Additional File 1: Methods

Hybridoma generation

Female BALB/c mice were immunized using cDNA encoding human (hu)CD27 (GenBank NM_001242) subcloned in pCI-neo vector (Promega).[1] hCD27-specific B cells were obtained by panning on irradiated Chinese hamster ovary (CHO)-K1.huCD27 cell transfectants and immortalized by fusion with the Sp2/0-Ag14 mouse myeloma cell line by electro-cell fusion to generate hybridomas.[2 3]

Mouse tumor cell-lines

Merck & Co., Inc., Palo Alto, CA, USA cell line banks for MC38 (Developmental Therapeutics Program Tumor Repository, Frederick National Laboratory, Frederick, MD) and MB49 (University of Iowa) were authenticated by IDEXX CellCheck and screened for mycoplasma.

Blood and tissue sources

Blood from human healthy volunteers for flow cytometry was collected as part of the in-house healthy donor program at Merck & Co., Inc., Palo Alto, CA, USA. Blood was collected into EDTA tubes (BD Biosciences) and gently inverted 10 times to mix. Buffy coats were obtained from the Stanford Blood Center (Palo Alto, CA, USA).

Blood from rhesus monkeys was collected into EDTA tubes at Bioreclamation IVT (Hicksville, NY, USA) and shipped at 4°C overnight to Merck & Co., Inc., Palo Alto, CA, USA.

Fresh tumor tissues were obtained from commercial vendors in accordance with state and federal regulations. Specimens collected at the operation sites were shipped in AQIX media (AQIX, UK) overnight at 4°C to Merck & Co., Inc., Palo Alto, CA, USA.

CD27 binding by ELISA and flow cytometry

CHO-K1 cells were transfected with pMX-puro or pCI-neo vectors encoding CD27 of human, rhesus monkey, or mouse origin using Lipofectamine 2000 following the manufacturer's instructions. For ELISA, bound antibodies were detected with HRP-conjugated $F(ab')_2$ goat anti-human IgG (Jackson ImmunoResearch) and visualized with TMB chromogen (ThermoFisher). Plates were read on a Molecular Devices VersaMax plate reader at 450 nm/650 nm and data was analyzed in GraphPad Prism 7 using nonlinear regression.

For flow cytometry, transfected cells were detached with enzyme-free cell dissociation solution (Gibco, 13151-014). Detached cells were transferred to 96 wells round-bottom well plates at approximately $1x10^5$ cells/well. Primary antibodies were incubated for 30 minutes at 4°C and after wash steps with PBS/BSA 1%, binding of antibodies was detected using goat-anti-mouse IgG-FITC (BD Bioscience, 349031, 2 µl/well) or goat-anti-human IgG-FITC (γ-chain specific, Southern Biotech, 2040-02, 2 µl/well). After washing once, antibody binding was

detected using a FACSCanto II (BD Biosciences) and data was analyzed using FlowJo software (Tree Star Inc.). Debris, dead cells and doublets were excluded from the analysis.

To test antibody binding to CD27 on primary peripheral blood T cells, human or rhesus monkey whole blood was incubated with a cocktail of phenotyping antibodies (Additional File 1, Table 1) and MK5890 directly labeled with DyLight™ 650. Red blood cells were lysed with ACK Lysing Buffer (Gibco), washed, and resuspended in PBS containing Fixable Viability Dye eFluor506 (eBioscience). After washing, cells were fixed in 1% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 minutes, washed, and acquired in a BD Fortessa™ flow cytometer (BD Biosciences). Flow cytometry data was analyzed using FlowJo software (Tree Star Inc.).

Table 1 Phenotypic Antibodies Used for T Cell Binding Assays

Targeted Antigen	Fluorophore	Vendor	Catalog no.	Clone	Amount per test (µL)	Isotype
CD20	FITC	BD Biosciences	555622	2H7	5	Mouse IgG2b, κ
CD14	PerCP-Cy5.5	BioLegend	301824	M5E2	5	Mouse IgG2a, κ
CD3	Pacific Blue	BD Biosciences	558124	SP34-2	5	Mouse IgG1, λ
CD56	BV650	BD Biosciences	564057	NCAM16.2	5	Mouse IgG2b, κ
CD4	PE/CF594	BD Biosciences	562402	L200	0.1	Mouse IgG1, κ
CD8a	APC/Cy7	BioLegend	301016	RPA-T8	5	Mouse IgG1, κ
CD25	PE/Cy7	BD Biosciences	557741	M-A251	5	Mouse IgG1, κ

APC = allophycocyanin; BV = Brilliant Violet™; Cy7 = cyanine 7; FITC = fluorescein isothiocyanate; no. = number; PE = phycoerythrin; PerCP-Cy5.5 = peridinin-chlorophyll-protein-cyanine 5

Antibody affinity measurement

Human IgG1 Fc-tagged human or rhesus monkey CD27 made by transient transfection in HEK293F cells was amine coupled to a CM5 sensor chip using an amine coupling kit (GE Healthcare). Binding kinetics were measured on a Biacore T200 by injecting 2.5-fold dilution titration series of MK-5890 from 4.1 nM to 400 nM over the amine coupled antigen. The chip was regenerated with a 30 second injection of 3 M magnesium chloride. Biacore T200 evaluation software was used to fit data to a 1:1 binding model to determine the association rate (ka, M1s1) and dissociation rate (kd, s1) constants for calculating the equilibrium dissociation constant (KD, M).

Crystallization and X-ray structure determination

cDNA encoding residues 21-177 of human CD27 (Uniprot P26842-2) preceded by a heterologous leader sequence (MDPKGSLSWRILLFLSLAFELSYG) and a C terminal His_6 tag was cloned into pTT5 and the resulting plasmid was used to express the protein in Expi293 cells (Thermo) according to the manufacturer's instructions. The culture supernatant was harvested and clarified, and the protein purified via IMAC chromatography and polished by size exclusion chromatography. The resulting protein was >99% pure as estimated by SDS-PAGE and

analytical SEC. The MK-5890 Fab was expressed recombinantly in Expi293 cells and was purified from the clarified supernatant with Kappa Select resin (GE Healthcare).

The CD27 - Fab complex was formed by incubating at a molar ratio of 2:1 antigen and Fab, respectively, for 48 hours at 4 °C. The reaction mixture was then loaded on a Superdex 200 HiLoad 16/600 (120mL) column and fractions were pooled based on SEC-UPLC analysis. The complex pool was then dialyzed against 25 mM Tris•Cl pH 8.0, 0.1 M NaCl. The dialyzed material was centrifuged and filtered over a 0.22 μm syringe filter. The protein concentration was 15 mg/ml.

Screening for crystallization conditions was performed with either the protein sample as is or with cadmium as an additive.[4 5] For the latter, several samples of the complex with various concentrations of cadmium were prepared and screening was performed at the highest concentration of the additive which promoted no precipitation of the protein upon addition. Ultimately the sample that crystallized was prepared by the addition of 6.25 μL of a 40 mM cadmium chloride stock to a 25 μL aliquot of a CD27-Fab complex. The final sample protein concentration was 12.6 mg/mL and the cadmium concentration 8.0 mM. Samples were held at room temperature for approximately 1 hour prior to setup. Crystals were obtained only when cadmium was present. Initial screening was performed using a Topaz free interface diffusion (FID) microfluidic system, Topaz 4.96 chips (Fluidigm), which does not allow for UV imaging. Crystals were found from the Rigaku Wizard Cryo 1-2 screen and the Jena Classic HTS1 screen. After optimization, the conditions were 0.1M Tris•Cl pH 8.5 and 40% v/v PEG 400 at 18°C. Crystals were harvested straight from the crystallization drop using a 0.3mm mesh Litholoop (Molecular Dimensions Ltd, Suffold, UK) and flash frozen in liquid nitrogen.

Data collection was performed at the Industrial Macromolecular Crystallography Association (IMCA) beam line, sector 17 of the Advanced Photon Source (APS) at the Argonne National Laboratory (ANL, Lemont, IL). Data were collected at a wavelength of 1.0Å using a Pilatus 6M detector (Dectris AG, Baden-Dättwil, Switzerland). The data were processed using the autoPROC[6] automated processing software with calls to XDS[7] for indexing and integration, AIMLESS for scaling[7], POINTLESS for space group determination,[8 9] and STARANISO for applying anisotropic diffraction limits.

The structure of the resolved crystals was solved by molecular replacement using the program MOLREP. PDB entry 2XTJ and 5TL5 were used to search for the Fab and antigen, respectively. Using default parameters, the two variable regions together were located first, then the constant regions were added. No unambiguous solution could be found at this stage for the antigen, even when using the Fab coordinates as a fixed model. The Fab model was rebuilt with the proper sequence and refined. A solution then could be found for the antigen with MOLREP using the Fab coordinates as a "fixed model".[10] Several rounds of refinement and rebuilding were performed. All refinement steps were done with the program autoBUSTER and COOT[11] was used for model rebuilding. Additional steps of rebuilding and refinement led to final values of R_{free} and R_{work} of 22.4% and 20.0%, respectively. The final model contains CD27 residues 26

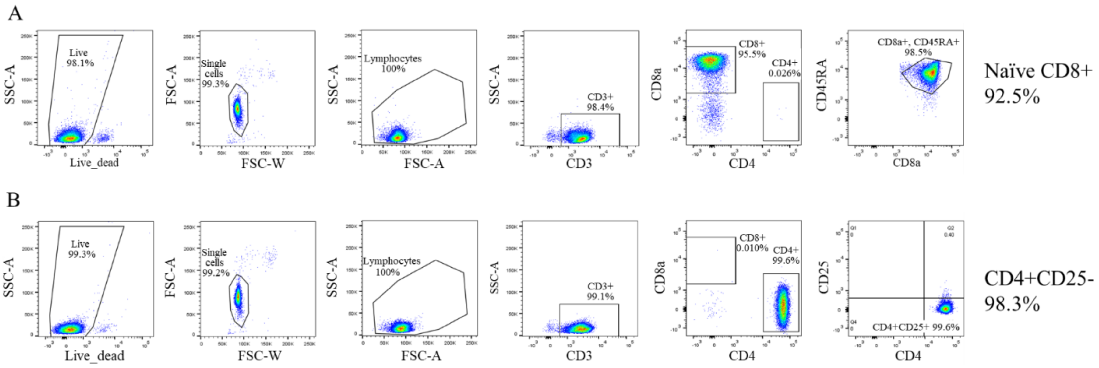
to 120, Fab light chain residues 1 to 212, Fab heavy chain residues 1 to 131 and 137 to 217, 6 Cadmium cations, one PEG 400 molecule and 286 waters. Electron density consistent with presence of glycosylation can be found near Asn75 side-chain, but the map quality is insufficient for unambiguous interpretation and the model does not contain coordinates for a covalently bound carbohydrate.

Isolation of primary T Cells

Human T cells were enriched from buffy coats using either the RosetteSep Human CD4+ or CD8+ T Cell Enrichment Cocktail (Stem Cell Technologies) according to manufacturer's instructions, followed by lysing of red blood cells using ACK Lysing Buffer. Naïve CD8+ T cells were isolated using Human Naïve CD8+ T Cell Enrichment Set (BD Biosciences). Naïve CD8+ T cell purities in assay development studies were typically greater than 90% (Fig 1A). Untouched CD4+CD25- T cells were isolated by removing the CD25+ population using the Human CD4+CD25+ Regulatory T Cell isolation kit (Miltenyi Biotec). CD4+CD25- T cell purities in assay development studies were usually greater than 95% (Fig 1B). Cells were either used immediately or viably frozen for use in future experiments.

To obtain CD4+CD25- cells from undiluted rhesus monkey whole blood, it was separated on a 60% percoll gradient. Following centrifugation, untouched CD4+CD25- T cells were isolated from the mononuclear cell layer by removing the CD25+ population with the NHP CD4+CD25+ Regulatory T Cell isolation kit (Miltenyi Biotec).

Figure 1: Evaluation of human CD8+ and CD4+CD25- T cell purity following isolations



Example flow cytometry plots showing naïve CD8+T cell purity (A) and CD4+CD25- T cell purity (B) after purification from human donor 62 buffy coat as described above. Purity = (#cells of interest/#live singlets) x 100.

Costimulation of primary T Cells

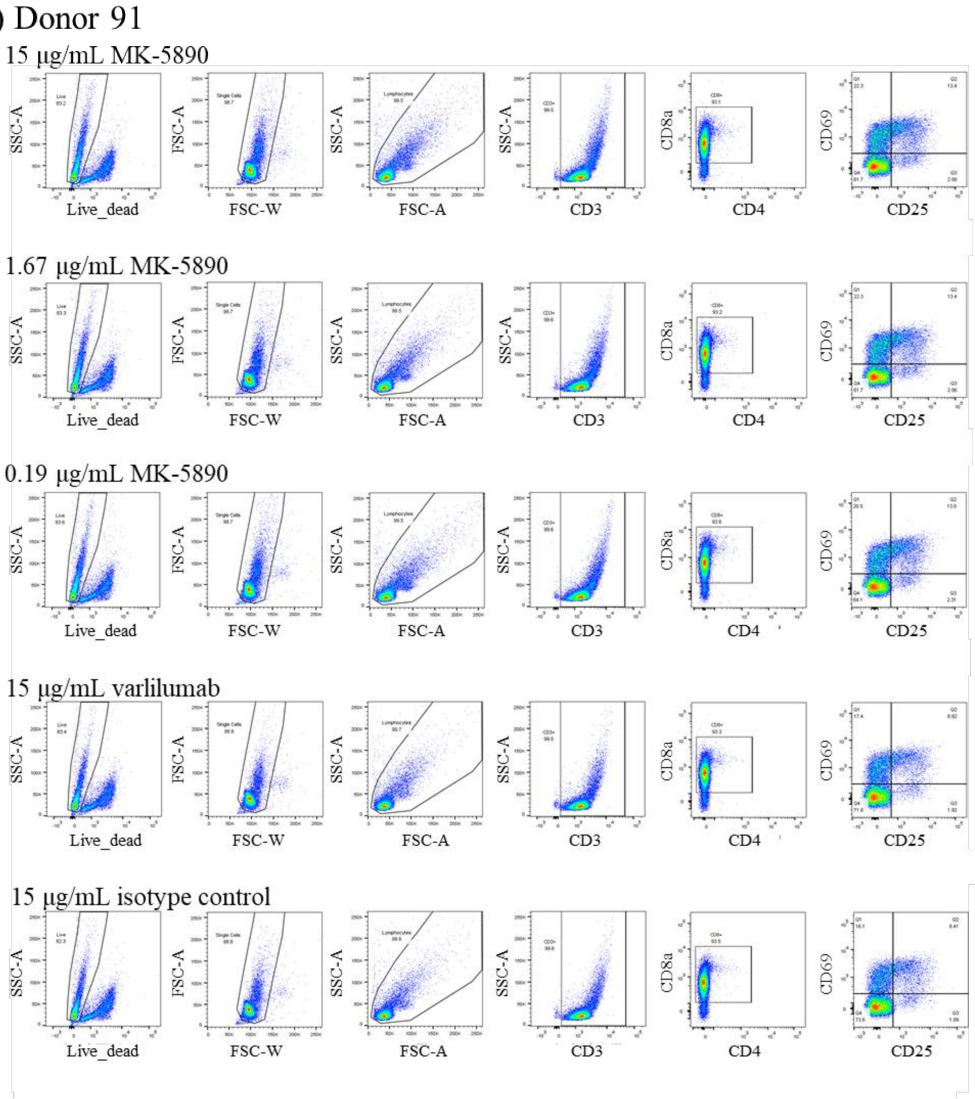
Antibodies used for phenotyping of T cell costimulation as described in the Methods section are described in Table 2, and representative plots are shown in Fig. 2.

Table 2 Phenotypic Antibodies Used for T cell Co-stimulation Assays

Targeted Antigen	Fluorophore	Vendor	Catalog no.	Clone	Amount per test (µL)	Isotype
CD69	FITC	BD Biosciences	557049	FN50	20	Mouse IgG1, κ
CD4	PE/CF594	BD Biosciences	562402	L200	0.5	Mouse IgG1, κ
CD25	PE/Cy7	BD Biosciences	557741	M-A251	5	Mouse IgG1, κ
CD8a	APC/Cy7	BioLegend	301016	RPA-T8	5	Mouse IgG1, κ
CD3	Pacific Blue	BD Biosciences	558124	SP34-2	5	Mouse IgG1, λ

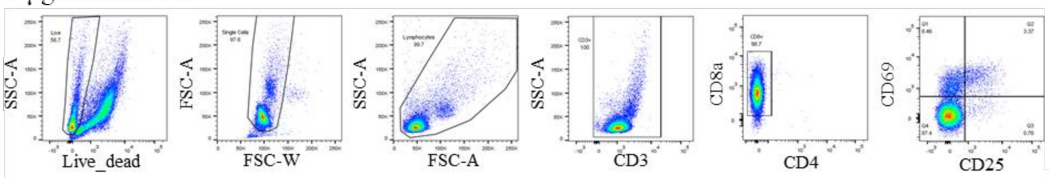
APC = allophycocyanin; Cy7 = cyanine 7; FITC = fluorescein isothiocyanate; no. = number; PE/CF594 = phycoerythrin-CF594; PE = phycoerythrin

Figure 2: Representative flow cytometry plots for phenotyping of CD25+CD69+ CD8 T cells in costimulation assay

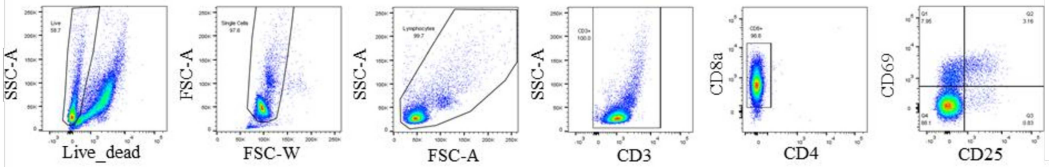


B) Donor 11

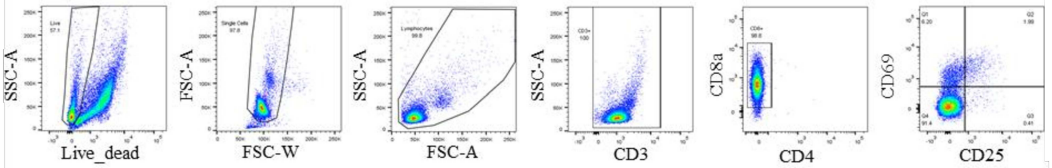
15 µg/mL MK-5890



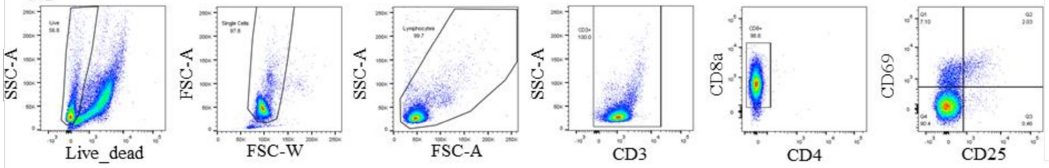
1.67 µg/mL MK-5890



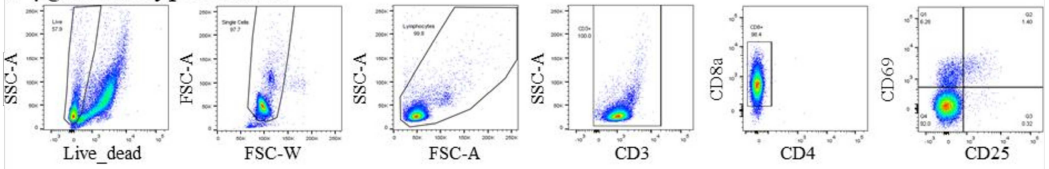
0.19 µg/mL MK-5890



15 µg/mL varlilumab

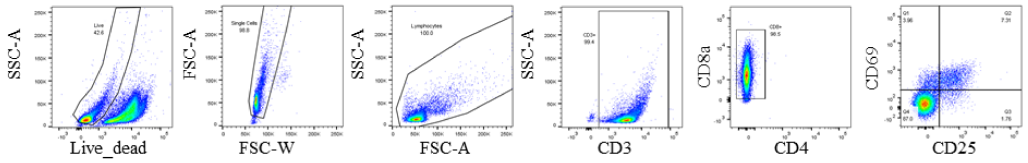


15 µg/mL ısotype control

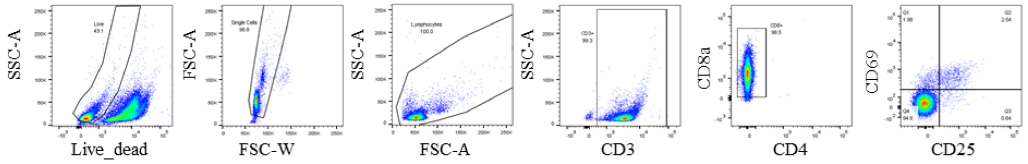


C) Donor 816

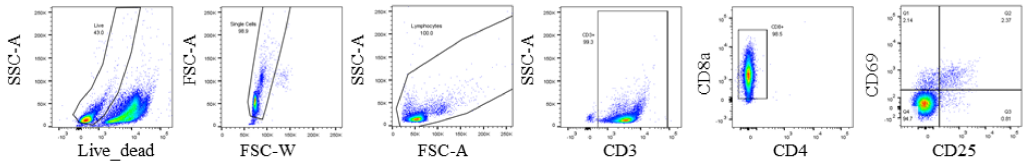
15 µg/mL MK-5890



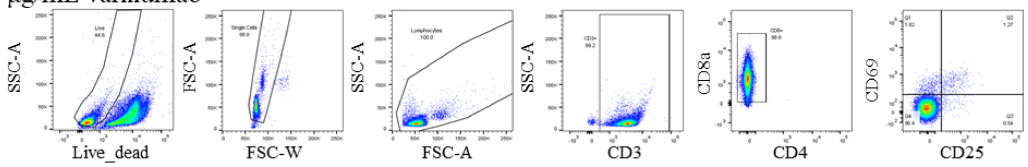
1.67 µg/mL MK-5890



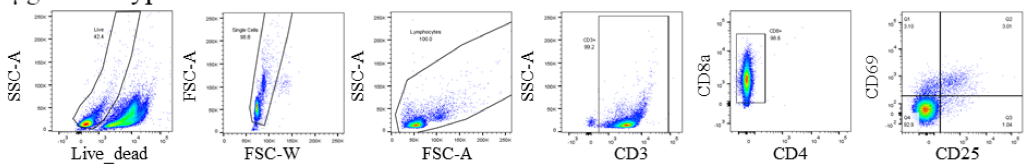
0.19 µg/mL MK-5890



15 µg/mL varlilumab



15 µg/mL isotype control



Bead-coupled MK-5890

For cross-linking studies, MK-5890 or isotype control antibodies were coupled to Dynabeads (M-450 Epoxy, Life Technologies) overnight. Bovine serum albumin was added to block any remaining unbound sites on the beads. The beads were washed and re-suspended to approximately 1.5×10^5 beads per µL in 0.1 M sodium phosphate buffer.

Chemokine assessment from human CD27 knock-in mouse MC-38 tumor model

5 mg/kg of MK-5890, MK-5890 hIgG2 or isotype control mAb was administered i.v. and terminal blood samples were collected by cardiac puncture from 5 animals per group at 3hrs post dose and from 5 animals per group at 48hrs post-dose. Blood was placed into serum separation tubes (Sarstedt Serum Separator Microtubes with Gel) and centrifuged at 10,000 rpm for 5 minutes at 4°C. Harvested serum was stored at 80°C.

Serum was analyzed by electrochemiluminescence using a custom chemokine U-plex panel (Mesoscale) according to manufacturer's instructions. Analytes included MCP1, MIP1α, MIP1β, MIP2, IP-10, TNFα, IFNγ, IL6, IL16, and KC/GRO. ** and *** indicate a p-value <0.01 and <0.001 respectively as analyzed by the non-parametric unpaired Mann-Whitney U-test

Blood and tumor processing and flow cytometry for human CD27 knock-in mouse MC-38 tumor model

5 mg/kg of MK-5890 (huIgG1), MK-5890 hIgG2, or anti-RSV human immunoglobulin G, subclass 1 (hIgG1) isotype control mAb were administered intraperitoneally on Days 0, 3, and 7 for a total of 3 doses. 5 mg/kg murinized anti-GITR antibody mDTA-1 was administered subcutaneously on Day 0. Blood and tumors were extracted on Study Day 10 and processed for analysis of T cell subpopulations by flow cytometry.

An aliquot of anti-coagulant containing blood (100 µL) was transferred to 96 well deep well plate and red blood cells were lysed using 1 mL of 1x ammonium chloride potassium lysis buffer (BioWhittaker), incubated at room temperature for 5 minutes, and then centrifuged at approximately 4°C and 1200 rpm for 5 minutes. The red blood cell lysis was repeated, followed by a wash in flow cytometry buffer (Invitrogen). The cell pellet was stained for flow cytometry analysis as described below. A second aliquot of (100 µL) blood sample for each animal was treated the same way but used to determine cell counts on a Vi-Cell™ machine.

Collected tumors were placed in a C-tube (Miltenyi) containing 5 mL of DMEM and dissociated on gentleMACS™ (Miltenyi) using the 'imp_mouse_Tumor1' program. Dissociated tumor samples were strained through 70 µM Fisherbrand™ Cell Strainers. Following a wash, samples were re-suspended in 1 mL of flow cytometry buffer. Cell numbers in each sample were counted using a Vi-Cell™ machine. About 5×10^6 cells were used for staining. When the cell numbers were less than 5×10^6, the entire sample was used for staining.

For surface marker staining, cells were incubated for 5 minutes at room temperature with mouse Fc Block (anti-mouse CD16/CD32 antibody). Cells were stained for 30 minutes at room temperature with a cocktail of fluorescently labeled cell surface antibodies listed in Table 3 below. Cells were washed 3 times with flow cytometry buffer and processed for intracellular staining with FoxP3-PE following kit provided instructions (Invitrogen).

After intracellular staining, cells were washed 3 times and re-suspended in flow cytometry buffer for detection using the BD LSRFortessa™ flow cytometer. Data was analyzed using FlowJo v10.1.

Table 3 Phenotypic Antibodies Used for Flow Cytometry Analysis in Mouse Tumor Models

Targeted Antigen	Fluorophore	Vendor	Catalog no.	Clone	Amount per test (µL)[1]	Isotype
CD16/CD32 (Fc block)	none	BD Biosciences	553142	2.4G2	0.05	Rat IgG2b
Live-Dead Fixable stain	Aqua	Life Technologies	L34966	NA	0.25	NA
CD45	BV711	BD Biosciences	563709	30-F-11	0.25	Rat IgG2b
CD11b	BUV395	BD Biosciences	563553	M1/70	0.25	Rat IgG2b
CD3	APC-Cy7	BD Biosciences	557596	145-2C11	1.0	Armenian hamster IgG1
TCRβ	APC-Cy7	BioLegend	109220	H57-597	1.0	Armenian hamster IgG2
CD90.2	BV786	BD Biosciences	564365	53-2.1	0.25	Rat IgG2a
CD4	BV650	BD Biosciences	563747	RM4-5	0.5	Rat IgG2a
CD8	BUV737	BD Biosciences	564297	53-6.7	0.5	Rat IgG2a
CD27	BV605	BD Biosciences	563365	LG3A10	0.5	Armenian hamster IgG1
CD25	BV421	BD Biosciences	562606	PC61	0.5	Rat IgG1
CD19	APC	BD Biosciences	550992?	1D3	0.25	Rat IgG2a
NK1.1	PE-Cy7	BD Biosciences	552878	PK136	0.5	Mouse IgG2a
Fox-P3 (Intracellular staining kit)	PE	Invitrogen	72-5775-40	FJK-16S	0.5	Rat IgG2a

[1] The volume of antibody is provided per 100 µL total volume.

APC = allophycocyanin; BV = Brilliant Violet™; BUV = Brilliant™ ultraviolet; Cy7 = cyanine 7; no. = number; PE = phycoerythrin; NA = not applicable

Dosing solution, monitoring, sample collection and analysis during toxicity study in rhesus monkey

The stock test article formulation consisted of MK-5890 (50 mg/mL), L histidine (10 mM), L methionine (10 mM), 7% sucrose, and 0.02% polysorbate 80. The vehicle formulation was identical to the test article formulation without MK-5890. The dosing volume was 0.6 mL/kg (0 and 30 mg/kg/week doses) or 0.2 mL/kg (0.1 and 3 mg/kg/week doses), which was administered as an intravenous bolus with an infusion rate of ~12 mL/minute.

Monitoring throughout the study included clinical observation, body weight, food consumption, ophthalmic examination, electrocardiographic examination, clinical pathology (including non GLP special chemistry examination of cytokines (IFNγ, IL-6, IL-2, IL-1β, TNFα, IL-10)), and anatomic pathology evaluation. In addition, target engagement (receptor occupancy), immunophenotyping, and immunogenicity (ADA) assays were performed. Immunophenotyping of peripheral blood lymphocytes (total and subsets of T cells including CD4, CD8, NKT cells, and regulatory T cells; as well as B cells and NK cells) was conducted

pretest and on Study Day 1, 4, 8, 15, 22, 25, 52, 80, 107. A target engagement (TE) assay, which relies on an APC-labeled anti-CD27 antibody that competes with MK-5890 for binding to CD27, was used to quantitate CD27 receptor availability after administering MK-5890. MK-5890 TE was assessed pretest and on Day 8, 15, 22, 25 in all dose groups, and on Day 108 in the 0.1 mg/kg, 3 mg/kg, and 30 mg/kg once weekly dose groups. Plasma drug concentrations in treated and control samples were determined. Whole blood samples from nonfasted or fasted animals were collected from the femoral artery/vein or saphenous vein into serum separator tubes and processed to serum for analysis. Measurement of serum MK-5890 was conducted using a bridging immunoassay utilizing streptavidin-coated microplates. Standards, controls and samples were incubated with a mixture of biotin-labeled mouse anti-human Ig kappa light chain and ruthenylated mouse anti-human IgG CH2 domain antibodies, followed by detection using a Meso Scale Discovery (MSD) Sector S 600 Analyzer.

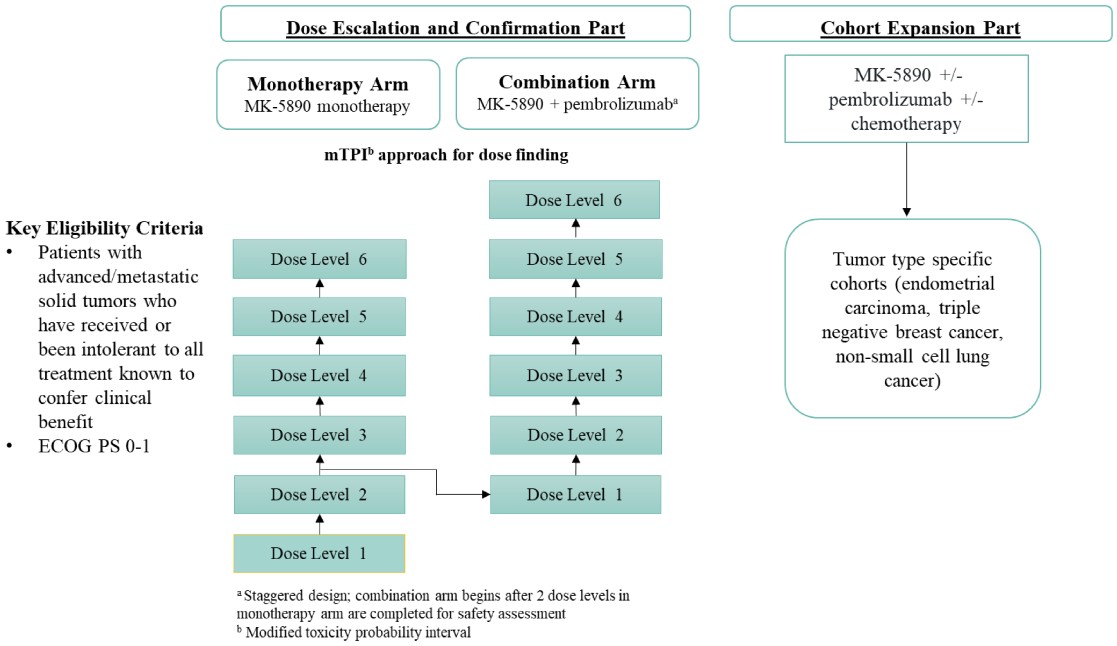
Chemokine assessment from Rhesus pharmacokinetic study

A single 3 mg/kg dose of MK-5890 or anti-RSV human immunoglobulin G, subclass 1 (hIgG1) isotype control mAb was administered i.v. as a bolus on Day 0 to male rhesus monkeys as part of a pharmacokinetics/pharmacodynamics study. Sera from blood collected 24 hours pre-dose, 3 hours post-dose, and 48 hrs post-dose were analyzed by electrochemiluminescence using a non-human primate chemokine V-plex panel for testing MIP-1β, Eotaxin-3, TARC, IP-10, MIP-1α, IL-8, MCP-1, MDC, MCP-4 (Mesoscale) according to manufacturer's instructions.

Phase I clinical study design, pharmacokinetic and pharmacodynamic analyses

The Phase 1 clinical study is outlined in Figure 3. The protocol and its amendments were approved by the relevant institutional review boards, and the study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. All patients signed written informed consent prior to having any study procedures performed.

Figure 3. A Phase 1 Study of MK-5890 as Monotherapy and in Combination with Pembrolizumab in Participants with Advanced Solid Tumors (overall design)



Blood for MK-5890 pharmacokinetics was collected prior to dosing, at the end of the MK-5890 infusion (+10 minutes), and 2 hours after the start of the MK-5890 infusion (+/-10 minutes) on Day 1, and once daily on Days 2, 3, 5, 8, 15 of Cycles 1-4; prior to dosing on Day 1 and Cycles 5 and 6, and every 4 cycles thereafter. MK-5890 quantitation in human serum was determined using a validated immunoassay to measure MK-5890 concentrations.

Blood for CD27 target engagement (receptor occupancy) was collected prior to dosing, at the end of MK-5890 infusion (+10 minutes), and 2 hours after the start of the MK-5890 infusion (+/-10 minutes) on Day 1 and once daily on Day 2, 3, 5, 8 and 15 of Cycles 1-4. In cycles 5 and 6, blood was additionally collected prior to dosing. CD27 target engagement was assessed using a fit-for-purpose validated free receptor flow cytometry assay method to measure unoccupied CD27 cell surface receptors on T-cell subpopulations; post-dose levels were compared with levels at baseline. Blood for immunophenotyping was collected prior to dosing on Day 1 and once daily on Day 8 and 15 of Cycles 1-4. In cycles ≥5 blood was additionally collected prior to dosing. Immunophenotyping of total and subsets of CD4+, CD8+ and regulatory T cells as well as B- and NK- cells was performed using fit-for-purpose validated assays. Serum for cytokine analyses was collected prior to dosing and 2 hours after the start of MK-5890 infusion on Day 1 and once daily on Day 2 and 8 of Cycles 1-4. In cycles ≥5 serum was additionally collected prior to dosing and 2 hours after start of MK-5890 infusion on day 1 only. MIP-1α, MIP-1β, MCP-1,

and IP-10 were analyzed using fit-for-purpose validated immunoassays to measure the abundance of these cytokines in serum.

Quantification and statistical analyses

The EC50 and IC50 values were calculated using GraphPad Prism 7. Non-linear regression (four-parameter) fitting was used for graphing and calculation. Average, median, and standard deviation values were calculated using Microsoft Excel.

Statistical analyses for mouse tumor model studies

To compare two treatment groups on a given day in the mouse tumor model studies, a generalization of the nonparametric Mann-Whitney test that allows for right-censored data was used: the Peto and Peto version of the Gehan-Breslow test. Two-sided p-values were estimated from 20,000 random reassignments of animals between the two treatments being compared. To control the familywise error rate across all time points for a given pair of treatments, p-values were multiplicity adjusted by Holm's method. A p-value of less than 0.05 was used to define statistical significance. Comparisons between treatments were made at each day of follow-up, using tumor volume at that day.

Analysis of tumor volumes: Follow-up of individual animals was terminated early because of excessive tumor burden or other reasons (Table 4). Depending on the reason and tumor size at the last measurement (see below), the last observed tumor volume was treated as a lower bound on volume at all later days for that animal (right-censored data).

For descriptive purposes, volumes for each day and treatment group were summarized by their median. To allow for censoring, a distribution function for each day and treatment group was estimated by the Kaplan-Meier method, with confidence band obtained using the beta product confidence procedure. The median was estimated as the 50th percentile of the distribution function, with confidence interval obtained by inverting the confidence band. A 68% confidence level was used, to be comparable to the common "mean ± SE" format for summarizing data, since the latter is approximately a 68% confidence interval for the mean.

When follow-up of an animal was terminated early, the reason was categorized and the animal's tumor volume data were handled as follows:

Table 4 Categorization of Early Terminations for Statistical Analyses

Reason for early termination	In statistical analysis:
Tumor burden	Right-censor at last measured value
Tumor ulceration; Found dead, metastases found; Found dead, unknown reason; Protocol deviation; Administrative; Accident	Right-censor at last measured value, provided this exceeded a threshold (1000 mm3). Otherwise omit animal at later times.
Weight loss/ill; Acute treatment toxicity; Found dead, with evidence of illness	Omit animal at later times

The threshold for using the last measured volume as a lower bound on later volumes was based on a judgment that once a tumor's volume exceeded that threshold, it was unlikely to shrink. To reduce the potential for bias, medians and group comparisons were not calculated at a time point if more than 25% of animals in a group had missing values. Censored values were not considered missing when calculating this percentage.

Table 5 P-values for comparisons of tumor volume between treatments by day for Figure 3A Study 16-M320-7830

Comparison	Day 7	Day 10	Day 13	Day 16	Day 20	Day 24	Day 27
Isotype ctrl vs muDX400	0.0020	0.0020	0.0008	0.0005	0.0005	0.0008	0.0008
Isotype ctrl vs MK-5890	0.0030	0.0011	0.0005	0.0005	0.0005	0.0011	0.0011
Isotype ctrl vs varlilumab	0.2867	0.4411	0.0778	0.0778	0.0441	0.0236	0.0176
MK-5890 vs varlilumab	0.2869	0.0360	0.0030	0.0304	0.0780	0.0780	0.0293
Isotype ctrl vs MK-5890 huIgG2	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
MK-5890 vs MK-5890 huIgG2	0.0704	0.0704	0.0186	0.0093	0.0027	0.0014	0.0010

Table 6 P-values for comparisons of tumor volume between treatments by day for Figure 3B Study 16-M320-7862

Comparison	Day 7	Day 10	Day 13	Day 16	Day 19	Day 22	Day 25
Isotype ctrl vs muDX400	0.0840	0.0045	0.1401	0.1029	0.1528	0.2070	0.1528
Isotype ctrl vs MK-5890	0.0708	0.0680	0.0708	0.1134	0.4852	0.3393	0.2308
Isotype ctrl vs varlilumab	1.0000	0.7252	1.0000	1.0000	1.0000	1.0000	1.0000
Isotype ctrl vs MK-5890 + muDX400	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
Isotype ctrl vs MK-5890 huIgG2 + muDX400	0.0864	0.0045	0.0404	0.0675	0.1569	0.1569	0.0675
Isotype ctrl vs varlilumab + muDX400	0.0560	0.0014	0.0025	0.0025	0.0025	0.0018	0.0016
muDX400 vs MK-5890 + muDX400	0.0138	0.0108	0.0036	0.0093	0.0063	0.0009	0.0093
muDX400 vs MK-5890 huIgG2 + muDX400	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
muDX400 vs varlilumab + muDX400	0.9084	0.8715	0.8715	0.8659	0.8715	0.5436	0.6824
MK-5890 vs MK-5890 + muDX400	0.0199	0.0072	0.0072	0.0072	0.0072	0.0072	0.0072
MK-5890 vs MK-5890 huIgG2 + muDX400	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
varlilumab vs varlilumab + muDX400	0.3966	0.2205	0.1635	0.2205	0.2205	0.1635	0.1264

References for Additional File 1 Methods

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