Supporting Information

Ruffell et al. 10.1073/pnas.1104303108

SI Materials and Methods

Flow Cytometry. Freshly resected tissue was manually minced and then incubated for 45 min at 37 °C in DMEM (Invitrogen) with 2.0 mg/mL Collagenase A (Roche) and 50 units/mL DNase I (Roche). Single cell suspensions were prepared by filtering through 70-μm nylon strainers (BD Biosciences), and $<10^6$ cells were incubated for 30 min on ice with Fc Receptor Binding Inhibitor (eBioscience) diluted 1/10 in PBS containing Live Dead Aqua (1:500, Invitrogen). Cells were then incubated for 30 min in PBS containing 1.0 mM EDTA and 5% FCS along with manufacturers' suggested dilutions of fluorescently labeled primary monoclonal antibodies (Table S2). After washing once, cells were fixed with BD Cytofix for 30 min on ice, washed again, and stored at 4 °C until analysis with an LSRII flow cytometer (BD Bioscience). Before intracellular FoxP3 staining, cells were instead fixed with the FoxP3 Fixation/Permeabilization system (eBioscience) according to the manufacturer's instructions.

Immunohistochemistry. Sections (5 μm) of formalin-fixed, paraffin-embedded tissues were deparaffinized with xylene, rehydrated, immersed in antigen retrieval citra (BioGenex), then heated for 7 min at maximum power in a microwave, followed by a 30-min incubation in the heated buffer. After washing 3 times in PBS and surrounding tissues with a hydrophobic Super Pap Pen (The Binding Site), peroxidase activity and nonspecific binding was blocked with appropriate components of the Thermo Scientific Ultravision Detection Kit according to the manufacturer's instructions. After a second blocking step with PBS containing 5% goat serum, 2.5% BSA, and 0.1% Tween-20, unlabeled primary antibodies (Table S3) were diluted according to manufacturers' recommendations and added to sections overnight at 4 °C. After washing, antibodies were detected by using the appropriate components of the Thermo Scientific Ultravision Detection Kit according to manufacturer's instructions. After development with liquid DAB, slides were washed in H_2O, counterstained briefly with 1% Methyl Green, dehydrated, and mounted with Cytoseal (Thermo Scientific). Representative images and quantitative image analysis was done by using the Aperio ScanScope CS Slide Scanner (Aperio Technologies) system with a 20× or 40× objective to capture whole-slide images. Positive staining was assessed with the nuclear default algorithm (Aperio).

Immunofluorescence. Sections (10 μm) of PFA fixed, sucrose protected, OCT embedded tissues were thawed at 37 °C for 10 min, permeabilized with 100% ice-cold acetone for 10 min, washed in PBS, and then blocked with goat blocking buffer for 2 h. To use two primary antibodies from the same species, one antibody was added at a 100-fold reduced dilution overnight at 4 °C in 0.5× blocking buffer, and after washing, slides were incubated with a biotinylated anti-mouse or rabbit secondary (Vector Laboratories) for 30 min. The signal from the diluted antibody was then amplified with a TSA indirect kit (Perkin-Elmer) according to manufacturer's instructions. After extensive washing, additional primary antibodies were added overnight at 4 °C. After another round of washing, goat anti-mouse Alexa 488, donkey anti-rabbit or anti-mouse Alexa 546, and streptavidin Alexa 647 (1/500, Invitrogen) were used to detect all three antibodies. To use three primary murine antibodies, slides were blocked with 5.0 μg/mL mouse IgG1 and IgG2a (BioLegend) for 30 min after detection of the first primary antibody with anti-mouse Alexa 647. Slides were then incubated with two primary antibodies directly conjugated to either FITC or Cy3 for 3 h, washed, and incubated with goat anti-FITC Alexa 488 (Invitrogen). Slides were mounted with ProLong Gold with DAPI anti-fade mounting medium (Invitrogen) overnight, and images were acquired by using a LSM510 Confocal Laser Scanning Microscope (Carl Zeiss).

Real-Time PCR. Culture and exposure of human BC cell lines to CTX agents was performed as described (1). mRNA was isolated from cells by using the RNeasy mini kit (Qiagen), contaminating DNA was removed by DNase I (Invitrogen) digestion, and reverse transcription into cDNA was performed by using SuperScript III (Invitrogen) according to the manufactures' directions. Real-time PCR was performed by using the Taqman system after a preamplification step with TaqMan PreAmp (Applied Biosystems) according to the manufacturer's instructions.

1. DeNardo DG, et al. (2011) Leukocyte complexity in breast cancer predicts overall survival and functionally regulates response to chemotherapy. *Cancer Discovery* 1: 54–67.

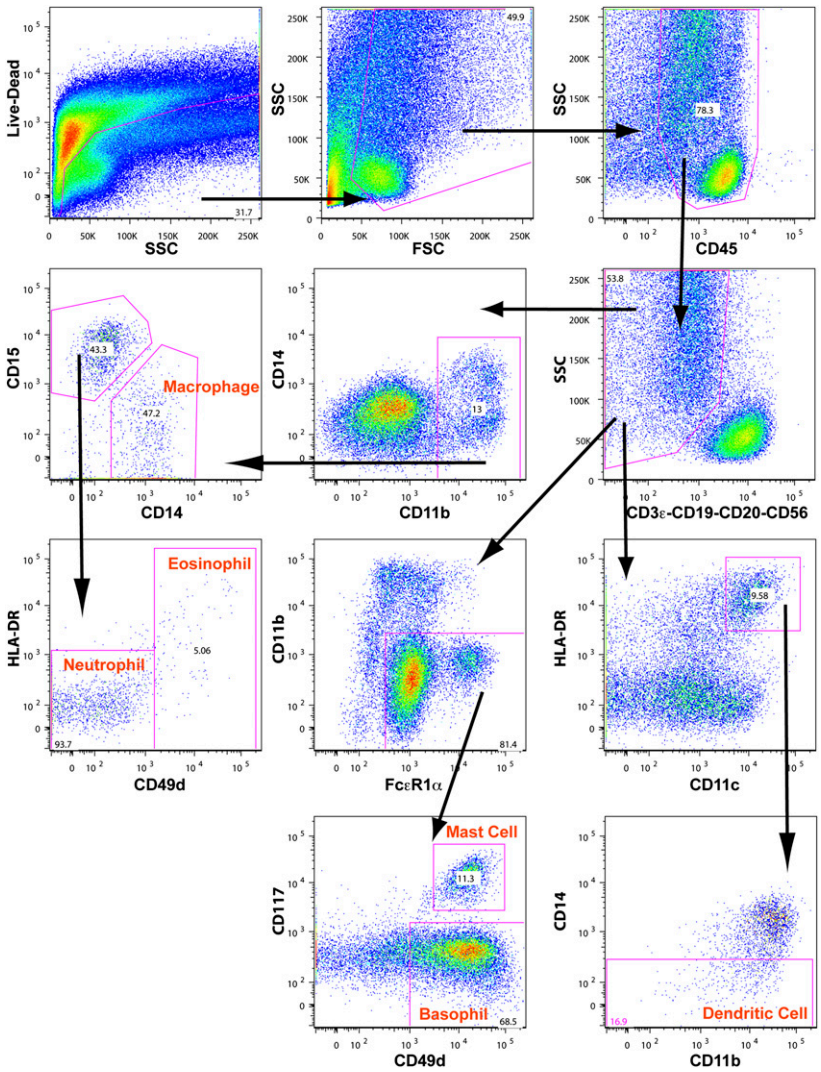

Fig. S1. Gating strategy for identification of myeloid-lineage populations. Starting from the upper left, arrows indicate directionality of subgates. Markers are indicated to the left and bottom of each polychromatic dot plot. Identified populations are marked in red text.

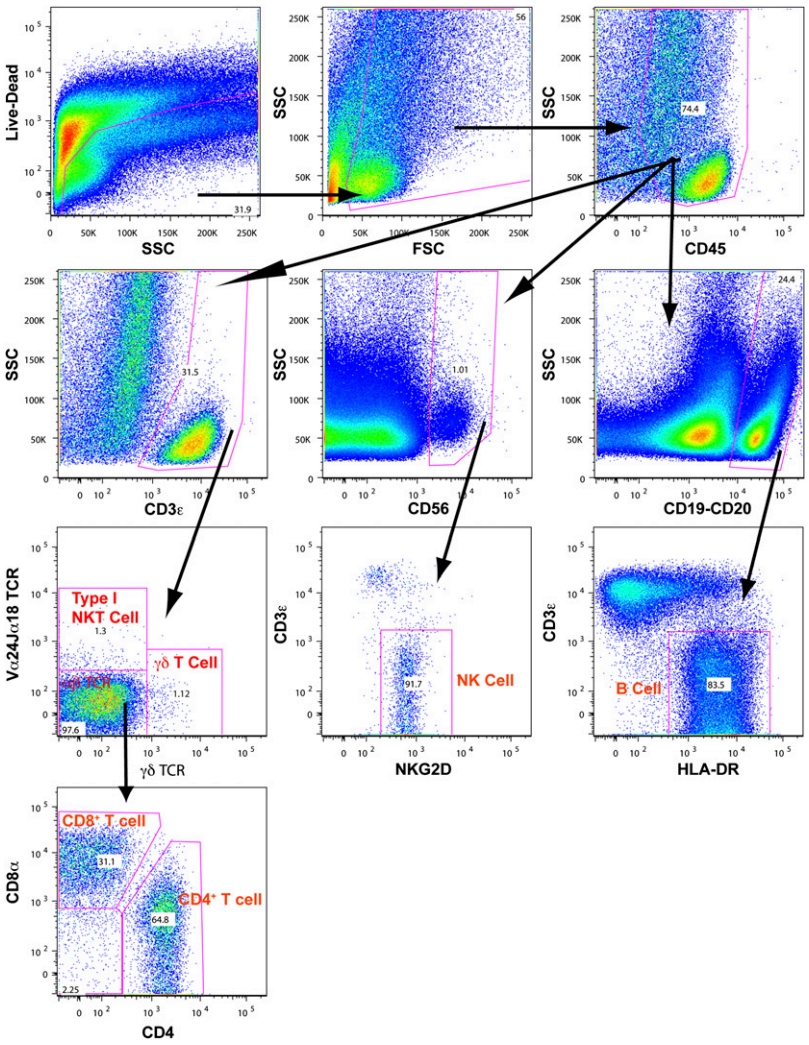

Fig. S2. Gating strategy for identification of lymphocyte populations. Starting from the upper left, arrows indicate directionality of subgates. Markers are indicated to the left and bottom of each polychromatic dot plot. Identified populations are marked in red text.

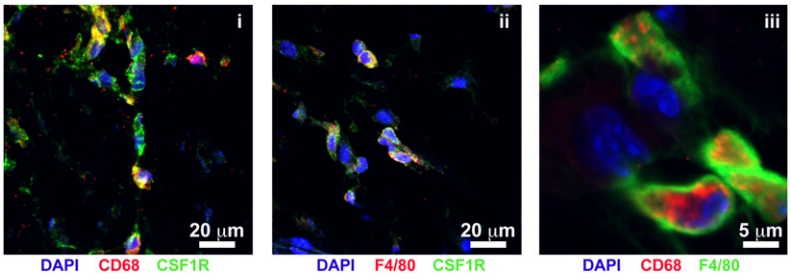

Fig. S3. Immunofluorescent staining in murine mammary tumors for CD68 (red; *i* and *iii*), CSF1R (green; *i* and *ii*) or F4/80 (red, *ii*; green, *iii*).

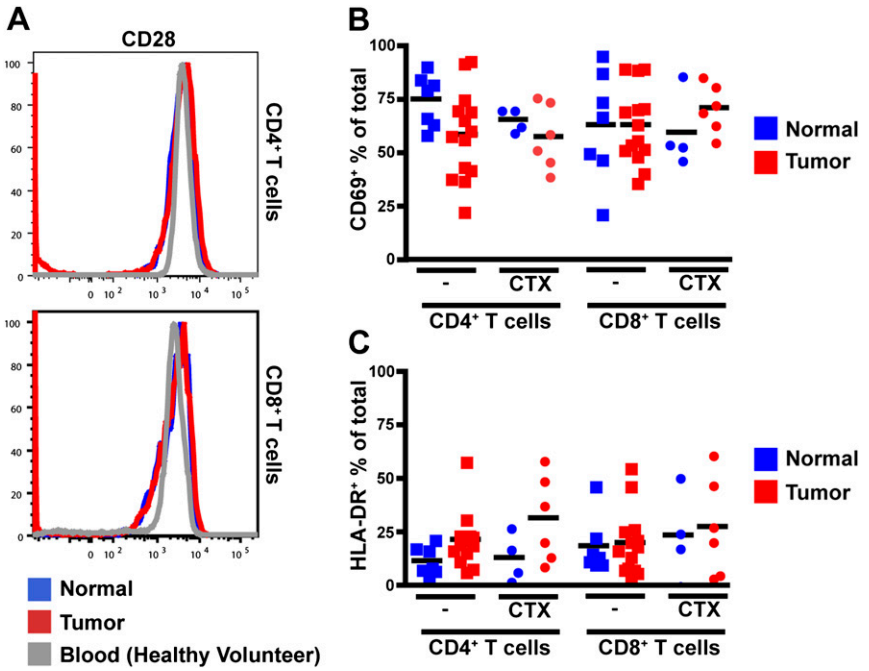

Fig. S4. Activation marker expression on T lymphocytes. (*A*) Representative histograms of CD28 expression for $CD3^+CD4^+$ (*Upper*) or $CD3^+CD8^+$ (*Lower*) T cells isolated from a single CTX-treated patient with both normal (blue) and tumor (red) tissue. (*B* and *C*) Percent of CD69 expressing (*B*) or HLA-DR expressing (*C*) T cells from all samples.

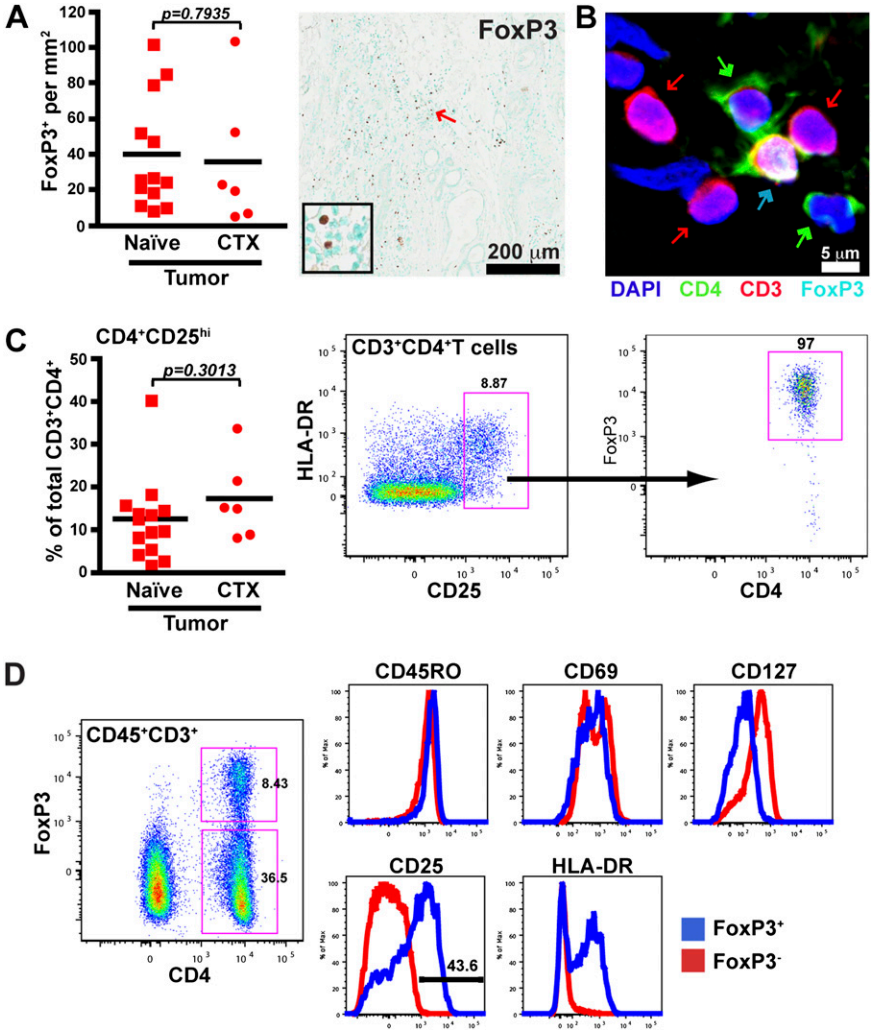

Fig. S5. Presence of $CD4^+FoxP3^+$ regulatory T cells within tumors. (*A*) Number of FoxP3 positive cells per area as determined by automated counting (*Left*) with a representative stained section shown (*Right*). (*B*) Immunofluorescent staining of tumors for CD4 (green), CD3 (red), and FoxP3 (teal). Arrows indicate $CD3^+CD4^-FoxP3^-$ (red), $CD3^+CD4^+FoxP3^-$ (green), and $CD3^+CD4^+FoxP3^+$ (teal) cells. (*C*) Percent of $CD25^{hi}$ cells within the $CD3^+CD4^+$ T-cell population (*Left*) with a representative polychromatic dot plot demonstrating FoxP3 staining within this population (*Right*). (*D*) Representative histograms of $CD3^+CD4^+FoxP3^-$ (red) and $CD3^+CD4^+FoxP3^+$ cells (blue) showing expression of CD45RO, CD69, CD25, CD127, and HLA-DR.

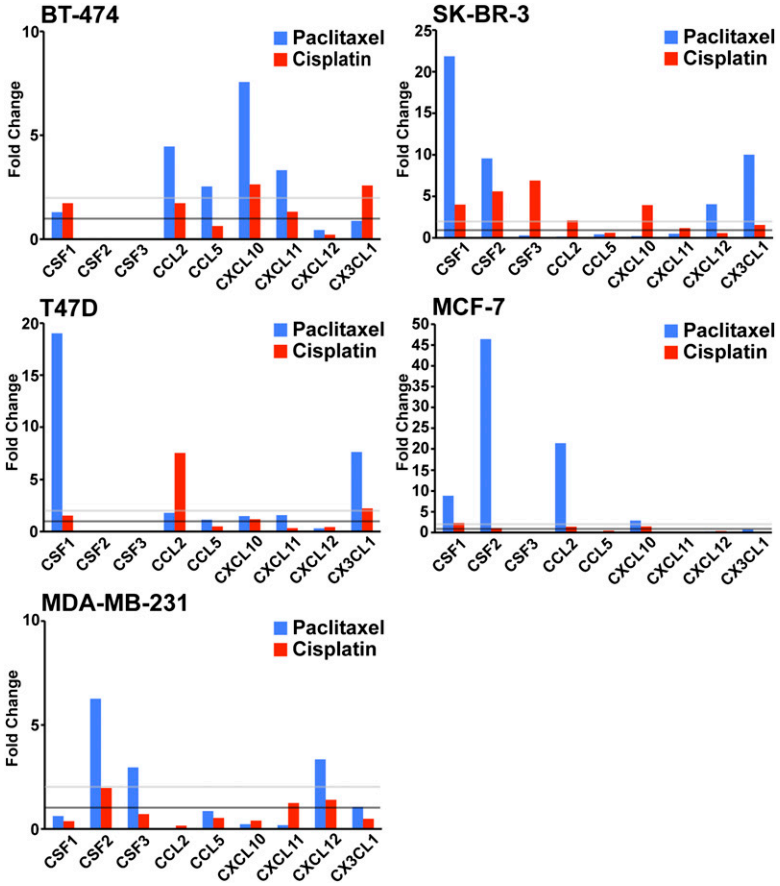

Fig. S6. CTX induces chemokine gene expression changes in human breast cancer cell lines. Relative gene expression compared with untreated controls after in vitro exposure to 25 nM paclitaxel or cisplatin for 24 h. Data represent an average of three replicates. Absence of bars for treated samples indicates undetectable gene expression in control. Fold change of 1× is shown with black horizontal bar, and 2× change is shown with gray horizontal bar.

Table S1. Patient information for evaluated tissue samples. ER, PR, and Her2 status is shown as grade, with percent positivity for ER/PR, and Her2 (evaluated by FISH) shown in parentheses

Age	Histology	Grade	ER	PR	Her2	Tumor size, cm	Node	Neoadjuvant Therapy
63	IDC	3	0 (0)	0 (0)	0 (1.1)	1.6	–	None
56	IDC	3	3+ (95)	3+ (90)	0	2.6	+	None
57	IDC	2	3+ (99)	2–3+ (25)	0	0.9	+	None
61	IDC	3	3+ (99)	3+ (60)	2+ (1.0)	4.8	+	None
78	IDC	3	3+ (30)	1–3+ (40)	3+	2.2	+	None
54	ILC	2	3+ (30)	3+ (30)	0	7.0	+	None
78	IDC	3	3+ (100)	0 (0)	2+ (1.0)	2.2	–	None
55	ILC	3	3+ (95)	0 (0)	2+ (1.6)	7.3	+	None
76	IDC	2	0 (0)	0 (0)	2+ (1.2)	5.1	–	None
65	IDC	2	1–3+ (90)	1–3+ (60)	0	9.0	+	None
74	IDC	2	1+ (1)	0 (0)	2+ (–)	0.8	+	None
65	ILC	2	3+ (100)	1–3+ (60)	0	1.1	–	Tamoxifen
59	ILC	2	3+ (100)	1+ (10)	0	7.0	+	None
71	ILC	1	3+ (95)	0 (0)	0	3.0	–	None
73	IDC	2	3+ (100)	2–3+ (50)	3+	9.8 (4.2)	+ (+)	Docetaxel, Carboplatin, Trastuzumab
66	IDC	3	0 (0)	0 (0)	0 (1.7)	10 (9.6)	+ (+)	Valproic Acid, 5-FU, Epirubicin, Cyclophosphamide
49	IDC	3	3+ (90)	0 (0)	2+ (2.3)	4.3 (4.3)	– (–)	Paclitaxol, Doxorubicin, Cyclophosphamide
39	IDC	2	3+ (95)	0 (0)	2+ (1.1)	8.0 (9.9)	+ (+)	Docetaxel, Doxorubicin, Cyclophosphamide
38	IDC	2	0 (0)	0 (0)	3+	3.5 (9.2)	+ (+)	Doxorubicin, Cyclophosphamide, Trastuzumab, Lapatinib
51	IDC	3	2+ (20)	0 (0)	2+ (1.0)	3.9 (4.2)	– (–)	Valproic Acid, 5-FU, Epirubicin, Anastrozole

Tumor size and node status are shown following neoadjuvant CTX in parentheses.

Table S2. Antibodies used for flow cytometry

Antigen	Clone	Fluorophore	Company	Catalog no.
CD3ε	OKT3	PerCP-eFluor 710	eBioscience	46-0037-42
CD3ε	OKT3	PerCP-Cy5.5	eBioscience	45-0037-42
CD4	RPA-T4	Qdot 655	Invitrogen	Q10007
CD4	RPA-T4	PE	BioLegend	300508
CD8α	RPA-T8	APC-eFluor 780	eBioscience	47-0088-42
CD8α	RPA-T8	Qdot 605	Invitrogen	Q10009
CD11b	ICRF44	PE-Cy7	eBioscience	25-0118-42
CD11c	3.9	Alexa 700	eBioscience	56-0116-73
CD14	61D3	Qdot 655	Invitrogen	Q10056
CD15	W6D3	Alexa 647	BioLegend	323012
CD19	HIB19	Alexa 700	eBioscience	56-0199-73
CD19	HIB19	PerCP-Cy5.5	eBioscience	45-0199-73
CD20	2H7	Alexa 700	BioLegend	302322
CD20	2H7	PerCP-Cy5.5	BioLegend	302326
CD25	BC96	PE-Cy5	BioLegend	302608
CD25	BC96	PE-Cy7	eBioscience	25-0259-42
CD27	O323	Alexa700	eBioscience	56-0279-73
CD28	CD28.2	PE-Cy7	eBioscience	25-0289-42
CD45	HI30	Qdot 705	Invitrogen	Q10062
CD45	HI30	APC-780	eBioscience	47-0459-42
CD45RA	HI100	Qdot 605	Invitrogen	Q10047
CD45RO	UCHL1	Alexa700	BioLegend	304218
CD49d	9F10	PE-Cy5	BioLegend	304306
CD56	HCD56	PE-Cy5	BioLegend	318308
CD56	HCD56	PerCP-Cy5.5	BioLegend	318322
CD69	FN50	FITC	eBioscience	11-0699-73
CD85g	17G10.2	APC	eBioscience	17-5179-42
CD86	IT2.2	PE-Cy5	eBioscience	15-0869-73
CD117	YB5.B8	PE	eBioscience	12-1179-42
CD127	eBioRDR5	650NC	eBioscience	95-1278-42
CCR4	TG6	PE-Cy7	BioLegend	335405
CCR5	HEK/1/85a	PE	BioLegend	313708
CCR7	TG8	Alexa 647	BioLegend	335603
CXCR3	TG1	Alexa 647	BioLegend	334903
FcεR1	AER-37	FITC	eBioscience	11-5899-73
FoxP3	PCH101	PE-Cy5	eBioscience	15-4776-42
HLA-DR	L243	eFluor450	eBioscience	48-9952-42
γδ TCR	B1.1	Alexa 647	BioLegend	331214
Vα24Jα18	6B11	PE	eBioscience	12-5806-42

Table S3. Antibodies used for immunohistochemistry and immunofluorescence

Antigen	Species	Dilution	Company	Catalog no.
CD3	Rabbit	1/150	Thermo Scientific	RM-9107-S1
CD4	Mouse	1/20	Thermo Scientific	MS-1528-S1
CD8	Mouse	1/100	Thermo Scientific	MS-457-S1
CD20	Mouse	1/50	Abcam	Ab9475
CD31 FITC	Mouse	1/10	BioLegend	303104
CD45	Mouse	1/100	eBioscience	14–0459-82
CD45 FITC	Mouse	1/10	BioLegend	304003
CD68	Mouse	1/100	Thermo Scientific	MS-397-P
CSF1R	Rabbit	1/100	Abcam	61137
Pan-keratin Alexa 488	Mouse	1/100	Cell Signaling	4523
FoxP3	Mouse	1/40	eBioscience	14-4777-82
Granzyme B	Mouse	1/50	Thermo Scientific	MS-1157-S1