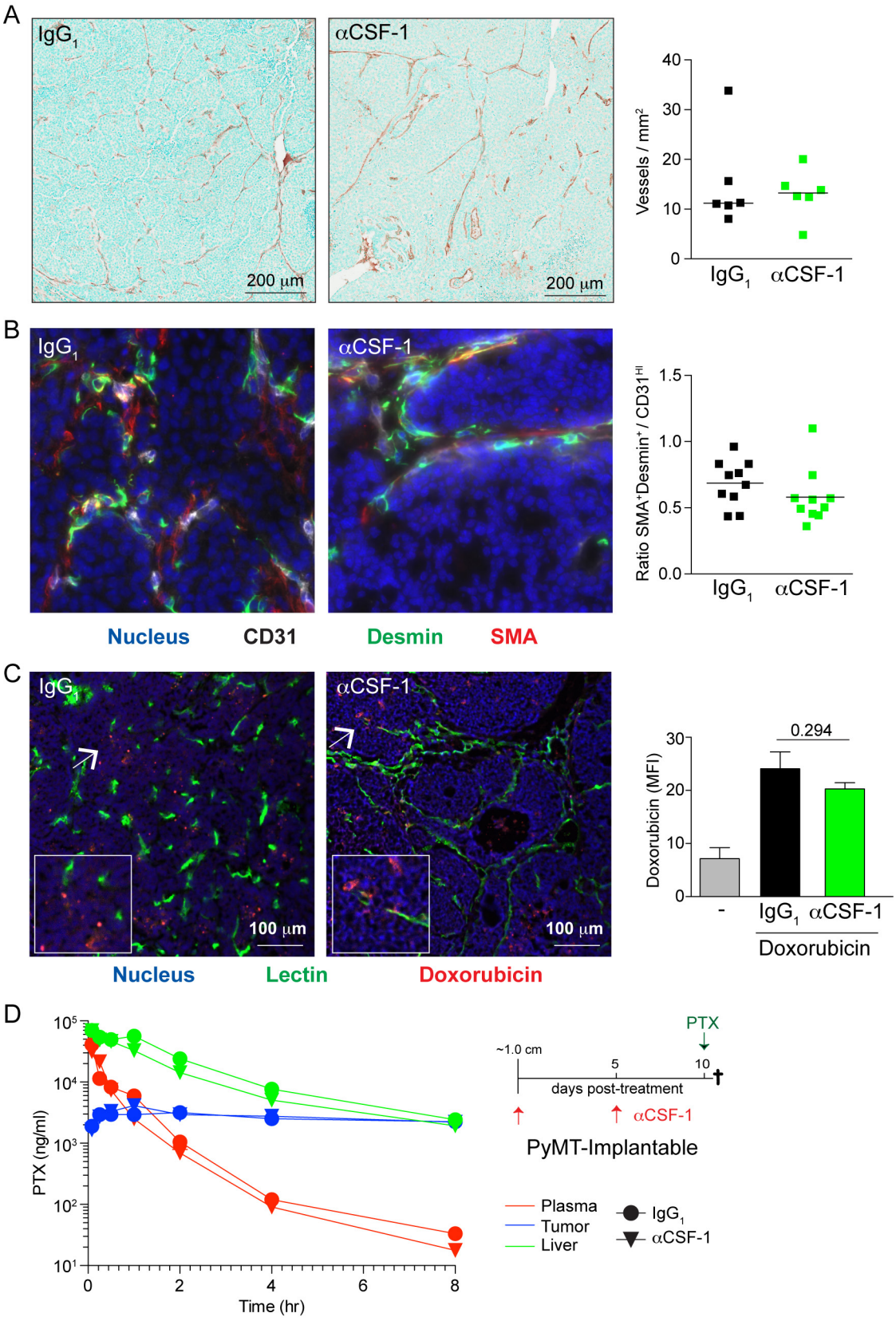
SUPPLEMENTAL FIGURES



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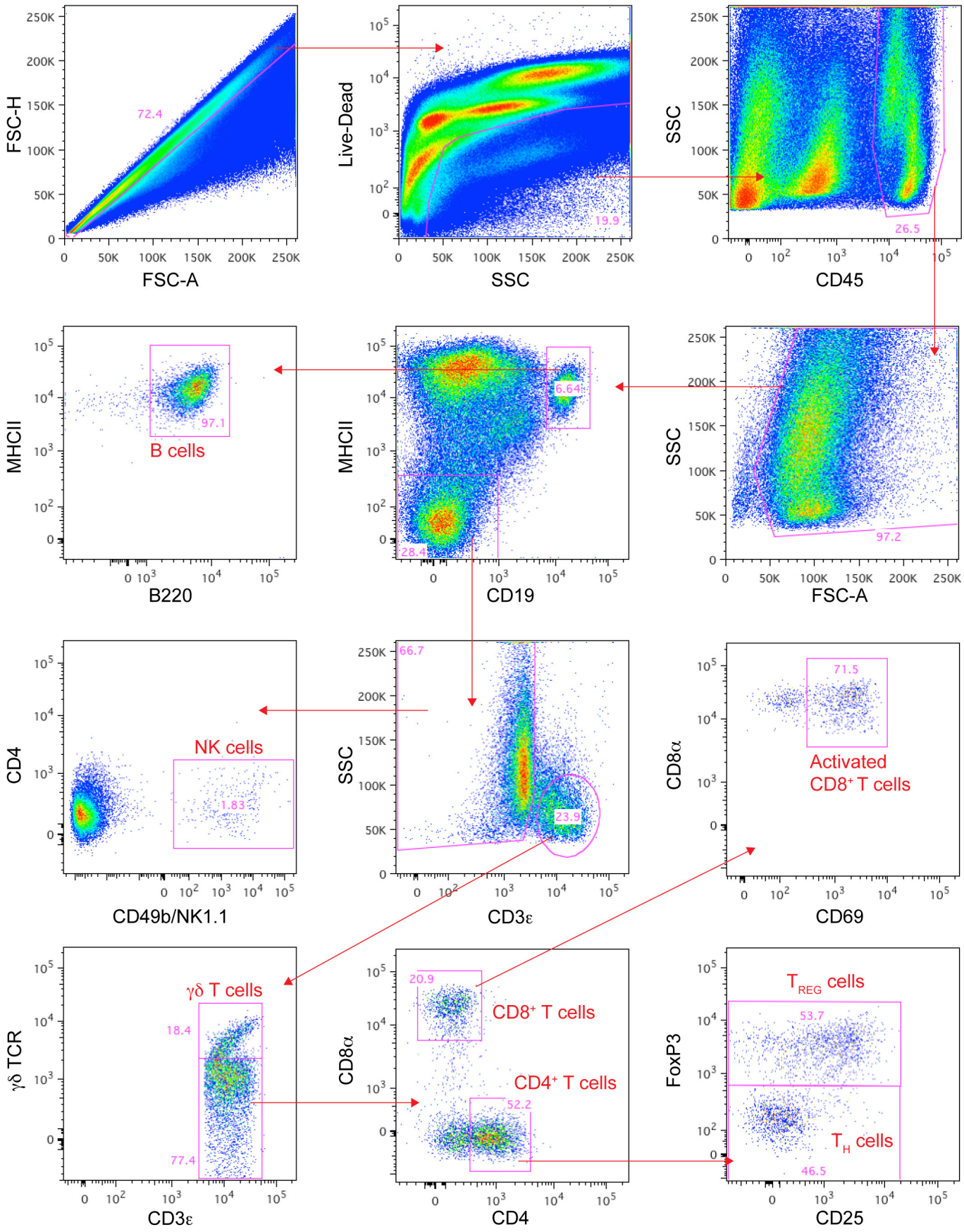
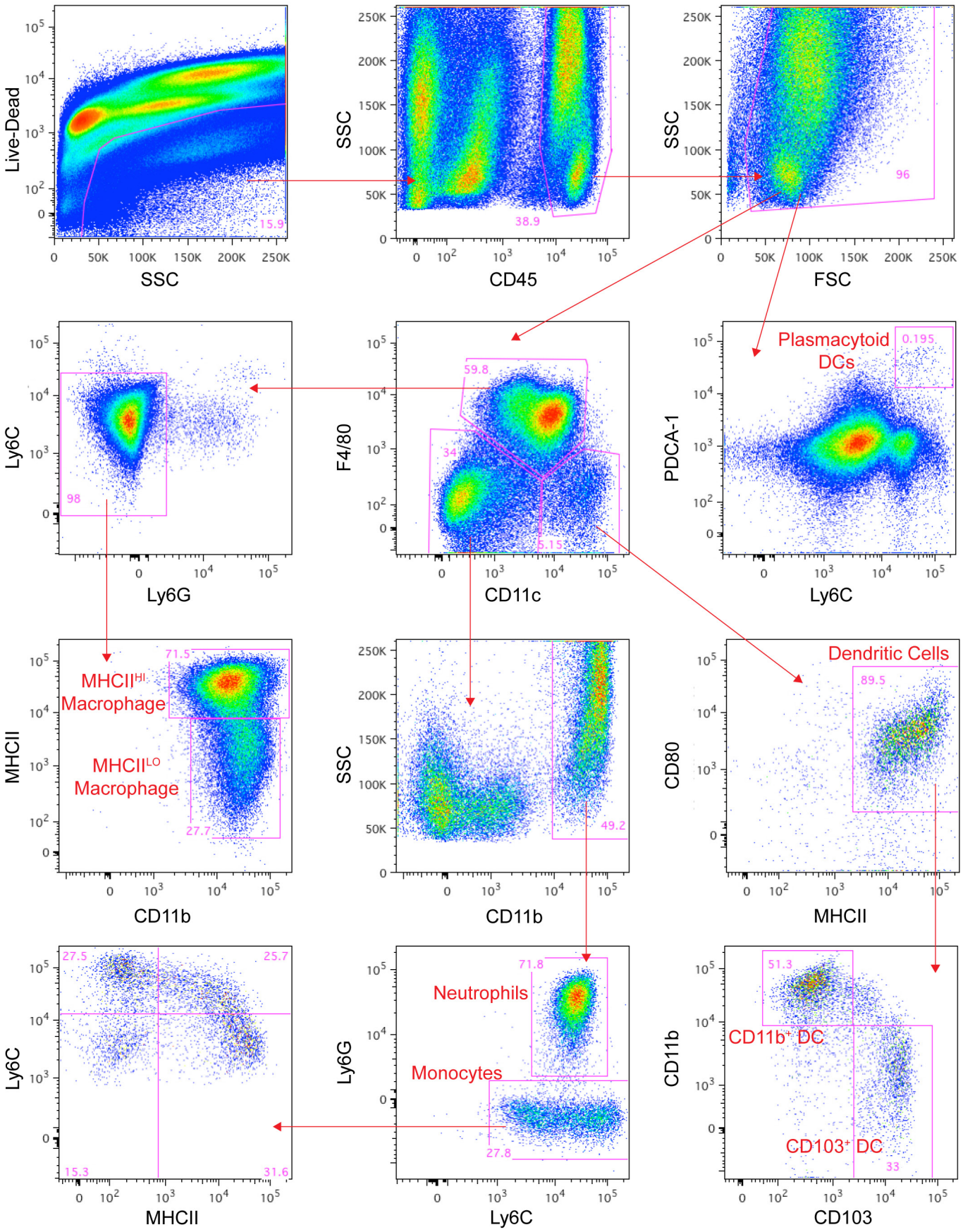

FSC-H
FSC-A
72.4
Live-Dead
SSC
19.9
SSC
CD45
26.5
MHCII
B220
97.1
B cells
MHCII
CD19
6.64
28.4
SSC
FSC-A
97.2
CD4
CD49b/NK1.1
NK cells
1.83
SSC
CD3ε
66.7
23.9
CD8α
CD69
71.5
Activated
CD8⁺ T cells
γδ TCR
CD3ε
γδ T cells
18.4
77.4
CD8α
CD4
20.9
CD8⁺ T cells
CD4⁺ T cells
52.2
FoxP3
CD25
T REG cells
53.7
T H cells
46.5

F


Live-Dead
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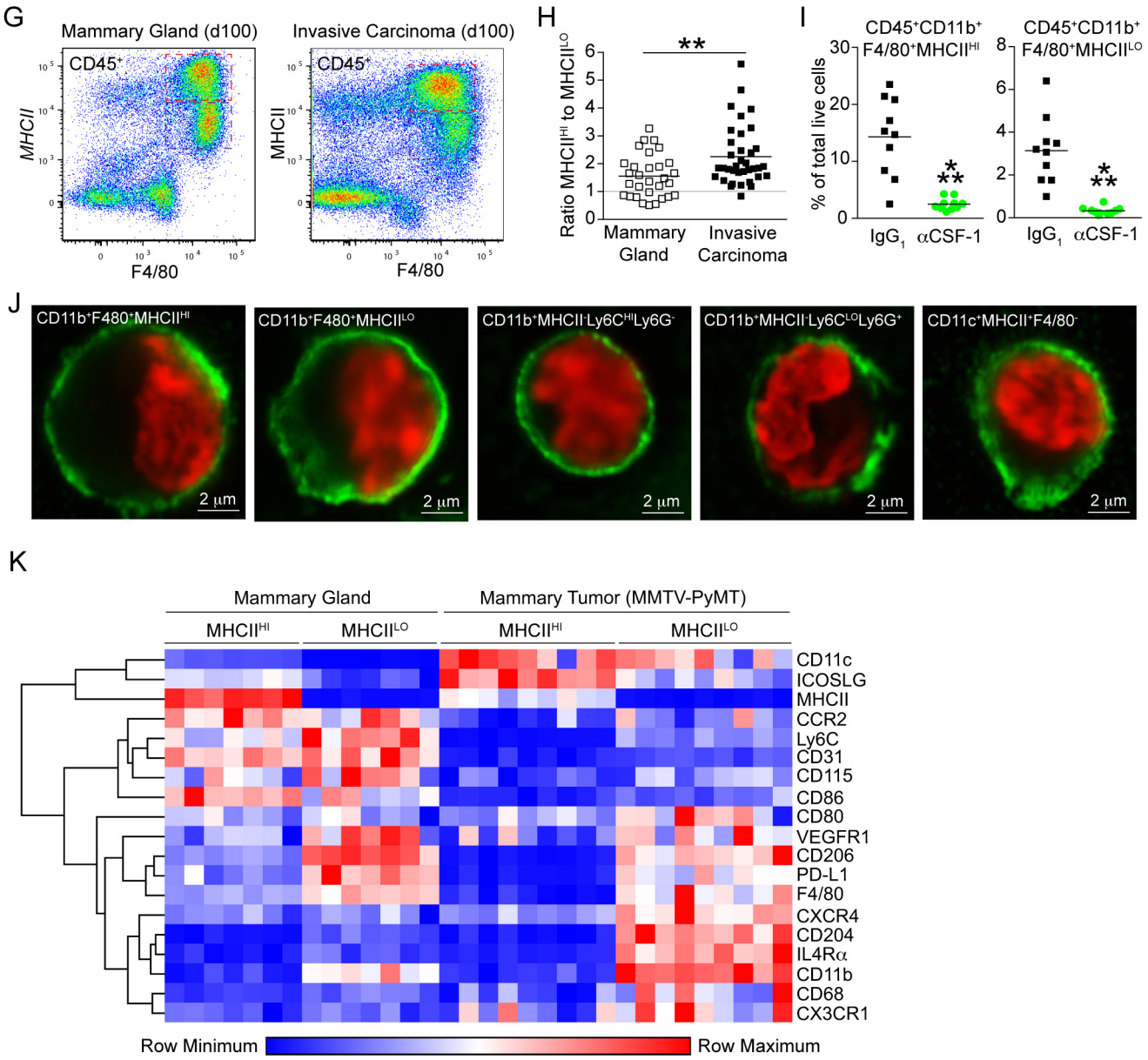
15.9
SSC
CD45
38.9
SSC
FSC
96
Ly6C
Ly6G
98
F4/80
CD11c
59.8
34
5.15
PDCA-1
Ly6C
Plasmacytoid DCs
0.195
MHCII
CD11b
MHCII^HI Macrophage
MHCII^LO Macrophage
71.5
27.7
SSC
CD11b
49.2
CD80
MHCII
Dendritic Cells
89.5
Ly6C
MHCII
27.5
25.7
15.3
31.6
Ly6G
Ly6C
Neutrophils
Monocytes
71.8
27.8
CD11b
CD103
CD11b+ DC
CD103+ DC
51.3
33


G
Mammary Gland (d100)
Invasive Carcinoma (d100)
CD45+
MHCII
F4/80
H
Ratio MHCIIHI to MHCIILO
**
Mammary Gland
Invasive Carcinoma
I
CD45+CD11b+ F4/80+MHCIIHI
CD45+CD11b+ F4/80+MHCIILO
% of total live cells
IgG1
αCSF-1
J
CD11b+F480+MHCIIHI
CD11b+F480+MHCIILO
CD11b+MHCII-Ly6CHILy6G-
CD11b+MHCII-Ly6CLOLy6G+
CD11c+MHCII+F4/80-
2 μm
K
Mammary Gland
Mammary Tumor (MMTV-PyMT)
MHCIIHI
MHCIILO
CD11c
ICOSLG
MHCII
CCR2
Ly6C
CD31
CD115
CD86
CD80
VEGFR1
CD206
PD-L1
F4/80
CXCR4
CD204
IL4Rα
CD11b
CD68
CX3CR1
Row Minimum
Row Maximum

L

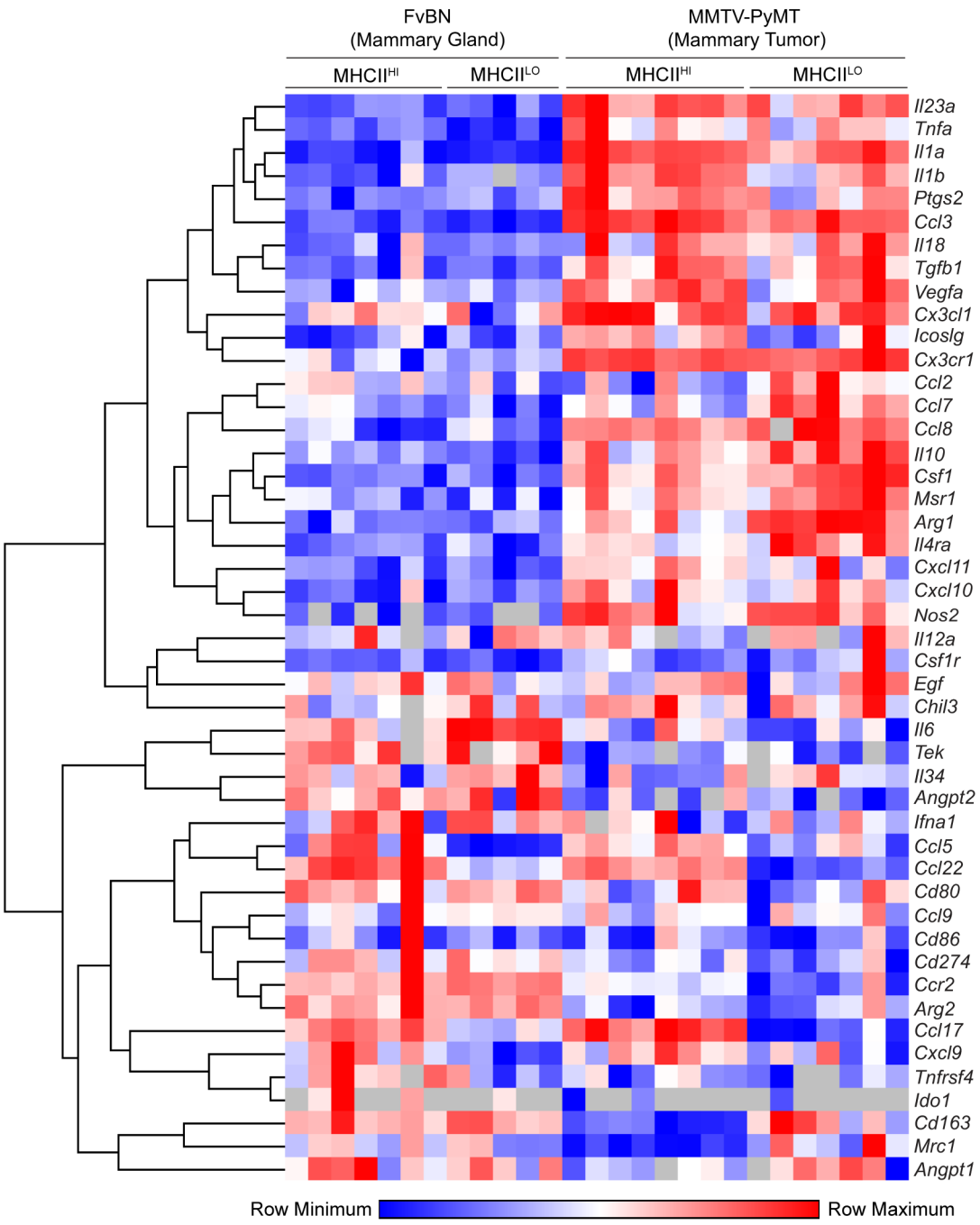

FvBN
(Mammary Gland)
MMTV-PyMT
(Mammary Tumor)
MHCII^HI
MHCII^LO
MHCII^HI
MHCII^LO
Il23a
Tnfa
Il1a
Il1b
Ptgs2
Ccl3
Il18
Tgfb1
Vegfa
Cx3cl1
Icoslg
Cx3cr1
Ccl2
Ccl7
Ccl8
Il10
Csf1
Msr1
Arg1
Il4ra
Cxcl11
Cxcl10
Nos2
Il12a
Csf1r
Egf
Chil3
Il6
Tek
Il34
Angpt2
Ifna1
Ccl5
Ccl22
Cd80
Ccl9
Cd86
Cd274
Ccr2
Arg2
Ccl17
Cxcl9
Tnfrsf4
Ido1
Cd163
Mrc1
Angpt1
Row Minimum
Row Maximum

Figure S1, related to Figure 1: (A) Representative images of CD31 immunoreactivity (left panels) with quantitation of vessel density (right panel) for mammary tumors from MMTV-PyMT mice treated with IgG_1 control or αCSF-1 mAb for 20 days. (B) Representative immunoflorescent images displaying CD31 (white), desmin (green) and α-smooth muscle actin (red) staining in mammary tumros of MMTV-PyMT mice treated for 20 days. The ratio of pericytes ($EpCAM^-CD45^-PDGFRa^-Desmin/SMA^+$) to endothelial cells ($CD31^{HI}CD45^-EpCAM^-$), as determined by flow cytometry, is shown to the right. (C) Uptake of doxorubicin 30 minutes post i.v injection in MMTV-PyMT mice treated for 10 days with IgG_1 control or αCSF-1. Representative confocal images are shown to the left with FITC-lectin marking the vasculature in green and doxorubicin fluorescence shown in red. Quantitation of doxorubicin fluorescence is shown to the right compared to non-injected controls. Data is displayed as mean ± SEM with n≥3 mice per group. Significance determined by unpaired *t*-test with Welch's correction. (D) Pharmacokinetics of paclitaxel in mice bearing orthotopic MMTV-PyMT tumors treated with IgG_1 control (circles) or αCSF-1 mAb (triangles) for 10 days. Concentration of paclitaxel (ng/ml of plasma or tissue homogenate) is shown for plasma (red), tumors (blue) and liver (green) up to 8 hours post i.v. injection. Data is displayed as mean ± SEM with n≥5 mice per time point. (E) Gating strategy for the identification of lymphoid-lineage populations. Starting from the upper left, arrows indicate directionality of sub-gates. Markers are indicated to the left and bottom of each polychromatic dot plot. Identified populations are marked in red text. (F) Gating strategy for identification of myeloid-lineage populations. Starting from the upper left, arrows indicate directionality of sub-gates. Markers are indicated to the left and bottom of each polychromatic dot plot. Identified populations are marked in red text. (G) Flow cytometry plots of $CD45^+$ cells displaying expression of MHCII and F4/80 in mammary glands and MMTV-PyMT mammary carcinomas at 100 days of age. Dotted boxes display gating used to define $MHCII^{HI}$ and $MHCII^{LO}$ populations for B-F. (H) Ratio of $MHCII^{HI}$ to $MHCII^{LO}$ macrophages in mammary glands and mammary carcinomas in sets of age matched mice between days 100-110. Significance determined by unpaired *t*-test with $^{**}p < 0.01$. (I) Infiltration by $MHCII^{HI}$ (left) and $MHCII^{LO}$ (right) macrophages in mammary tumors from MMTV-PyMT animals treated with IgG_1 or αCSF-1 mAb for 20 days determined by flow cytometry and shown as a percent of total live cells. Significance determined by unpaired *t*-test with Welch's correction, with $^{***}p < 0.001$. (J) Confocal microscopy images of FACS-sorted populations from MMTV-PyMT mammary tumors stained with β-actin (green) and DAPI (red). (K) Relative expression of cell surface markers on macrophage subsets determined by flow cytometry using mean fluorescence intensity. Data is displayed as a heat map with hierarchical clustering. CD115 expression levels were measured by intracellular staining. (L) Relative gene expression in macrophage subsets determined by real time PCR. Data is displayed as a heat map with hierarchical clustering. Genes undetectable in select populations are displayed in gray.

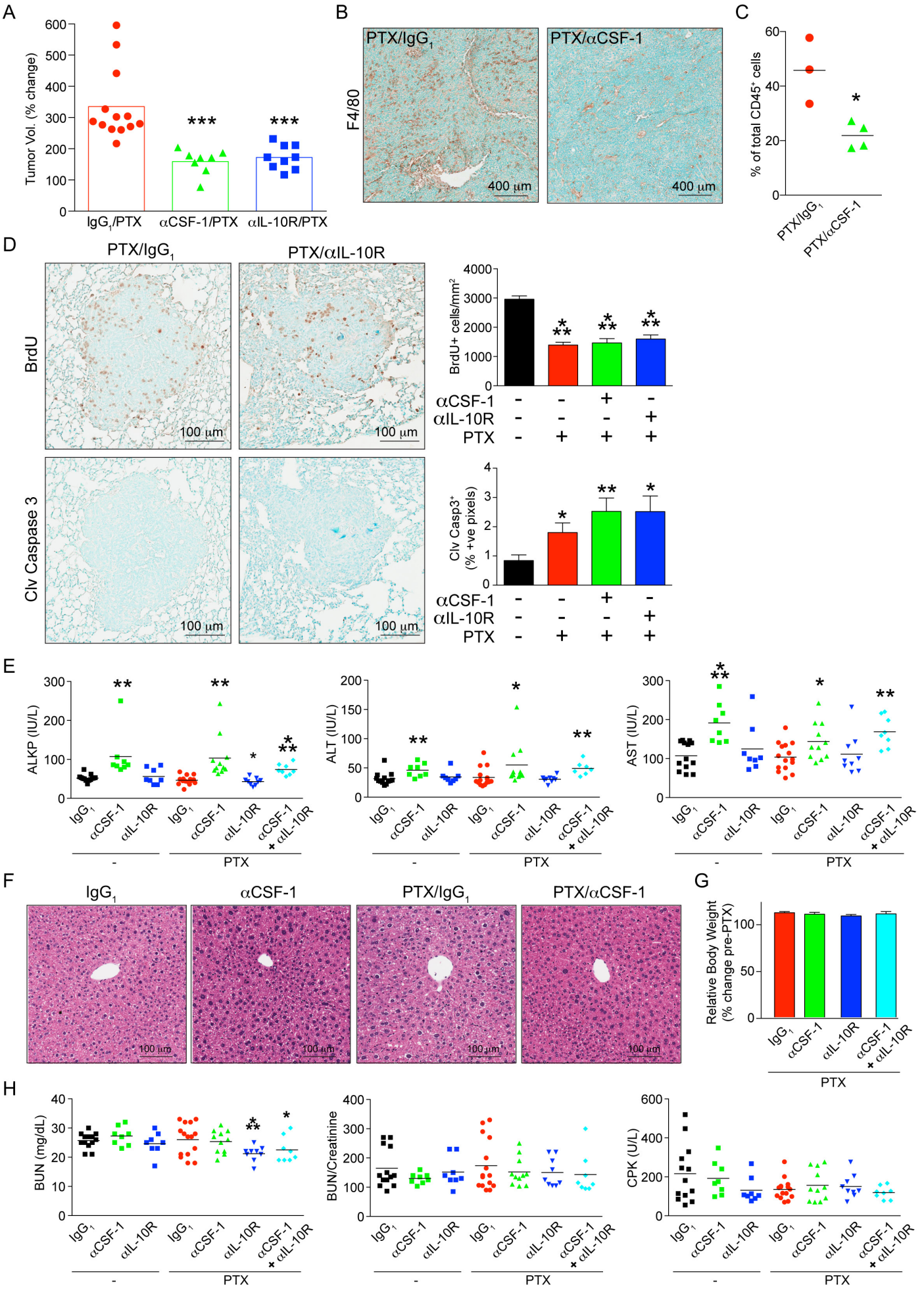

A
Tumor Vol. (% change)
600
500
400
300
200
100
0
***
***
IgG1/PTX
αCSF-1/PTX
αIL-10R/PTX
B
F4/80
PTX/IgG1
PTX/αCSF-1
400 μm
400 μm
C
% of total CD45+ cells
60
40
20
0
*
PTX/IgG1
PTX/αCSF-1
D
PTX/IgG1
PTX/αIL-10R
BrdU
Clv Caspase 3
100 μm
100 μm
100 μm
100 μm
BrdU+ cells/mm2
4000
3000
2000
1000
0
αCSF-1 - - + -
αIL-10R - - - +
PTX - + + +
Clv Casp3+ (% +ve pixels)
4
3
2
1
0
αCSF-1 - - + -
αIL-10R - - - +
PTX - + + +
E
ALKP (IU/L)
ALT (IU/L)
AST (IU/L)
IgG1
αCSF-1
αIL-10R
αCSF-1 + αIL-10R
-
PTX
F
IgG1
αCSF-1
PTX/IgG1
PTX/αCSF-1
100 μm
G
Relative Body Weight (% change pre-PTX)
100
50
0
PTX
H
BUN (mg/dL)
BUN/Creatinine
CPK (U/L)
IgG1
αCSF-1
αIL-10R
αCSF-1 + αIL-10R
-
PTX

Figure S2, related to Figure 2: (A) Relative tumor volume after 3 rounds of PTX in C3(1)-TAg transgenic mice following combination therapy with αCSF-1 mAb or αIL-10R mAb. Significance determined by unpaired *t*-test with Welch's correction with ***p < 0.001. (B) F4/80 immunohistochemistry in C3(1)-TAg tumors following treatment with PTX in combination with IgG_1 or αCSF-1 mAb. (C) Percent of total $CD45^+$ cells in C3(1)-TAg tumors comprised of $CD11b^+F4/80^+MHCII^+Ly6C^-$ macrophages. Data generated by flow cytometry. Significance determined by an unpaired *t*-test with *p < 0.05. (D) Proliferation (BrdU positivity) and cell death (cleaved caspase 3 positivity) in metastatic foci of MMTV-PyMT animals treated with combinatorial chemotherapy. Representative images are show on left, with quantitation shown on right. Data is displayed as mean ± SEM with n≥9 mice per group. Significance determined by unpaired *t*-test with Welch's correction with *p < 0.05, **p < 0.01, ***p < 0.001. (E) Liver function tests based on serum isolated from MMTV-PyMT transgenic mice treated with combinatorial CTX. Significance determined by an unpaired *t*-test with Welch's correction with *p < 0.05, **p < 0.01, ***p < 0.001. (F) Representative H&E sections of liver from mice in A with no evidence of tissue pathology. (G) Percent change in body weight following 3 doses of PTX in the different treatment groups. Data is displayed as mean ± SEM. (H) Renal function tests (BUN, BUN/Creatinine) and CPK measurements for muscle damage in serum of mice. Significance determined by unpaired *t*-test with Welch's correction with *p < 0.05, **p < 0.01.

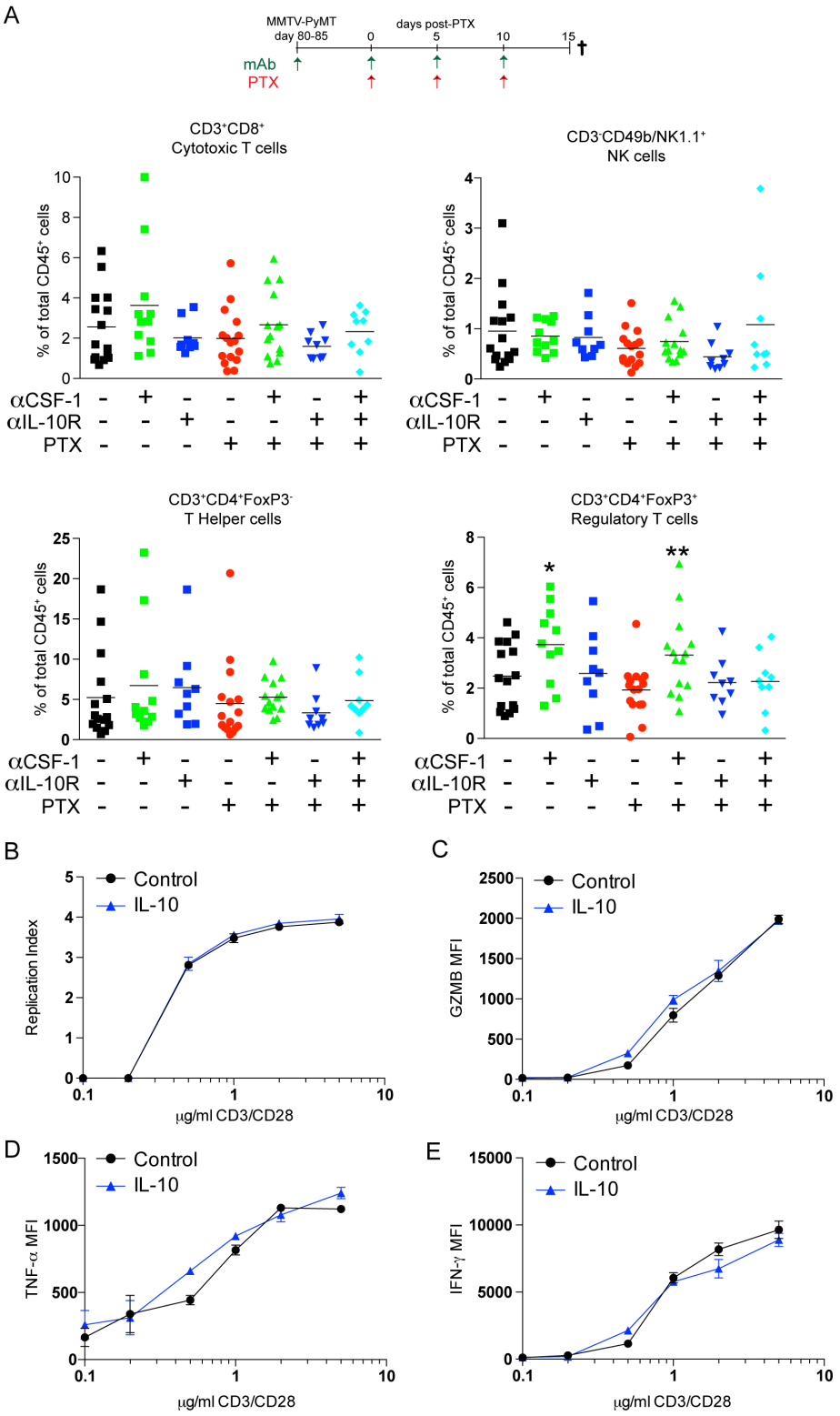


Figure S3, related to Figure 3: (A) Immune populations within the tumors of MMTV-PyMT animals treated with 3 rounds of PTX were identified via polychromatic flow cytometry and are displayed as a percentage of total $CD45^+$ cells. Significance is shown compared to the IgG_1 control group for PTX-naïve mice and to the PTX/IgG_1 group for PTX-treated mice, and was determined by an unpaired *t*-test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B-E) Purified splenic $CD8^+$ T cells were stimulated with αCD3/αCD28 for 48 hrs in the presence of 10 ng/ml IL-10 and measured for fold expansion by replication index (A), expression of GZMB (B), expression of TNF-α (C), or expression of IFN-γ (D). Samples were assayed in triplicate and one of three representative experiments is shown. Data is displayed as mean ± SEM.

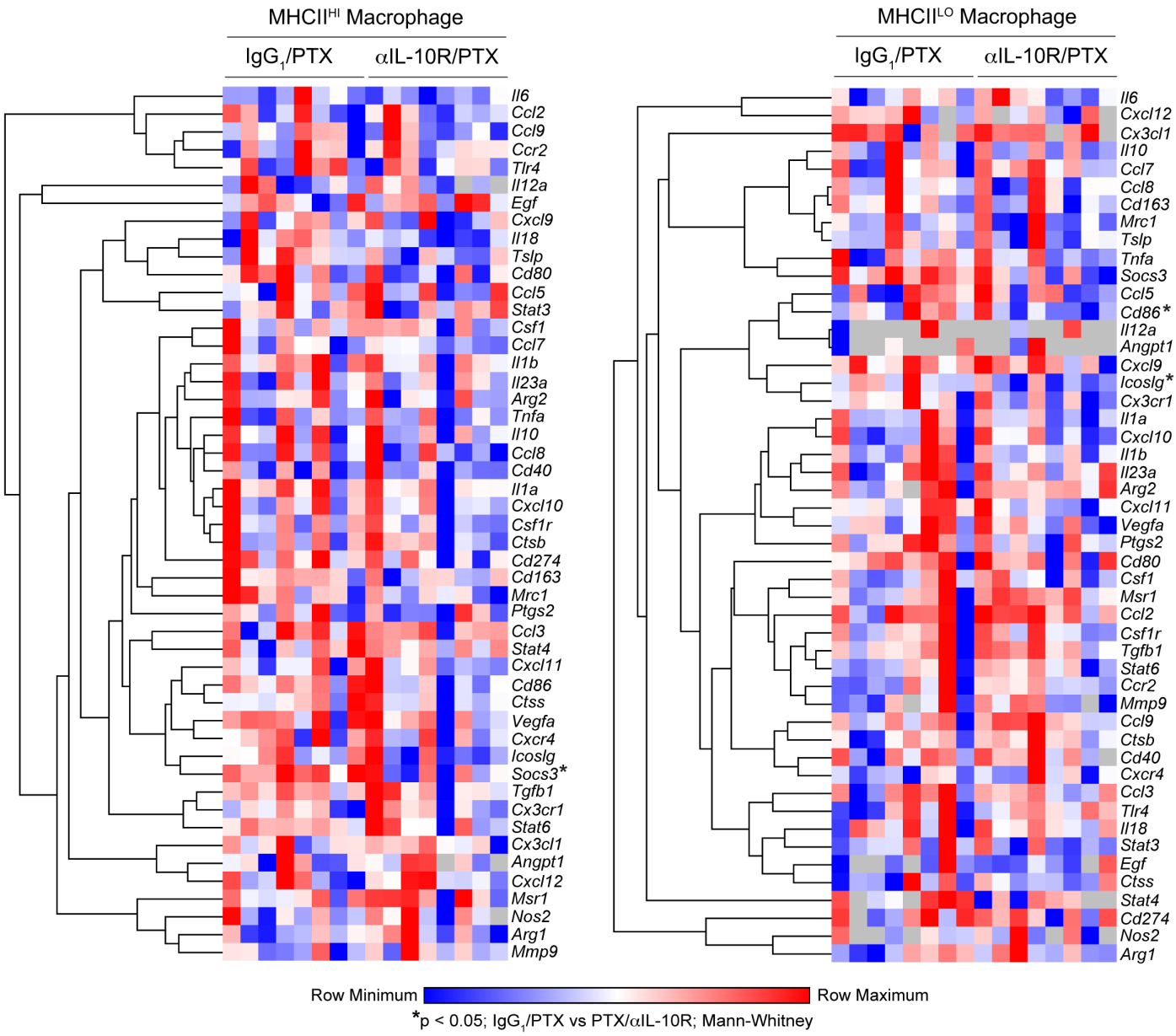


Figure S4, related to Figure 4: Gene expression by real time PCR in MHCII^{HI} and MHCII^{LO} macrophage subsets isolated from MMTV-PyMT animals treated with IgG_1/PTX or αIL-10R/PTX. Data was analyzed by comparative threshold cycle method using *Tbp* as a reference gene and is represented by a heat map with hierarchical clustering. Genes undetectable in select populations are displayed in gray. Significance determined by Mann-Whitney with *$p < 0.05$.

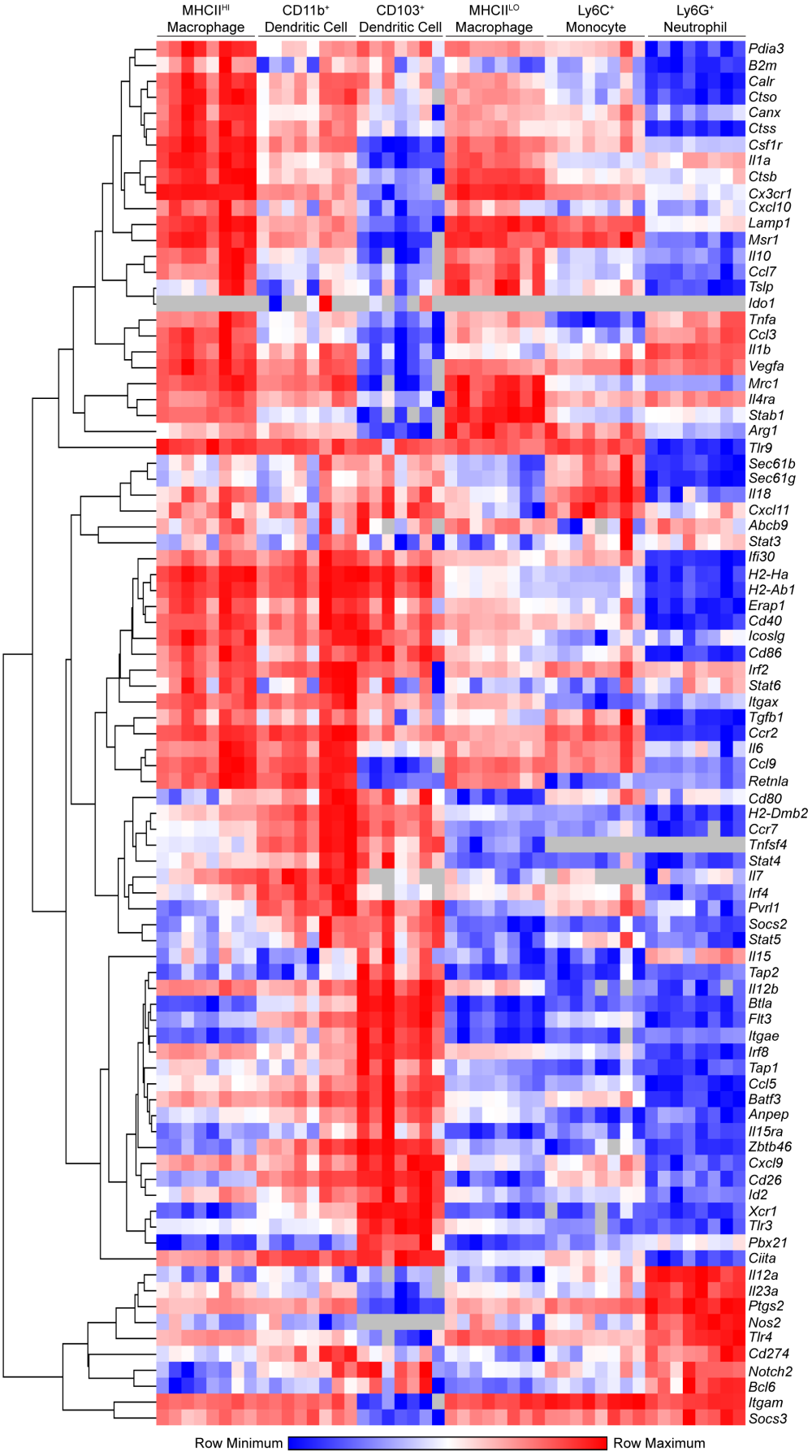


Figure S5, related to Figure 5: Real time PCR analysis of FACS-sorted myeloid populations from mammary tumors of untreated, end-stage MMTV-PyMT mice (>100 days). Data is displayed as a heat map with hierarchical clustering. Genes undetectable in select populations are displayed in gray.

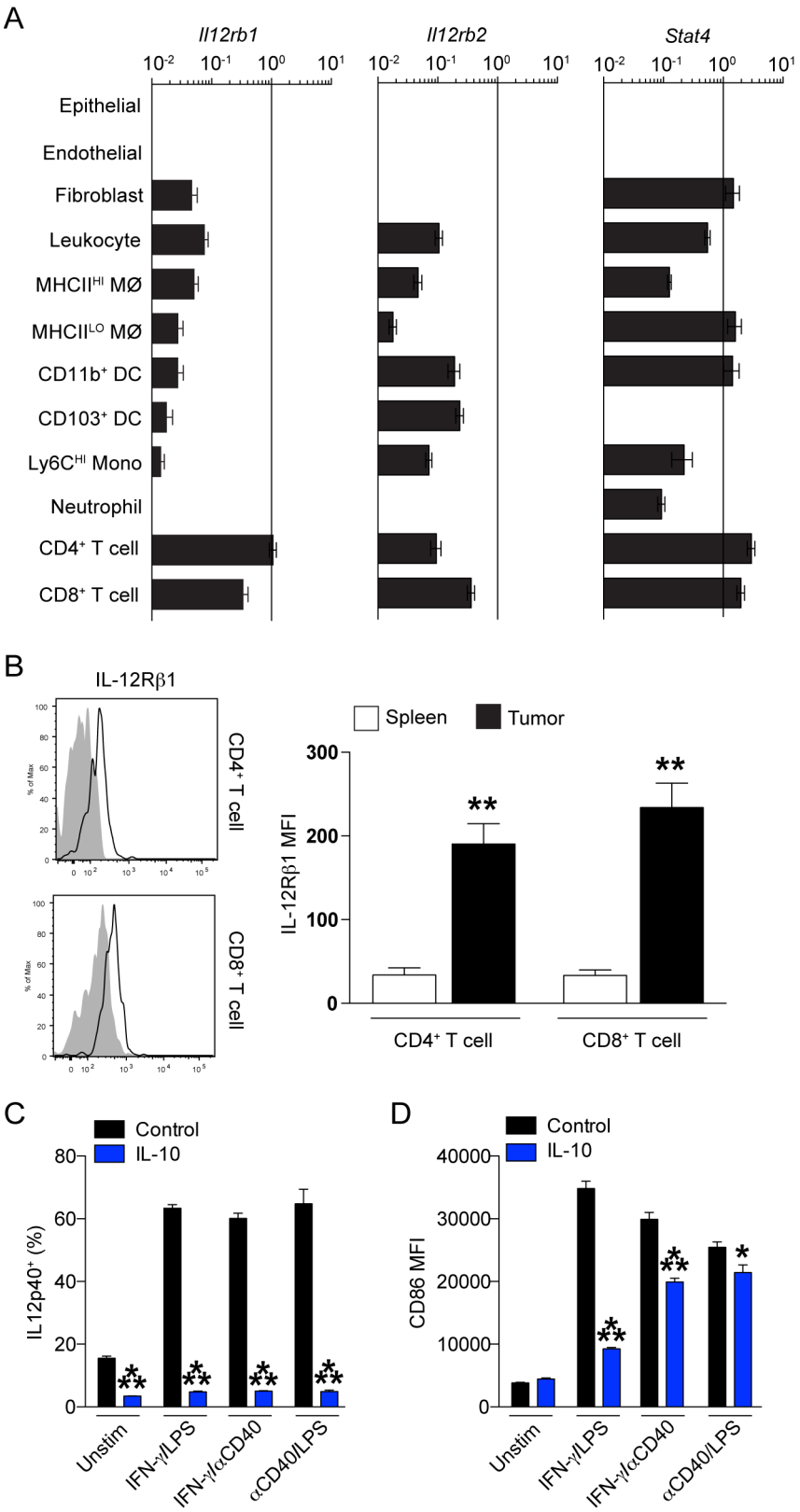

A
Il12rb1
Il12rb2
Stat4
Epithelial
Endothelial
Fibroblast
Leukocyte
MHCII^HI MØ
MHCII^LO MØ
CD11b+ DC
CD103+ DC
Ly6C^HI Mono
Neutrophil
CD4+ T cell
CD8+ T cell
B
IL-12Rβ1
CD4+ T cell
CD8+ T cell
Spleen
Tumor
IL-12Rβ1 MFI
C
Control
IL-10
IL12p40+ (%)
Unstim
IFN-γ/LPS
IFN-γ/αCD40
αCD40/LPS
D
Control
IL-10
CD86 MFI
Unstim
IFN-γ/LPS
IFN-γ/αCD40
αCD40/LPS

Figure S6, related to Figure 6: (A) Real time PCR analysis of *Il12rb1*, *Il12rb2*, and *Stat4* from FACS-sorted leukocyte populations from the tumors of untreated, end-stage MMTV-PyMT mice (>100 days). Data is normalized to *Tbp* expression and is displayed as mean ± SEM with n=8 mice per cell type. MØ, macrophage; mono, monocyte; DC, dendritic cell. (B) Surface expression of IL12Rβ1 as measured by mean fluorescence intensity (MFI) minus background in T lymphocytes from MMTV-PyMT mammary tumors and normal spleens. Data is displayed as mean ± SEM with n=4 mice per group. Significance was determined by an unpaired *t*-test with $^{**}p < 0.01$. Representative histograms of T lymphocytes from MMTV-PyMT mammary tumors are shown to the left. (C) Percent of BMDCs expressing IL-12p40 by intracellular flow cytometry following 24 hr stimulation with IFN-γ/LPS, IFN-γ/αCD40 or αCD40/LPS. Cells were pre-treated for 24 hours with 10 U/ml IL-10 prior to stimulation. (D) Surface expression of CD86 on the BMDCs from C, as determined by mean fluorescence intensity. For C-D, samples were assayed in quadruplicate and one of two representative experiments is shown. Data is displayed as mean ± SEM. Significance determined by unpaired *t*-test relative the control group, with $^{*}p < 0.05$, $^{***}p < 0.001$.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Quantitation of metastatic burden

Following resection, lungs from transgenic MMTV-PyMT animals were injected with neutral buffered formalin via the trachea and incubated overnight in formalin prior to ethanol dehydration and paraffin embedding. Lungs were sectioned and haematoxylin and eosin staining was performed on slides throughout lungs stationed 100 μm apart for analysis. Frequency and size of the metastatic foci were determined by manual circling in a blinded fashion using Imagescope software.

Flow cytometry and fluorescence activated cell sorting

Isolation and staining of stromal cell populations was performed as described using antibodies from BioLegend and eBioscience (Ruffell et al., 2013). Ex vivo intracellular staining (Liu and Whitton, 2005) for IL-12p40 was performed on isolated cells 6 hours following an intravenous injection of 0.25 mg brefeldin A (Sigma-Aldrich). Gating strategies can be found in supplemental Figure S2-3. For flow cytometry, data was collected with either a LSRII or Fortessa flow cytometer (BD Bioscience) with analysis performed using FlowJo (Tree Star Incorporated). Fluorescent-activated cell sorting (FACS) was conducted on a FACSAriaII or InFlux (BD Biosciences). Sorted cells were either used immediately in vitro or flash frozen in liquid nitrogen as a cell pellet for subsequent gene expression analysis.

Real-time PCR

Mice were cardiac-perfused to clear peripheral blood. Tumor tissues were snap-frozen and pulverized in liquid nitrogen for whole tissue analysis, or were used to purify immune populations by FACS. Total mRNA was prepared using RNeasy Micro/Mini kit guidelines (Qiagen) followed by RNA quantitation using a NanoDrop ND-1000 (Thermo Fisher Scientific). Contaminating DNA was removed with DNAse I (Life Technologies), and then SuperScript III (Life Technologies) was used to reverse transcribe purified RNA into cDNA according to manufacturer's directions. Real-time PCR for gene expression was performed using individual TaqMan Assays or microfluidic TaqMan Low Density Arrays (Life Technologies). A preamplification step (Life Technologies) was used prior to analysis of FACS-sorted populations. The comparative threshold cycle method was used to calculate fold change in gene expression, which was normalized to either a single (*Tbp*) or multiple (*Actb*, *Gusb*, *Gapdh*, *Pgk1*, *Tfrc*) reference genes.

Immunohistochemistry

Immunohistochemistry was performed as described for mouse (Ruffell et al., 2013) and human tissue (Ruffell et al., 2012). Citrate retrieval was used for BrdU (BU1/75; 1:200; Serotec), cleaved Caspase 3 (1:200; Cell Signaling), granzyme B (1:200; Novus Biologicals), and IL-10 (1:100; R&D Systems). Proteinase K retrieval (Dako) was used for CD31 (MEC13.3; 1:100; eBioscience) and F4/80 (CI:A3-1;

1:500; Serotec). All slides were digitally scanned using the Aperio ScanScope CS Slide Scanner with a 20X objective. Automated quantitative image analysis was performed using Imagescope (Aperio) and the provided positive pixel (cleaved caspase 3) or nuclear stain (BrdU) detection algorithm. CD31 and granzyme B quantitation was done by manual counting in a blinded fashion.

Immunofluorescence

Animals were injected intravenously with 8.0 mg/kg doxorubicin and 100 μl of 1.0 mg/ml fluorescein-labeled *Lycopersicon esculentum* Lectin (Tomato Lectin, Vector Laboratories) for 30 min and 3 min, respectively, prior to cardiac perfusion with PBS containing 4% paraformaldehyde (PFA, Electron Microscopy Sciences) and embedding tissue into OCT. Alternatively, animals were cardiac perfused with PBS containing 10 U/ml of Heparin and tissue was directly embedded into OCT. Unfixed tumors were permeabilized with 100% ice-cold acetone for 10 min, washed in PBS, and then blocked with goat blocking buffer for 1 hr. Primary antibodies including α-smooth muscle actin Cy3 (1:1000, Sigma), desmin (1:500, Millipore/Upstate), CD31 (1:100, BioLegend), F4/80 (1:100, BioLegend), CD103 (1:100, BioLegend), and pan-Keratin (1:100, Cell Signaling) were diluted in 0.5x blocking buffer and incubated on sections overnight at 4°C. For immunofluorescent staining of human breast cancer tissues, 5 μm sections of formalin fixed, paraffin embedded tissue were deparaffinized with xylene, rehydrated, and subjected to antigen retrieval with heated citrate buffer (BioGenex). After 1 hr in horse serum blocking buffer, CD163 (1:50, Thermo Scientific) and IL-10 (1:100; R&D Systems) antibodies were applied overnight at 4°C. After washing, secondary antibodies from Life Technologies were used at 1:500 for 30 min at room temperature, followed by incubation with 1.0 μg/ml Hoechst 33342 (Life Technologies) for 15 min. Slides were then washed and mounted with ProLong Gold anti-fade mounting medium (Life Technologies). Sorted cells were affixed to slides by a Shandon Cytospin, fixed/permeabilized with 100% ethanol, and then stained for β-actin (1:1000; Sigma-Aldrich) as described above except with the use DAPI as a nuclear stain. Images were taken with a LSM510 Confocal Laser Scanning microscope (Carl Zeiss), Nikon C1si Spectral Confocal Microscope, or Ariol automated scanning microscope (Leica). Fluorescent quantitation was performed with ImageJ.

Flt-3 Ligand Bone Marrow DCs

Bone marrow was harvested from FVB/n female mice and red blood cells lysed with Pharmlyse (BD Biosciences). Remaining cells were plated at $2x10^6$ per ml in RPMI1640 containing 2.0 mM L-glutamine and 25 mM HEPES, supplemented with 10 mM Sodium Pyruvate, nonessential amino acids, 100 U/ml penicillin/streptocmycin, 55 μM B-ME, and 10% fetal calf serum (Life Technologies). Recombinant human Flt-3 Ligand Immunoglobulin (Flt-3L-Ig; BioXCell) was added at 100 ng/ml and cells were incubated untouched for 9 days. Cells in suspension were removed by pipetting (>90% $CD11c^+$), resuspended at 10^6 per ml in RPMI1640 with 100 ng/ml Flt-3L-Ig, and incubated for 24 hours with 1.0-10 U/ml IL-10 (Peprotech). Cells were then stimulated for an additional 24 hours with a combinational of IFN-γ (40 ng/ml;

Peprotech), LPS-EB Ultrapure (100 ng/ml; InvivoGen), or αCD40 (10 μg/ml; FGK4.5; BioXCell). For intracellular flow cytometry, Brefeldin A (3.0 μg/ml; eBioscience) was added 4 hours prior to harvesting the cells.

T cell activation

Splenic $CD8^+$ T cells were purified by magnetic negative selection (Stem Cell Technologies) to over 95% purity and labeled with Cell Trace Violet (Invitrogen). $CD8^+$ T cells (10^5) were then placed a 96-well plate coated with 5.0 μg/ml αCD3 (145.2C11) and αCD28 (PV-1) antibodies in RPMI1640 containing 2.0 mM L-glutamine and 25 mM HEPES, supplemented with 10 mM Sodium Pyruvate, 100 U/ml penicillin/streptocmycin, 55 μM B-ME, and 10% fetal calf serum (Life Technologies) for 48 hrs at 37°C. Brefeldin A (3.0 μg/ml; eBioscience) was added 4 hours prior to harvesting the cells for intracellular staining with GZMB PE, IFN-γ APC, and TNF-α PE-Cy7. Data acquisition was performed on a BD Fortessa flow cytometer with analysis using FlowJo software.

T cell suppression assay

Splenic $CD8^+$ T cells were purified by magnetic negative selection (Stem Cell Technologies) to over 95% purity and labeled with Cell Trace Violet (Invitrogen). $CD8^+$ T cells (10^5) were then placed in a 96-well plate coated with 5 μg/ml αCD3 (145.2C11) and αCD28 (PV-1) antibodies. After a brief spin, FACS-sorted macrophages from MMTV-PyMT late stage tumors were added into wells at various ratios with or without 10 μg/ml of blocking/neutralizing antibodies. Cells were cultured together in RPMI1640 containing 2.0 mM L-glutamine and 25 mM HEPES, supplemented with 10 mM Sodium Pyruvate, 100 U/ml penicillin/streptocmycin, 55 μM B-ME, and 10% fetal calf serum (Life Technologies) for 60 hrs at 37°C. Cells were then stained with CD8-PE, CD11b-APC and 7AAD prior to analysis on an LSRII or Fortessa flow cytometer. The replication index was determined using FlowJo software.

Quantitation of paclitaxel

Mice bearing orthotopic mammary tumors were intravenously injected with 10 mg/kg paclitaxel and, following a cardiac puncture blood draw, were perfused to clear peripheral blood. Tumor and liver tissues were snap-frozen in liquid nitrogen. Liquid chromatography-mass spectrometry was performed on homogenized samples by Integrated Analytical Solutions (Berkeley, CA) using the injected compound as a reference sample.

Supplemental References

Liu, F., and Whitton, J. L. (2005). Cutting edge: re-evaluating the in vivo cytokine responses of CD8+ T cells during primary and secondary viral infections. J Immunol *174*, 5936-5940.

Ruffell, B., Affara, N. I., Cottone, L., Junankar, S., Johansson, M., DeNardo, D. G., Korets, L., Reinheckel, T., Sloane, B. F., Bogyo, M., and Coussens, L. M. (2013). Cathepsin C is a tissue-specific regulator of squamous carcinogenesis. Genes Dev *27*, 2086-2098.