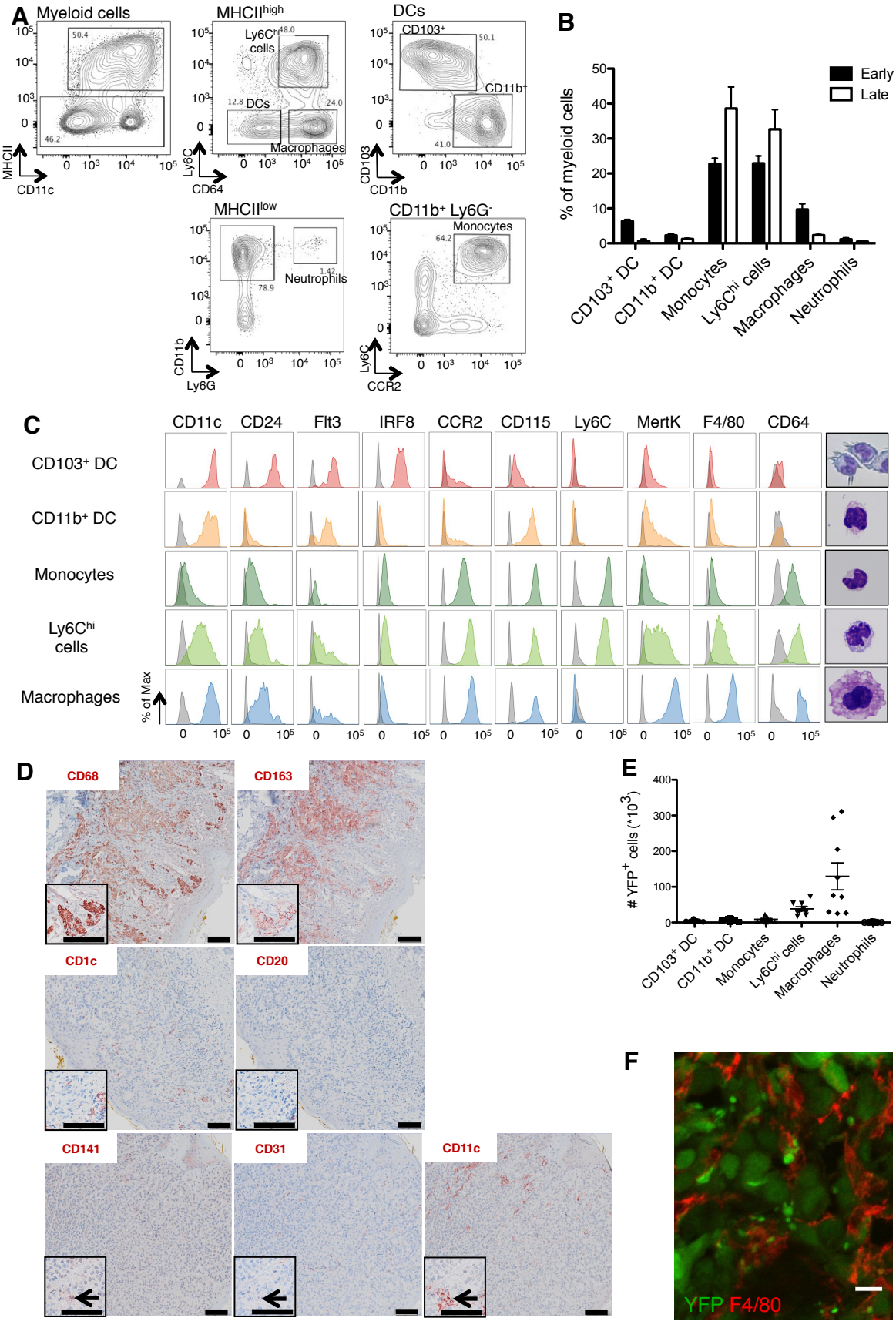
Supplemental Figure 1 (related to Figure 1)



Supplemental Figure 1 (related to Figure 1)

(A) Gating strategy. Myeloid cells were gated on $CD11b^+$ and/or $CD11c^+$ cells within $CD45^+$ cells.

(B) Frequency of each myeloid cell population among total myeloid cells infiltrating B16 tumors, at early (day 10) and late (day 16) stages of tumor growth. Bar graph shows the mean ± SEM of 3-5 mice per group.

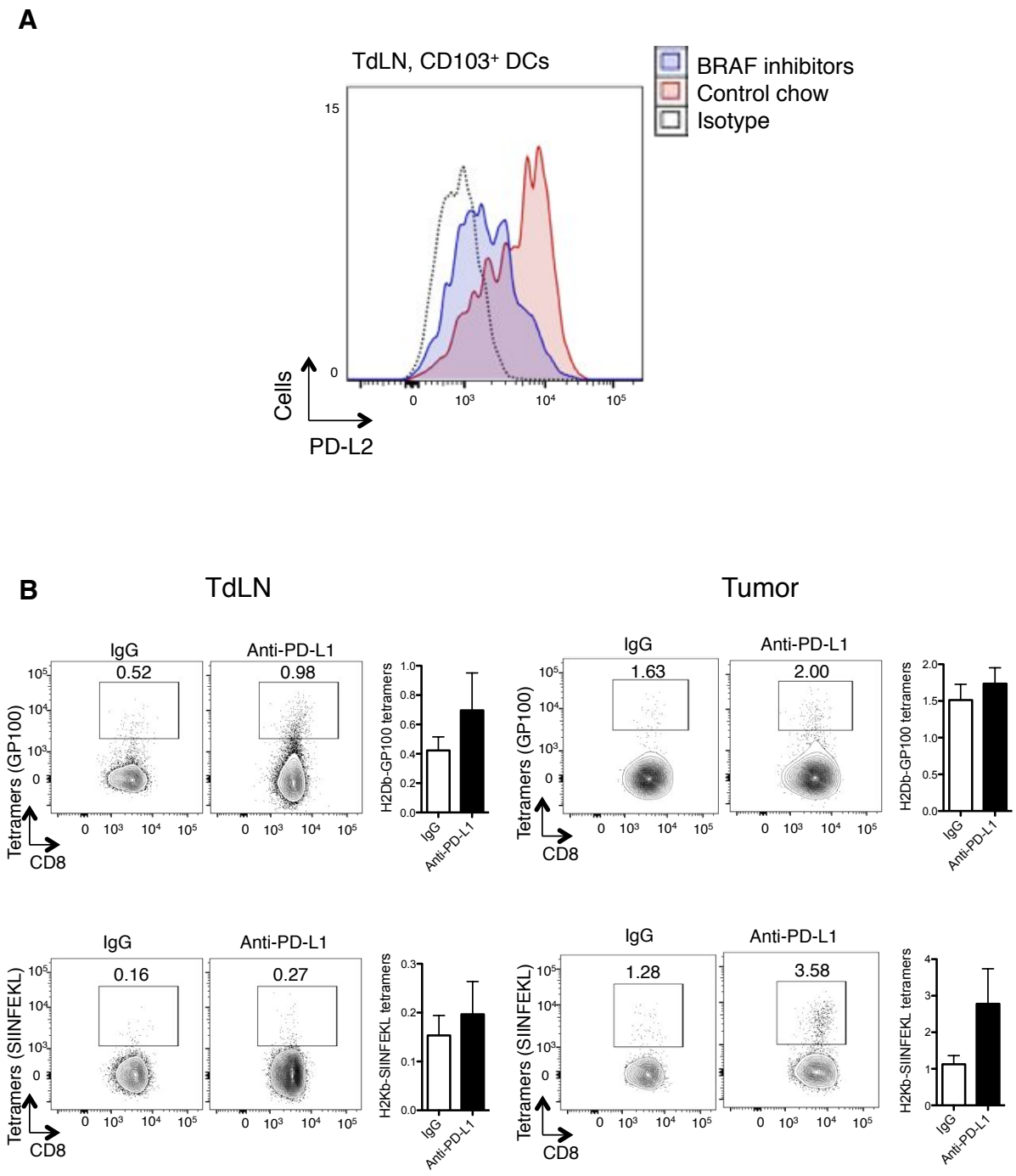
(C) Expression of a panel of DC and macrophage markers compared with respective isotypes (gray shaded). GFP expression was used to assess CD115 expression in Mafia mice (see material section) and cells were cultured O/N with 10ng/ml GM-CSF for Flt3 expression analysis. Morphological characteristics of each cell population sorted from B16 tumors and analyzed by Wright-Giemsa staining after cytospin are displayed in the right panel.

(D) Representative brightfield images of macrophages ($CD68^+$, $CD163^{+/-}$), $CD1c^+$ DC ($CD1c^+$ $CD20^-$) and $CD141^+$ DC ($CD141^+$ $CD31^-$ $CD11c^+$) accumulating in human primary melanoma. 20x magnification. The lower left inserts show a higher magnification (40x). Scale bars: 100 μ m.

(E) Absolute numbers of YFP^+ myeloid cells infiltrating B16-YFP tumors, analyzed on day 15 after tumor challenge. Shown is the mean ± SEM of 2 independent experiments with a total of 9 mice.

(F) Representative confocal image of YFP uptake by macrophages or monocyte-derived cells ($F4/80^+$, red) in B16-YFP tumor frozen at day 15 after tumor challenge. Scale bar, 10µm.

Supplemental Figure 2 (related to Figure 2)

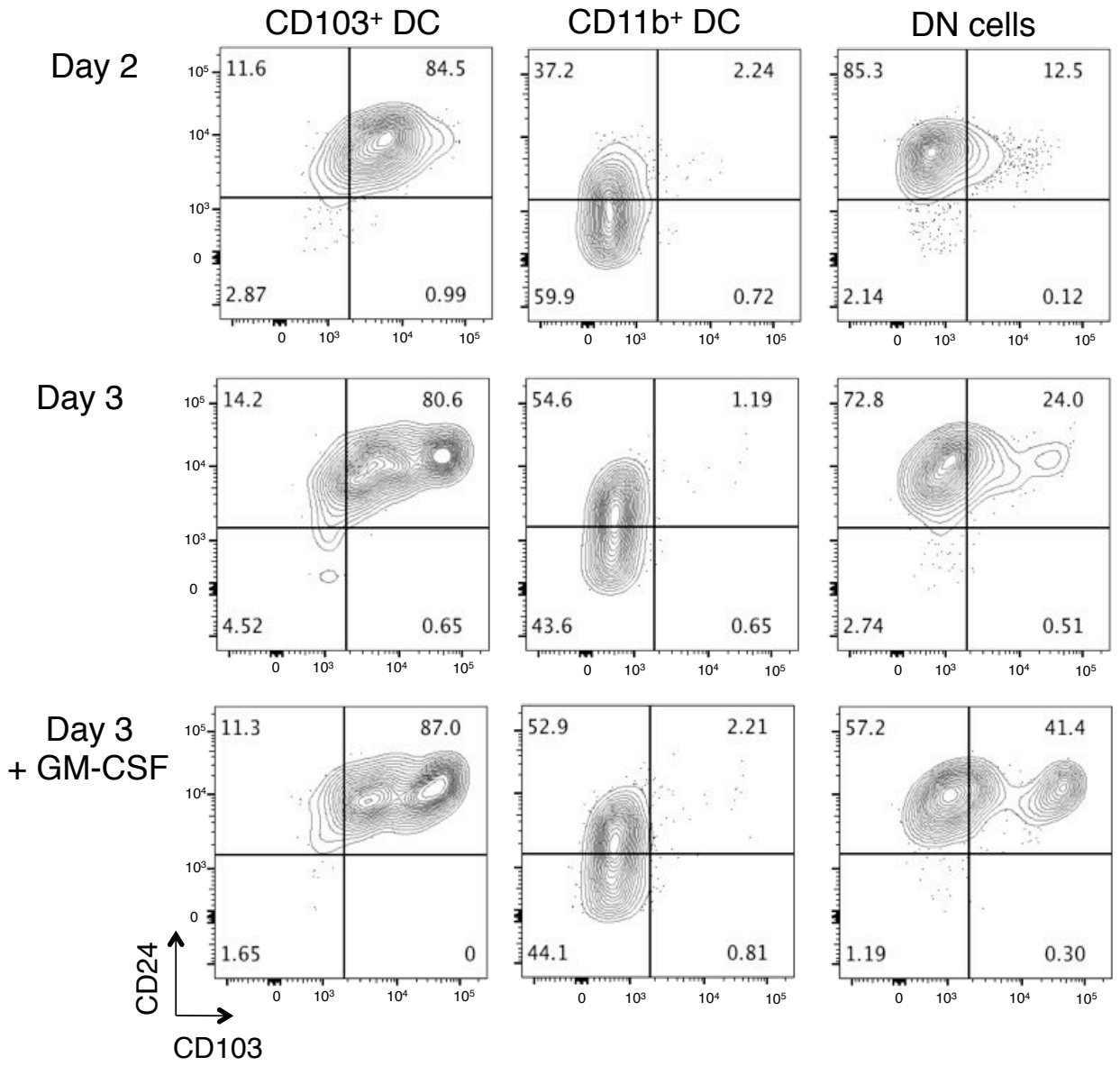


Supplemental Figure 2 (related to Figure 2)

(A) Twenty-eight days after tamoxifen (4-HT) topical application, *Braf*-mutant mice were treated as in Figure 2B with BRAF inhibitors or control chow for 4 days. Histogram shows expression of PD-L1 on migratory $CD103^+$ DC in the TdLN analyzed at day 32. Shown are representative histograms of 2 independent experiments.

(B) B16-tumors bearing WT mice were treated with anti-PD-L1 Abs or control IgG as in Figure 2C. Two days after the last injection of anti-PD-L1 Abs/IgG, TdLN and tumors were harvested and tumor antigen-specific $CD8^+$ T cells were quantified using GP100-specific and SIINFEKL-specific tetramers, in B16 and B16-OVA tumor-bearing mice, respectively. Representative dot plots (left panels), and bar graphs show the mean proportion of tetramers positive $CD8^+$ T cells ± SEM (right panels) of 2 independent experiments with a total of 3-6 mice per group.

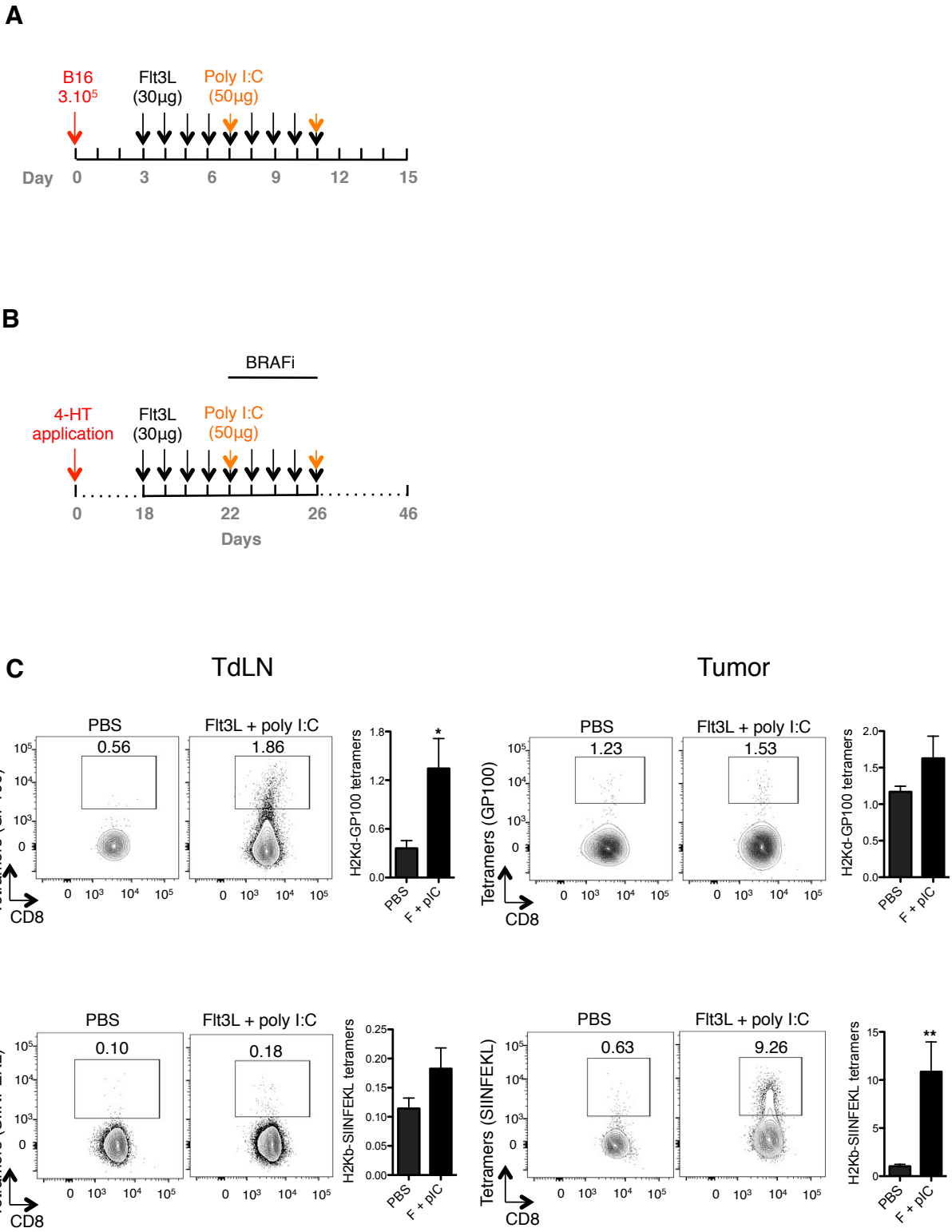
Supplemental Figure 3 (related to Figure 3)



Supplemental Figure 3 (related to Figure 3)

(A) B16-tumors bearing mice were injected daily with Flt3L from day 3 to day 9 after tumor challenge. One day after the last Flt3L injection, DN cells, $CD103^+$ DC and $CD11b^+$ DC were isolated from the tumor lesions and cultured in vitro on a bone marrow stromal cell layer in the presence of Flt3L +/- GM-CSF for 2 and 3 days. Shown are representative plots of one experiment in triplicate/condition.

Supplemental Figure 4 (related to Figure 4)



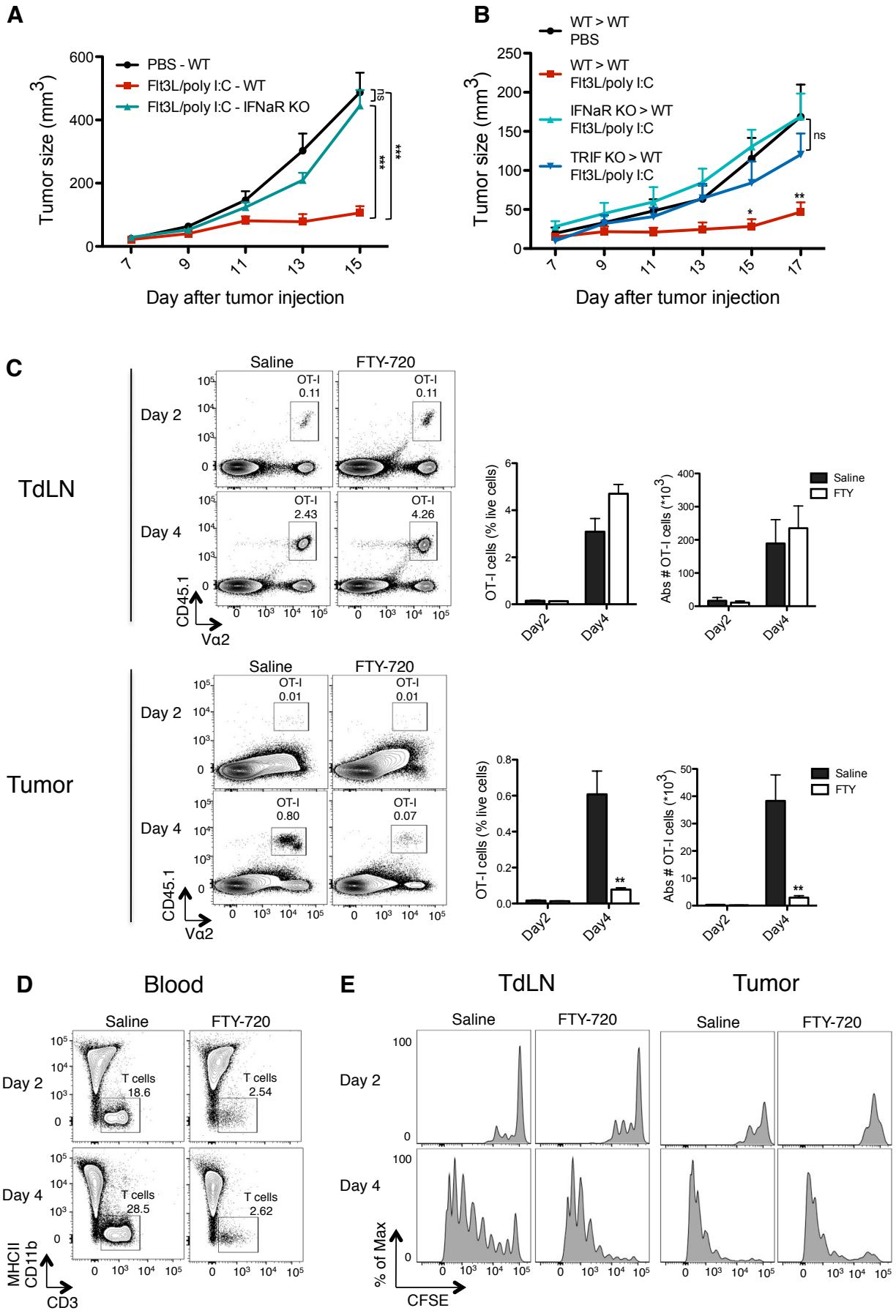
Supplemental Figure 4 (related to Figure 4)

(A) Graphical timeline of the Flt3L±poly I:C treatment scheme in B16 tumor-bearing mice.

(B) Graphical timeline of the Flt3L±poly I:C treatment scheme in Braf-mutant tumor-bearing mice. Eighteen days after tamoxifen application (4-HT), when tumors were palpable, mice were injected with Flt3L+polyI:C mice and fed with BRAF inhibitors (BRAFi) admixed chow at the indicated time points.

(C) B16-tumors bearing mice were treated with Flt3L-poly I:C or PBS as in Figure S4A. Two days after the last injection TdLN and tumors were harvested and tumor antigen-specific $CD8^+$ T cells were quantified using gp100-specific and SIINFEKL-specific tetramers, in B16 and B16-OVA tumor-bearing mice, respectively. Shown are representative dot plots (left panels), and bar graphs showing the mean proportion of tetramers positive $CD8^+$ T cells ± SEM (right panels), of 2 independent experiments with a total of 3-6 mice per group.

Supplemental Figure 5 (related to Figure 5)



Supplemental Figure 5 (related to Figure 5)

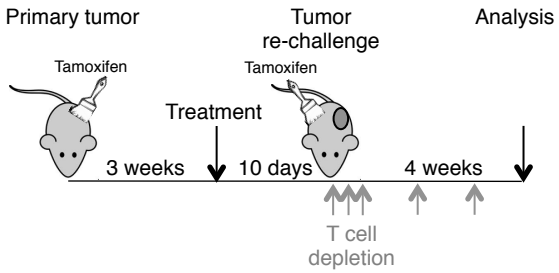
(A) B16 tumor-bearing WT and *Ifnar*$^{-/-}$ mice were treated as in Figure S4A with PBS or Flt3L-poly I:C. Tumor growth was followed for 15 days. Shown is the mean tumor growth ± SEM of 4 independent experiments with a total of 12-15 mice.

(B) Bone marrow chimeric mice in which lethally irradiated C57BL/6 WT mice were reconstituted with bone marrow cells isolated from *Ifnar*$^{-/-}$, *Trif*$^{-}$ or WT mice. Eight to twelve weeks later, mice were injected with B16 tumors and were treated as described in Figure S4A except that first Flt3L injection started on day 5 instead of day 3 because of the smaller B16 tumor growth observed in bone marrow chimeric mice. Graph shows the mean tumor growth ± SEM of 2 independent experiments with a total of 4-8 mice per group.

(C-E) Mice bearing B16-OVA tumors received daily Flt3L injections from day 5 to 9 post-tumor challenge followed by one intratumoral injection of poly I:C at day 9. Two hours after the poly I:C injection, tumor-bearing mice were injected with $3x10^6$ CFSE-labeled naïve tumor antigen-specific $CD8^+$ T cells (OT-I) in the presence or absence of the S1P receptor antagonist FTY-720 as described in the methods. (C) Representative dot plots (left panel) and bar graphs show the numbers of OT-I cells (right panels) in TdLN and tumors 2 or 4 days after adoptive cell transfer. Graphs show the mean ± SEM of 2 independent experiments with a total of 4-5 mice per group. For OT-I cell infiltration in the tumor, data is shown at day 4 (as analyzed in Figure 5I) and at day 2 after adoptive cell transfer. (D) Efficacy of the inhibition of T cell egress from LN by FTY-720 was assessed by checking T cell frequencies in the blood (among live cells) 2 and 4 days after adoptive cell transfer and start of FTY-720 treatment. Shown are representative dot plots of 2 experiments with a total of 2-3 mice per group. (E) The proliferation of OT-I T cells in the TdLN and at the tumor site was determined by CFSE dilution. Histograms are representative of two independent experiments with a total of 4-5 mice per group.

Supplemental Figure 6 (related to Figure 6)

A



Supplemental Figure 6 (related to Figure 6)

(A) Cartoon scheme showing that Braf-mutant tumors were first induced in adult mice upon topical 4-HT administration. Three weeks later when tumors were palpable, mice received, in addition to BRAF inhibitors admixed chow, Flt3L, polyI:C and anti-PD-L1 Ab (referred to as “tritherapy”), anti-PD-L1 Ab alone or control IgG at the indicated time points. Mice were re-challenged with 4-HT topical administration on the opposite flank 10 days after the end of the treatment, in addition to anti-CD4 and CD8 Ab or control IgG.

Supplemental Experimental Procedures

Mice

All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai. Transgenic mice expressing the MHC class I restricted T cell receptor specific for the octamer SIINFEKL peptide ovalbumin$_{257-264}$ (OT-I mice) or the gp100$_{25-33}$ (pmel mice) were purchased from Jackson laboratories. *Batf3*$^{-/-}$ mice generated as described (Hildner et al., 2008) were obtained from Dr. Kenneth Murphy (Washington University) and backcrossed on the C57BL/6 background for >10 generations in our facility. Macrophage Fas-Induced Apoptosis (MAFIA) mice (Burnett et al., 2004) were kindly provided by Dr. Shu-Hsia Chen (Mount Sinai). *Ifnar*$^{-/-}$ mice (Muller et al., 1994) and *Trif*$^{-/-}$ mice (Yamamoto et al., 2003) were provided by Dr. Adolfo Garcia-Sastre (Mount Sinai).

Bone marrow transplantation in mice

Eight-wk-old CD45.1^{+} C57BL/6 mice were lethally irradiated with 1,200 rad delivered in two doses of 600 rad each, 3h apart, and injected i.v. with 2x10^{6} CD45.2^{+} BM cells isolated from WT, *Ifnar*$^{-/-}$ or *Trif*$^{-/-}$ mutant mice. Levels of blood donor chimerism were analyzed by measuring the percentage of CD45.2^{+} cells among each blood cell population 6-10 weeks after transplantation.

Antibodies, multimer staining and flow cytometry

The following antibodies were obtained from BioLegend: anti-CD64-APC (X54-5/7.1), anti-I-A/I-E-AlexaF700 (M5/114.15.2), anti-CD103-PerCP/Cy5.5 (2E7), anti–Ly6C-PB (HK1.4), anti-CD45-BV510 (30-F11), anti-F4/80-PE (BM8), anti-CD3-AlexaF700 (17A2), anti-CD62L-FITC (MEL-14), antiCD44-BV785 (IM7). The following antibodies were obtained from eBioscience: anti-CD11b-APC-eF780 (M1/70), anti-CD11c-PE-

Cy7 (N418), anti-CD24-PE-Cy7 (M1-69), anti-EpCAM-PE-Cy7 (G8.8), anti-F4/80-PE (BM8), anti-CD40-APC (1C10), anti-CD86-FITC (GL1), anti-PDL1-PE (MIH5), anti-CD4-PerCP/Cy5.5 (RMA4-5), anti-CD8-APC-eF780 (53-6.7), anti-TNFα-APC (MP6-XT22), anti-FoxP3-PE (FJK-16s), anti-Vα2 TCR-APC (B20.1). Other reagents were Cytofix/Cytoperm solution (BD Biosciences), Live/Dead Fixable Blue (Life Technologies), and DAPI (Sigma-Aldrich). For intracellular staining, cells were stimulated with 100 ng/ml PMA (Sigma-Aldrich) and 0.5 mg/ml ionomycin (Sigma-Aldrich) at 37°C for 3 hrs, adding Brefeldin A (10 mg/ml; Sigma-Aldrich) to allow accumulation of intracellular cytokines. After staining of surface markers, cells were fixed and permeabilized (eBiosciences), followed by staining with anti-IFNγ, TNFα and Foxp3. Tetramers H-2Db-gp100-APC and dextramers H-2Kb-SIINFEKL-APC were purchased from MBL and Immudex, respectively. One to two million cells were stained with the multimers for 30-60min at RT before adding surface antibodies. Samples were acquired on a Fortessa (BD) and data were analyzed using FlowJo software (Tree Star).

Time of Flight mass cytometry (CyTOF)

All mass cytometry reagents were purchased from Fluidigm Inc. (former DVS) unless otherwise noted. Tumor samples were dissociated into single-cell suspensions, washed with PBS containing 0.1% BSA and blocked with a commercial Fc-blocking reagent (BD Bioscience) to minimize non-specific antibody binding. The cells were then stained with a panel of metal-labeled antibodies against 26 cell surface markers (see table below) for 30 minutes on ice, and then washed. All antibodies were either purchased pre-conjugated to metal tags, or conjugated in-house using MaxPar X8 conjugation kits according to the manufacturer's instructions. After antibody staining, the cells were incubated with cisplatin for 5 minutes at RT as a viability dye for dead cell exclusion. The cells were then fixed and permeabilized with a FoxP3/Transcription Factor staining kit (eBioscience) according to the manufacturer's

protocol and stained with metal-labeled antibodies against transcription factors. The cells were then washed and incubated in PBS containing 1.6% formaldehyde and 125nM Ir nucleic acid intercalator to label all nucleated cells. Immediately prior to acquisition, the cells were washed in PBS, then in diH20 and resuspended in diH20 containing a 1/10 dilution of EQ 4 Element Calibration beads. The samples were acquired on a CyTOF2 Mass Cytometer in sequential 10 min acquisitions at an acquisition rate of <500 events/s. The resulting FCS files were concatenated and normalized using a bead-based normalization algorithm in the CyTOF acquisition software and uploaded to Cytobank for analysis. FCS files were manually pre-gated on Ir193 $DNA^{+}CD45^{+}$ events, excluding $cisplatin^{+}$ dead cells, doublets and DNA-negative debris. The gated $CD45^{+}$ population was then clustered based on all labeled phenotypic markers using spanning-tree progression analysis of density-normalized events (SPADE). Putative cell populations on the resulting SPADE trees were manually annotated based on the expression of key canonical markers.

Antibodies used for CyTOF analysis

Isotype	Antibody	Clone	Source
Cd (eFluorNC650)	Thy1.2	53-2.1	eBioscience
141 Pr	Ly6G	1A8	Biolegend
142 Nd	CD11c	N418	Fluidigm
143 Nd	TCRb	H57-597	Fluidigm
146 Nd	CD8	53-6.7	Fluidigm
147 Sm	CD45	30-F11	Fluidigm
148 Nd	CD11b	M1/70	Fluidigm
149 Sm	CD19	6D5	Fluidigm
150 Nd	CD24	M1/69	Fluidigm
151 Eu	CD25	3C7	Fluidigm
152 Sm	Siglec F	E50-2440	BD Bioscience
153 Eu	CD317	927	Biolegend
154 Sm	TER119	TER119	Fluidigm
156 Gd	CD64	X54-5/7.1	Biolegend
158 Gd	FoxP3	FJK-16s	Fluidigm
159 Tm	F4/80	BM8	Fluidigm
160 Gd	CD62L	MEL-14	Fluidigm
161 Dy	Tbet	eBio4B10	eBioscience
162 Dy	Ly6C	HK1.4	Biolegend
163 Dy	RORgt	B2D	eBioscience
164 Dy	CD103	2E7	Biolegend
165 Ho	EpCAM	G8.8	Fluidigm
166 Er	CD117	2B8	Fluidigm
167 Er	Gata3	TWAJ	Fluidigm
168 Er	MertK	Polyclonal	Abgent
169 Tm	GFP	5F12.4	Fluidigm
170 Er	NK1.1	PK136	Fluidigm
171 Yb	CD44	IM7	Fluidigm
172 Yb	CD4	RM4-5	Fluidigm
174 Yb	MHCII	M5/114.15.2	Fluidigm
175 Lu	CD135	A2F10	eBioscience
176 Yb	B220	RA3-6B2	Fluidigm

In vitro antigen presentation assay

$CD8^+$ OVA-specific T cells were isolated from the spleen of OT-I mice. $CD8^+$ T cells were negatively enriched with a $CD8^+$ T cell isolation kit, according to the manufacturer's instructions (Miltenyi) with the addition of anti-CD11c-biotin to the antibody cocktail. 10 x 10^7/ml enriched T cells were labeled with 1 µM CFSE (Life Technologies) in PBS/0.1% BSA for 10 min at 37°C. The reaction was quenched with FCS and cells were washed 2-3 times with complete RPMI. 150,000 T cells were added to each well containing APCs (96-well plate). Activation and division of OVA-specific T cells was determined by flow cytometry after culture at 37°C and 5% CO_2 for 3 days. For analysis, cells were stained with Vα2-, TCR-, CD8-specific antibodies and DAPI before acquisition on Fortessa (BD).

In vitro culture of DC progenitors

DN cells were cultured in the presence of the BM support cells. 4.5x10^6 $CD45.1^+$ BM cells were seeded into six-well plates in RPMI medium (Cellgro) supplemented with L-glutamine, penicillin-streptomycin, non-essential amino acids, 10% FCS (Cellgro) and β-mercaptoethanol (Millipore). After 2 days, 5x10^3 sorted $CD45.2^+$ $CD103^+$ DCs, $CD11b^+$ DCs and DN cells from Flt3L-treated B16 tumors (5-7 tumors were pooled) were added to each well and cultured in complete medium with 100ng/ml Flt3L (Celldex) +/- 10ng/ml GM-CSF (Peprotech). Their $CD45.2^+$ progeny were phenotypically assessed by flow cytometry 2 and 3 days after being seeded.

Purification of $CD8^+$ T cells and adoptive transfer

$CD8^+$ T cells were enriched (>90%) from spleen and LN of OT-I or GP100 mice by negative selection using anti-F4/80, anti-MHCII (TIB120), anti-B220 (RA3-6B2), anti-CD4 (GK1.5) and anti-NK1.1 (PK136) mAb (produced in-house) followed by anti-rat Dynabeads (Life Technologies). 10-20^7/ml $CD8^+$ T cells were labeled with 5 µM

CellTrace Violet or CFSE (Life Technologies) for 10 min at 37°C. 2-4x10^6 antigen-specific CD45.1$^+$ CD8$^+$ T cells were transferred into tumor-bearing C57Bl/6 CD45.2$^+$ mice, at day 7 or 9 after tumor challenge, as indicated in the figure legends), 2h after PBS or poly I:C injection. In vivo proliferation was analyzed by FACS on day 2, 3 or 4 after T cell transfer in the tumor and TdLN, as indicated in the figure legends.

FTY-720 treatment

FTY-720 was purchased from Sigma, and injected i.p. (200µl) at a final concentration of 100µg/ml in saline every day over the indicated time periods.

Immunohistochemistry

YFP$^+$ B16 and Tomato$^+$ *Braf*-mutant tumors and TdLN were harvested and fixed with 0.05 M phosphate buffer containing 0.1M L-lysine (pH 7.4), 2mg/ml NaIO4, and 10mg/ml paraformaldehyde over night at 4°C and then equilibrated in 10, 20, then 30% sucrose solution for 2h. Tissues were then frozen in OCT and stored at -80°C. 10µm-tissue sections were blocked for 1h in PBS solution supplemented with 2% fetal calf serum and 0.5% bovine serum albumin. Sections were then stained with conjugated primary antibodies, purchased from Biolegend: anti-CD3-AlexaFluor(AF)488 or AF647 (17A2), anti-CD11c-AF488 or AF594 (N418), F4/80-AF647 (BM8) and anti-CD31-AF647 (MEC13.3). Each antibody incubation step was conducted for a minimum of 2h in a dark, humidifier chamber at 4°C. For patient tumors, de-identified paraffin-embedded melanoma samples were obtained from the Biorepository at Mount Sinai School of Medicine. All samples had appropriate IRB approval and informed consent from study participants. Five-µm-thick formalin-fixed and paraffin-embedded (FFPE) tissue sections were deparaffinized prior to incubation in antigen retrieval solution (Dako, S2369 and S2367) at 95°C for 30 minutes. Tissue sections were incubated in 3% hydrogen peroxide and in serum-free protein block solution (Dako, X0909) before adding the primary antibodies. After

signal amplification using biotinylated-secondary antibody and streptavidin-horseradish peroxidase, chromogenic revelation was performed and slides counterstained with hematoxylin. Slides were scanned for digital imaging and quantification (Olympus whole-slide scanner with Olyvia software). After scanning, the slide was bleached to remove the chromogen stain and subjected to the next round of staining (MICSSS method, Remark et al., submitted). The following Abs were used for macrophages and DC characterization: CD1c (clone 2F4), CD11c (clone EP1347Y), CD31 (clone JC70), CD68 (clone KP1), CD141 (clone EPR4051) and CD163 (clone 10D6). After image acquisition, each stain was artificially attributed a color code and images were overlaid using Adobe Photoshop CS6. Tissue-associated immune cell densities were then measured as previously described (Remark et al., 2013). Immune cell density was expressed as an absolute number of positive cells/mm^2.

Image acquisition and analysis

Fluorescent microscopy images were acquired with either a Zeiss LSM780 confocal microscope equipped with a motorized stage for tiled imaging or a Zeiss Axioplan widefield microscope (Microscopy CORE, Icahn School of Medicine at Mount Sinai). Linear unmixing was performed when needed to get rid of autofluorescence or avoid spectral overlap. Images were prepared using ImageJ software and quantifications of T cell densities and T cell distance to $CD31^+$ vessels were performed using Cell Profiler software. Brightfield microscopy images were acquired using a Nikon Eclipse Ci-E microscope operated with Nikon NIS Elements BR software.

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