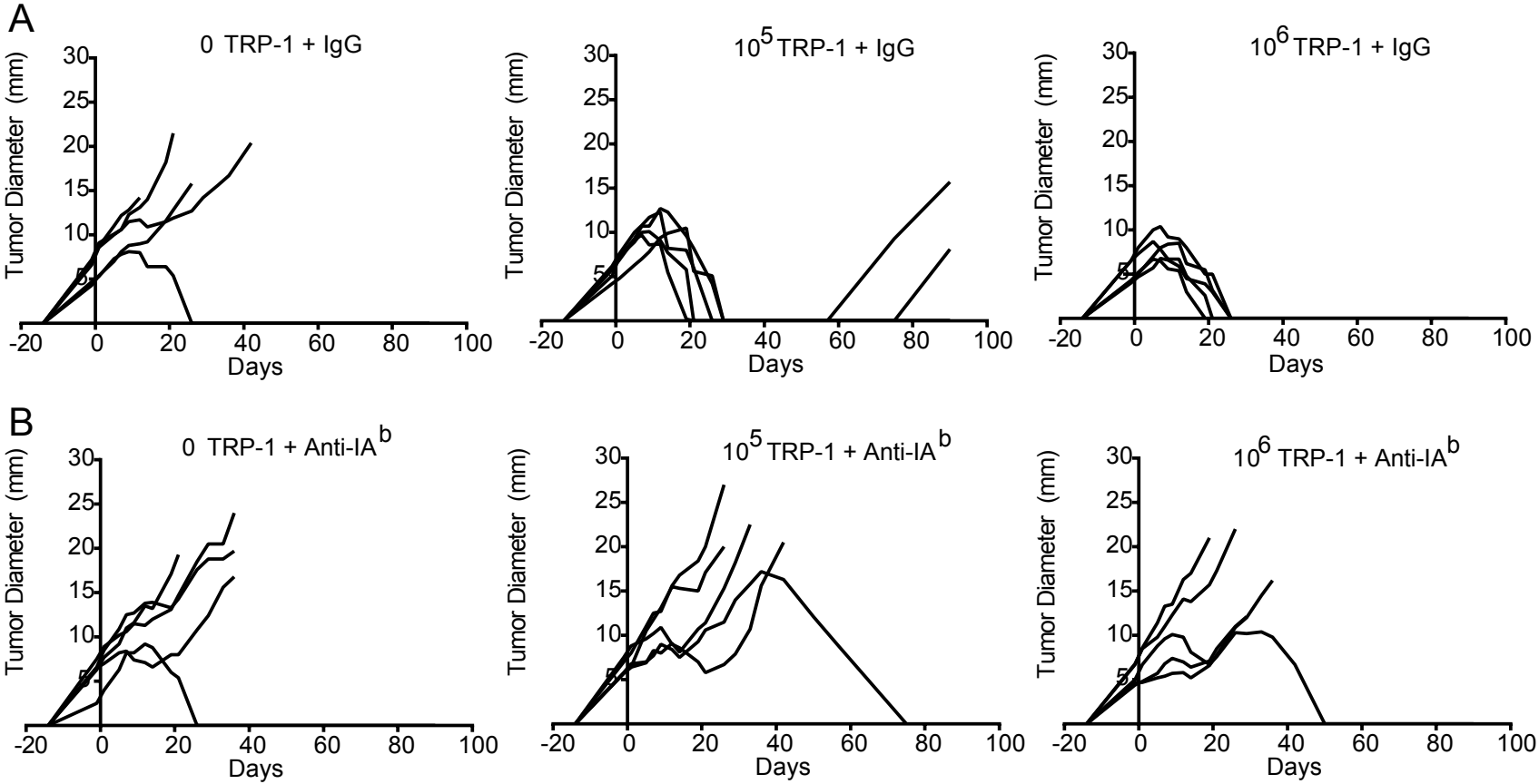
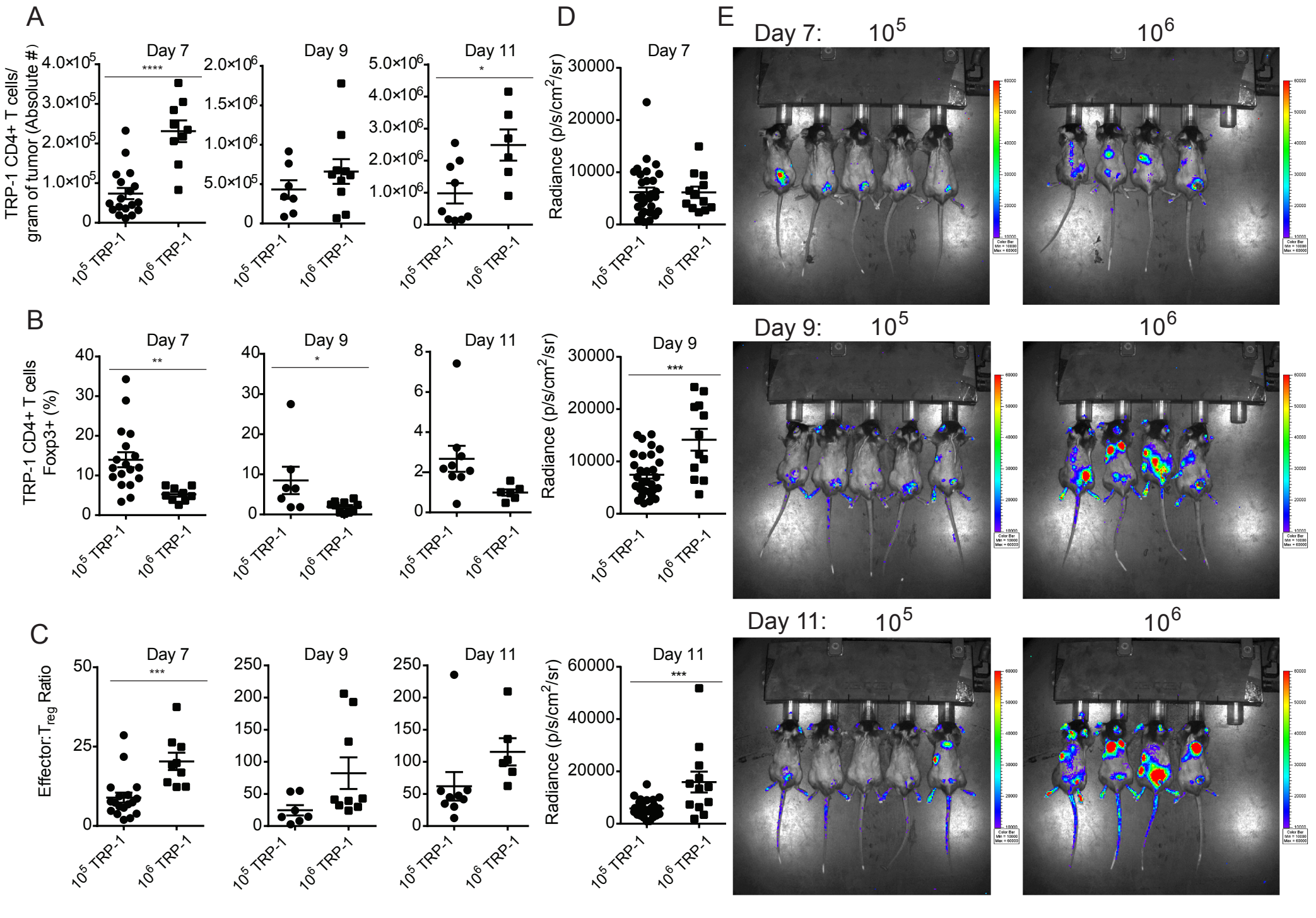
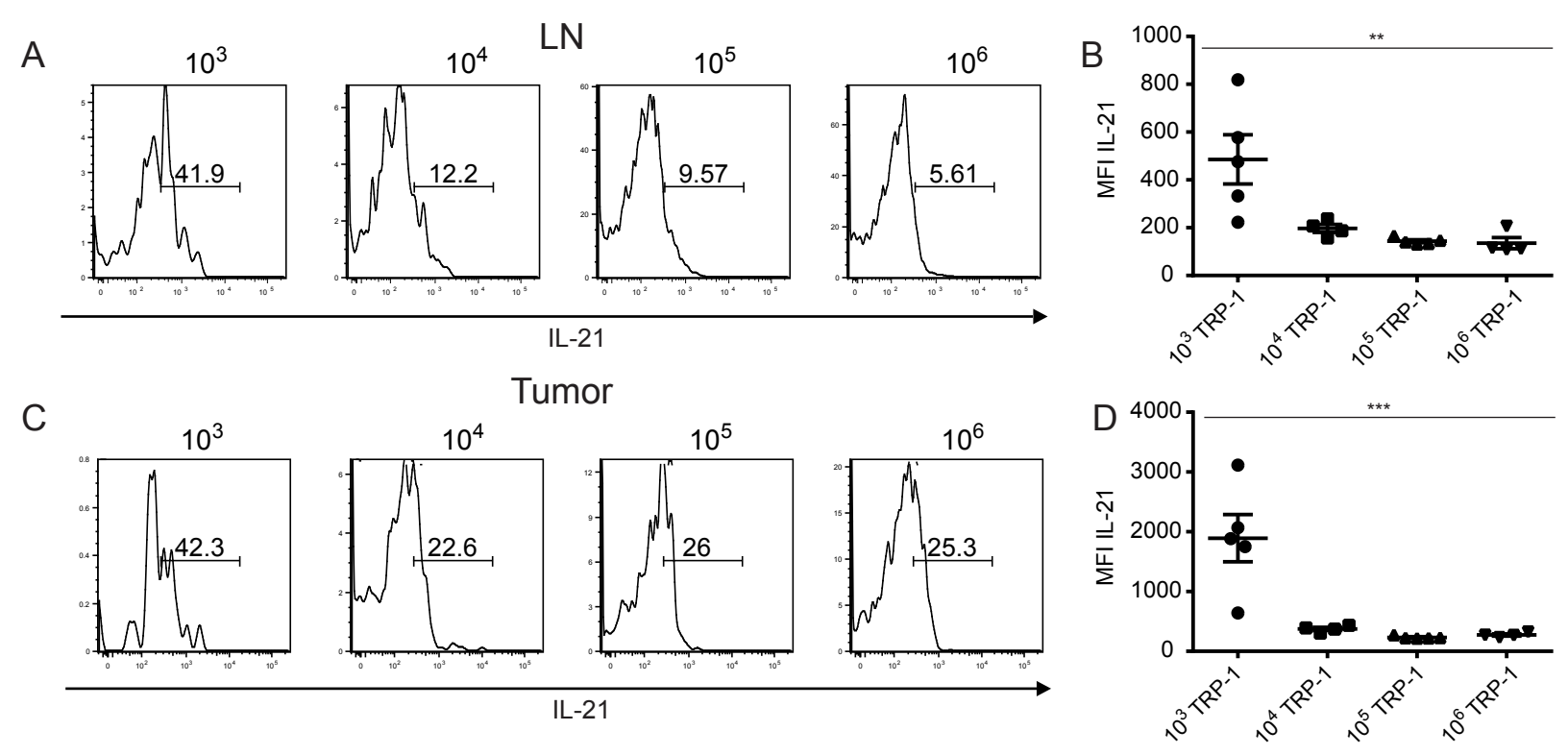
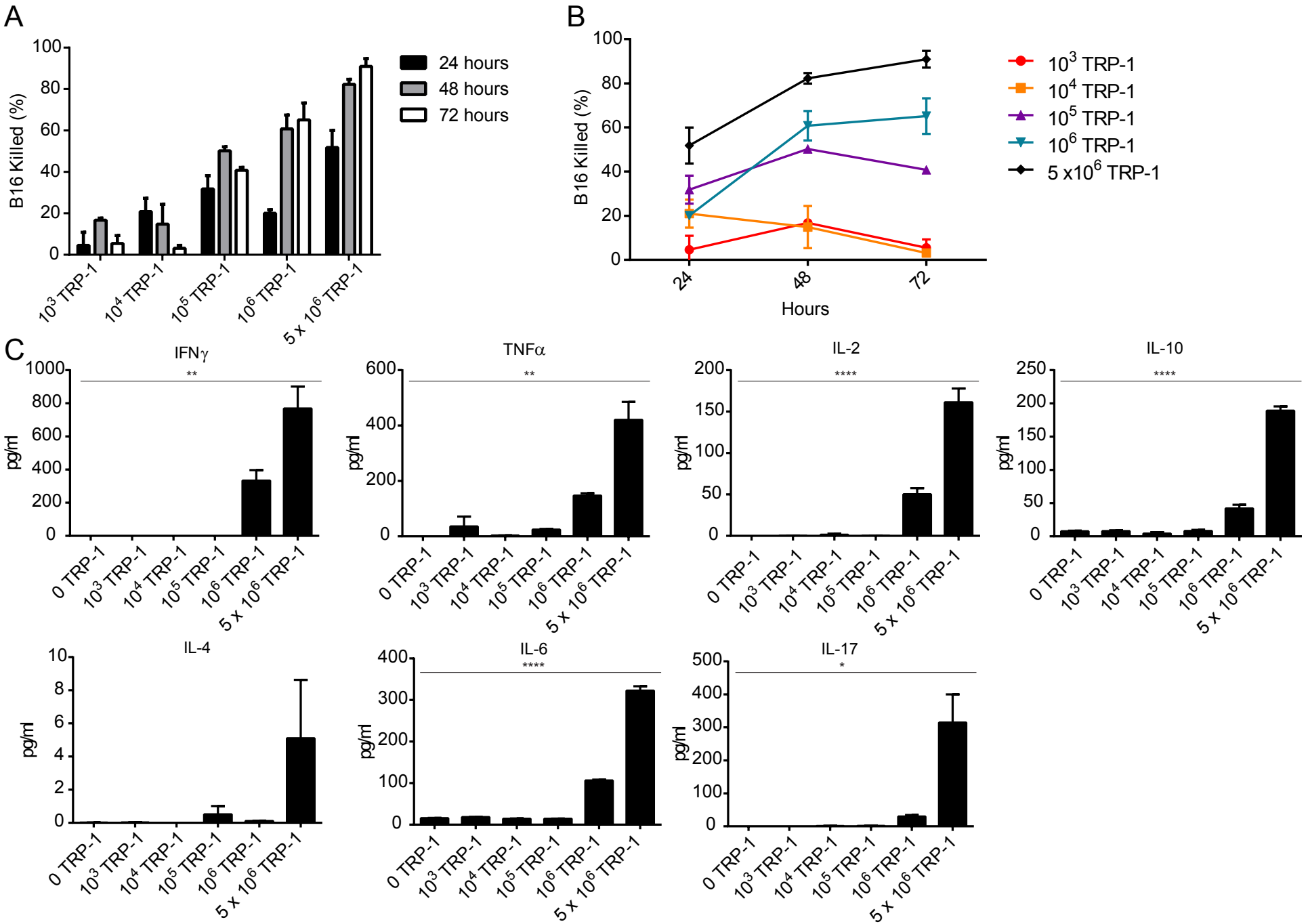
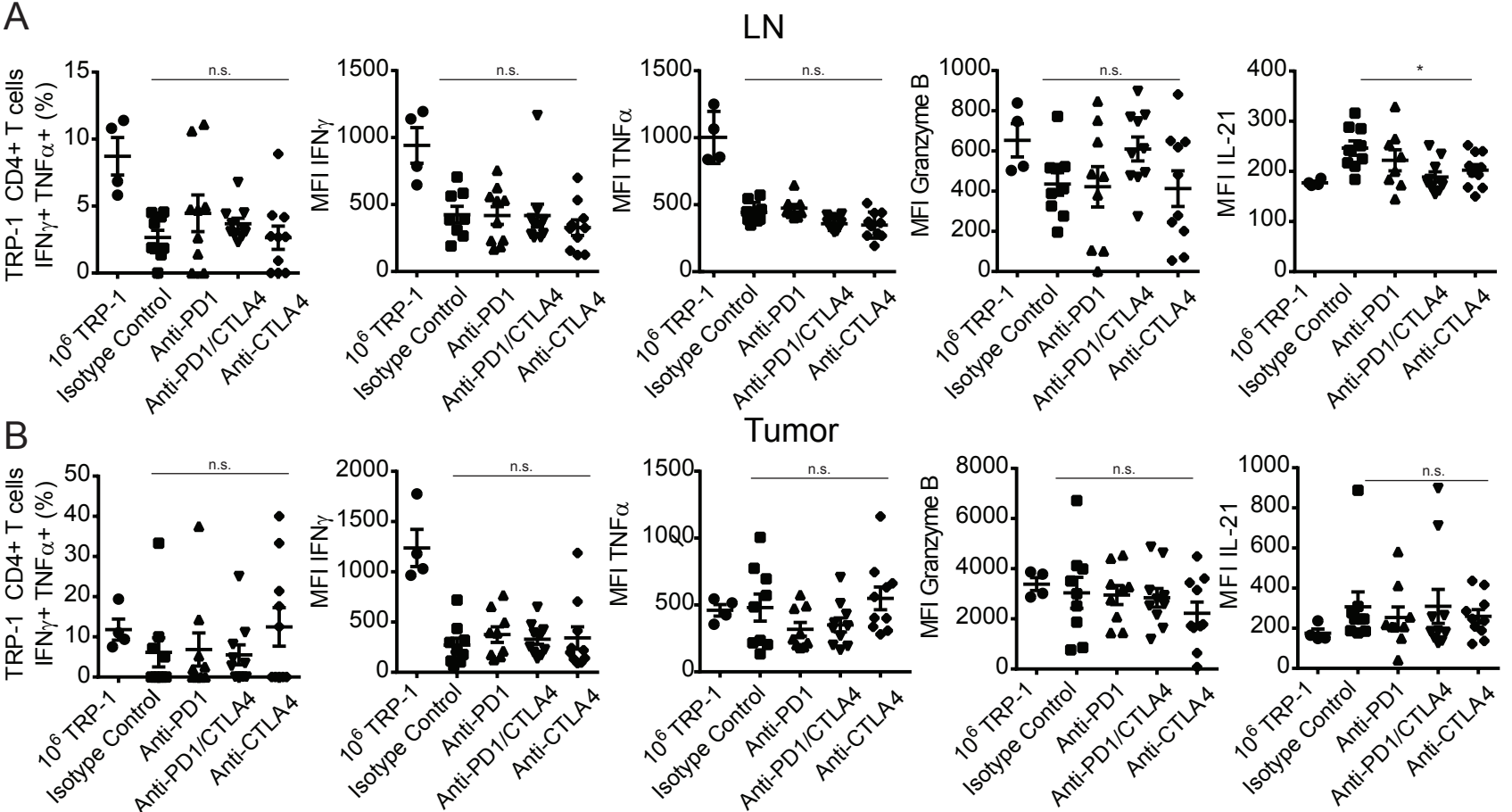
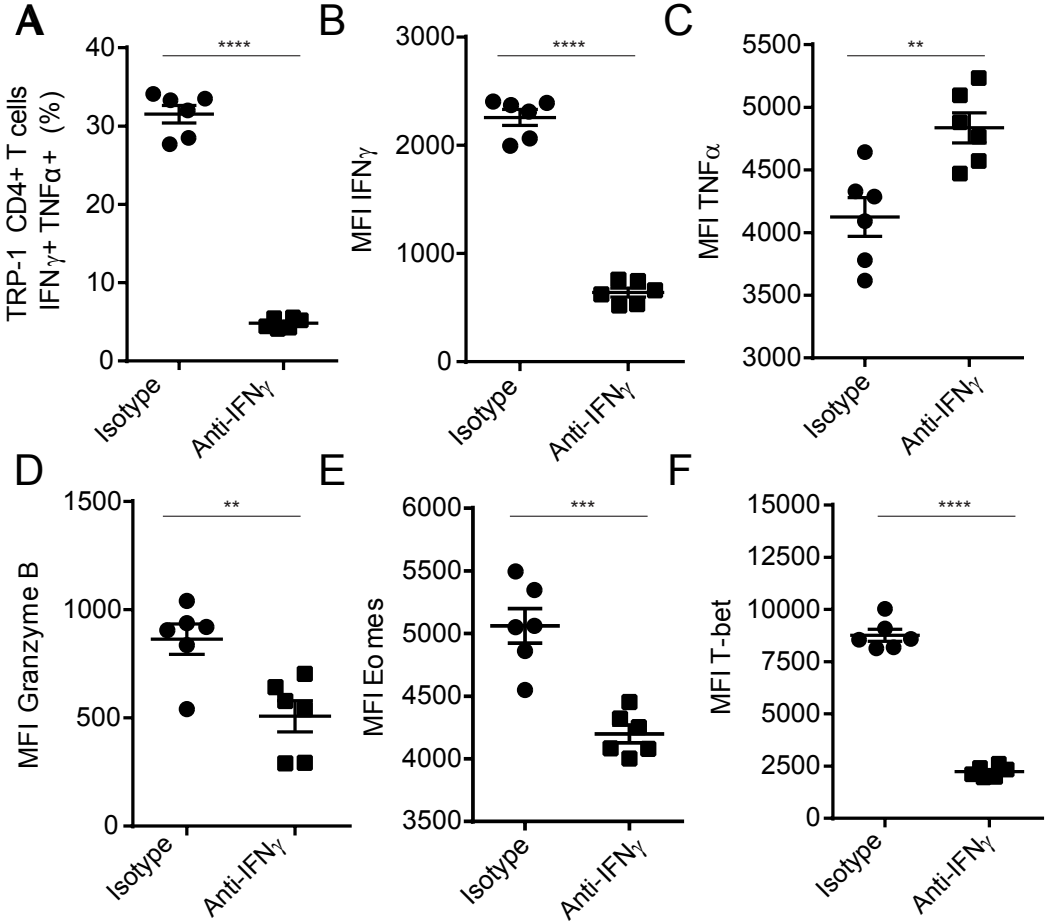

A
0 TRP-1 + IgG
10^5 TRP-1 + IgG
10^6 TRP-1 + IgG
B
0 TRP-1 + Anti-IA^b
10^5 TRP-1 + Anti-IA^b
10^6 TRP-1 + Anti-IA^b
Tumor Diameter (mm)
Days
30
25
20
15
10
5
-20
0
20
40
60
80
100

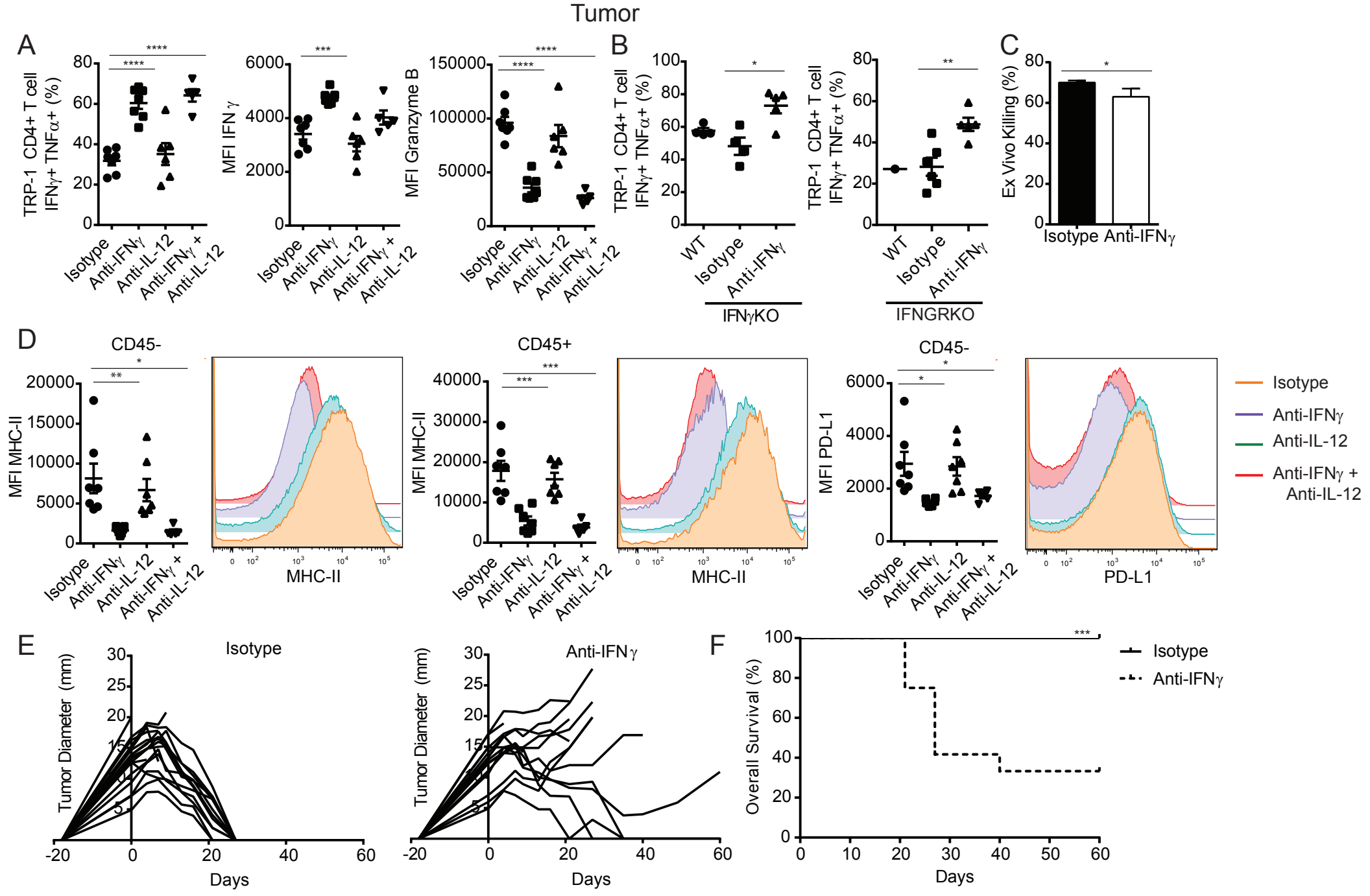

A
TRP-1 CD4+ T cells/ gram of tumor (Absolute #)
Day 7
Day 9
Day 11
****
*
4.0×10^5
3.0×10^5
2.0×10^5
1.0×10^5
0
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
5.0×10^6
4.0×10^6
3.0×10^6
2.0×10^6
1.0×10^6
10^5 TRP-1
10^6 TRP-1
D
Day 7
Radiance (p/s/cm^2/sr)
30000
20000
10000
0
B
TRP-1 CD4+ T cells Foxp3+ (%)
Day 7
Day 9
Day 11
**
*
40
30
20
10
0
8
6
4
2
Day 9
***
C
Effector:T_reg Ratio
Day 7
Day 9
Day 11
***
***
50
25
0
250
200
150
100
50
60000
40000
20000
E
Day 7: 10^5
10^6
Day 9: 10^5
10^6
Day 11: 10^5
10^6


A
LN
10^3
10^4
10^5
10^6
41.9
12.2
9.57
5.61
IL-21
B
MFI IL-21
**
1000
800
600
400
200
0
10^3 TRP-1
10^4 TRP-1
10^5 TRP-1
10^6 TRP-1
C
Tumor
10^3
10^4
10^5
10^6
42.3
22.6
26
25.3
IL-21
D
MFI IL-21
***
4000
3000
2000
1000
0
10^3 TRP-1
10^4 TRP-1
10^5 TRP-1
10^6 TRP-1


A
B16 Killed (%)
24 hours
48 hours
72 hours
10^3 TRP-1
10^4 TRP-1
10^5 TRP-1
10^6 TRP-1
5 x 10^6 TRP-1
B
B16 Killed (%)
Hours
10^3 TRP-1
10^4 TRP-1
10^5 TRP-1
10^6 TRP-1
5 x10^6 TRP-1
C
IFNγ
TNFα
IL-2
IL-10
IL-4
IL-6
IL-17
pg/ml
0 TRP-1
10^3 TRP-1
10^4 TRP-1
10^5 TRP-1
10^6 TRP-1
5 x 10^6 TRP-1


A
LN
TRP-1 CD4+ T cells IFNγ+ TNFα+ (%)
MFI IFNγ
MFI TNFα
MFI Granzyme B
MFI IL-21
n.s.
*
B
Tumor
10^6 TRP-1
Isotype Control
Anti-PD1
Anti-PD1/CTLA4
Anti-CTLA4


A
TRP-1 CD4+ T cells IFNγ+ TNFα+ (%)
40
30
20
10
0
****
Isotype
Anti-IFNγ
B
MFI IFNγ
3000
2000
1000
0
****
Isotype
Anti-IFNγ
C
MFI TNFα
5500
5000
4500
4000
3500
3000
**
Isotype
Anti-IFNγ
D
MFI Granzyme B
1500
1000
500
0
**
Isotype
Anti-IFNγ
E
MFI Eo mes
6000
5500
5000
4500
4000
3500
***
Isotype
Anti-IFNγ
F
MFI T-bet
15000
12500
10000
7500
5000
2500
0
****
Isotype
Anti-IFNγ


Tumor
A
TRP-1 CD4+ T cell IFNγ+ TNFα+ (%)
MFI IFN γ
MFI Granzyme B
Isotype
Anti-IFNγ
Anti-IL-12
Anti-IFNγ + Anti-IL-12
B
TRP-1 CD4+ T cell IFNγ+ TNFα+ (%)
WT
Isotype
Anti-IFNγ
IFNγKO
IFNGRKO
C
Ex Vivo Killing (%)
Isotype
Anti-IFNγ
D
CD45-
CD45+
MFI MHC-II
MFI PD-L1
MHC-II
PD-L1
Isotype
Anti-IFNγ
Anti-IL-12
Anti-IFNγ + Anti-IL-12
E
Isotype
Anti-IFNγ
Tumor Diameter (mm)
Days
F
Overall Survival (%)
Days
Isotype
Anti-IFNγ

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Related to Figure 2. Tumor rejection is dependent on TRP-1 CD4+ T cell interaction with MHC-II

(A) MHC-II deficient C57BL/6J mice were implanted with 2.5 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 0, 10^5, or 10^6 TRP-1 CD4+ T cells with co-transferred splenocytes derived from MHC-II deficient mice. One cohort of mice received intra-peritoneal injection of 200 μg of an MHC-II (IA^b) blocking antibody on the day prior to adoptive transfer and every other day until 21 days post-transfer.

(B) One cohort of mice received intra-peritoneal injection of 200 μg of an IgG isotype control beginning on the day prior to adoptive transfer and every other day until 21 days post-transfer. Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. Mice were euthanized when tumor diameter reached 2.5 cm. Representative data of at least 3 independent experiments (n= 5 mice per group).

Figure S2. Related to Figure 2. During the peak of the anti-tumor immune response T cell infiltration into the tumor is greatest when TRP-1 CD4+ T cells are present at high precursor frequencies

(A) 10^5 or 10^6 TRP-1 CD4+ T cells were adoptively transferred IV into C57BL/6 mice bearing 14 day old B16 melanoma tumors. Total cell quantity transferred was

normalized to 30 x 10^6 per mouse using naïve splenocytes. Absolute number of TRP-1 CD4+ T cells infiltrating the tumor as determined by quantifying TRP-1 by flow cytometry and counting on a Guava cytometer and normalizing to tumor mass.

(B) Percentage of Foxp3+ T cells within tumor infiltrating TRP-1 CD4+ T cell population.

(C) Ratio of absolute number of TRP-1 CD4+ T cell effectors to absolute number of TRP-1 CD4+ regulatory T cells.

(D) 10^5 or 10^6 TRP-1 CD4+ T cells expressing a luciferase reporter were adoptively transferred I.V. into C57BL6 mice bearing 14 day old B16 melanoma tumors. Total cell quantity transferred was normalized to 30 x 10^6 per mouse using naïve splenocytes. On days 7, 9, and 11 mice were injected with D-luciferin substrate and imaged on an IVIS 200. Radiance quantified within tumor region of interest.

(E) Representative images.

Figure S3. Related to Figure 3. At low precursor frequencies tumor antigen specific CD4+ T cells produce IL-21

(A) D7 p.t. lymphocytes restimulated with PMA/ionomycin, representative histograms of IL-21 expression in TRP-1 CD4+ T cell LN population.

(B) MFI of IL-21 in TRP-1 CD4+ T cell LN population.

(C) D7 p.t. lymphocytes restimulated with PMA/ionomycin, representative histograms of IL-21 expression in tumor infiltrating TRP-1 CD4+ T cell population.

(B) MFI of IL-21 in tumor infiltrating TRP-1 CD4+ T cell population.

Figure S4. Related to Figure 4. Large numbers of TRP-1 CD4+ T cells sustain high levels of B16 killing over time

(A) TRP-1 CD4+ T cells were isolated via MACS purification from TRP-1 TCR transgenic mice and directly co-embedded at indicated quantities with equivalent numbers of B16 targets (50,000) in a collagen fibrin gel based *ex vivo* killing assay. At 24, 48, and 72 hours gels were lysed, plated for B16 colony forming units, and killing was evaluated by staining for CFU 7 days later. Percentage of B16 killed as a function of concentration. (B) Percentage of B16 killed as a function of time. (C) Supernatants overlaying the gel based killing assay were collected upon gel lysis at 48 hours. Using a CBA kit, cytokine concentrations in the supernatants were quantified. Data are represented as mean ± SEM.

Figure S5. Related to Figure 6. Combining PD-1 blockade with anti-CTLA-4 does not improve effector function in the exhausted tumor specific CD4+ T cells

(A) Mice with established tumors received transfer 10^3 or 10^6 TRP-1 CD4+ T cells. Beginning on the day of transfer, mice in the 10^3 group received anti-PD-1 alone or in combination with anti-CTLA-4 or an isotype control every 3 days. Day 7 p.t. lymphocytes restimulated with PMA/ionomycin, summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells and MFI of IFNγ, TNFα, granzyme-B and IL-21 expression in TRP-1 CD4+ T cell population respectively from LN and (B) tumor.

Figure S6. Related to Figure 7. Neutralization of IFN-γ *in vitro* decreases Th1 differentiation and effector function

(A) Irradiated feeder APCs were pulsed for 2 hours with TRP-1 peptide and plated with MACS separated naïve TRP-1 CD4+ T cells in media containing IFN-γ neutralizing antibody or an isotype control in a 96 well plate. 5 days after initiation of culture lymphocytes were stimulated with PMA/ionomycin and evaluated for effector function and transcription factor expression. Summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells (B) MFI IFNγ (C) MFI TNFα (D) MFI Granzyme B (E) MFI EOMES (F) MFI Tbet. Each culture condition was performed with six replicates. Data are representative of two independent experiments. Data are represented as mean ± SEM.

Figure S7. Related to Figure 7. Blockade of IFNγ increases CD4+ T cell cytokine secretion in the tumor but does not result in an increase of anti-tumor function

(A) 10^6 TRP-1 CD4+ T cells were adoptively transferred I.V. into C57BL/6 mice,(B) IFN**γ** deficient and IFNGR deficient mice, respectively, bearing 14 day old B16 melanoma tumors, following 600 cGy of sub-lethal irradiation. Beginning on day of adoptive transfer and every other day mice were injected I.P. with isotype control neutralizing antibody. Day 7 tumor infiltrating lymphocytes were stimulated with PMA/ionomycin; summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells, and MFI IFNγ, granzyme-B respectively.

(C) Day 7 TRP-1 CD4+ T cells were sorted from tumor and embedded in an ex vivo killing assay, killing percentage

(D) MFI MHC-II and PD-L1 on CD45- and CD45+ tumor populations

(E) 10^6 TRP-1 CD4+ T cells were adoptively transferred IV into C57BL6 bearing 14 day old B16 melanoma tumors, following 600 cGy of sub-lethal irradiation. Beginning on day of adoptive transfer and then every other day mice were injected with control or IFNγ neutralizing antibody. Tumor diameter was measured with calipers every 3-5 days to track tumor growth, tumor growth curves and (F) survival curves.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice and tumors

All mouse procedures were performed in accordance with institutional protocol guidelines at Memorial Sloan-Kettering Cancer Center (MSKCC) under an approved protocol. C57BL/6J, IFNγ$^{-/-}$ (Stock 002287), IFNγR$^{-/-}$ (Stock 003288)(Stock 6–8-wk-old males were obtained from The Jackson Laboratory. MHC-II deficient (ABBN12) and MHC-II sufficient control male mice were obtained from Taconic Biosciences, Inc. TRP-1 CD4$^+$ TCR transgenic mice were obtained from the N. Restifo laboratory (National Institutes of Health, Bethesda, MD). TRP-1 CD4$^+$ TCR-Luciferase reporter mice were obtained from the J. Allison laboratory (MD Anderson Cancer Center, Houston, TX). TRP-1 CD4$^+$ TCR transgenic were crossed to RAG1$^{-/-}$ TRP-1$^{-/-}$ CD45.1 background. The B16-F10 mouse melanoma line was originally obtained from I. Fidler (M.D. Anderson Cancer Center, Houston, TX). Tumor implantation was via intradermal injection of a 50 µl bolus containing 2.5 x 10^5 B16-F10 and PBS in the flank of shaved recipients. Tumor

growth was tracked every 3-5 days via caliper measurement of diameter. Mice received adoptive transfer of tumor specific T cells on day 14 after implantation. Mice were euthanized when tumors reached 2 cm in diameter.

Irradiation and adoptive transfer

Recipient mice received 600 cGy total body irradiation from a ^{137}Cs source several hours prior to adoptive transfer. Donor cells were isolated from lymph node and spleen of male TRP-1 TCR transgenic mice (6-12 week old) or TRP-1 TCR-Luciferase reporter mice when indicated. Purification was by positive selection magnetic cell sorting using CD4 beads (L3T4) (Miltenyi Biotech) according to the manufacturer's instructions. Dose groups were washed twice with PBS, re-suspended at 30 million cells per 200 µl, and injected intravenously via tail vein. Open repertoire splenocytes were derived from the ACK lysis buffer (Lonzo) incubated spleens of naïve donors. When indicated, CD4+ T cells were labeled with 5 µM CFSE (Life technologies) prior to transfer according to manufacturers instructions.

***In Vivo* and *Ex Vivo* killing assays**

For *in vivo* killing assays splenocytes from C57BL/6J mice were labeled with 5 or 0.5 µM CFSE. $CFSE^{high}$ splenocytes were loaded with 20 µM of TRP-1 peptide (Genemed Synthesis, Inc) for 2 hours at 37°C. On day 6 after adoptive transfer 5×10^5 cells of a 50:50 mixture of TRP-1 peptide pulsed and un-pulsed splenocytes were transferred via tail vein injection in a 200 µl bolus. The following day, mice were sacrificed and spleens

were removed, and the percentage of loaded and unloaded splenocytes was analyzed by flow cytometry. Cytotoxicity was calculated using the following equation:

In vivo killing percentage =

100% (1−((unloaded/loaded)$_{control}$/(unloaded/loaded)$_{experimental}$)).

For clonogenic *ex vivo* killing assays wells in a 48-well tissue culture plate were filled sequentially with 5 µl PBS containing 0.1 U thrombin, 100 µl PBS containing 1 mg/ml of human fibrinogen, 1 mg/ml of rat tail collagen I, 10% FBS, and B16 cells, with or without TRP-1 cells at a 1:10 ratio or at the T cell quantity indicated. The plates were incubated for 20 min at 37°C in a 95% air/5% CO_2 humidified atmosphere to allow the fibrin to gel. Gels were overlaid with 0.5 ml RPMI 1640 supplemented with 10% FCS, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 µM β-mercaptoethanol and incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere. These gels are 0.1 ml in volume and ∼1,500 µm in height. 24 hours later, the gels were lysed by sequential collagenase (2.5 mg/ml) and trypsin (2.5 mg/ml; Sigma-Aldrich) digestion. The lysed gels were then diluted and the recovered melanoma cells were plated in 6-well plates for colony formation. After 7 days in culture, the plates were fixed with formaldehyde, stained with 2% methylene blue, and the colonies were counted manually. For the preparation of targets, B16 cells were incubated overnight with 10 ng/ml of recombinant IFNγ (PeproTech) and single cell suspension were prepared using Cellstriper (Cellgro) before the assay. Target cells killing was calculated using the equation: 1 − [melanoma + T cells]/[melanoma alone]. The killing coefficient *k* was calculated applying the equation: $b = b_0\, e^{-(kp - g)t}$ in which b is the tumor concentration

at any time, b_0 is the initial tumor concentration, p is the T cell concentration, *k* is the second-order rate constant for T cell killing of tumor, and *g* is the first-order rate constant for tumor growth. [1]

***In vivo* neutralization via monoclonal antibody**

Anti-PD-1(RMP1-14), anti-MHC-II (M5/114), anti–CTLA-4 (9D9,) anti–IFNγ (XMG1.2), anti-IL-12p40 (C17.8) and respective isotype controls (2A3, LTF-2, HRPN, MPC-11) were purchased from BioXCell. For cytokine neutralization experiments mice received intra-peritoneal injection of 200µg/dose of isotype control, anti-IFNγ, or anti-IL-12p40 antibody with the exception of the wildtype control group. Treatment was initiated the day before adoptive transfer and was administered every other day. For checkpoint blockade experiments mice received intra-peritoneal injection of 250 µg/dose of anti-PD-1, 100 µg/dose of anti-CTLA-4, or their respective isotype controls, with the exception of the 10^6 adoptive transfer control group. Treatment was initiated the day of adoptive transfer and was administered every 3 days.

***In vivo* T cell imaging**

Fully shaved mice were anaesthetized and injected retro-orbitally 2 minutes prior to imaging with 1.5 mg D-Luciferin K+ reconstituted according to manufacturers instructions (PerkinsElmer). Mice were imaged every 2-3 days on an IVIS 200 (Xenogen). Data was acquired and analyzed using LivingImage software (Xenogen).

***Ex vivo* restimulation, quantification, and flow cytometry**

Cells from tumor draining lymph nodes, spleens, and tumors were prepared by mechanical dissociation in 40 μM filters and red blood cells were removed by incubation in ACK Lysing Buffer (Lonza). When tumor mass exceeded .1 grams live lymphocytes were isolated using Percoll (GE Healthcare) gradient centrifugation. Pre- and post Percoll isolation samples were taken to determine total enrichment. Sample cellularity was quantified prior to enrichment steps using a Guava EasyCyte (EMD Millipore) and CytoSoft software. (EMD Millipore). Absolute number was determined by gating on the population of interest, back calculating, and multiplying by absolute number. For samples receiving re-stimulation, cells were stimulated in RPMI 1640 supplemented with 10% FCS, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 μM β-mercaptoethanol with 500 ng/ml PMA and 1 μg/ml ionomycin and incubated at 37°C. After 2 hours, 10 μg/ml monensin and 1x GolgiPlug (BD Biosciences) were added to the culture and incubated for an additional 3 hours at 37°C. Surface staining was performed with an initial 1 hour incubation FcBlock biotin (clone 2.4G2; BD Biosciences) and CD45.1 biotin (clone 2.4G2; BD). A secondary surface stain with fluorophore conjugated streptavidin and antibodies followed for 45 minutes. All intracellular staining was conducted using the Foxp3 fixation/permeabilization buffer (eBioscience) according to the manufacturer's instructions. Flow cytometry was performed on an LSRII (BD Biosciences). FlowJo software (version 9.4.10; Tree Star) was used for all flow cytometry analysis. FACS sorting was conducted on a FACSAria II cell sorter (BD Biosciences).

Flow cytometry antibodies

Biolegend – Biotin CD45.1, Pe-Cy7 PDL1, APC-Cy7 MHC-II (I-A/I-E)

BD Biosciences - FITC Ki67, FITC Streptavidin, FITC Cd11b, FITC CD44, Alexa488 TNF, PE Streptavidin, PE - CD80, PerCP CD3e, PerCP CD4, PerCP CD8, Pe-Cy7 CD4, Pe-Cy7 Cd11c, Pacific Blue CD8a, V450 CD80, Alexa700 IFNγ, APC-Cy7 CD45

eBioscience - FITC ICOS, PE-BTLA, PE Eomesodermin, PE CD86, PeCy7 PD-1, eFluor 450 LAG-3, eFluor 450 MHC-II (I-A/I-E), eFluor 450 Eomesodermin, APC IL-21, APC CTLA-4, APC eFluor 780 CD27, APC eFluor780 CD8a, APC eFluor780 CD62L

LifeTechnologies - PE-TexasRed Granzyme B

Lymph node and tumor cytokine quantification

Inguinal tumor draining lymph node was isolated and homogenized using a Retsch Mixer Mill in 150 µl PBS containing Complete EDTA-free protease inhibitor (Roche) resuspended according to the manufacturer instructions. 500 µl of tumor volume was massed and homogenized in 500 µl PBS + protease inhibitor via Retsch Mixer Mill. Homogenate supernatant was clarified twice via centrifugation at 2000 rpm. Cytokine was quantified on a Luminex Magpix using a Milliplex MAP Kit (Millipore). For tumor samples cytokine was normalized to mass of isolated tissue.

***In vitro* supernatant cytokine quantification**

The 1 ml of media overlaying the collagen-fibrin gels of *in vitro* clonogenic killing assays was collected at 24 or 48 hours prior to digesting gels. The supernatant was frozen down and thawed to assess cytokine production. Cytokine was quantified using a CBA Th1/Th2/Th17 Kit (BD Biosciences) on the LSRII (BD Biosciences).

***In vitro* TRP-1 CD4+ T cell differentiation assays**

TRP-1 CD4+ T cells were isolated as previously described by CD4 positive separation on MACS columns. 50,000 TRP-1 CD4+ T cells were co-plated with 25,000 irradiated splenocytes that had been pulsed with TRP-1 peptide for 2 hours at 37°C and washed twice, in RPMI 1640 supplemented with 10% FCS, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 μM β-mercaptoethanol supplemented with 20 U/ml of rhIL-2 and incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere. 50 μg per well of IFNγ neutralizing antibody (XMG1.2) or an isotype control (HRPN)were added on the first day of culture. T cell differentiation was evaluated 5 days after initiation of culture.

RT-PCR

CD4+ T cells were sorted on a FACS AriaII (BD Biosciences) directly into Trizol LS (Life technologies) and total RNA was obtained via Trizol purification according to the manufacturers instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. All primers and probes were from TaqMan Gene Expression Assays (Applied Biosystems).

Real-time PCR reactions were prepared with 3 µl cDNA according to the manufacturer's instructions. All amplifications were done using the ABI 7500 Real Time PCR system (Applied Biosystems). Each gene was amplified in triplicate and cDNA concentration differences were normalized to GAPDH. Relative gene expression of the target genes were shown by 2 − ΔCt (ΔCt = Ct(target gene) − Ct(GAPDH)) using mean Ct (threshold cycle) of triplicates.

Statistical Analysis

All values shown in graphs represent the mean ± SEM, with the exception of *ex vivo* clonogenic killing assays, which represent the mean ± SD. In graphs comparing titration of 4 different precursor frequencies, significance is only shown for extreme groups for ease of interpretation. Statistical differences were determined using ANOVA and *post hoc* Bonferroni adjustment for multiple comparisons. Statistics for comparing 2 groups were determined by a 2-tailed Student's *t* test. * $P < 0.05$ was considered statistically significant. ** $p < 0.01$, *** $p< 0.001$, **** $p < 0.0001$. Significance of survival curves was evaluated by log rank (Mantel Cox) test. All graphs and statistical calculations were done using Prism software (GraphPad Software). PLSR and heat maps were generated using MatLab (MathWorks) reflecting the following equation $Y_{T\,cells}$: $Y_{T\ \ cells} = \beta_0+\Sigma_{i,j}\ \beta_{i,j}.X_{i,j,k}$ where $X_{i,j,k} = \alpha_0+\Sigma_{i,j}\ \alpha_{i,j}.log_{10}(M_{i,j,k})$.

SUPPLEMENTAL REFERENCES

1. Li, Y., et al., *Determination of the Critical Concentration of Neutrophils Required to Block Bacterial Growth in Tissues.* The Journal of Experimental Medicine, 2004. **200**(5): p. 613-622.