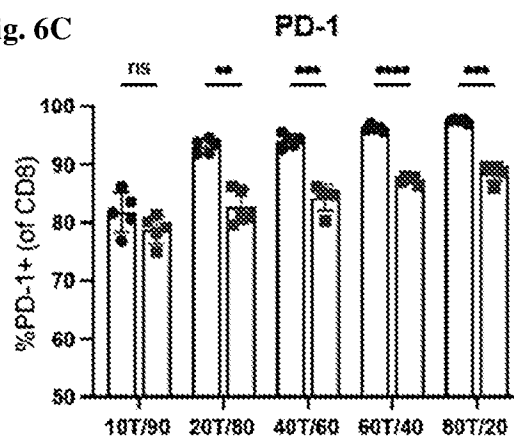


Fig. 6D

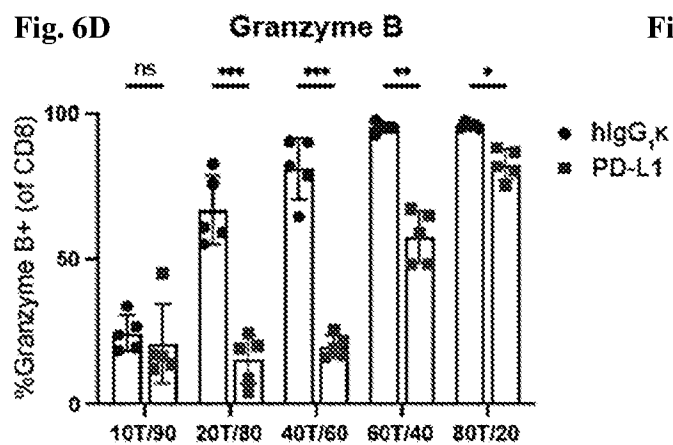


Fig. 6E

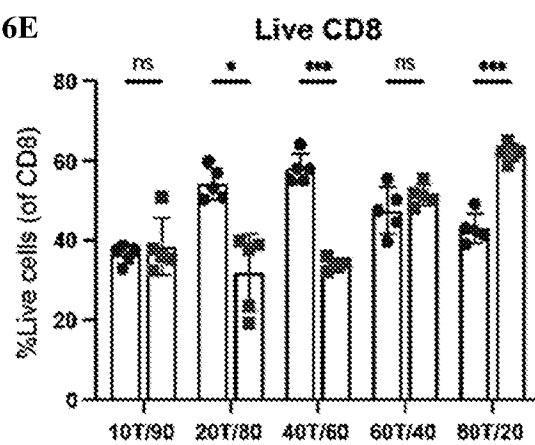


Fig. 6F

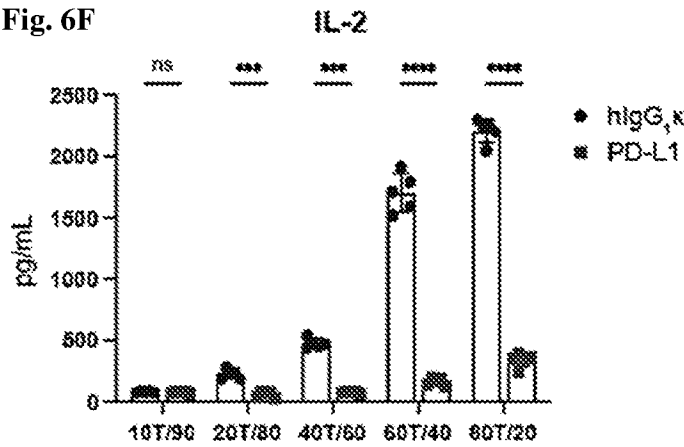


Fig. 6G

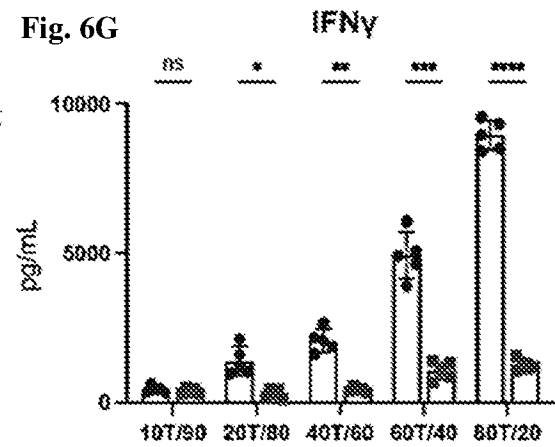


Fig. 6H

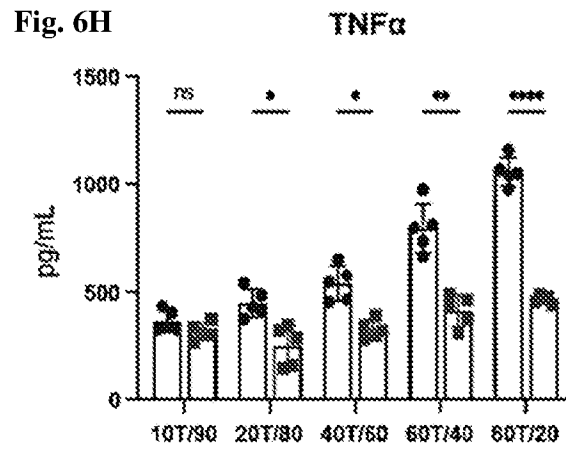


Fig. 6I

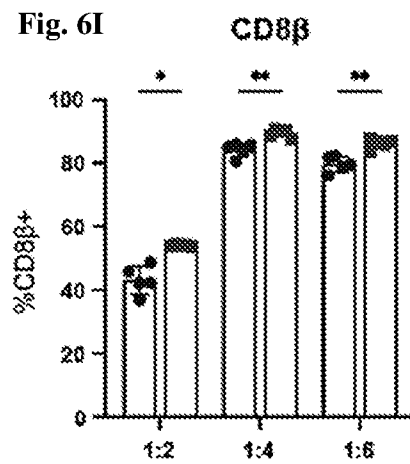


Fig. 6J

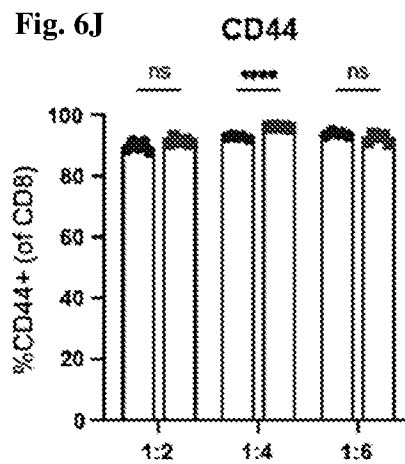


Fig. 6K

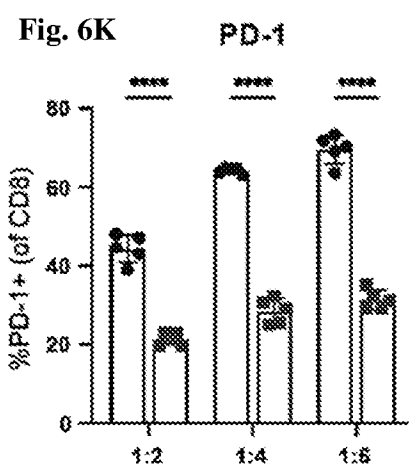


Fig. 6L

Granzyme B

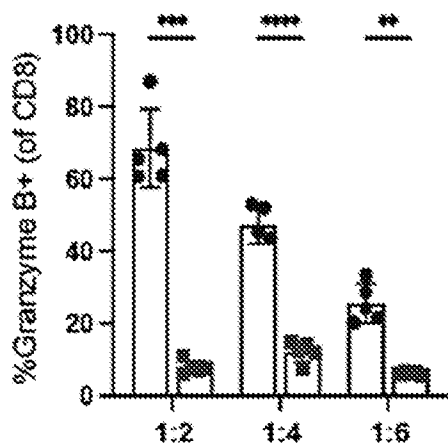


Fig. 6M

Live CD8

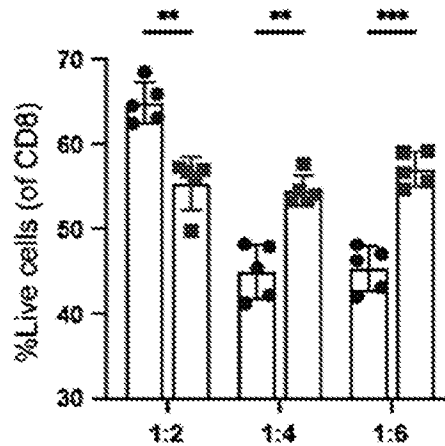


Fig. 6N

IL-2

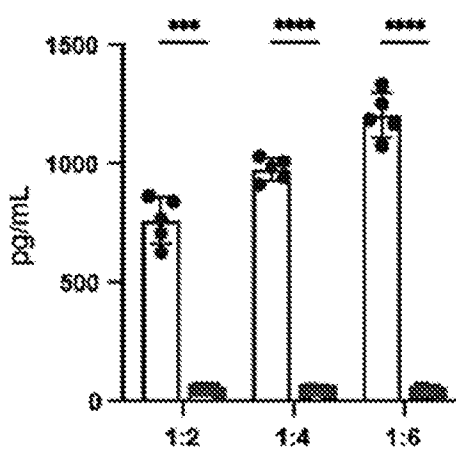


Fig. 6O

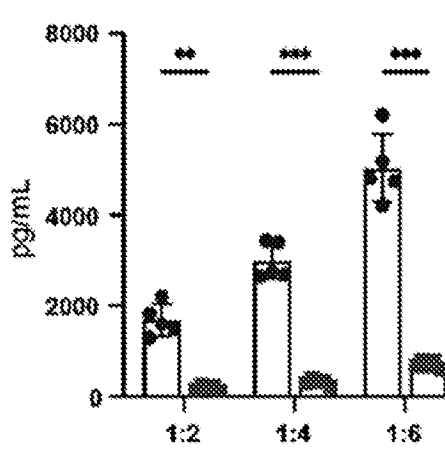
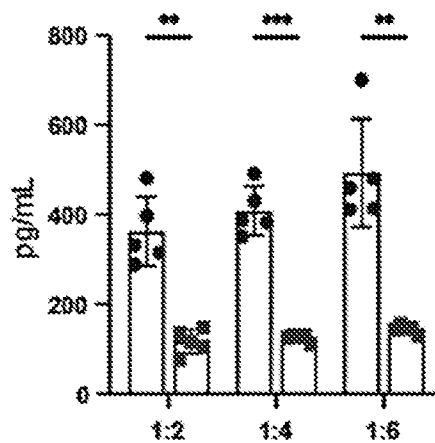
IFN γ 

Fig. 6P

TNF α 

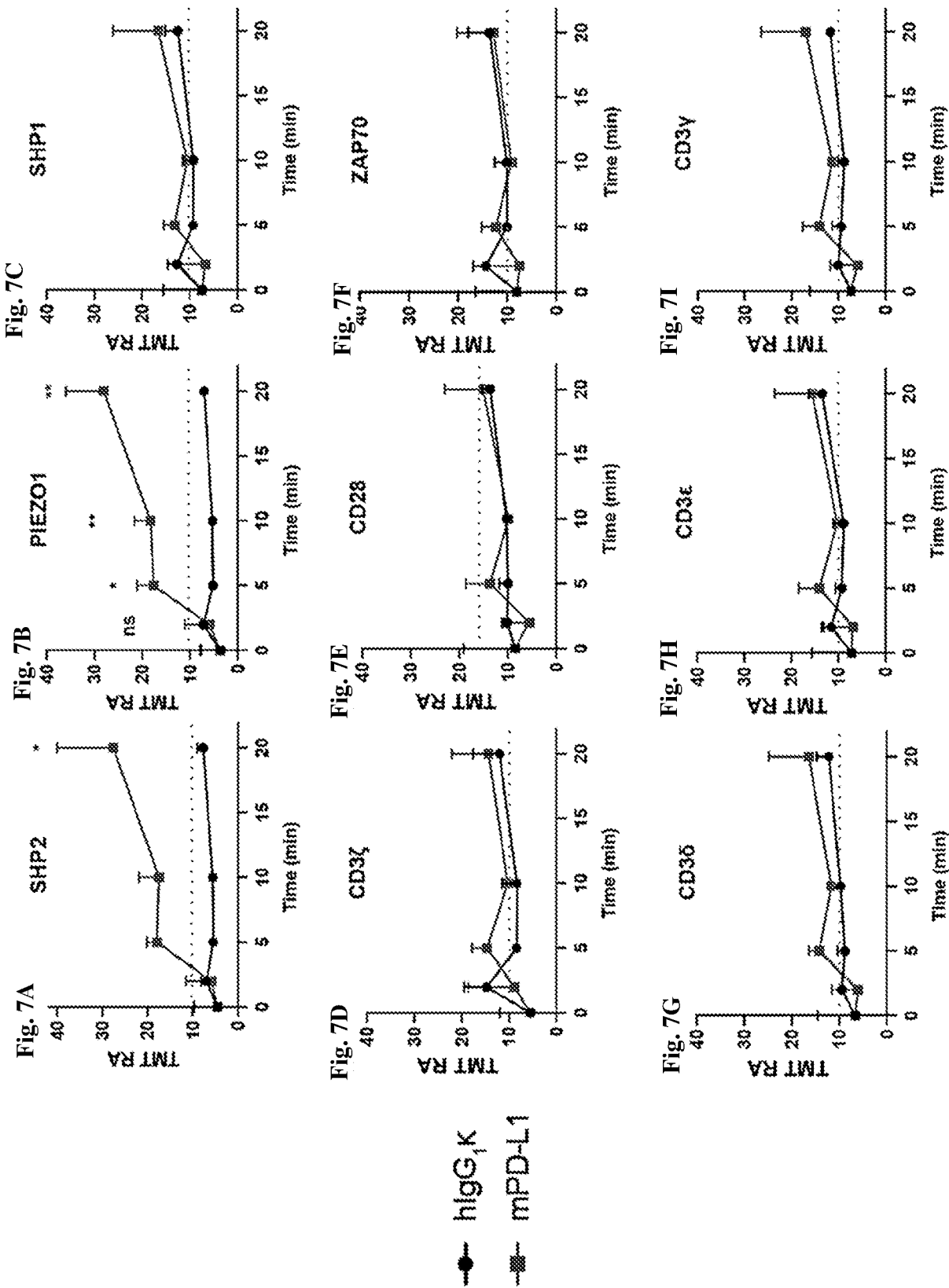


Fig. 8A

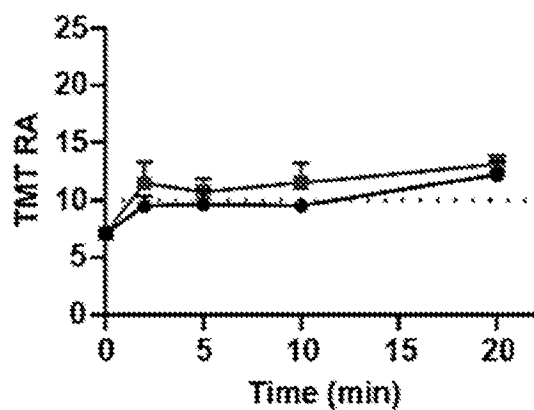
CD3 δ 

Fig. 8B

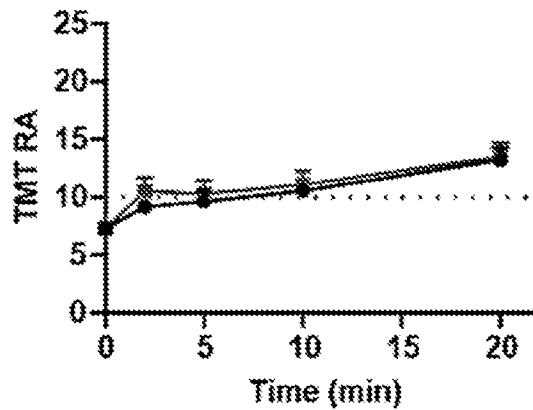
CD3 ϵ 

Fig. 8C

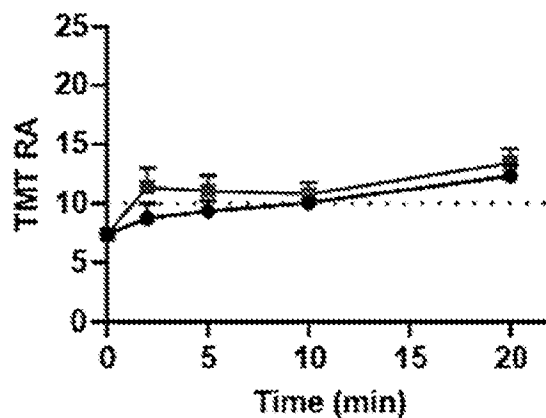
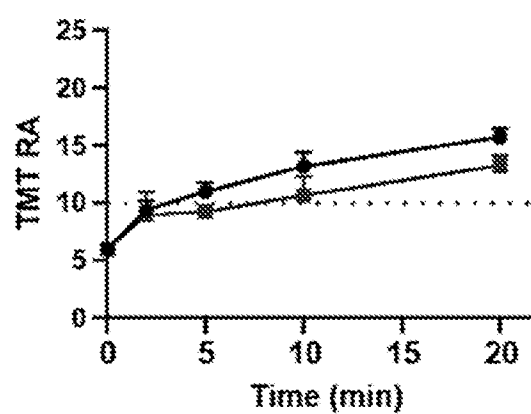
CD3 γ 

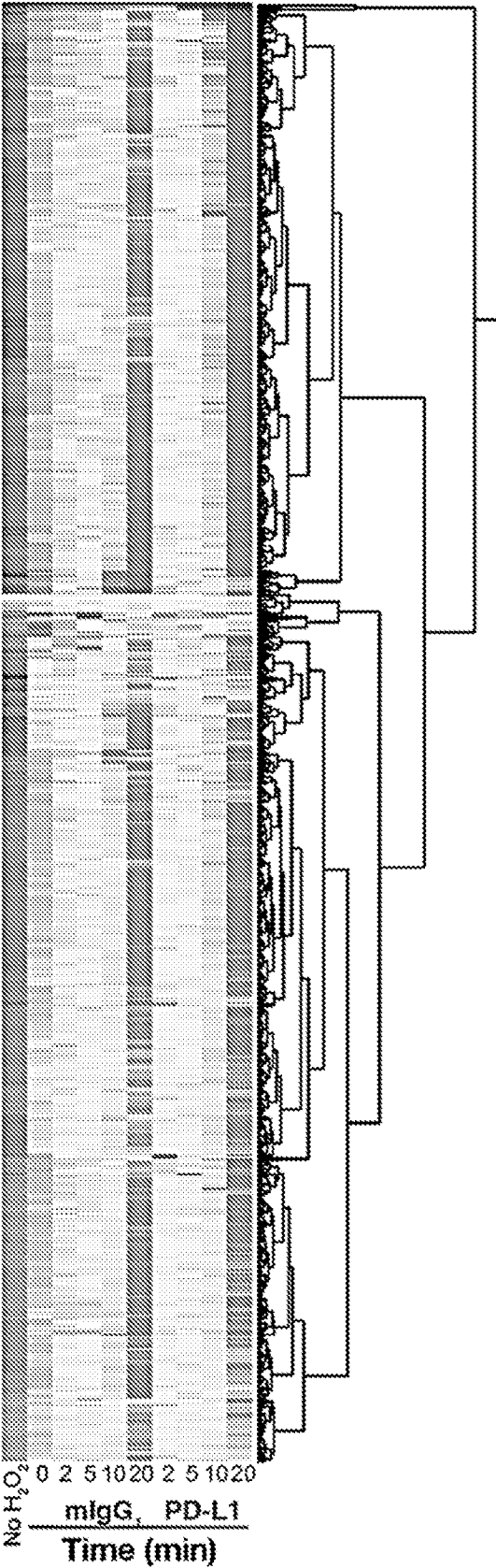
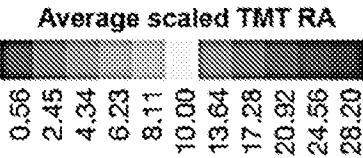
Fig. 8D

ZAP70



● mIgG₁
■ PD-L1

Fig. 8E



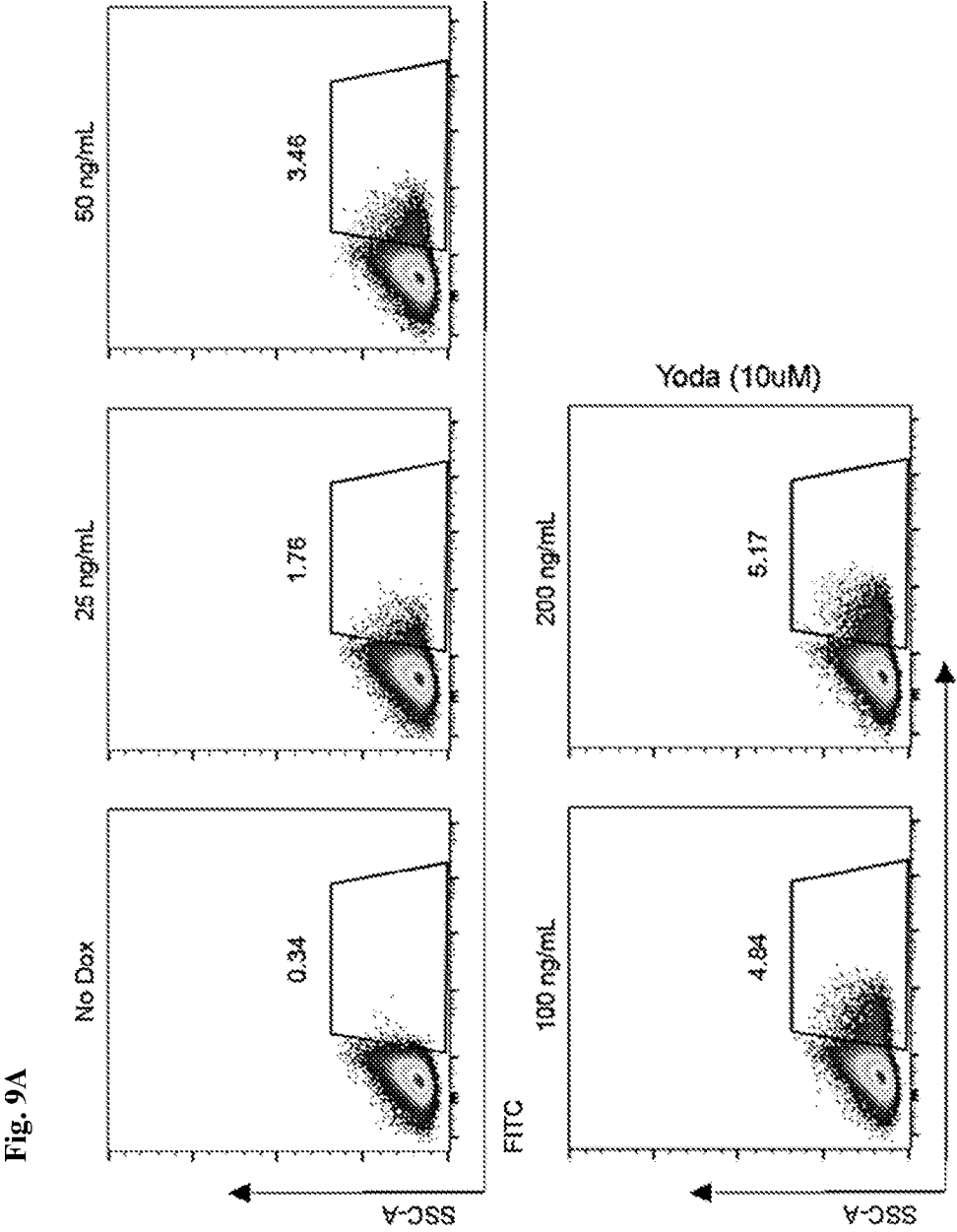


Fig. 9B

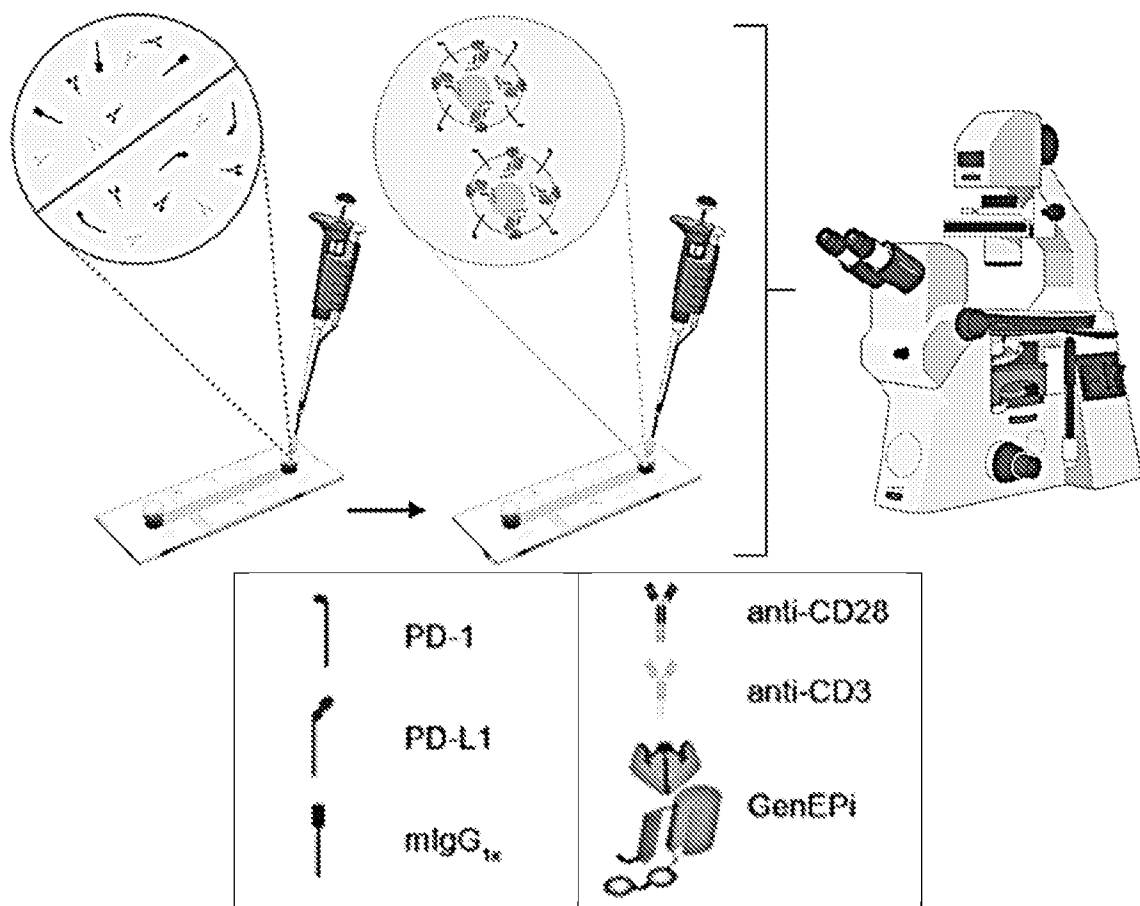
TCR and PD-L1 or
control channel coatingPD-1-expressing GenEPi
Jurkat flow throughTIRF time
lapse imaging

Fig. 9C

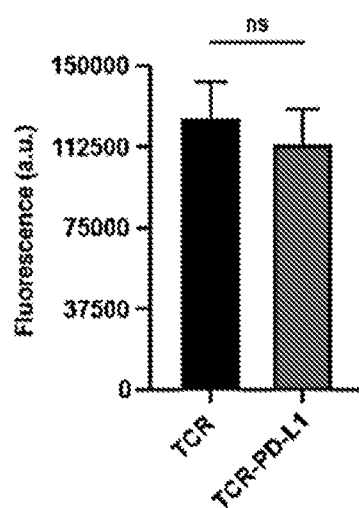
Local
PD-1

Fig. 9D

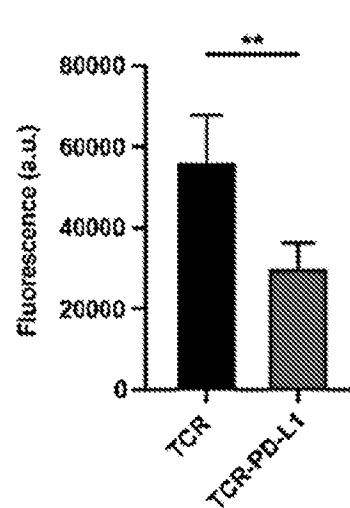
Local
PIEZO1 activity

Fig. 9E 10 s acquisition

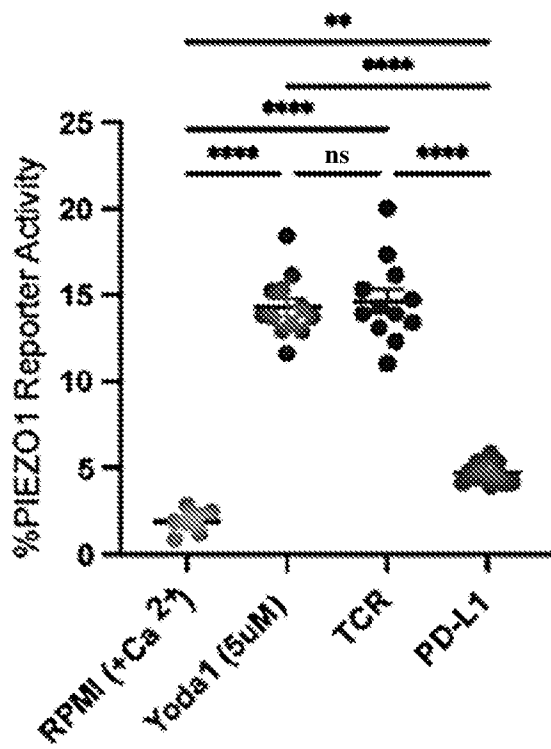


Fig. 9F 30 s acquisition

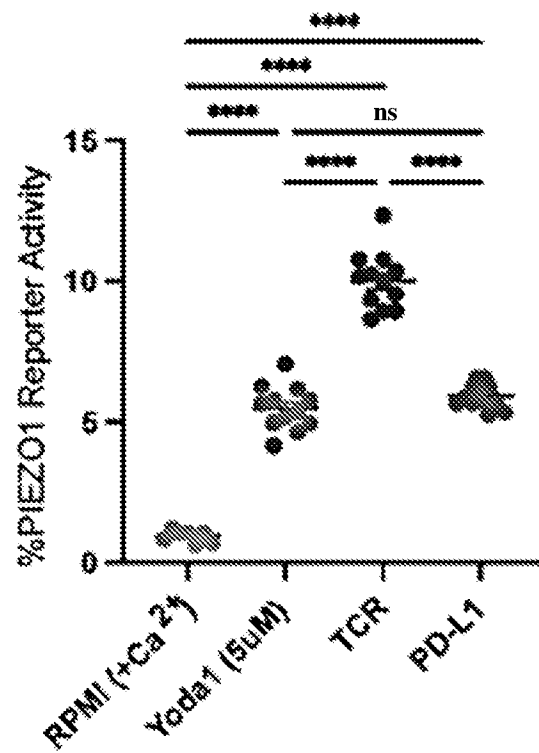


Fig. 9G

29/41

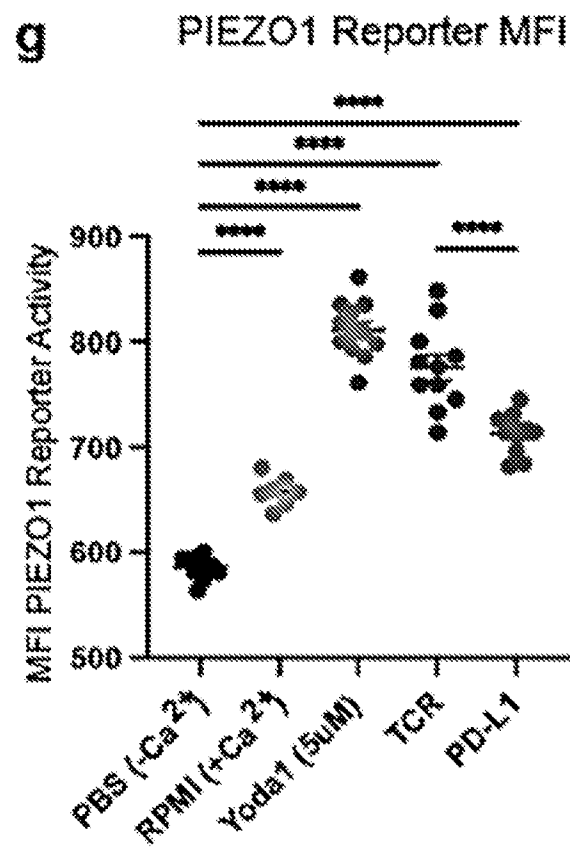


Fig. 10A

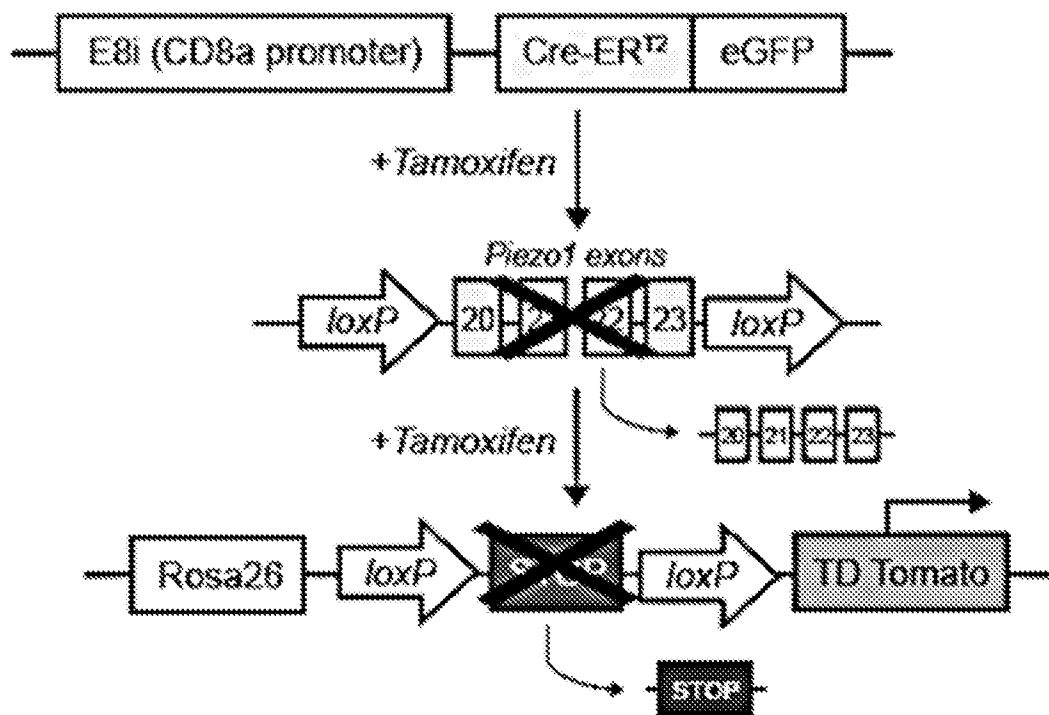


Fig. 10B

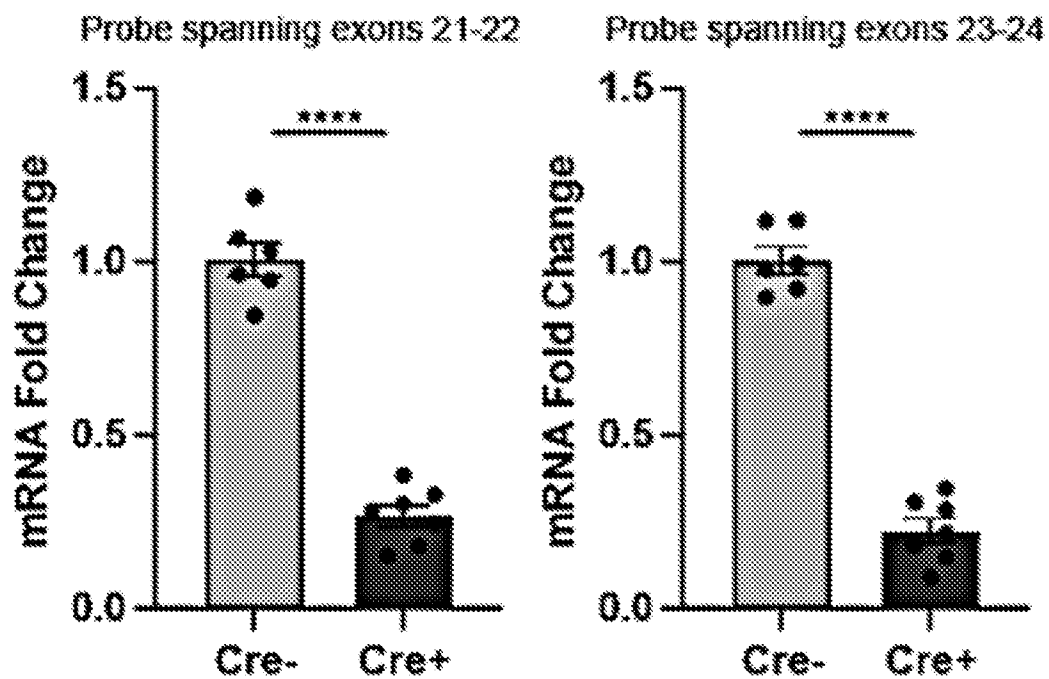


Fig. 10C

In vivo tamoxifen dosing

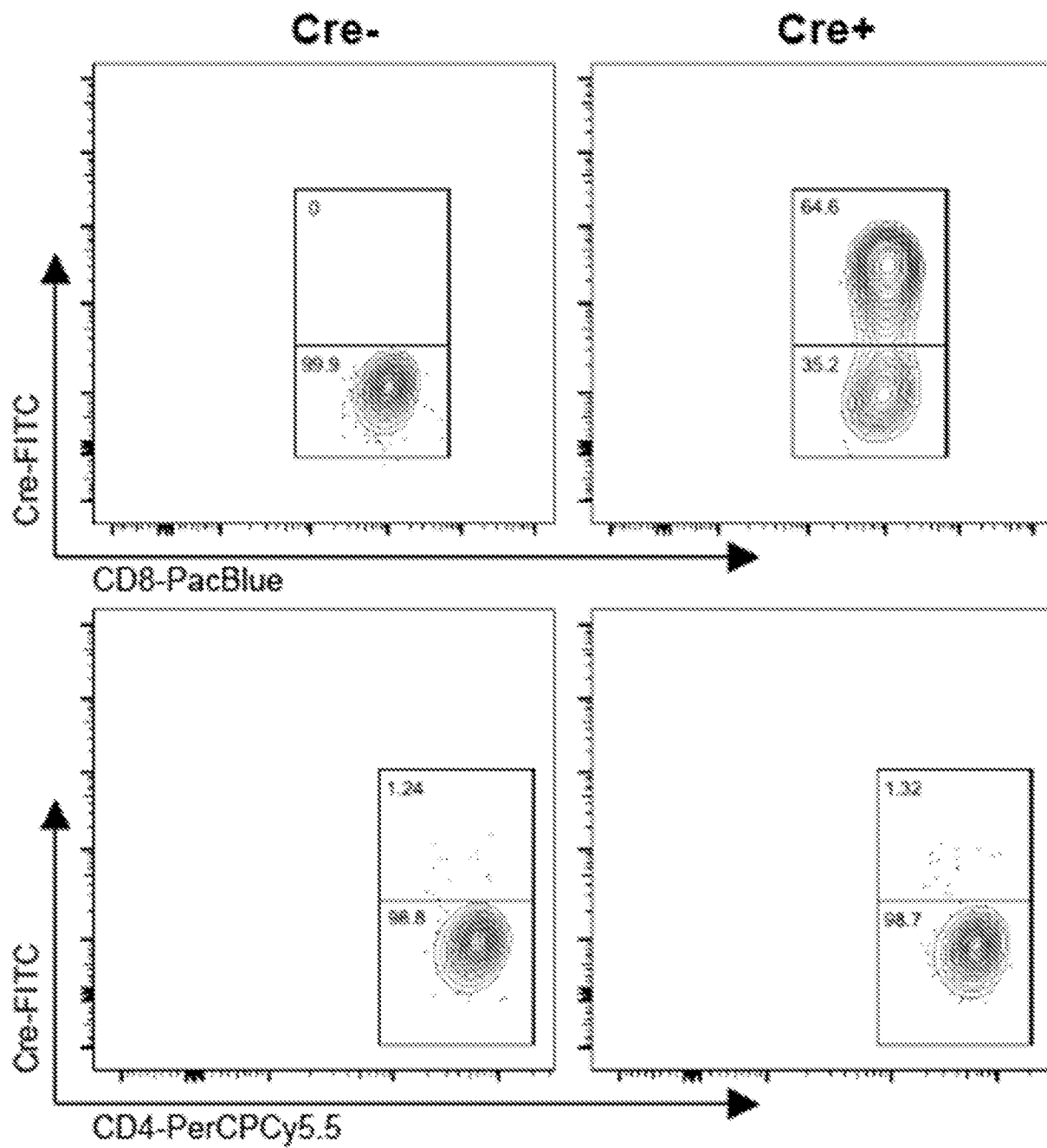


Fig. 10C cont.

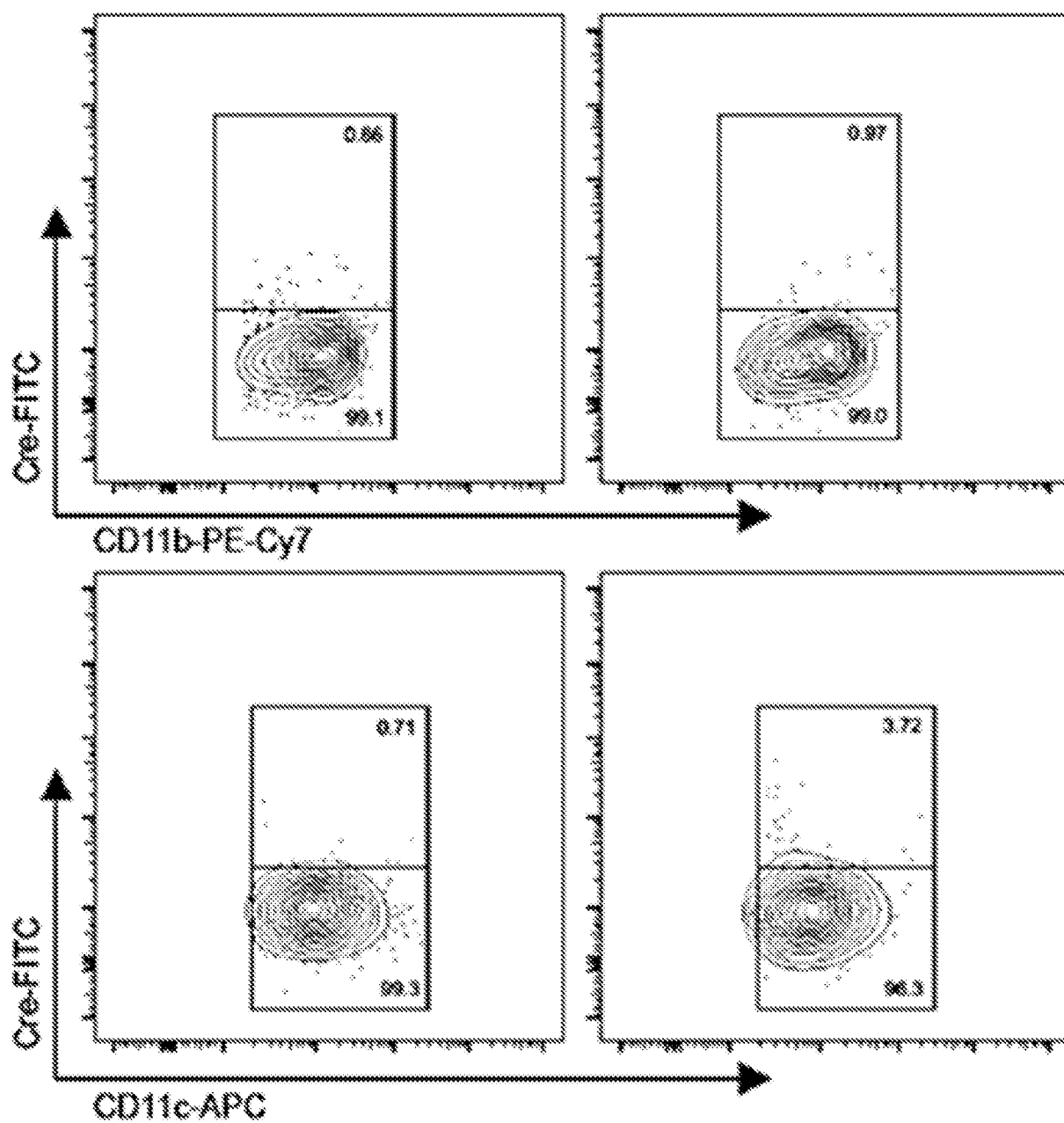


Fig. 10D

In vitro 4-OH dosing

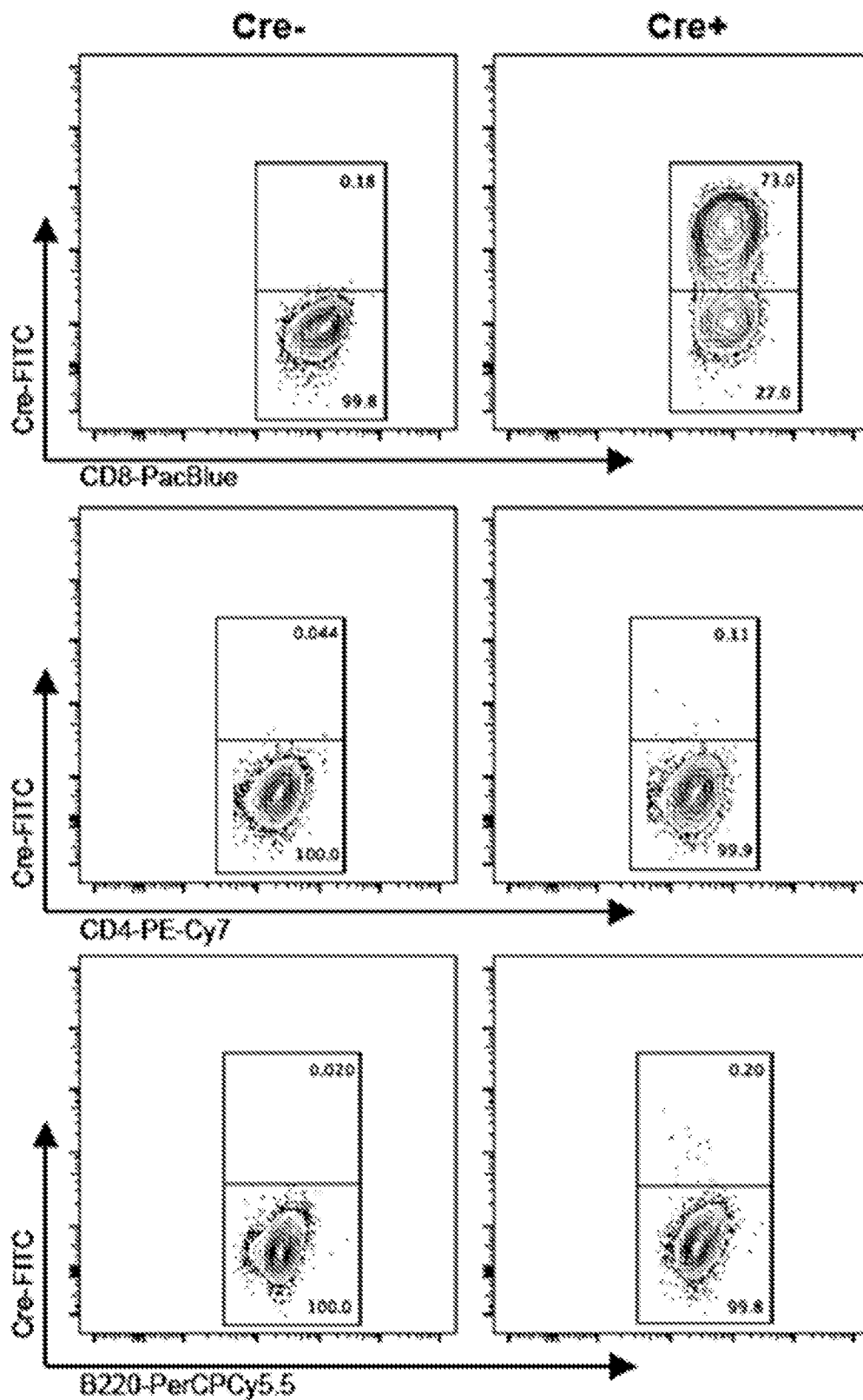


Fig. 10E

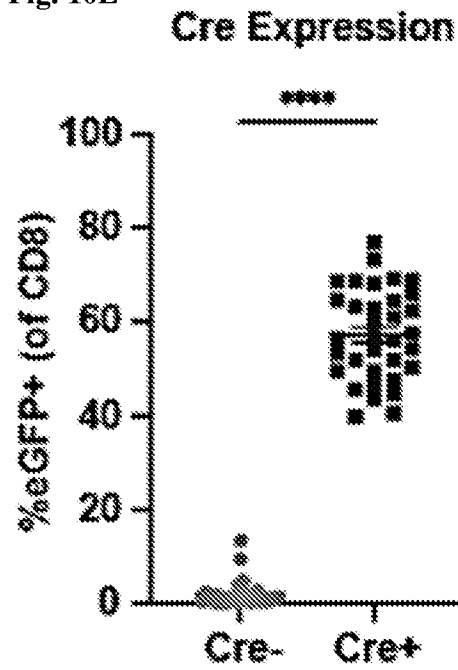


Fig. 10F

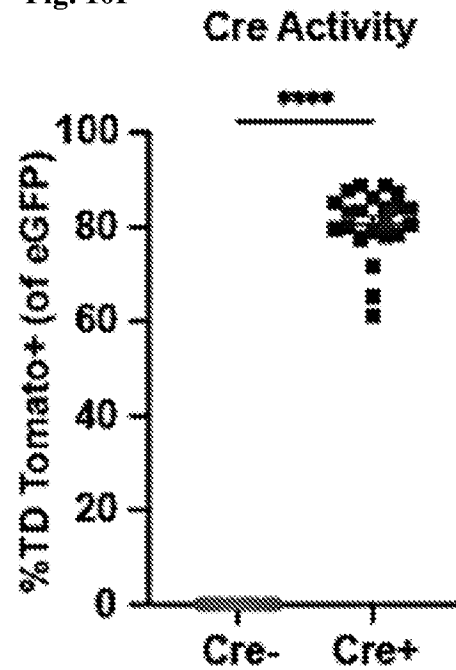
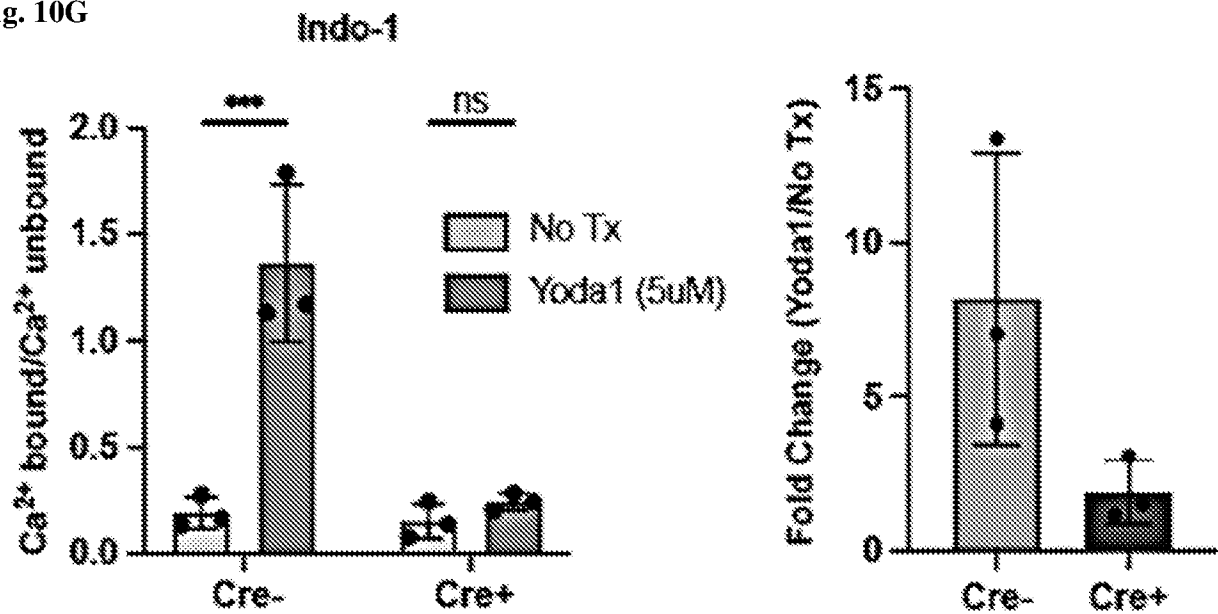


Fig. 10G



● Cre+
■ Cre-

Fig. 11A

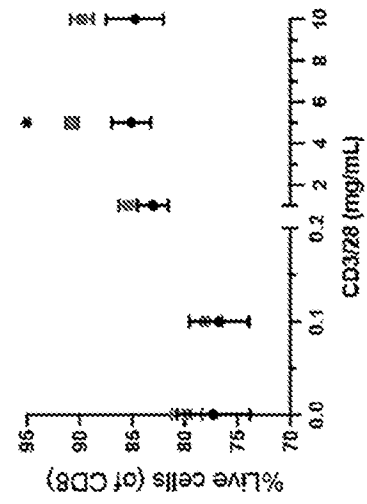


Fig. 11B

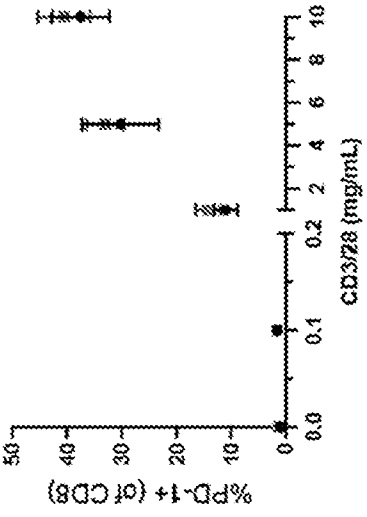


Fig. 11C

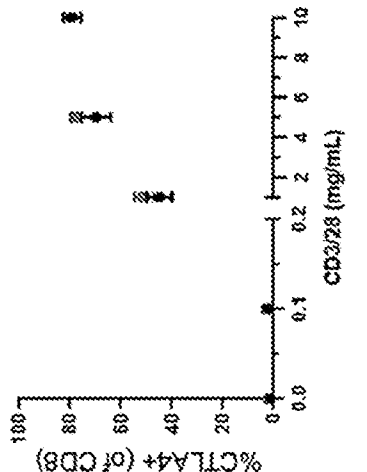


Fig. 11D

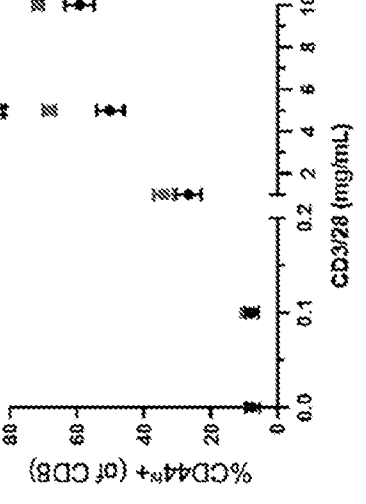


Fig. 11E

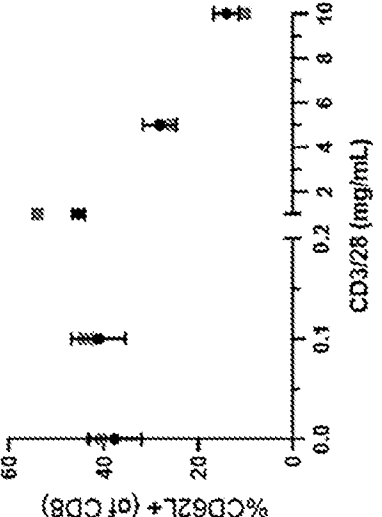
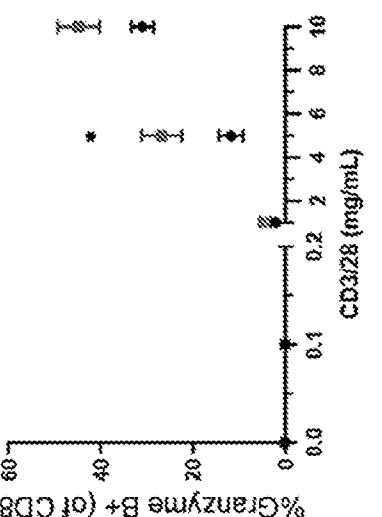


Fig. 11F



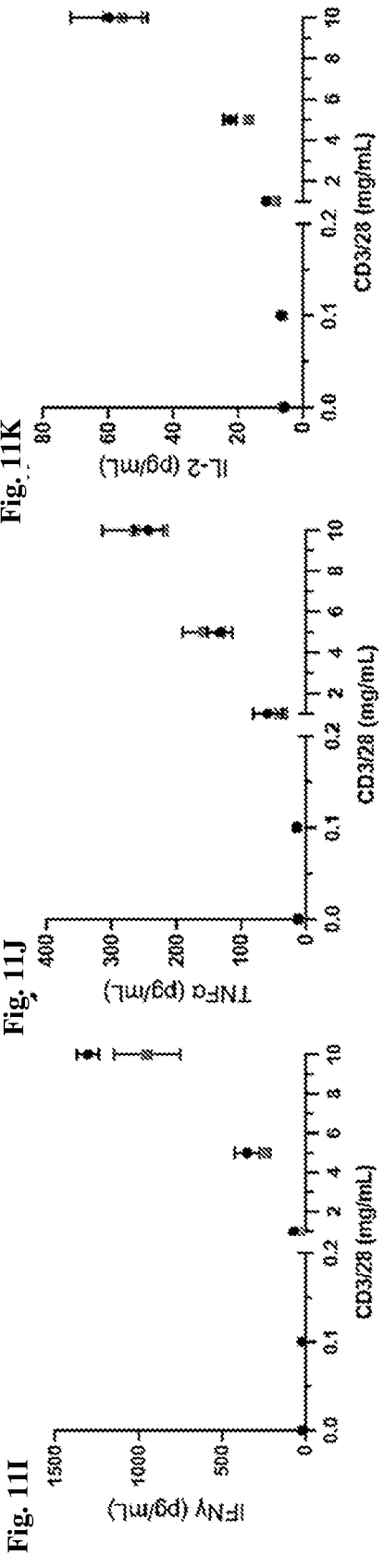
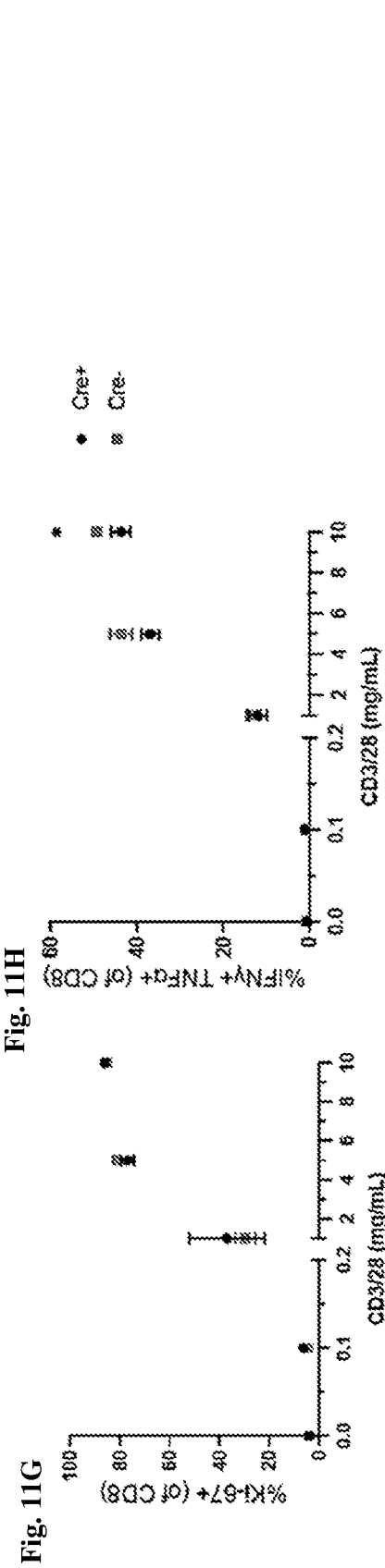
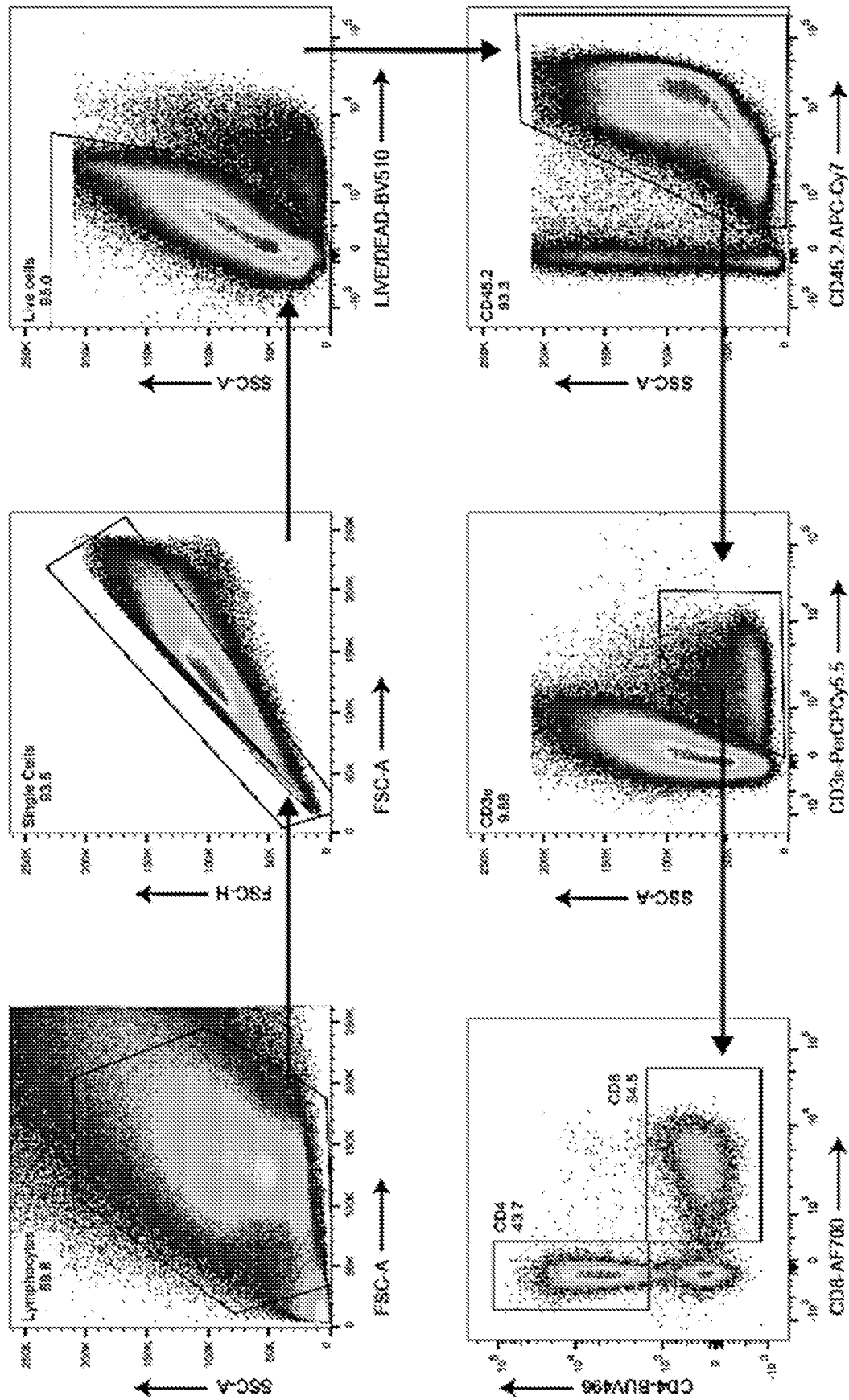
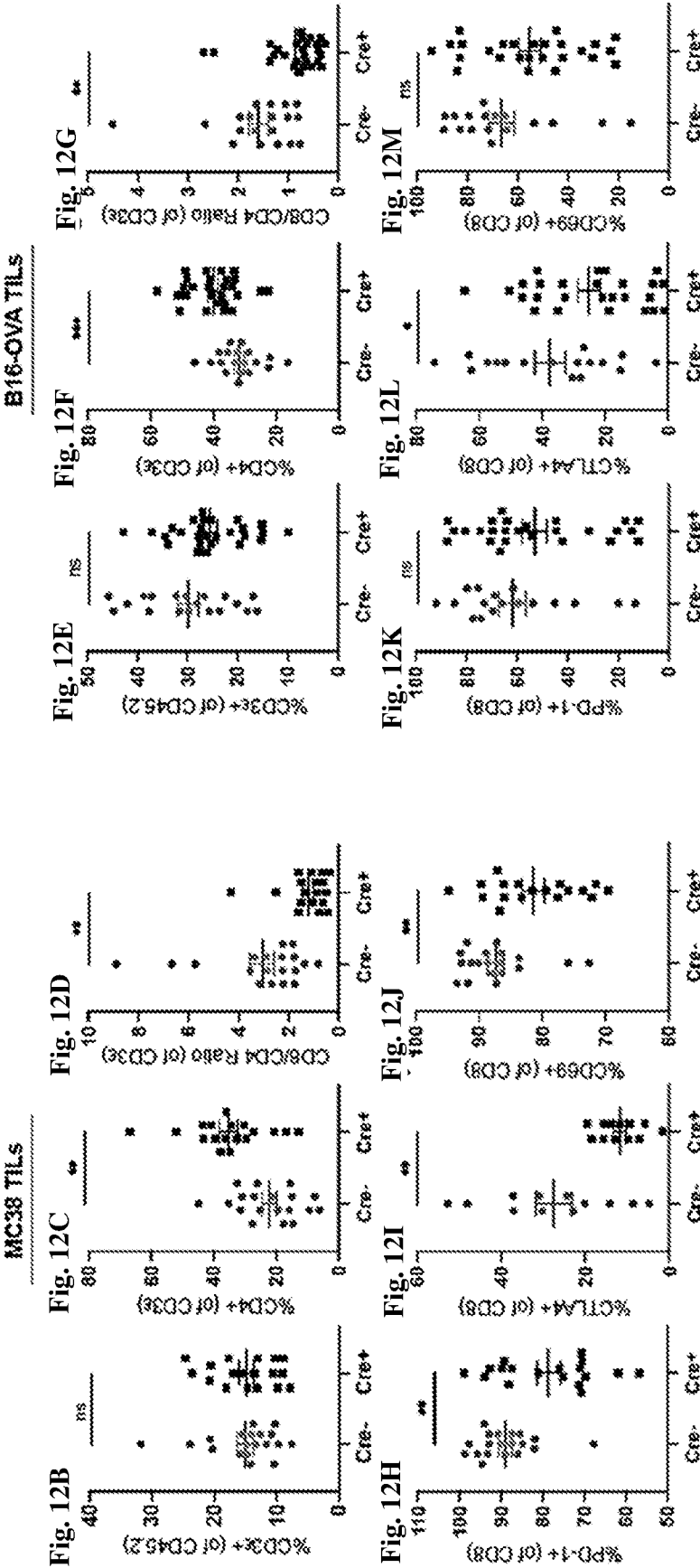
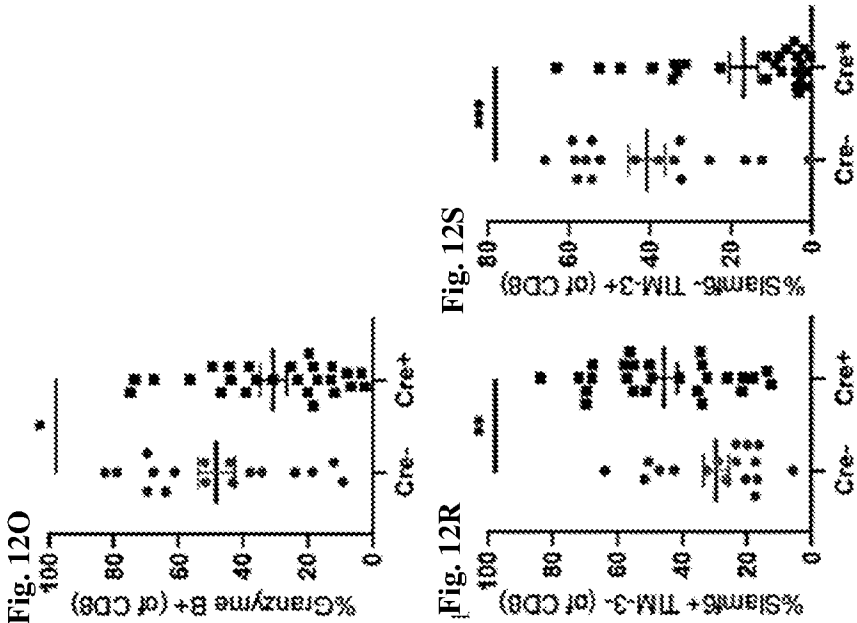


Fig. 12A

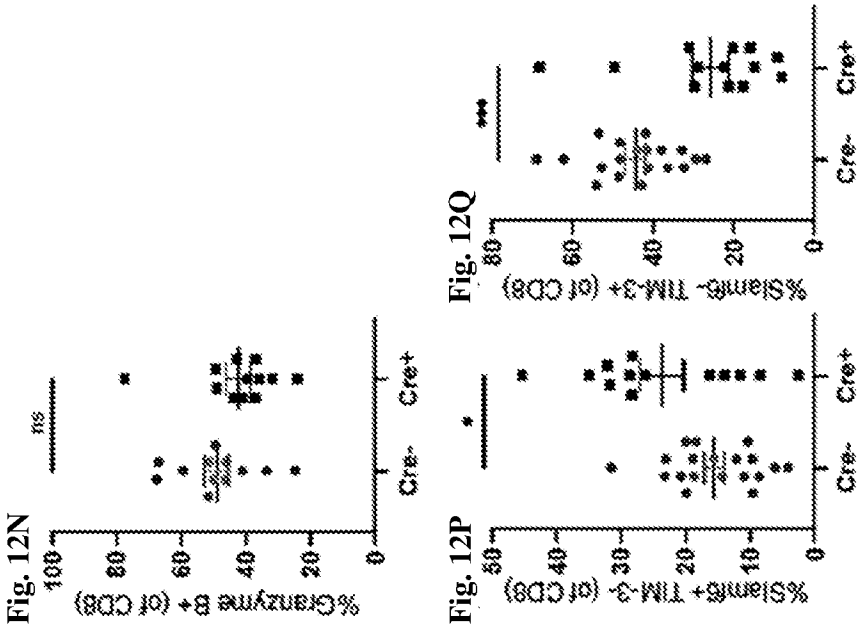


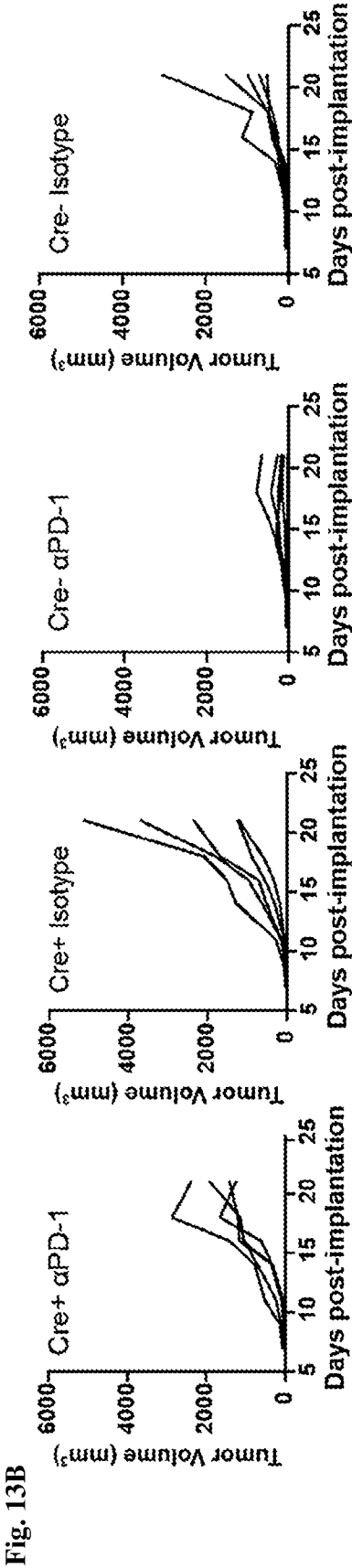
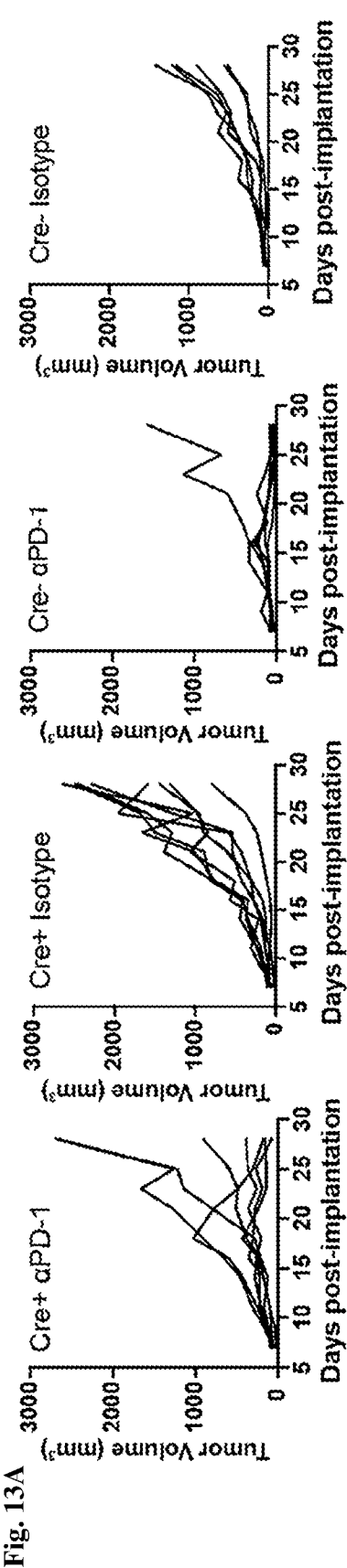


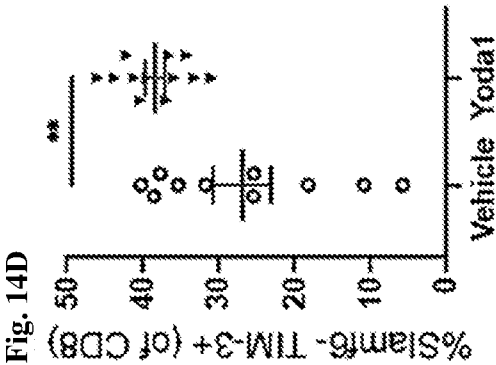
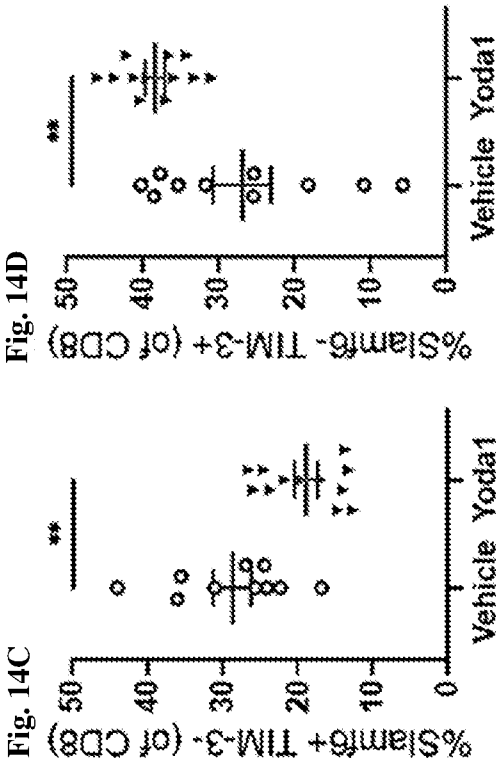
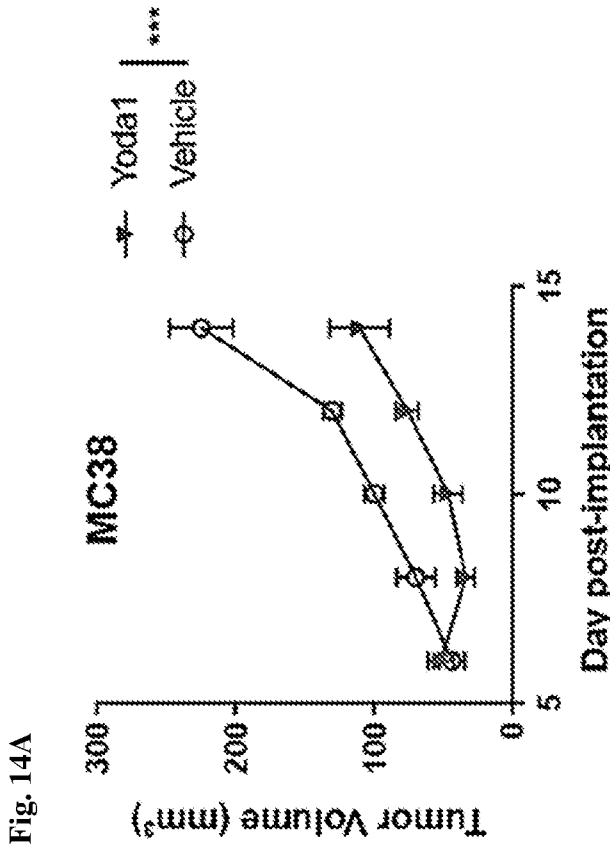
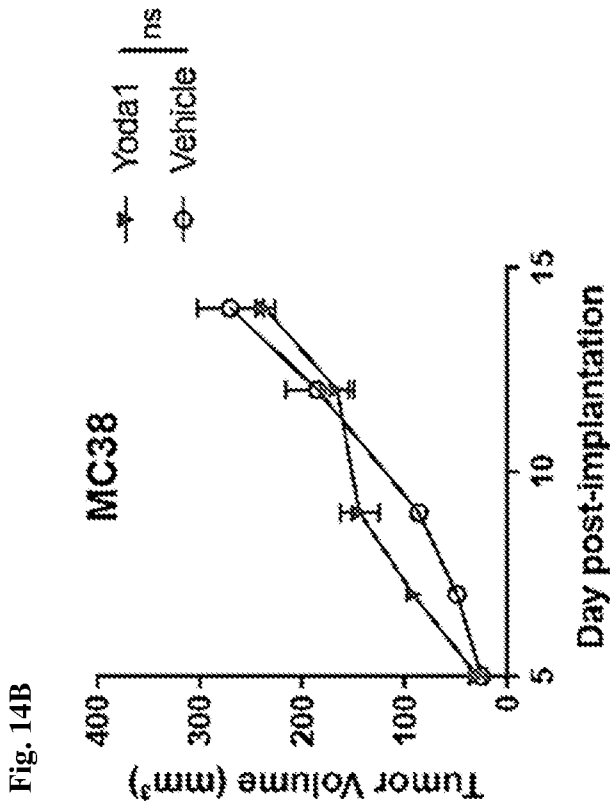
B16-OVA TILS



MC38 TILS







INTERNATIONAL SEARCH REPORT

International application No
PCT/US2023/032718

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	A61K31/341	A61K31/381
	A61K48/00	A61P35/00
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)		
A61K A61P		
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, 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	WO 2019/202473 A1 (UNIV LAUSANNE [CH]) 24 October 2019 (2019-10-24) claims 1-4, 10-15; 0031: p. 8, 1. 3 -----	1-6, 8, 9, 13-20, 22-26
A	WO 2020/028686 A1 (UNIV NEW YORK [US]) 6 February 2020 (2020-02-06) 0058- 0061 and Fig. 1B.; 0083 ----- -/--	1-9, 13-26
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
18 December 2023		29/02/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Dahse, Thomas

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2023/032718

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YU JIA-LIN ET AL: "Piezo-type mechanosensitive ion channel component 1 (Piezo1) in human cancer", BIOMEDICINE & PHARMACOTHERAPY, ELSEVIER, FR, vol. 140, 16 May 2021 (2021-05-16), XP086635114, ISSN: 0753-3322, DOI: 10.1016/J.BIOPHA.2021.111692 [retrieved on 2021-05-16] the whole document -----	1-9, 13-26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/032718

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.
 - b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13~~ter~~.1(a)).
☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2023/032718

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

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

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

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

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

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

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

see additional sheet

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:
2, 3, 19, 20 (completely); 1, 4-9, 13-18, 21-26 (partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2023/032718

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 2019202473	A1	24-10-2019	NONE	

WO 2020028686	A1	06-02-2020	NONE	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 2, 3, 19, 20 (completely); 1, 4-9, 13-18, 21-26 (partially)

directed to a compound/composition comprising an agent that increases or stabilizes the activity or expression of Piezo1 wherein said agent is small molecule agonist of PIEZO1, such as Yoda1, Jedi1 or Jedi2 (but not including the PIEZO1 modulator DHA), or uses thereof.

2. claims: 10-12 (completely); 1, 4-9, 13-18, 21-26 (partially)

directed to a compound/composition comprising an agent that increases or stabilizes the activity or expression of Piezo1 being gRNA fused to a transcription activator or a vector encoding a PIEZO1 protein, or uses thereof.

3. claims: 1, 4-9, 13-18, 21-26 (all partially)

directed to a compound/composition comprising an agent that increases or stabilizes the activity or expression of Piezo1 and is not covered by inventions I-II (such as e.g. DHA), or uses thereof.
