SI Methods

Cloning and Protein Production

The heavy chain and light chain variable regions of α4-1BB antibody (clone LOB12.3) were synthesized as gBlock gene fragments (Integrated DNA technologies) and cloned into the gWiz expression vector (Genlantis) using In-fusion cloning (Takara Bio). Antibodies were expressed as chimeras with a murine kappa light chain constant region and a murine IgG1 heavy chain constant region. Antibodies were encoded in a single expression cassette with a T2A peptide inserted between the light chain and heavy chain. αFITC (clone 4420) were constructed in the same fashion, but a murine IgG2c isotype with LALA-PG silencing mutations was used for the heavy chain constant region (90). For LAIR fusions, the murine LAIR1 gene was synthesized as a gBlock gene fragment (Integrated DNA technologies) and cloned as a fusion to the C-terminus of the heavy chain constant region separated by a flexible $(G_4S)_3$ linker. Plasmids were transformed into Stellar competent cells (Takara Bio) for amplification and isolated with Nucleobond Xtra endotoxin-free kits (Macherey-Nagel).

a4-1BB, a4-1BB-LAIR, αFITC, and αFITC-LAIR were produced using the Expi293 expression system (Gibco) following manufacturer's instructions. Briefly, 1 mg/L of DNA and 3.2 mg/L of ExpiFectamine 293 were individually diluted into OptiMEM media (Gibco) and then combined dropwise. This mixture was then added dropwise to Expi293F suspension cells and 18-24 hours later ExpiFectamine 293 Transfection enhancers 1 and 2 (Gibco) were added to the culture. 7 days after transfection supernatants were harvested and antibodies were purified using Protein G sepharose 4 Fast Flow resin (Cytiva).

TA99 was produced using a FreeStyle 293-F stable production line generated in-house. Cells were expanded and then seeded at a density of 1 M/mL and supernatant was harvested 7 days later. 9D9 was produced using a CHO DG44 stable production line gifted to us by David Hacker. Cells were expanded and then seeded at a density of 0.5M/mL and supernatant was harvested 7 days later. Both TA99 and 9D9 were purified using rProtein A Sepharose Fast Flow resin (Cytiva).

Following purification, proteins were buffer exchanged into PBS (Corning) using Amicon Spin Filters (Sigma Aldrich), 0.22 µm sterile filtered (Pall), and confirmed for minimal endotoxin (<0.1 EU/dose) using the Endosafe LAL Cartridge Technology (Charles River). Molecular weight was confirmed with SDS-PAGE. Proteins were run alongside a Novex Sharp Pre-Stained Protein Standard (Invitrogen) on a NuPAGE 4 to 12% Bis-Tris gel (Invitrogen) with 2-(*N*-morpholino) ethanesulfonic acid (MES) running buffer (VWR) and stained for visualization with SimplyBlue Safe Stain (Life Technologies). Proteins were confirmed to be free of aggregates by size exclusion chromatography using a Superdex 200 Increase 10/300 GL column on an Äkta Explorer FPLC system (Cytiva). All proteins were flash frozen in liquid nitrogen and stored at -80°C.

IVIS

Proteins were labeled with Alexa Fluor 647 NHS Ester (Life Technologies) and a Zeba desalting column (Thermo Scientific) was used to remove excess dye. Total molar amount of dye injected per sample was normalized between groups before injection. 20 µg of αFITC mIgG2c LALA-PG and a molar equivalent of αFITC-LAIR mIgG2c LALA-PG were used for *in vivo* retention studies.

C57Bl/6 albino mice were inoculated with 10^6 B16F10-Trp2 KO cells and labeled proteins were injected i.t. on day 7. Fluorescence at the site of the tumor was measured longitudinally using the IVIS Spectrum Imaging System (Perkin Elmer). One week prior to study initiation, mice were switched to an alfalfa-free casein chow (Test Diet) to reduce background fluorescence. Total radiant efficiency was calculated after subtracting background fluorescence and normalizing to the maximum value for each protein using Living Image software (Caliper Life Sciences).

Tumor Cytokine/Chemokine Analysis

Tumors were excised, weighed, mechanically dissociated, and incubated in tissue protein extraction reagent (T-PER, Thermo Fisher Scientific) with 1% Halt protease and phosphatase inhibitors (Thermo Fisher Scientific) for 30 minutes at 4°C while rotating. The lysates were then centrifuged and supernatants filtered through a Costar 0.22 µm SpinX filter (Corning) to remove any remaining debris. Lysates were flash frozen and stored at -20°C until time of analysis. Lysates were analyzed with the 13-plex mouse Cytokine Release Syndrome LEGENDplex panel and the Mouse/Rat Total/Active TGF-β1 LEGENDplex kit (Biolegend). Data was collected on a BD LSR II cytometer (BD Biosciences).

Collagen I ELISA

96 well plates precoated with rat collagen I (Gibco) were blocked overnight with PBSTA (PBS (Corning) + 2% w/v BSA (Sigma Alrich) + 0.05% v/v Tween-20 (Millipore Sigma)) at 4°C. After washing with 3 times PBST (PBS (Corning) + 0.05% v/v Tween-20 (Millipore Sigma)) and 3 times with PBS (Corning), a4-1BB and a4-1BB-LAIR were incubated in PBSTA overnight at 4°C while shaking. Wells were washed 3 times with PBST and 3 times with PBS and then

incubated with goat αmIgG1-Horseradish peroxidase (HRP) (1:2000, Abcam) in PBSTA for 1 hour at RT while shaking. Wells were again washed 3 times with PBST and 3 times with PBS and then 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher) was added for 5-15 min, followed by 1 M sulfuric acid to quench the reaction. Absorbance at 450 nm (using absorbance at 570 nm as a reference) was measured on an Infinite M200 microplate reader (Tecan). Binding curves were generated with GraphPad Prism software V9. K_D values were calculated using a nonlinear regression fit for one site total binding with no non-specificity and curves were normalized to the B_{max} values.

Surface 4-1BB Binding Assay

The gene for murine 4-1BB (OriGene) was cloned into the pIRES2 expression vector, which encodes for GFP downstream of the inserted 4-1BB gene using an IRES site, using In-Fusion cloning (Takara Bio). Freestyle 293-F cells were transiently transfected by mixing 1 mg/mL of plasmid DNA and 2 mg/mL of polyethylenimine (Polysciences) in OptiPRO Serum Free Medium (Gibco) and, after incubating, adding dropwise to the cells. 3-5 days after transfection, cells were harvested and pelleted in V-bottom 96 well plates. Cells were titrated with a4-1BB or a4-1BB-LAIR and incubated for 3 hours shaking at 4°C. Cells were washed with PBSA (PBS (Corning) + 0.1% BSA (Sigma Aldrich)) and incubated with αmIgG1-APC (diluted 1:250, clone M1-14D12, Biolegend) for 30 minutes shaking at 4°C. Data was collected on a BD LSR II cytometer (BD Biosciences). Binding curves were generated with GraphPad Prism software V9. K_D values were calculated using a nonlinear regression fit for one site total binding with no non-specificity and curves were normalized to the B_{max} values.

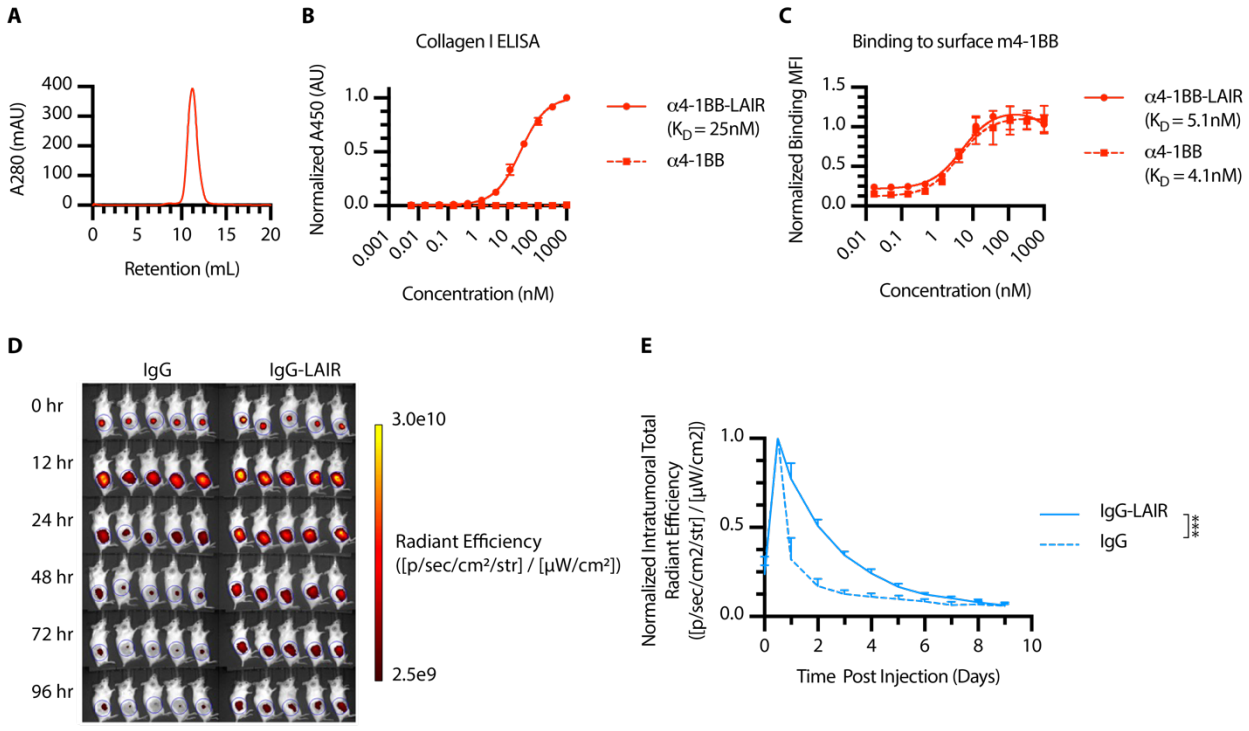

Figure S1. α4-1BB-LAIR behaves as expected *in vitro* and *in vivo*

(A) SEC chromatogram of α4-1BB-LAIR. **(B)** Equilibrium binding curve of α4-1BB-LAIR and α4-1BB to collagen I coated plates (mean ± S.D., n = 4). **(C)** Equilibrium binding curve of α4-1BB-LAIR and α4-1BB to HEK cells expressing murine 4-1BB (mean ± S.D., n = 4). **(D-E)** Mice were inoculated with 1 x 10^6 B16F10-Trp2 KO cells on day 0, injected with 20 μg of fluorescently labeled control IgG or equimolar amount of IgG-LAIR, and fluorescence was measured longitudinally via IVIS. **(D)** example fluorescence images from select timepoints and **(E)** Quantification of normalized radiant efficiency (mean ± S.D.) in mice receiving IgG or IgG-LAIR (n = 5). Retention data were compared using two way ANOVA with Tukey's multiple hypothesis testing correction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

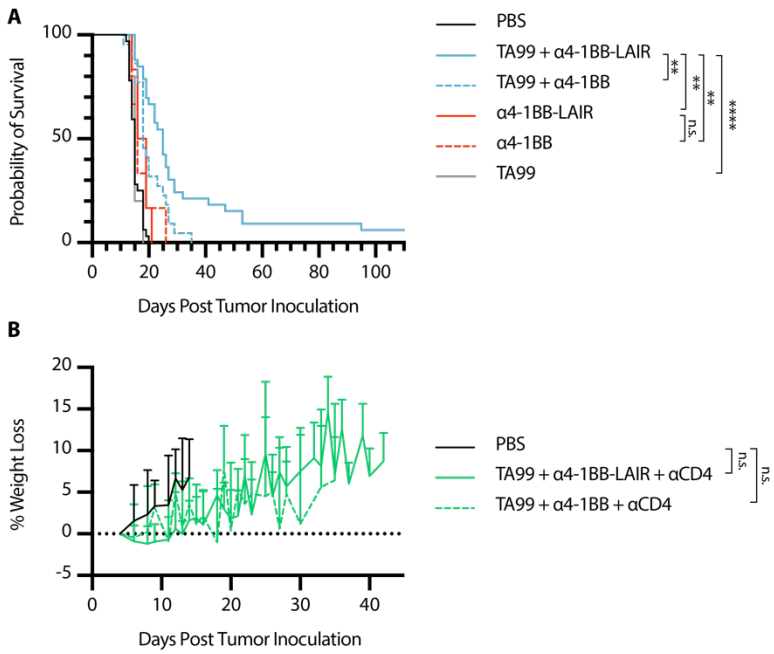

Figure S2. Monotherapies are not efficacious and Tx + αCD4 exhibits no toxicity

(A) Overall survival of mice treated with PBS (n = 32), TA99 + α4-1BB-LAIR ("Tx", n = 33), TA99 + α4-1BB (n = 22), TA99 (n = 5), α4-1BB (n = 6), or α4-1BB-LAIR (n = 6) with treatment schedule outlined in Fig. 1A (six independent studies). **(B)** Weight loss of mice treated with PBS (n = 10), TA99 + α4-1BB-LAIR + αCD4 (n = 10), or TA99 + α4-1BB + αCD4 (n = 9) from survival study shown in Fig. 1D (two independent studies). Survival was compared using the log-rank Mantel-Cox test and weight loss data were compared using two-way ANOVA with Tukey's multiple hypothesis testing correction. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

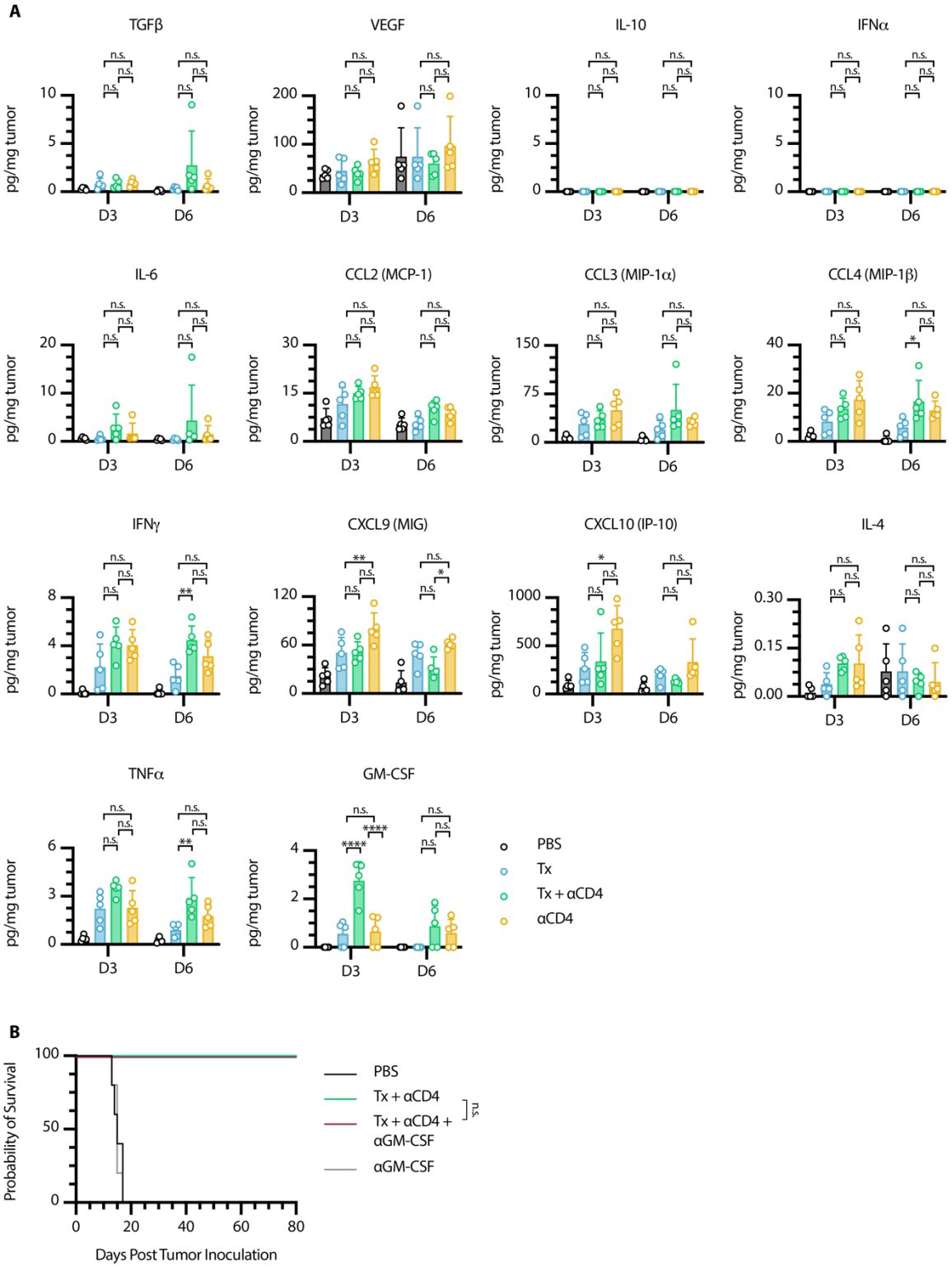

Figure S3. Tumor supernatant cytokine/chemokine analysis does not explain differences in efficacy

(A) Measured levels of indicated soluble cytokines/chemokines in tumor supernatant 3 and 6 days after first α4-1BB-LAIR treatment (n = 5). **(B)** Survival of mice treated with PBS (n = 5), Tx + αCD4 (n = 7), Tx + αCD4 + αGM-CSF (n = 7), or αGM-CSF (n = 5). Chemokine/cytokine measurements were compared using two-0way ANOVA with Tukey's multiple hypothesis testing correction. Survival was compared using the log-rank Mantel-Cox test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

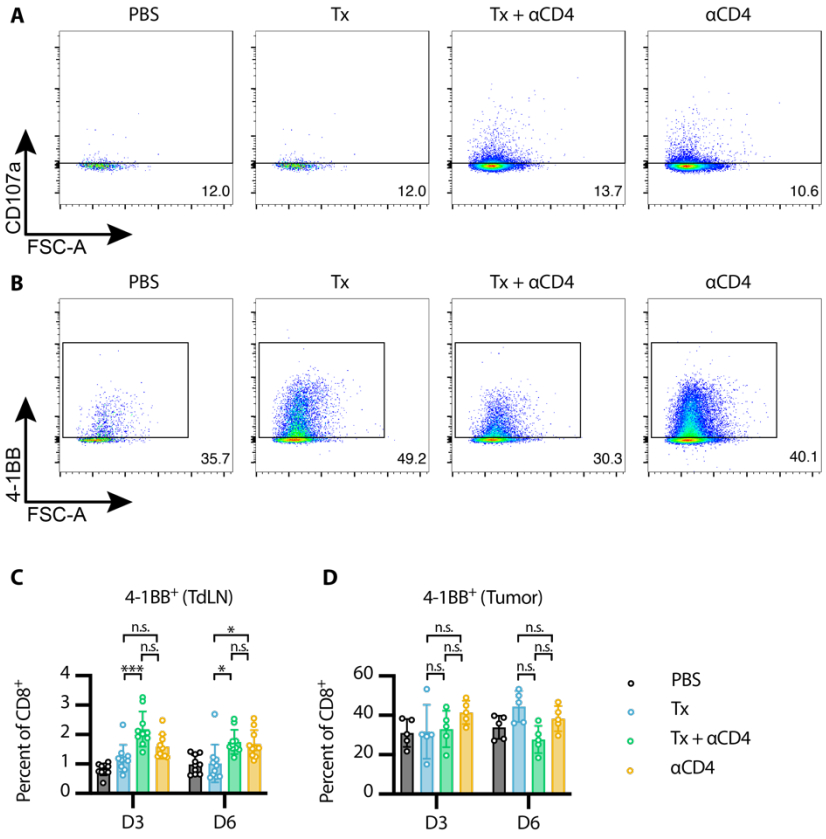

Figure S4. 4-1BB expression on CD8$^+$ TILs uniform across treatment groups

(A) Representative gating of CD107a$^+$ CD8$^+$ T cells in the tumor 6 days after first α4-1BB-LAIR treatment (gated on single cell/live/CD45$^+$/CD3$^+$NK1.1$^-$/CD8$^+$). **(B)** Representative gating of 4-1BB$^+$ CD8$^+$ T cells in the tumor 6 days after first α4-1BB-LAIR treatment (gated on single cell/live/CD45$^+$/CD3$^+$NK1.1$^-$/CD8$^+$). Flow cytometry quantification (mean±SD) of 4-1BB$^+$ CD8$^+$ T cells in the **(C)** TdLN and **(D)** tumor 3 and 6 days after first α4-1BB-LAIR treatment (gated on single cell/live/CD45$^+$/CD3$^+$NK1.1$^-$/CD8$^+$, n = 5-10, two independent experiments). Flow data were compared using two-way ANOVA with Tukey's multiple hypothesis testing correction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

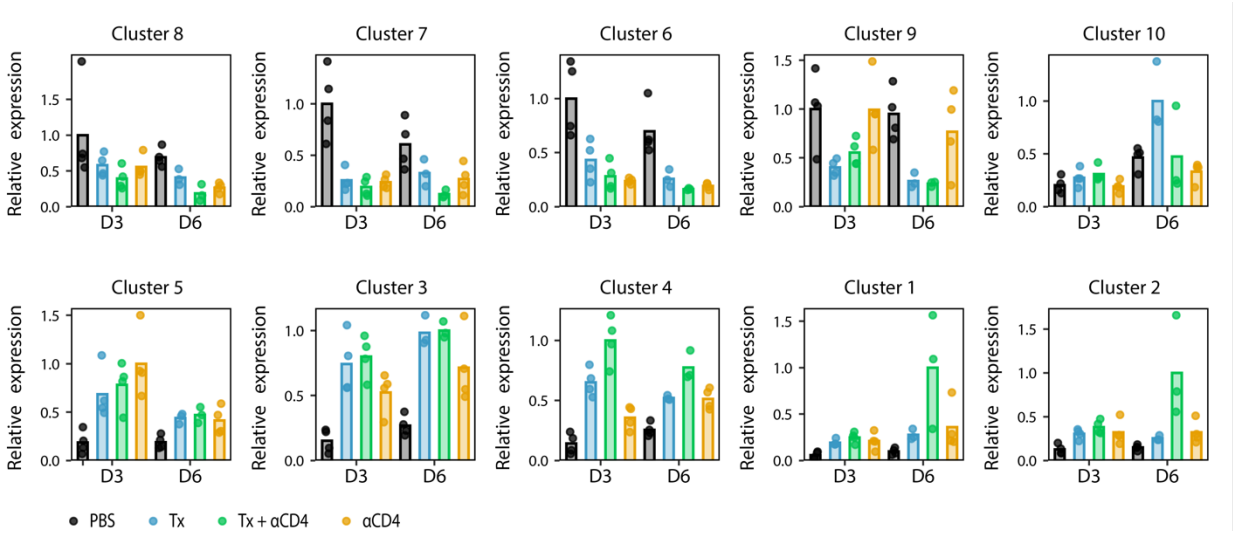

Figure S5. Gene clusters exhibit differential expression among various experimental cohorts

Normalized expression of all gene clusters identified in Fig 4A for different experimental cohorts.

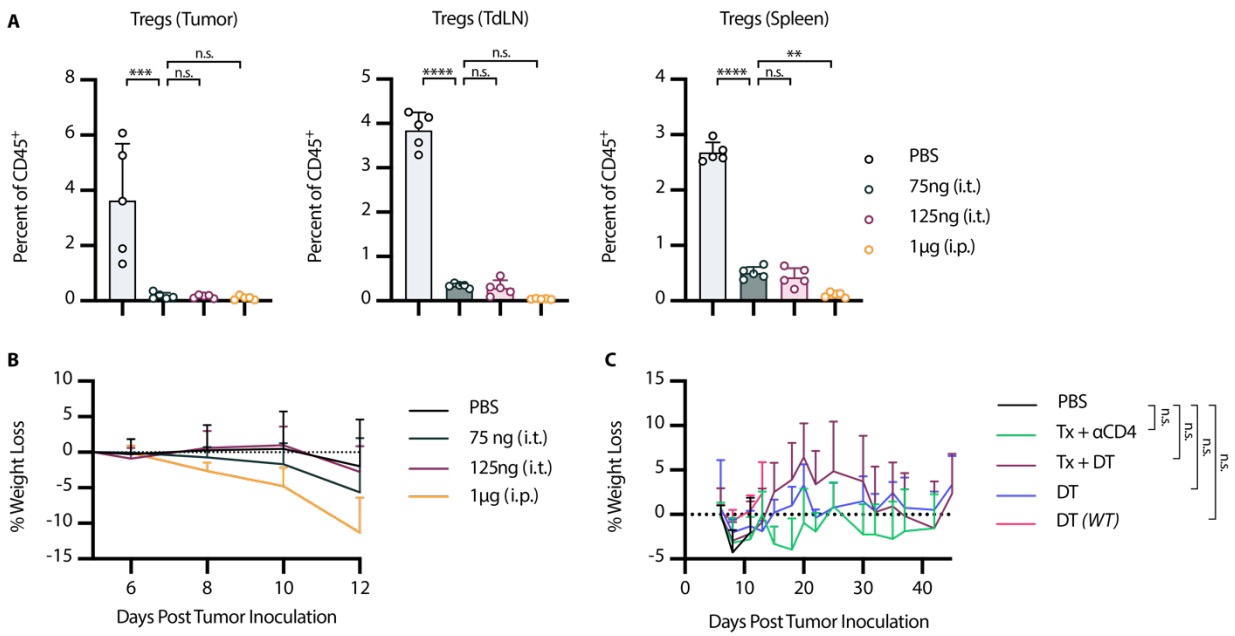

Figure S6. Low dose 75 ng (i.t.) DT depletes Tregs to a similar extent as 125 ng (i.t.)

Foxp3-DTR Mice were inoculated with 1 x 10^6 B16F10 cells on day 0. **(A)** Mice were treated on days 6, 8, and 10 with either 125 ng DT (i.t.), 75 ng DT (i.t.), or 1 µg DT (i.p.). Flow cytometry quantification (mean±SD) of Tregs in tumor, TdLN, or spleen on day 12 (gated on single cell/live/$CD45^+$/$CD3^+NK1.1^-$/$CD4^+$/GFP(*Foxp3*)$^+$, n = 5) **(B)** Weight loss (mean+SD) of mice from **(A)**. **(C)** Weight loss data from survival study shown in Fig 6C. Flow data were compared using one way ANOVA with Tukey's multiple hypothesis testing correction and weight loss data were compared using two-way ANOVA with Tukey's multiple hypothesis testing correction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

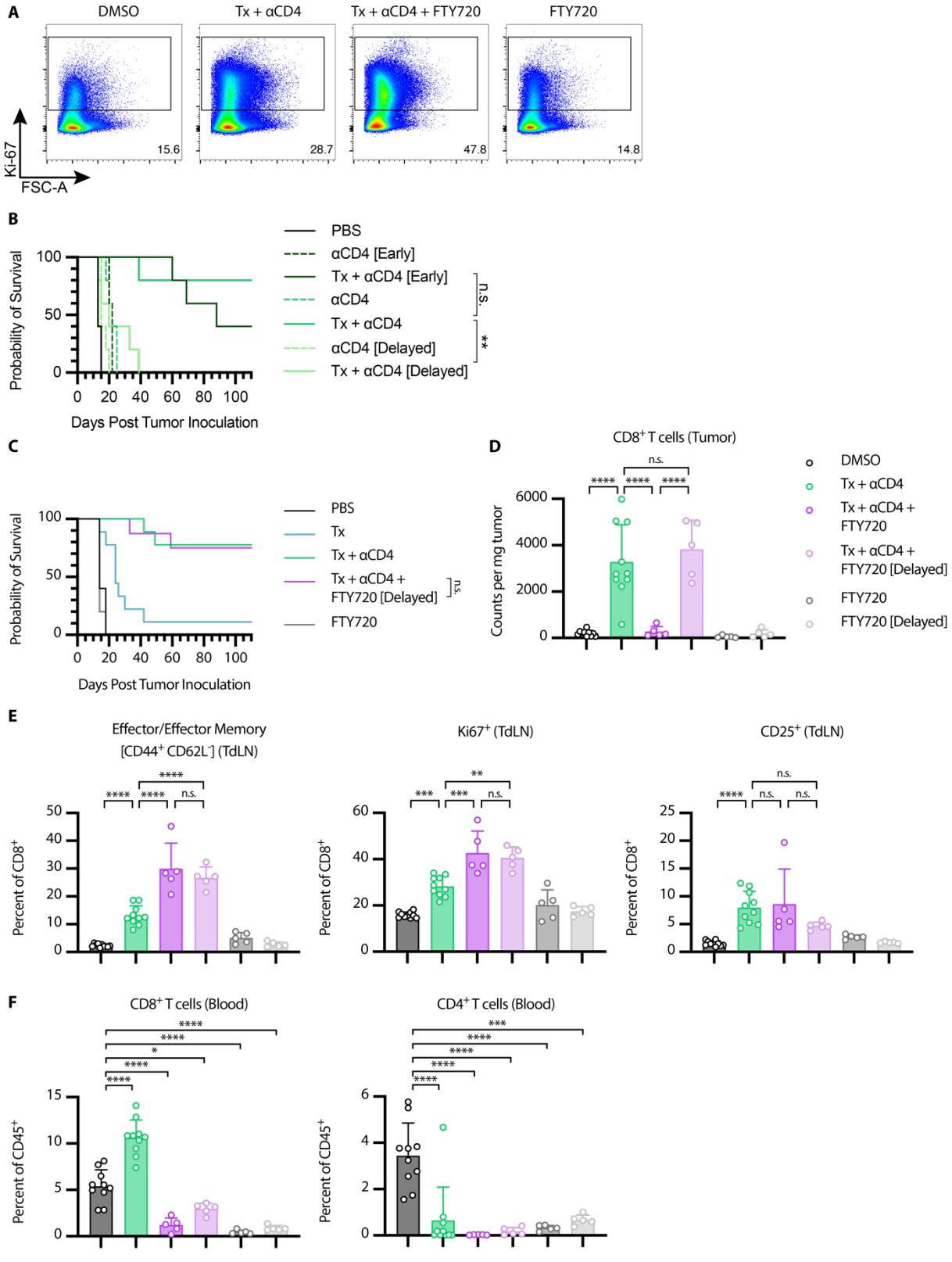

Figure S7. Delayed FTY720 initiation does not affect therapeutic efficacy of Tx + αCD4

(A) Representative gating for Ki67$^+$ CD8$^+$ T cells in TdLN 6 days after first α4-1BB-LAIR treatment (gated on single cell/live/CD45$^+$/CD3$^+$NK1.1$^-$/CD8$^+$). **(B-E)** Delayed FTY720 refers to FTY720 initiation concurrent with α4-1BB-LAIR treatment, while FTY720 refers to FTY720 initiation concurrent with αCD4. **(B)** Overall survival of mice treated with PBS/DMSO (n = 5), Tx (n = 9), Tx + αCD4 (n = 9), Tx + αCD4 + delayed FTY720 (n = 8), or delayed FTY720 (n = 5). Mice were treated with the same dose/dose schedule as in Fig. 1A, with delayed FTY720 treatment initiated on day 6 and continued every other day until day 34. **(C)** Flow cytometry quantification (mean±SD) of CD8$^+$ T cells in the tumor 6 days after first α4-1BB-LAIR treatment (gated on single cell/live/CD45$^+$/CD3$^+$NK1.1$^-$/CD8$^+$, n = 5-10, two independent experiments). **(D)** Flow cytometry quantification (mean±SD) of effector/effector memory (CD44$^+$ CD62L$^-$), CD25$^+$, and Ki67$^+$ CD8$^+$ T cells in the TdLN 6 days after first α4-1BB-LAIR treatment (gated on single cell/live/CD45$^+$/CD3$^+$NK1.1$^-$/CD8$^+$, n = 5-10, two independent experiments). **(E)** Flow cytometry quantification (mean±SD) of CD8$^+$ T cells and CD4$^+$ T cells in the blood 6 days after first α4-1BB-LAIR treatment (gated on single cell/live/CD45$^+$/CD3$^+$NK1.1$^-$/CD8$^+$, n = 5-10, two independent experiments). **(F)** Overall Survival of mice treated with PBS, Tx + αCD4, or αCD4 with αCD4 initiated on day 4 as outlined in figure 1A, day 10 ("delayed"), or day -8 ("early") (n = 5). Survival data were compared using log-rank Mantel-Cox test and flow cytometry data were compared using one-way ANOVA with Tukey's multiple hypothesis testing correction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

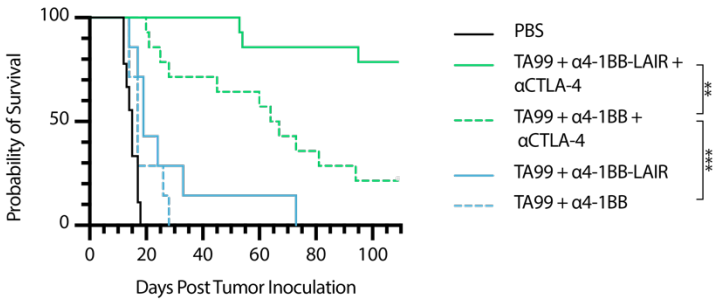

Figure S8. Non-collagen anchoring TA99 + α4-1BB + αCTLA-4 is also efficacious

Mice were inoculated with 1 x 10^6 B16F10 cells on day 0. Overall survival of mice treated either with PBS (n = 9, two independent studies), Tx (n = 7), TA99 + α4-1BB-LAIR + αCTLA-4 (n = 14, two independent studies), TA99 + α4-1BB-LAIR + αCTLA-4 (n = 14, two independent studies), TA99 + α4-1BB-LAIR (n = 7), or TA99 + α4-1BB (n = 7). Mice were treated with the same dose/dose schedule as in Fig. 1A with 200 μg αCTLA-4 (i.p.) given on days 6, 9, 13, 16, 20, 23, and 27. Survival data were compared using log-rank Mantel-Cox. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

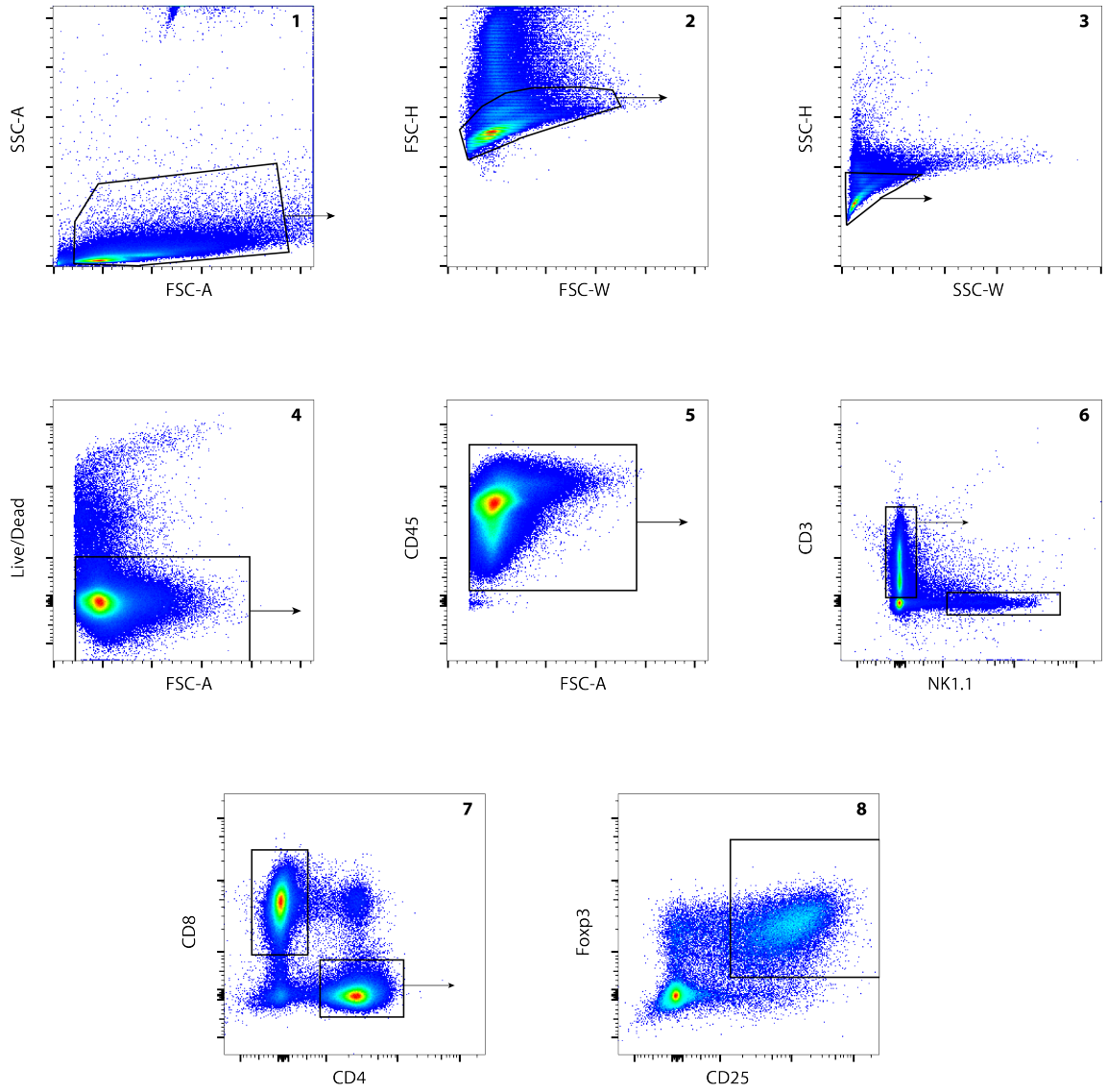

Figure S9. Example gating

Gating strategy for $CD8^+$ T cells, $CD4^+$ T cells, and $Foxp3^+$ $CD25^+$ Tregs, shown on a TdLN sample. Identical gating strategies were used for tumor, spleen, and blood samples.

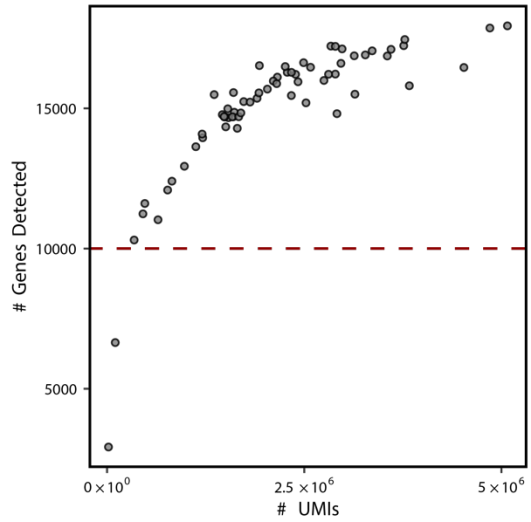

Figure S10. Low read samples removed from RNA-sequencing analysis

Plot of number of genes detected versus number of unique reads per sample for all tumor and TdLN bulk-RNA seq samples. Samples with less than 10,000 unique genes detected were excluded from analysis. Two samples (one Tx D6 and one Tx + αCD4 D6) met this exclusion criteria.

SI Tables

Table S1. Amino acid sequence list

Key: signal peptide, variable region, constant region, linker, LAIR

α4-1BB Light Chain (murine kappa constant region)	MSVLTQVLALLLLWLTGARCADIQMTQSPASLSASLEEI VTITCQASQDIGNWLAWYHQKPGKSPQLLIYGSTSLADG VPSRFSGSSSGSQYSLKISRLQVEDIGIYYCLQAYGAPWT FGGGTKLELKRADAAPTVSIFPPSSEQLTSGGASVVCFLN NFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSM SSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

α4-1BB Heavy Chain (murine IgG1 constant region)	MKWSWVFLFLMAMVTGVNSDVQLVESGGGLVQPGRSL KLSCAASGFIFSYFDMAWVRQAPTKGLEWVASISPDGSI PYYRDSVKGRFTVSRENAKSSLYLQMDSLRSEDTATYY CARRSYGGYSEIDYWGQGVMVTVSSATTKGPSVYPLAP GSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVH TFPAVLQSDLYTLSSSVTVPSSTWPSQTVTCNVAHPASST KVDKKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITL TPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTKPRE EQINSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIE KTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITNFF PEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLN VQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSPGK
α4-1BB-LAIR Heavy Chain (murine IgG1 constant region)	MKWSWVFLFLMAMVTGVNSDVQLVESGGGLVQPGRSL KLSCAASGFIFSYFDMAWVRQAPTKGLEWVASISPDGSI PYYRDSVKGRFTVSRENAKSSLYLQMDSLRSEDTATYY CARRSYGGYSEIDYWGQGVMVTVSSATTKGPSVYPLAP GSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVH TFPAVLQSDLYTLSSSVTVPSSTWPSQTVTCNVAHPASST KVDKKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITL TPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTKPRE EQINSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIE KTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITNFF PEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLN VQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSPGKGG GGSGGGGSGGGGSQEGSLPDITIFPNSSLMISQGTFVTVV CSYSDKHDLYNMVRLEKDGSTFMEKSTEPYKTEDEFEIG PVNETITGHYSCIYSKGITWSERSKTLELKVIKENVIQTPA PGPTSDTSWLKTYSIY
αFITC Light Chain (murine kappa constant region)	MSVLTQVLALLLLWLTGARCADVVMTQTPLSLPVSLGD QASISCRSSQSLVHSNGNTYLRWYLQKPGQSPKVLIYKV SNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQS THVPWTFGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGAS VVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSK DSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSF NRNEC
αFITC Heavy Chain (murine IgG2c constant region, **LALA-PG silencing**	MKWSWVFLFLMAMVTGVNSDVKLDETGGGLVQPGRP MKLSCVASGFTFSDYWMNWVRQSPEKGLEWVAQIRNK PYNYETYYSDSVKGRFTISRDDSKSSVYLQMNNLRVED MGIYYCTGSYYGMDYWGQGTSVTVSAKTTAPSVYPLAP VCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHT FPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTK VDKKIEPRGPTIKPCPPCKCPAPN**AA**GGPSVFIFPPKIKDV LMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQT

mutations bolded and underlined)	QTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNK DL**G**APIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLT CMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSY FMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFS RTPGK
αFITC-LAIR Heavy Chain (murine IgG2c constant region, **LALA-PG silencing mutations bolded and underlined**)	MKWSWVFLFLMAMVTGVNSDVKLDETGGGLVQPGRP MKLSCVASGFTFSDYWMNWVRQSPEKGLEWVAQIRNK PYNYETYYSDSVKGRFTISRDDSKSSVYLQMNNLRVED MGIYYCTGSYYGMDYWGQGTSVTVSAKTTAPSVYPLAP VCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHT FPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTK VDKKIEPRGPTIKPCPPCKCPAPN**AA**GGPSVFIFPPKIKDV LMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQT QTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNK DL**G**APIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLT CMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSY FMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFS RTPGKGGGGSGGGGSGGGGSQEGSLPDITIFPNSSLMISQ GTFVTVVCSYSDKHDLYNMVRLEKDGSTFMEKSTEPYK TEDEFEIGPVNETITGHYSCIYSKGITWSERSKTLELKVIK ENVIQTPAPGPTSDTSWLKTYSIY