Supplemental Data

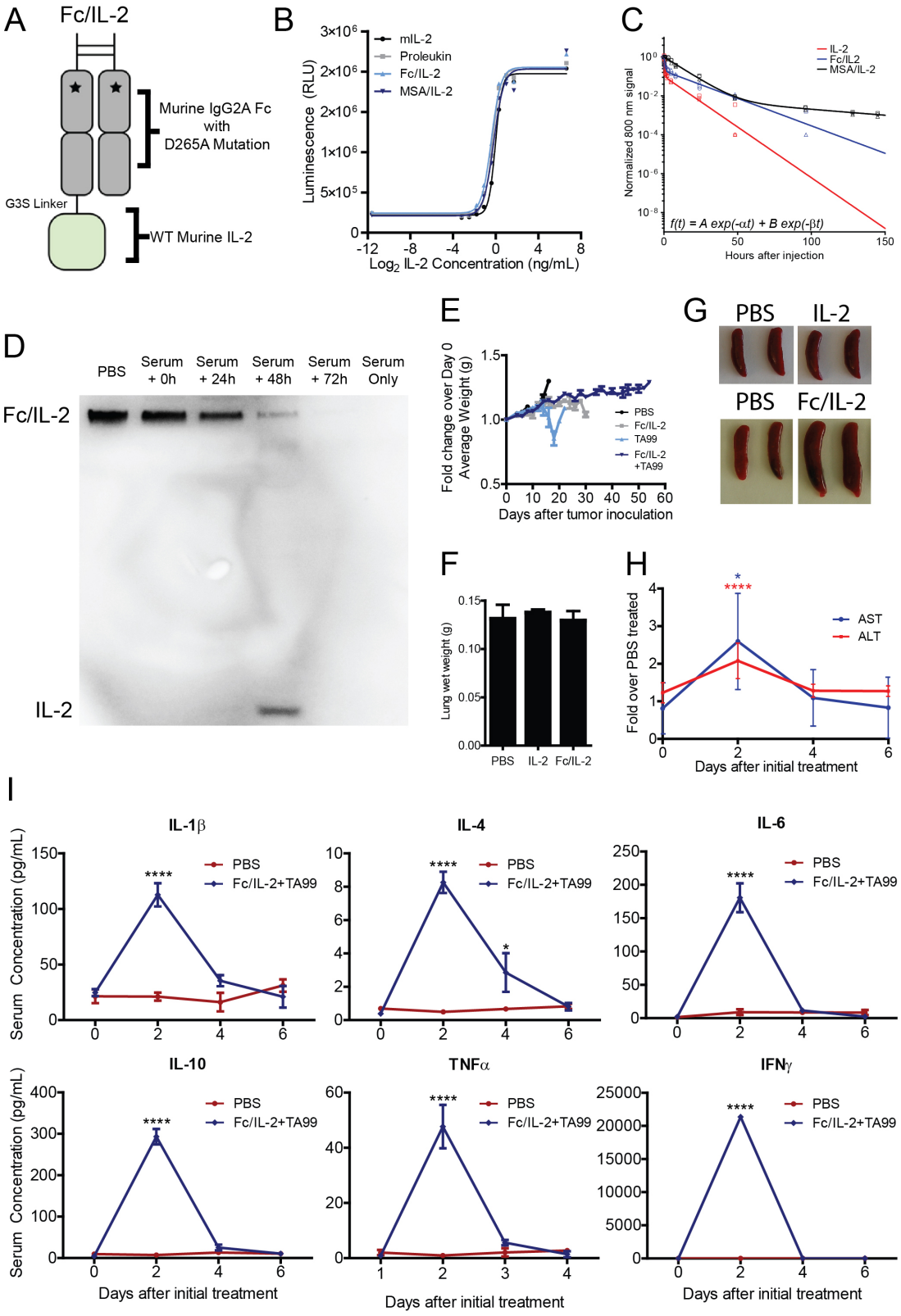


Figure S1, related to Figure 1. IL-2 fused to Fc creates a functional form of IL-2 with extended serum half-life and induces transient systemic toxicity

(A) Schematic of Fc/IL-2. Wild-type murine IL-2 is fused to the C-terminus of a murine IgG2a Fc fragment containing a D265A mutation (indicated by asterisks), where the protein fusion is monomeric with respect to IL-2.

(B) CTLL-2 cells were cultured in media with murine IL-2 produced in *E. coli* (mIL-2), human IL-2 produced in *E.* coli (Proleukin), Fc/IL-2 or MSA/IL-2, both produced in HEK cells, at the indicated concentrations on an IL-2 basis. Proliferation analyzed by CellTiter-Glo Luminescent Cell Viability Assay. n = 3 per group.

(C) Normalized 800 nm signal of blood samples taken at various time points from mice injected retro-orbitally with 25 μg of IL-2 or 50 μg of Fc/IL-2 or MSA/IL-2 labeled with IRDye 800CW. Mean fluorescence intensity data were fit to a bi-phasic exponential decay. See Table S1 for fitted parameter values. n = 3 per group.

(D) Equal amounts of Fc/IL-2 were incubated in serum isolated from C57BL/6 mice at 37 °C for the indicated time. A western blot against IL-2 was performed to detect Fc/IL-2 and any degradation products in these samples, compared to an equal amount of Fc/IL-2 from the stock solution in PBS, as well as serum alone. Representative blot is shown.

(E) Weight progression of C57BL/6 mice bearing B16F10 tumors during the course of treatment with PBS, Fc/IL-2 and/or TA99. n = 5 per group.

(F) C57BL/6 mice were injected i.v. with a single bolus of PBS, 12 μg IL-2, or 50 μg Fc/IL-2. 4 days after injection, lung weight was determined by lyophilization. n = 3 per group.

(G) C57BL/6 mice were injected i.v. with a single bolus of PBS, 12 μg IL-2, or 50 μg Fc/IL-2. 4 days after injection, spleens were extracted and photographed. 2 representative spleens are shown.

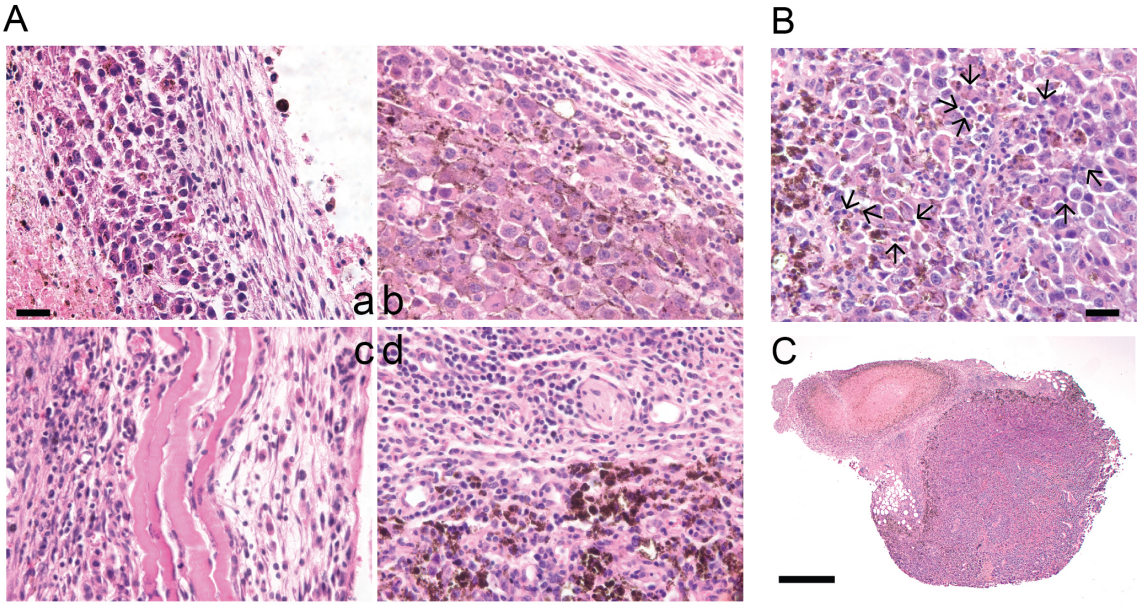
(H) C57BL/6 mice bearing B16F10 tumors were injected i.v. with a single bolus of PBS or Fc/IL-2+TA99. At the indicated time points, blood was drawn, serum isolated, and activity of liver enzymes AST and ALT was assayed. n = 3 per group.

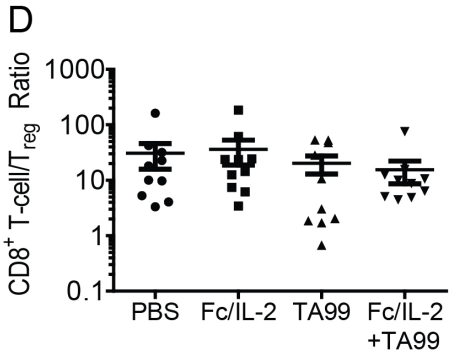
(I) C57BL/6 mice bearing B16F10 tumors were injected i.v. with a single bolus of PBS or Fc/IL-2+TA99. At the indicated time points, blood was drawn, serum isolated, and the serum concentration of various inflammatory cytokines was assayed. n = 3 per group. Data are mean ± SEM.

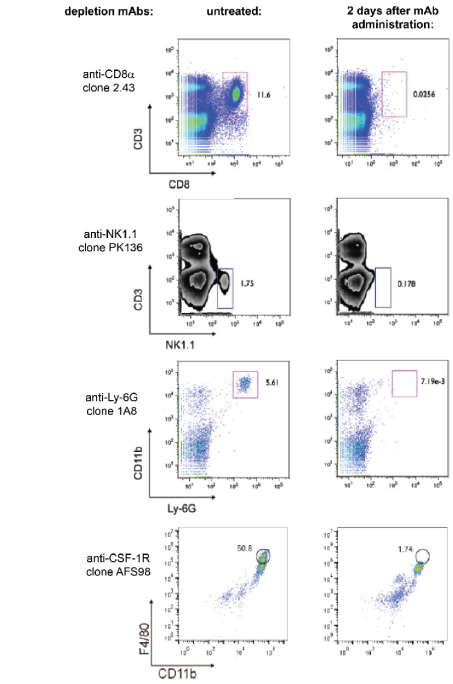
Table S1, related to Figure 1. Pharmacokinetics parameters of IL-2, Fc/IL-2, and MSA/IL-2 administered as single *i.v.* bolus.

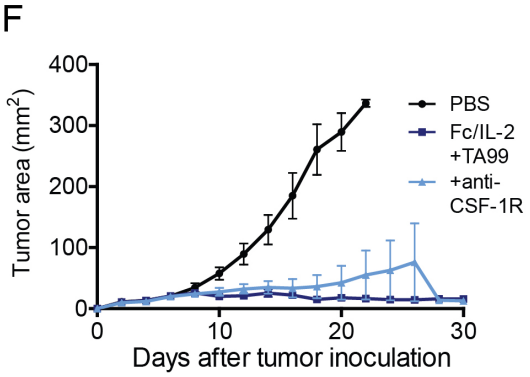
	A	α (hr^{-1})	B	β (hr^{-1})	$\tau_{1/2,\alpha}$ (hr)	$\tau_{1/2,\beta}$ (hr)
IL-2	0.88 ± 0.02	2.61 ± 0.25	0.12 ± 0.02	0.44 ± 0.03	0.3 ± $2.8x10^{-2}$	4.8 ± 1.8
Fc/IL-2	0.86 ± 0.054	1.99 ± 0.086	0.14 ± 0.054	0.056 ± $8.7x10^{-3}$	0.4 ± 0.01	12.5 ± 1.3
MSA/IL-2	0.99 ± $7.6x10^{-4}$	0.11 ± $4.8x10^{-3}$	$8.2x10^{-3}$ ± $7.6x10^{-4}$	0.014 ± 0.0014	6.3 ± 0.3	49.9 ± 4.9

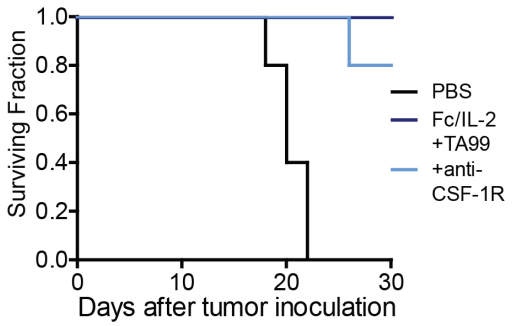
C57BL/6 mice were injected *i.v.* with a single bolus of 25 μg IL-2, 50 μg Fc/IL-2, or 50 μg MSA/IL-2 labeled with IRDye800 (*n* = 3). Serum levels were fit to a bi-phasic exponential decay of form: $MFI(t) = Ae^{-\alpha t} + Be^{-\beta t}$. Fast- and slow half-lives, $\tau_{1/2,\alpha}$ and $\tau_{1/2,\beta}$, were calculated as $\ln 2/\alpha$ and $\ln 2/\beta$, respectively.


A
a b
c d
B
C


D
CD8+ T-cell/Treg Ratio
1000
100
10
1
0.1
PBS
Fc/IL-2
TA99
Fc/IL-2 +TA99


E
depletion mAbs:
untreated:
2 days after mAb administration:
anti-CD8α clone 2.43
CD3
CD8
11.6
0.0256
anti-NK1.1 clone PK136
CD3
NK1.1
1.75
0.178
anti-Ly-6G clone 1A8
CD11b
Ly-6G
5.61
7.19e-3
anti-CSF-1R clone AFS98
F4/80
CD11b
50.8
1.74


F
Tumor area (mm2)
400
300
200
100
0
0
10
20
30
Days after tumor inoculation
PBS
Fc/IL-2 +TA99
+anti-CSF-1R


Surviving Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Days after tumor inoculation
PBS
Fc/IL-2 +TA99
+anti-CSF-1R

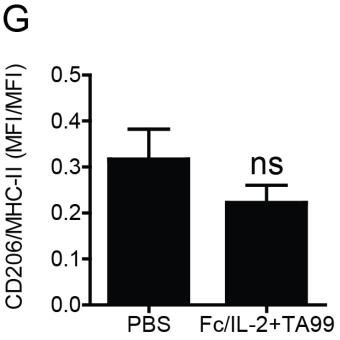

G
CD206/MHC-II (MFI/MFI)
0.5
0.4
0.3
0.2
0.1
0.0
ns
PBS
Fc/IL-2+TA99

Figure S2, related to Figure 3. Fc/IL-2+TA99 induces immune infiltrates within B16F10 tumors; specific antibodies deplete corresponding immune cell population *in vivo*

(A-B) Representative images of H&E stained tumor sections. (A) Tumor periphery of different treatment conditions: a) PBS, b) Fc/IL-2, c) TA99, d) Fc/IL-2+TA99. (B) Interior of tumor treated with Fc/IL-2+TA99. Arrows indicate points of lymphocyte:tumor cell engagement. Magnification 40x. Scale bar 25 microns.

(C) Representative image of H&E stained tumor section treated with Fc/IL-2+TA99. Entire tumor section shown. Magnification 4x. Scale bar 600 microns.

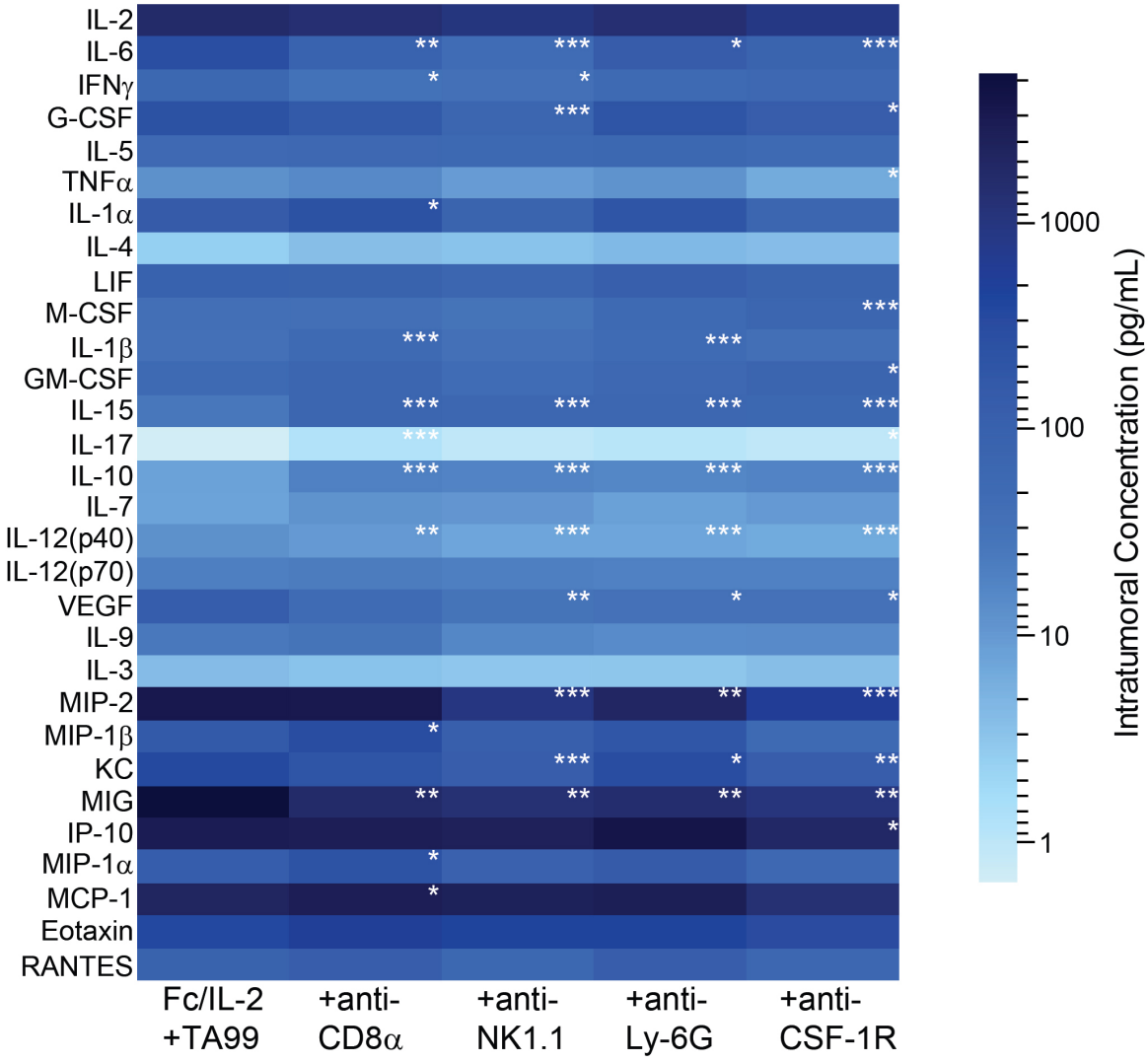
(D) Analysis of the intratumoral effector $CD8^+$ T-cell/T_{reg} ratio with the indicated treatments. The ratio is not significantly altered by any of the treatments. n = 10 per group.

(E) 2 days after depleting antibody administration, RBC-lysed single-cell suspensions of the spleen were stained with fluorescently labeled anti-CD3ε, anti-CD8α, and anti-NK1.1 to assess $CD8^+$ and $NK1.1^+$ depletion. RBC-lysed peripheral blood cells were stained with fluorescently labeled anti-CD11b and anti-Ly-6G to assess $Ly\text{-}6G^+$ depletion. Peritoneal macrophages were stained with fluorescently labeled anti-CD11b and anti-F4/80 to assess macrophage depletion.

(F) Tumor growth and survival curves of C57BL/6 mice bearing subcutaneous B16F10 tumors treated with PBS, Fc/IL-2+TA99, or Fc/IL-2+TA99 with anti-CSF-1R antibody. n = 5 per group.

(G) B16F10 tumors were treated once with PBS or Fc/IL-2+TA99 and harvested 3 days later to analyze the polarization of intratumoral $CD11b^{+}F4/80^{+}$ macrophages as a ratio of the MFIs of CD206 and MHC-II. ns indicates not significant. n = 3 per group. Data are mean ± SEM.

A


IL-2
IL-6
IFNγ
G-CSF
IL-5
TNFα
IL-1α
IL-4
LIF
M-CSF
IL-1β
GM-CSF
IL-15
IL-17
IL-10
IL-7
IL-12(p40)
IL-12(p70)
VEGF
IL-9
IL-3
MIP-2
MIP-1β
KC
MIG
IP-10
MIP-1α
MCP-1
Eotaxin
RANTES
Fc/IL-2 +TA99
+anti-CD8α
+anti-NK1.1
+anti-Ly-6G
+anti-CSF-1R
Intratumoral Concentration (pg/mL)
1000
100
10
1

B

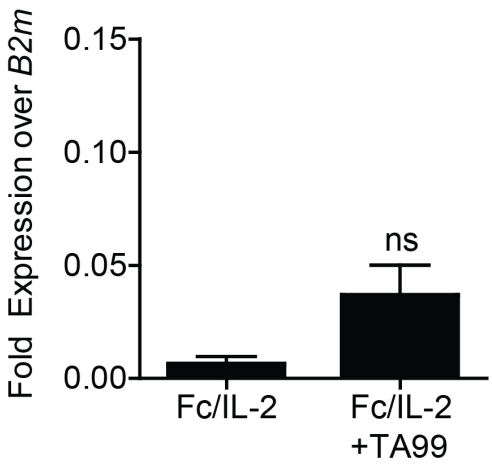

Fold Expression over B2m
0.15
0.10
0.05
0.00
ns
Fc/IL-2
Fc/IL-2 +TA99

Figure S3, related to Figure 4. $CD8^+$ T-cells, NK Cells, neutrophils, and macrophages are directly or indirectly involved in the intratumoral increase of various cytokines/chemokines induced by Fc/IL-2+TA99.

(A) Analysis of intratumoral concentrations of a panel of cytokines and chemokines in tumors treated with Fc/IL-2+TA99 and the indicated depletions. Color bar representing intratumoral concentration is on a log scale. *p < 0.05, **p < 0.01, ***p < 0.001 v. Fc/IL-2+TA99. n = 10 per group

(B) *Cxcl2* expression in $CD3^-NK1.1^+$ NK cells sorted from tumors treated with the indicated conditions, normalized to beta-2 microglobulin (*B2m*) expression. ns indicates not significant. n = 5 per group.

Data are mean ± SEM.

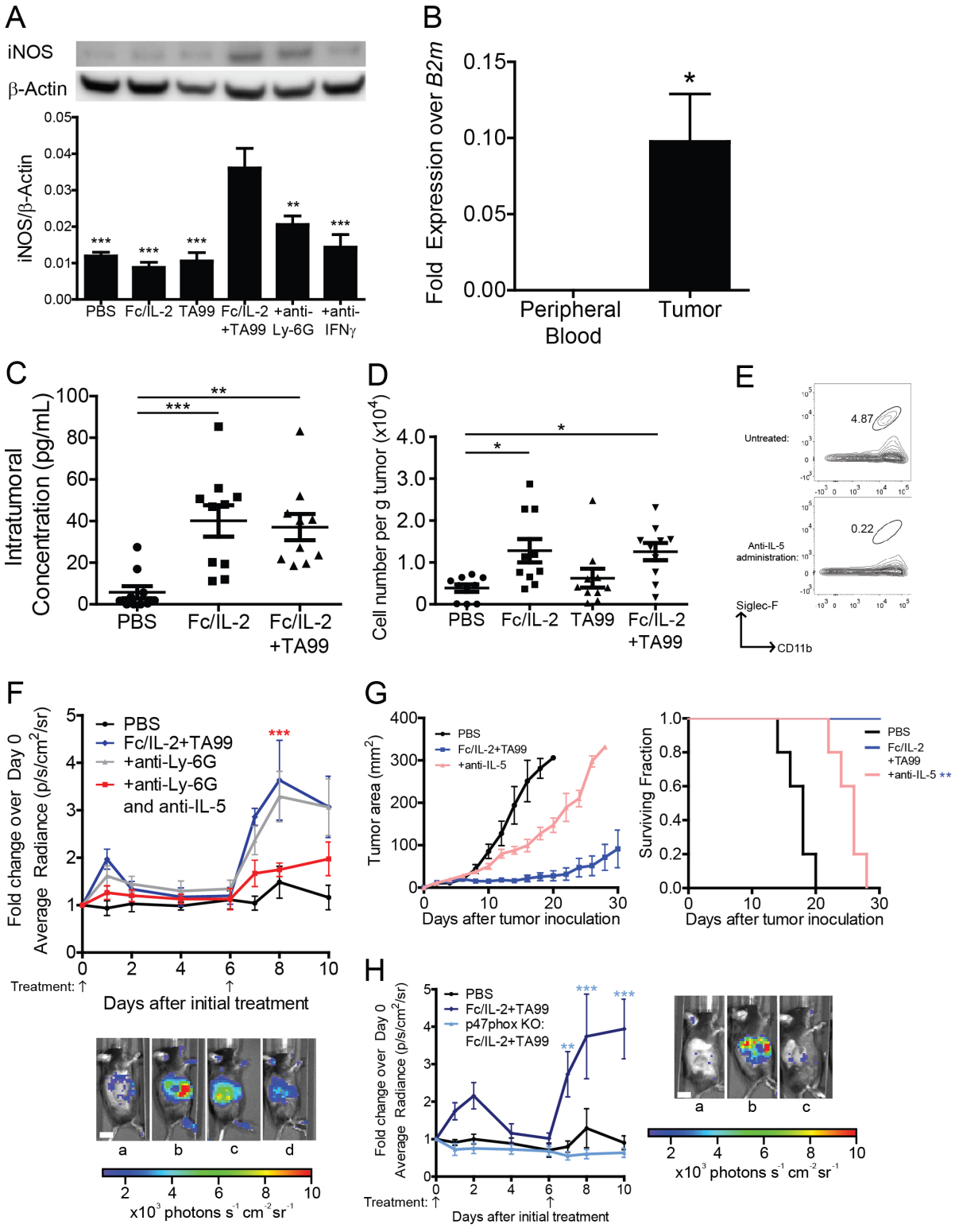

A
iNOS
β-Actin
iNOS/β-Actin
PBS
Fc/IL-2
TA99
Fc/IL-2 +TA99
+anti-Ly-6G
+anti-IFNγ
B
Fold Expression over B2m
Peripheral Blood
Tumor
C
Intratumoral Concentration (pg/mL)
D
Cell number per g tumor (x10^4)
E
Untreated:
Anti-IL-5 administration:
Siglec-F
CD11b
4.87
0.22
F
Fold change over Day 0 Average Radiance (p/s/cm^2/sr)
PBS
Fc/IL-2+TA99
+anti-Ly-6G
+anti-Ly-6G and anti-IL-5
Treatment:
Days after initial treatment
a
b
c
d
x10^3 photons s^-1 cm^-2 sr^-1
G
Tumor area (mm^2)
+anti-IL-5
Days after tumor inoculation
Surviving Fraction
Fc/IL-2 +TA99
+anti-IL-5 **
H
p47phox KO: Fc/IL-2+TA99

Figure S4, related to Figure 5. Fc/IL-2+TA99 induces iNOS production in neutrophils; eosinophils contribute to therapeutic efficacy through respiratory burst

(A) B16F10 tumors treated with the indicated conditions were assayed for iNOS as detected by western blot. β-Actin served as a loading control. Representative blot is shown. n = 5 per group. **p < 0.01, ***p < 0.001 v. Fc/IL2+TA99.

(B) *Nos2* expression in $CD11b^{+}Ly\text{-}6G^{+}$ neutrophils sorted from tumors or peripheral blood in mice treated with Fc/IL-2+TA99, normalized to beta-2 microglobulin (*B2m*) expression. n = 5 per group. *p < 0.05 v. Peripheral Blood.

(C) Analysis of intratumoral concentrations of IL-5 in tumors treated with PBS, Fc/IL-2, or Fc/IL-2+TA99.

(D). Analysis of $CD11b^{+}Siglec\text{-}F^{+}$ eosinophil infiltration into B16F10 tumors treated with PBS, Fc/IL-2, TA99, or Fc/IL-2+TA99, normalized to total tumor mass.
n = 10 per group. *p < 0.05, **p < 0.01, ***p < 0.001 between indicated groups.

(E) 2 days after two administrations of anti-IL-5 (clone TRFK5), RBC-lysed single-cell suspensions of the spleen were stained with fluorescently labeled anti-CD11b and anti-Siglec-F to assess eosinophil depletion.

(F) B16F10 tumors were treated with PBS, Fc/IL-2+TA99, and with depletions/neutralizations as indicated. On days of imaging, mice were injected with 200 mg/kg of luminol in PBS and average radiant luminescence was quantified. Representative images on day 8 are shown. (a) PBS; (b) Fc/IL-2+TA99; (c) Fc/IL-2+TA99+anti-Ly-6G; (d) Fc/IL-2+TA99+anti-Ly-6G+anti-IL-5. Arrows indicate points of

treatment. n = 5 per group. ***p < 0.001 Fc/IL-2+TA99 v. corresponding color group as shown in legend. Scale bar 1 cm.

(G) Tumor growth and survival curves of C57BL/6 mice bearing subcutaneous B16F10 tumors treated with PBS, Fc/IL-2+TA99, or Fc/IL-2+TA99 with anti-IL-5 antibody. n = 5 per group. **p < 0.01 v. corresponding color group as shown in legend.

(H) B16F10 tumors were treated with PBS or Fc/IL-2+TA99, in either WT C57BL/6 or p47phox KO mice. On days of imaging, mice were injected with 200 mg/kg of luminol in PBS and average radiant luminescence was quantified. Representative images on day 8 are shown. (a) PBS; (b) Fc/IL-2+TA99; (c) p47phox KO: Fc/IL-2+TA99. Arrows indicate points of treatment. n = 5 per group. **p < 0.01, ***p < 0.001 Fc/IL-2+TA99 v. p47phox KO: Fc/IL-2+TA99. Scale bar 1 cm.

Data are mean ± SEM.

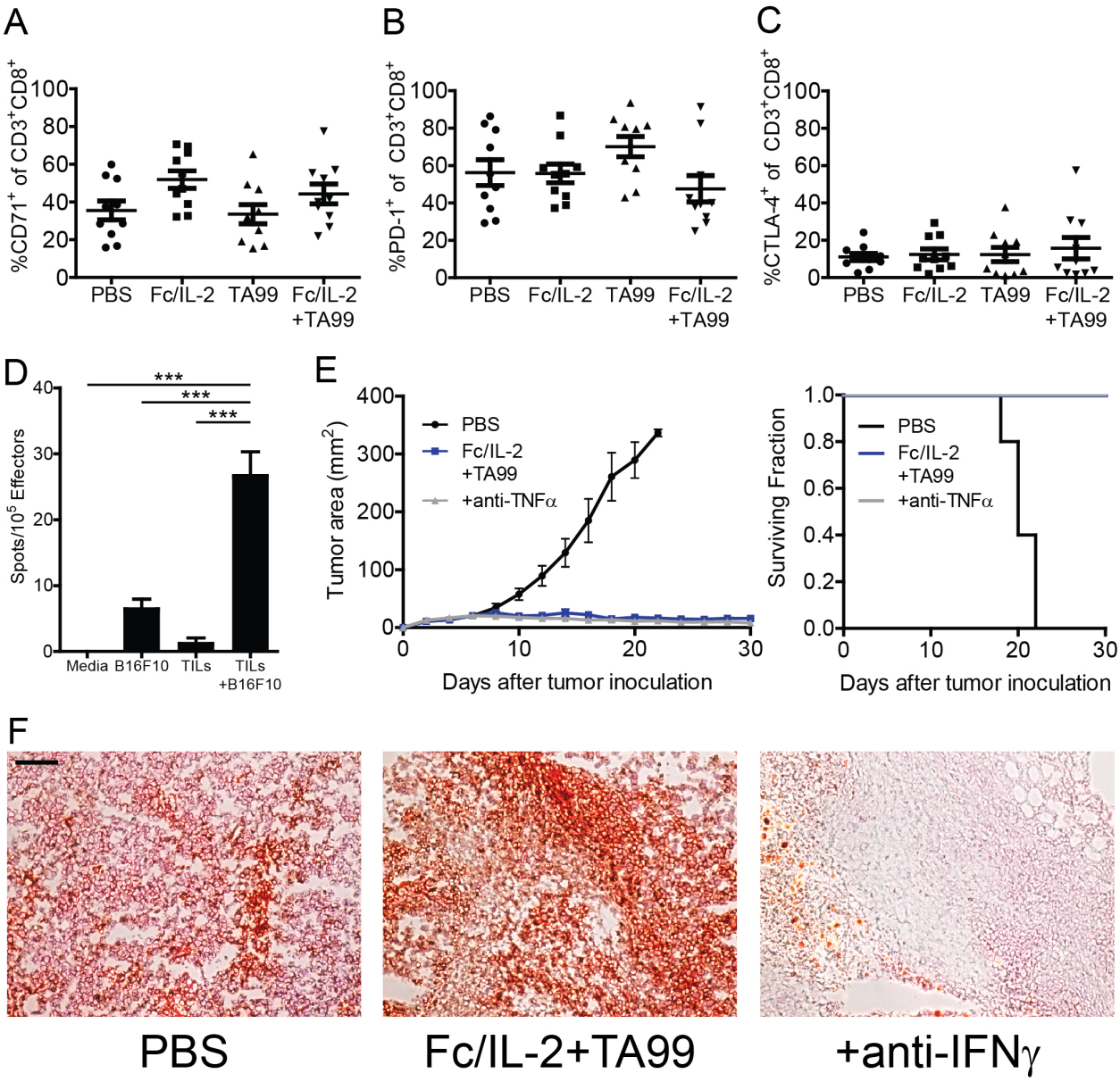

A
%CD71+ of CD3+CD8+
PBS
Fc/IL-2
TA99
Fc/IL-2 +TA99
B
%PD-1+ of CD3+CD8+
C
%CTLA-4+ of CD3+CD8+
D
Spots/10^5 Effectors
***
Media
B16F10
TILs
TILs +B16F10
E
Tumor area (mm2)
PBS
Fc/IL-2 +TA99
+anti-TNFα
Days after tumor inoculation
Surviving Fraction
F
PBS
Fc/IL-2+TA99
+anti-IFNγ

Figure S5, related to Figure 6. Fc/IL-2+TA99 weakly modulates $CD8^+$ T-cell replication and checkpoint blockade expression; Fc/IL-2+TA99 induces IFNγ dependent MHC-I expression on B16F10

(A-C) B16F10 tumors were treated once with PBS, Fc/IL-2 and/or TA99 and harvested 3 days later to analyze $CD8^+$ T-cell activation markers, as a percentage of the intratumoral $CD8^+$ population. The following conditions were investigated (A) $CD3^+CD8^+CD71^+$ cells; (B) $CD3^+CD8^+PD\text{-}1^+$ cells; (C) $CD3^+CD8^+CTLA\text{-}4^+$ cells. n = 10 per group.

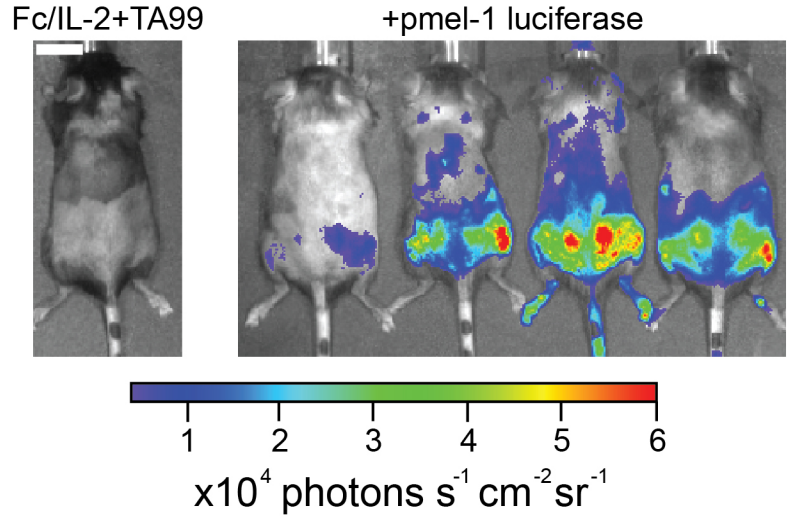
(D) ELISPOT of $CD3^+CD8^+$ T-cells sorted from B16F10 tumors treated with Fc/IL-2+TA99. ***p < 0.001 between indicated groups.

(E) Tumor growth and survival curves of C57BL/6 mice bearing subcutaneous B16F10 tumors treated with PBS, Fc/IL-2+TA99, or Fc/IL-2+TA99 with anti-TNFα antibody. n = 5 per group.

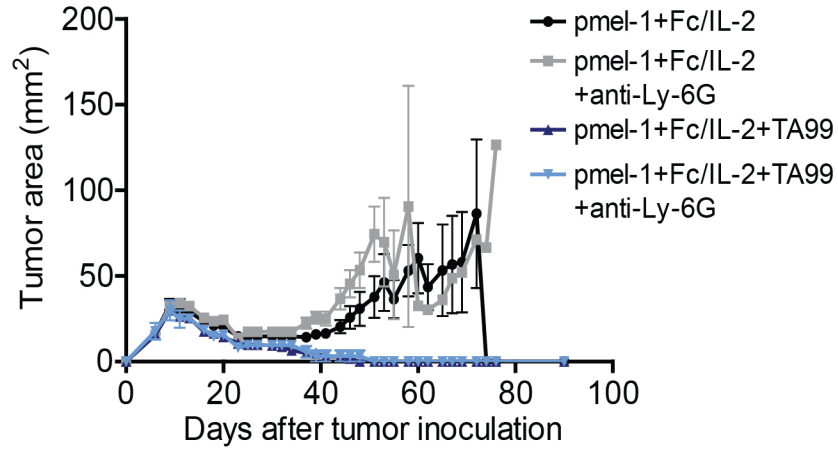
Data are mean ± SEM.

(F) Representative immunohistochemistry of MHC-I expression using NovaRed stain in B16F10 tumors with the indicated conditions. Magnification 10x. Scale bar 100 microns.

A


Fc/IL-2+TA99
+pmel-1 luciferase
1
2
3
4
5
6
x10^4 photons s^-1 cm^-2 sr^-1

B


Tumor area (mm^2)
200
150
100
50
0
0
20
40
60
80
100
Days after tumor inoculation
pmel-1+Fc/IL-2
pmel-1+Fc/IL-2 +anti-Ly-6G
pmel-1+Fc/IL-2+TA99
pmel-1+Fc/IL-2+TA99 +anti-Ly-6G

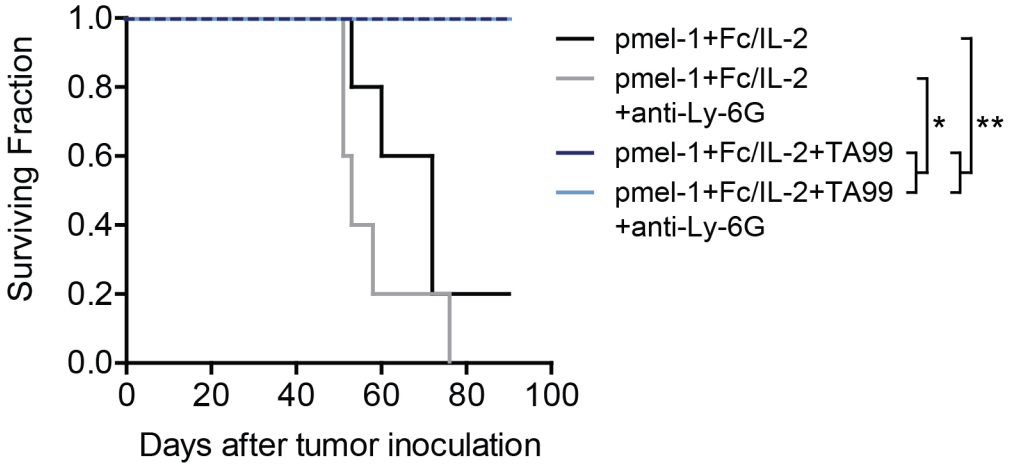

Surviving Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Days after tumor inoculation
pmel-1+Fc/IL-2
pmel-1+Fc/IL-2 +anti-Ly-6G
pmel-1+Fc/IL-2+TA99
pmel-1+Fc/IL-2+TA99 +anti-Ly-6G
*
**

Figure S6, related to Figure 7. pmel-1 $CD8^+$ T-cells treated with Fc/IL-2+TA99 persist after tumor rejection; neutrophils are dispensable for the efficacy of pmel-1+Fc/IL-2+TA99

(A) Representative pictures on day 140 after tumor inoculation showing luciferase signal from pmel-1 in B16F10-bearing C57BL/6 mice that had survived treatment with Fc/IL-2+TA99 (left, 1 mouse) or pmel-1+Fc/IL-2+TA99 (right, 4 mice). The posterior of the mice were shaved to better determine pmel-1 persistence throughout the body. Scale bar 1 cm.

(B) Tumor growth and survival curves of C57BL/6 mice bearing subcutaneous B16F10 tumors were treated with Fc/IL-2 or Fc/IL-2+TA99 with anti-Ly-6g antibody as indicated. All mice also received 5 Gy of total body irradiation 5 days after tumor inoculation and adoptive cell therapy of 10^7 $CD8^+$ pmel-1 T-cells 6 days after tumor inoculation. n = 4-5 per group. *$p < 0.05$, **$p < 0.01$ between indicated groups. Data are mean $\pm$ SEM.

Supplemental Experimental Procedures

Cloning

Vectors encoding the heavy and light chains of mouse TA99 antibody were gifts of Jeffrey V. Ravetch (The Rockefeller University). The DNA sequences encoding the heavy and light chains were cloned into gWIZ (Genlantis), yielding gWIZ-TA99-HC and gWIZ-TA99-LC. A vector encoding the scFv sequence of 237 mAb (Ward et al., 1989) was a kind gift from David M. Kranz (University of Illinois at Urbana-Champaign). The DNA sequences encoding the variable heavy and light chains were isolated and used to replace the variable regions of the previous gWIZ-TA99-HC and gWIZ-TA99-LC, yielding gWIZ-237-HC and gWIZ-237-LC, respectively. A vector encoding the heavy chain of mouse IgG2a from C57BL/6 mice was a gift of Jeffrey V. Ravetch (The Rockefeller University). A D265A mutation was introduced and the DNA encoding the non-lytic D265A Fc, henceforth referred to simply as Fc, was cloned into gWIZ. For cloning of Fc/IL-2, murine IL-2 (InvivoGen) with C-terminal 6xHis tags was subsequently cloned C-terminal to the Fc, separated by a short G_3S linker, yielding gWIZ-Fc/IL-2. To enable expression of monovalent heterodimeric Fc/IL-2, a vector encoding the Fc with a C-terminal FLAG tag, gWIZ-Fc/FLAG, was also constructed using similar methods. Plasmid DNA was transformed into XL-1 Blue (Agilent) for amplification and purified using EndoFree Plasmid Maxi Kit (Qiagen). For cloning of the recombinant fusion protein of mouse serum albumin fused to murine IL-2 (MSA/IL-2), the coding sequence for mouse serum albumin (Origene) was used to replace the sequence encoding the Fc in the existing gWIZ-Fc/IL-2 vector, generating the vector gWIZ-MSA/IL-2.

Protein Production

TA99, 237 mAb, Fc/IL-2, and MSA/IL-2 fusion proteins were produced using HEK293 cells (Life Technologies) according to manufacturer's instructions. HEK293 cells were transfected with gWIZ-TA99-HC and gWIZ-TA99-LC; gWIZ-237-HC and gWIZ-237-LC; gWIZ-Fc/IL-2 and gWIZ-Fc/FLAG; or gWIZ-MSA/IL-2 for TA99, Sm3e, Fc/IL-2 or MSA/IL-2, respectively, using polyethylenimine in FreeStyle 293 media supplemented with OptiPro (Life Technologies). TA99 and 237 mAb were purified by Protein A Agarose (Genscript), Fc/IL-2 was purified by TALON Metal Affinity Resin (Clontech) and followed by anti-FLAG M2 Affinity Gel (Sigma-Aldrich), and MSA/IL-2 was purified by TALON Metal Affinity Resin and further purified using a HiLoad™ 16/600 Superdex™ 200 pg column (GE Healthcare Life Sciences) on an ÄKTAFPLC protein purification system (GE Healthcare Life Sciences). All in-house produced protein were ensured to contain minimal levels of endotoxin (< 0.1 total EU/injection within 1 hour) using the QCL-1000 assay (Lonza).

Mice

p47phox KO mice were aged between 6-10 weeks of age and hmHER2 Tg mice were aged between 8-12 weeks of age before tumor induction. Generation of hmHER2 Tg mice was previously described (Wang et al., 2012). Generation of p47phox KO mice was previously described (Jackson et al., 1995) and kindly provided by Dr. Michael Yaffe (MIT). For *in vivo* luminescence studies, pmel-1 mice were back-crossed with C57BL/6 luciferase mice, kindly provided by Christopher Contag (Stanford), to generate luciferase expressing pmel-1 mice.

CTLL-2 Viability Assay

CTLL-2 cells (ATCC) were cultured in RPMI 1640 (ATCC), supplemented with fetal bovine serum, L-alanyl-L-glutamine, sodium pyruvate, and penicillin-streptomycin, all purchased from Life Technologies, as well as T-STIM with ConA (BD Biosciences). For maintenance, cells were passaged to a density of 10,000 cells/mL. For viability assays, cells were seeded at 150,000 cells/mL to which various forms of IL-2—murine IL-2 produced in *E. coli* (mIL-2), human, IL-2 produced in *E. coli* (Proleukin), Fc/IL-2 produced in HEK cells, or MSA/IL-2 produced in HEK cells, were added at indicated concentrations on an IL-2 basis, in a final volume of 100 μL. Cells were grown for 48 hours before assaying viability using CellTiter-Glo Luminescent Cell Viability Assay (Promega), according to manufacturer's instructions. Luminescence was measured using an Infinite M1000 microplate reader (Tecan) using an integration time of 1 second. A dose response curve was fit via nonlinear regression using GraphPad Prism 5 software (GraphPad).

Fc/IL-2 *in vitro* Serum Stability

Blood was drawn from mice retro-orbitally, allowed to clot, and serum isolated via centrifugation in Microvette 100 tubes (Sarstedt), according to manufacturer's instructions. Equivalent amounts of Fc/IL-2 were incubated in serum at 37 °C for up to 3 days. Incubations were staggered such that all incubations (0 days-3 days) finished on the same day. Protein was run on a 4-12% Bis-Tris protein gels (Life Technologies) and transferred to a nitrocellulose membrane using iBlot transfer stacks and the iBlot gel transfer device (Life Technologies), according to manufacturer's instructions. Membranes were blocked with non-fat dry milk in TBST, probed with biotinylated anti-IL-2 antibody (clone JES6-5H4; eBioscience), and subsequently probed with Avidin-HRP

(eBioscience). Detection of HRP chemiluminescence was performed using Thermo Scientific Super Signal West Dura Chemiluminescent Substrate (Fisher Scientific) and imaged using an ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Pharmacokinetic Studies

Wild-type murine IL-2 (Peprotech), Fc/IL-2, and MSA/IL-2 were labeled with IRDye® 800CW NHS Ester (Li-Cor) and degree of labeling determined, according to manufacturer's instructions. 25 μg of IL-2 or 50 μg of Fc/IL-2 or MSA/IL-2 was injected retro-orbitally into C57BL/6 mice and blood was drawn from the tail at indicated time points and collected in heparin coated tubes (VWR). Tubes were protected from light and kept at 4 °C until blood collection was complete. IR luminescence was measured using the Odyssey Infrared Imaging System (Li-Cor) and analyzed using ImageJ (NIH).

Tumor Cells

B16F10 cells (ATCC), RM9 cells (Baley et al., 1995), and Ag104A cells (Ward et al., 1989) were maintained in DMEM, supplemented with fetal bovine serum, L-alanyl-L-glutamine, and penicillin-streptomycin, all purchased from Life Technologies. D5-HER2 cells (Wang et al., 2012) were maintained in RMPI 1640, supplemented with fetal bovine serum, L-alanyl-L-glutamine, and pencillin-streptomycin, all purchased from Life Technologies. The RM9 prostate cancer cell line was a kind gift from Dr. Timothy C. Thompson (MD Anderson Cancer Center). The Ag104A fibrosarcoma cancer cell line was a kind gift from Dr. Hans Schreiber (University of Chicago).

Tumor Inoculation and Treatment

For induction of B16F10 tumors, 10^6 cells in 100 μL of PBS were injected subcutaneously into the flanks of C57BL/6 mice and allowed to establish for 6 days

before treatment. These tumors are on average 25-30 mm^2 in area and equivalently sized tumors could be achieved through other methods, such as inducing with 10^5 cells with a 10-14 day establishment period, based on the natural doubling time of B16F10 in C57BL/6 mice. Retro-orbital injection of PBS, IL-2 (6 μg; PeproTech), Fc/IL-2 (25 μg), MSA/IL-2 (30 μg), and/or TA99 (100 μg) was done on day 6, 12, 18, 24, and 30 after tumor inoculation for a total of five treatments. Daily intraperitoneal injections of Proleukin (36 μg), provided under a research grant from Prometheus Laboratories, started on day 6 and ended on day 30 after tumor inoculation. For induction of D5-HER2 tumors, hmHER2 Tg mice were inoculated subcutaneously in the flank with $3x10^3$ D5-HER2 cells. Beginning on day 1 after tumor induction, animals received intraperitoneal injections of either PBS, trastuzumab (Herceptin) biweekly (200 μg; Genentech), Fc/IL-2 weekly (25 μg), or the combination of the two. For induction of RM9 tumors, 250,000 cells in 50 μL of PBS were injected subcutaneously into the flanks of C57BL/6 mice. Retro-orbital injection of PBS, Fc/IL-2 (25 μg), and/or 3F8 (150 μg) was done on day 6, 12, 18, 24, and 30 after tumor inoculation for a total of five treatments. 3F8 (Zhang et al., 1998), a murine IgG3 antibody against the tumor antigen GD2, was a kind gift from Dr. Nai-Kong V. Cheung (Memorial Sloan Kettering Cancer Center). For induction of Ag104A tumors, 10^6 cells in 50 μL of PBS were injected subcutaneously into the flanks of C3H/HeN mice. Retro-orbital injections of PBS, Fc/IL-2 (10 μg), and/or 237 mAb (100 μg) was done on day 6, 12, 18, 24, and 30 after tumor inoculation for a total of five treatments.

Pulmonary Wet Weight

C57BL/6 mice were injected *i.v.* with a single bolus of PBS, 12 μg IL-2, or 50 μg Fc/IL-2. 4 days after injection, lungs were extracted, placed into scintillation vials (VWR), frozen in liquid nitrogen, and lyophilized for 48 hours at room temperature under vacuum. Pulmonary wet weight was calculated by subtracting the sample weight after lyophilization from the initial sample weight.

Liver Enzymes AST and ALT Activity Assay

C57BL/6 mice were induced with B16F10 as previously described. Mice were bled on the day of first treatment with PBS or Fc/IL-2+TA99 and bled every other day thereafter until the sixth day after treatment for a total of four blood draws. In all cases the blood was allowed to clot, serum isolated via centrifugation in Microvette 100 tubes (Sarstedt), according to manufacturer's instructions, and then immediately flash frozen in liquid nitrogen to allow for subsequent batch processing. Determination of AST and ALT activity was done using AST activity assay kit (Sigma-Aldrich) and ALT activity assay kit (Sigma-Aldrich), respectively, according to manufacturer's instructions.

Depletions, Neutralizations, and Knock-out Mice

With the exception of IFNγ depletion, tumor inoculation and treatment were done as previously described. All depleting or neutralizing antibodies were injected i.p., as indicated until the end of Fc/IL-2+TA99 treatment. Anti-CD8α (clone 2.43), anti-NK1.1 (clone PK136), and anti-Ly-6G (clone 1A8) were injected at a dose of 400 μg per antibody, 4 days after tumor inoculation and every 4 days thereafter. Anti-CSF-1R (clone AFS98) was injected at a dose of 300 μg, 4 days after tumor inoculation and every other day thereafter. Anti-TNFα (clone XT3.11) was injected at a dose of 200 μg, 5 days after tumor inoculation and every other day thereafter. Anti-IL-5 (clone TRFK5)

was injected at a dose of 1 mg, 1 day before each treatment of Fc/IL-2+TA99. Anti-MIP-2 (clone MAB452; R&D Systems) was injected at a dose of 150 μg, 5 days after tumor inoculation and every other day thereafter. To evaluate the role of IFNγ in the therapy, mice were inoculated with 10^6 B16F10 in 100 μL PBS and allowed to grow 8 days before administration of Fc/IL-2+TA99, which occurred every 6 days thereafter for a total of 5 treatments. We injected anti-IFNγ (clone XMG1.2) at a dose of 200 μg, 7 days after tumor inoculation and every other day thereafter. All antibodies were purchased from BioXCell unless otherwise indicated. Depletion of complement was achieved through the i.p. administration of cobra venom factor (CVF) from *Naja naja kouthia* (Millipore) at a dose of 30 μg, 1 day before each treatment of Fc/IL-2+TA99. In evaluating therapeutic efficacy in p47phox KO mice, B16F10 tumors were inoculated as previously described and treatment of Fc/IL-2+TA99 was initiated after tumors reached an average area of 25-30 mm^2, with subsequent treatments of Fc/IL-2+TA99 every 6 days for a total of 5 treatments.

Histology, Immunohistochemistry, and Immunofluorescence

Tumor inoculation and treatment were done as previously described. For H&E staining, animals were sacrificed four days after a single dose of PBS, Fc/IL-2, TA99, or Fc/IL-2+TA99, and B16F10 tumors were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

For immunohistochemistry of MHC-I expression, in addition to PBS and Fc/IL-2+TA99 treatment, a cohort of mice was also injected with anti-IFNγ antibody, previously described in conjunction with Fc/IL-2+TA99 but 5 days after tumor inoculation and every other day thereafter until euthanasia. Animals were sacrificed four days after a single

dose of Fc/IL-2+TA99, and B16F10 tumors were embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Inc.) and frozen in isopentane (Alfa Aesar) in liquid nitrogen. Sectioned specimens were air-dried and fixed in acetone for 20 minutes. Endogenous peroxidase activity was quenched with H_2O_2 (3% in PBS) for 15 minutes. Endogenous biotin and avidin was quenched with Avidin/Biotin Blocking Kit (Vector Labs), according to manufacturer's instruction. Samples were washed with TBST and incubated with a biotinylated anti-mouse H-2K^b/H-2D^b (clone 28-8-6; BD Pharmingen), 1:100 dilution, at 4 °C overnight. Slides were then incubated with ABC reagent from VECTASTAIN Elite ABC Kit (Vector Labs), according to manufacturer's instruction, and color development was done using VECTOR NovaRed Peroxidase Substrate Kit (Vector Labs), according to manufacturer's instruction. Slides were counterstained with hematoxylin. Images were captured with a Zeiss Axioplan II upright microscope (Zeiss). For immunofluorescence staining of different immune cells types infiltrating into the tumor, animals were sacrificed three days after a single dose of PBS or Fc/IL-2+TA99, and B16F10 tumors were embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Inc.) and frozen in isopentane (Alfa Aesar) in liquid nitrogen. Sectioned specimens were air-dried and fixed in ice-cold acetone for 5 minutes. Endogenous biotin and avidin was quenched with Avidin/Biotin Blocking Kit (Vector Labs), according to manufacturer's instruction. Samples were washed with TBST and incubated with anti-mouse CD8α (clone 53-6.7; Biolegend), anti-mouse F4/80 (clone BM8; Biolegend), anti-mouse Ly-6G (clone 1A8; Biolegend), or polyclonal anti-mouse NKp46 (R&D Systems), all biotinylated, 1:100 dilution, at 4 °C overnight. Slides were subsequently incubated with streptavidin conjugated to Alexa Fluor 647 (Life Technologies), at 1:400 dilution, at 4 °C

overnight. Nuclear staining was performed with Hoescht 33258 (Sigma-Aldrich) according to manufacturer's instructions. Images were captured with a Nikon A1R+ Ultra-Fast Spectral Scanning Confocal Microscope (Nikon) and images were analyzed using NIS Elements Advanced Research software (Nikon).

Flow Cytometry

Tumor inoculation and treatment were done as previously described. All relevant depletions were also performed as previously described. For analysis of infiltrating cells numbers and activation markers on NK cells and $CD8^+$ T-cells, mice were sacrificed and tumors harvested for analysis after 3 days of a single dosing of indicated treatment. For analysis of degranulation and IFNγ production of NK cells and $CD8^+$ T-cells, mice were sacrificed and tumors harvested for analysis after 2 days of a single dosing of indicated treatment. For analysis of neutrophil degranulation and activation markers, mice were sacrificed and tumors harvested for analysis after 24 hours of a single dosing of indicated treatment. For confirmation of systemic depletion of an immune cell population via administration of a depleting antibody, mice were sacrificed and either the spleen, blood, or peritoneal lavage were harvested for analysis after 2 days of a single dosing of the depleting antibody, except in the case of anti-IL-5, when the spleen was harvested 2 days after two doses of the depleting antibody. Single cell suspensions were prepared by mechanically dissociating tumors or spleens between two frosted glass slides. Cell suspensions were passed through 70 μm filters, pelleted, resuspended in 96 well plates, and stained. Red blood cells in spleen and peripheral blood samples were lysed using RBC Lysis Buffer (Biolegend). Cells were first treated with Zombie Aqua Fixable Viability Kit (Biolegend). Cells were subsequently treated with TruStain fcX (clone 95;

Biolegend) and then stained with antibodies against CD3ε (clone 145-2C11), CD8α (clone 53-6.7), CD4 (clone GK1.5), CD25 (clone 3C7), NK1.1 (clone PK136), Ly-6G (clone 1A8), CD11b (clone M1/70), F4/80 (clone BM8), Ly-6C (clone HK1.4), I-A/I-E (clone M5/114.15.12), CD206 (clone C068C2), CD63 (clone H5C6), Siglec-F (clone E50-2440; BD Biosciences), CD69 (clone H1.2F3), CD71 (clone RI7217), KLRG1 (clone 2F1/KLRG1), PD-1 (29F.1A12), CTLA-4 (clone UC10-4B9), all purchased from Biolegend unless otherwise indicated. For intracellular FoxP3 staining, samples were fixed, permeabilized, and stained with antibodies against FoxP3 (clone FJK-16s; eBioscience) or isotype control (clone eBR2a; eBioscience). For intracellular cytokine staining and CD107a staining, samples were first incubated with 0.5 μg/mL Brefeldin A (Sigma-Aldrich) and 0.5 μg/mL Monensin (Sigma-Aldrich) for 5 hours. During this incubation, cells were simultaneously stained with antibodies against CD107a (clone 1D4B; Biolegend). Following surface staining as described above, samples were fixed, permeabilized, and stained with antibodies against IFNγ (clone XMG1.2; Biolegend) or isotype control (clone RTK2071; Biolegend). Samples were analyzed on a BD LSR-II flow cytometer (BD Biosciences) and data analysis was performed using FlowJo software (Tree Star).

Serum and Intratumoral Cytokine Analysis

Tumor inoculation and treatment were done as previously described. All relevant depletions were also performed as previously described. All mice were sacrificed and tumors harvested for analysis 24 hours after a single dosing of PBS, Fc/IL-2, or Fc/IL-2+TA99. Tumors were homogenized using 1.00 mm zirconium beads in 2 mL tubes (KSE Scientific) using a Mini-Beadbeater-16 (Biospec Products) in 5 volumes of PBS

with Complete Protease Inhibitor Cocktail (Roche). Protein concentrations of all samples were normalized using a BCA Assay (Pierce) and snap-frozen in liquid nitrogen to allow for batch processing. Samples were evaluated in triplicate using the Mouse 32-Plex Cytokine/Chemokine Panel Luminex Assay as performed by Eve Technologies (Eve Technologies, Calgary, AB, Canada).

For analysis of cytokine levels in the serum, mice were bled on the day of first treatment with PBS or Fc/IL-2+TA99 and bled every other day thereafter until the sixth day after treatment, for a total of four blood draws. In all cases the blood was allowed to clot, serum isolated via centrifugation in Microvette 100 tubes (Sarstedt), according to manufacturer's instructions, and then immediately flash frozen in liquid nitrogen to allow for subsequent batch processing. Samples were evaluated in triplicate using the Mouse 32-Plex Cytokine/Chemokine Panel Luminex Assay as performed by Eve Technologies.

qPCR Analysis

Tumor inoculation and treatment were done as previously described. All relevant depletions were also performed as previously described. All mice were sacrificed and tumors harvested for analysis 24 hours after a single dosing of Fc/IL-2+TA99. Tumors were made into single cell suspensions and stained as previously described. Samples were then run on a BD FACSAria III sorter (BD Biosciences), isolating $CD11b^{+}F4/80^{+}$ macrophages, $CD11b^{+}Ly\text{-}6G^{+}$ neutrophils, or $CD3^{-}NK1.1^{+}$ NK cells. Cell lysis and RT-PCR were then performed using an Ambion Cells-to-C_t kit (Life Technologies) according to manufacturer's instructions. qPCR was performed using TaqMan probes purchased from Life Technologies for the murine genes *Cxcl2, Nos2,* and *B2m* as internal control,

and the TaqMan Universal PCR Master Mix (Life Technologies) according to manufacturer's instructions, on a Roche Lightcycler 480 (Roche).

Western Blot of iNOS

Tumor inoculation and treatment were done as previously described. All relevant depletions were also performed as previously described. All mice were sacrificed and tumors harvested for analysis 48 hours after a single dosing of Fc/IL-2+TA99. Tumors were homogenized using 1.00 mm zirconium beads in 2 mL tubes (KSE Scientific) using a Mini-Beadbeater-16 (Biospec Products) in 5 volumes of PBS with Complete Protease Inhibitor Cocktail (Roche). Protein concentrations of all samples were normalized using a BCA Assay (Pierce) and snap-frozen in liquid nitrogen to allow for batch processing. Protein was run on a 4-12% Bis-Tris protein gels (Life Technologies) and transferred to a nitrocellulose membrane using iBlot transfer stacks and the iBlot gel transfer device (Life Technologies), according to manufacturer's instructions. Membranes were blocked with non-fat dry milk in TBST and probed with HRP-conjugated anti-iNOS antibody (Santa Cruz Technologies) and HRP-conjugated anti-β-Actin antibody (Abcam). Detection of HRP chemiluminescence was performed using Thermo Scientific Super Signal West Dura Chemiluminescent Substrate (Fisher Scientific) and imaged using an ImageQuant LAS 4000 (GE Healthcare Life Sciences). Images were analyzed using densitometry via Image J (NIH), normalizing to β-Actin as a loading control.

Bioluminescent Imaging of Respiratory Burst *in vivo* using Luminol

Tumor inoculation, treatments, relevant depletions and neutralizations all performed as previously described. Mice were injected with luminol (Santa Cruz Biotechnologies)

resuspended in PBS at a concentration of 50 mg/mL and injected at a dosage of 200 mg/kg, i.p. and imaged with the Xenogen IVIS Imaging System 100 (Xenogen) under the following conditions: f/stop: 1; no optical filter; exposure time: 300 sec; binning: 4.

ELISPOT Assay

Tumor inoculation and treatment were done as previously described. All mice were sacrificed and tumors harvested for analysis 4 days after a single dosing of Fc/IL-2+TA99. Tumors were processed into single cell suspensions and stained as previously described. Samples were then run on a BD FACSAria III sorter (BD Biosciences), isolating $CD3^+CD8^+$ T-cells. Using a Mouse IFN-gamma ELISpot Kit (R&D Systems), following manufacturer's instructions, isolated $CD8^+$ T-cells were co-incubated with B16F10 cells that had been previously treated with 500 U/mL of IFNγ overnight and irradiated at 120 Gy on the day of the assay. Additionally, isolated $CD8^+$ T-cells, B16F10 cells, and media were analyzed individually. Spots were visualized and quantified using CTL-Immunospot S6 Macro Analyzer (C.T.L.).

Adoptive Cell Transfer of $CD8^+$ pmel-1 T-cells and Bioluminescence Imaging

Tumor inoculation was performed as previously described. 5 days after tumor inoculation, recipient mice underwent 5 Gy of total body irradiation. Mice receiving adoptive cell transfer treatment were given a single i.p. injection of 10^7 $CD8^+$ pmel-1 T-cells 6 days after tumor inoculation. Administration of Fc/IL-2 and/or TA99 to cohorts receiving these therapies was done as previously described. Depletion of neutrophils was done as previously described.

For preparation of $CD8^+$ pmel-1 T-cells, 48 hours before administration, splenocytes were harvested by mechanically disrupting the spleens of 6-10 week old pmel-1 mice.

Splenocytes were cultured in T-cell media (RPMI supplemented with 10% heat inactivated FBS, 20 mM HEPES, 1 mM sodium pyruvate, 0.055 mM 2-mercaptoethanol, 100 I.U. penicillin, 100 µg/mL streptomycin, 2 mM L-alanyl-L-glutamine, 1X MEM non-essential amino acids; Corning Cellgro) supplemented with 10 µg/mL murine IL-7 (Peprotech) and 2 µg/mL concanavalin A (Sigma-Aldrich). After 24 hours, $CD8^+$ T-cells were isolated using magnetic bead negative selection (Stemcell) and cultured in T-cell media supplemented with 10% T-STIM with ConA (BD Biosciences). Prior to being used as a supplement, 15 mg/mL methyl-alpha-D-mannopyranoside (Sigma-Aldrich) was added to the T-STIM with ConA. 24 hours later, the $CD8^+$ T-cells were harvested for administration and prior to injection, the cells were washed three times in PBS and passed through a 40 µm cell strainer (BD Biosciences).

For *in vivo* bioluminescent imaging, the same treatment was performed as outlined above, except pmel-1-luc mice were used as donors instead of pmel-1. On days where bioluminescence was measured, 200 µL of 15 mg/kg D-luciferin (Perkin-Elmer) was injected i.p. 10 minutes prior to imaging Xenogen IVIS Imaging System 100 (Xenogen).

Statistics

AST and ALT liver enzyme data, serum cytokine data, luminol chemiluminescence data, and pmel-1-luciferase bioluminescence data were analyzed using a 2-way ANOVA with Bonferroni post-test, survival data were determined using a log-rank Mantel-Cox test, flow cytometry data, western blot data, and ELISPOT were analyzed using a 1-way ANOVA with Tukey post-test for analyzing all possible comparisons or with Dunnett post-test for analyzing comparisons only to a specific group, and multiplex

cytokine/chemokine assay data were analyzed through false discovery rate control using the Benjamini-Hochberg procedure, with $\alpha = 0.05$

Figure 1C-D, 4F, 5F, 6H, S1B, S1D, S3B are representative of two independent experiments. Figure 3H, 6D-G are representative of the sum of two independent experiments. Figure 3B-G, 6A-C, S4A, S4D, S5A-C are representative of the sum of three independent experiments. Figure 5A is representative of the sum of four independent experiments. Figure 3A is representative of five independent tumor sections/condition. Figures S2A-C are representative of three independent tumor sections/condition. Figure S5F is representative of four independent tumor sections/condition. Otherwise, all data shown represent one independent experiment.