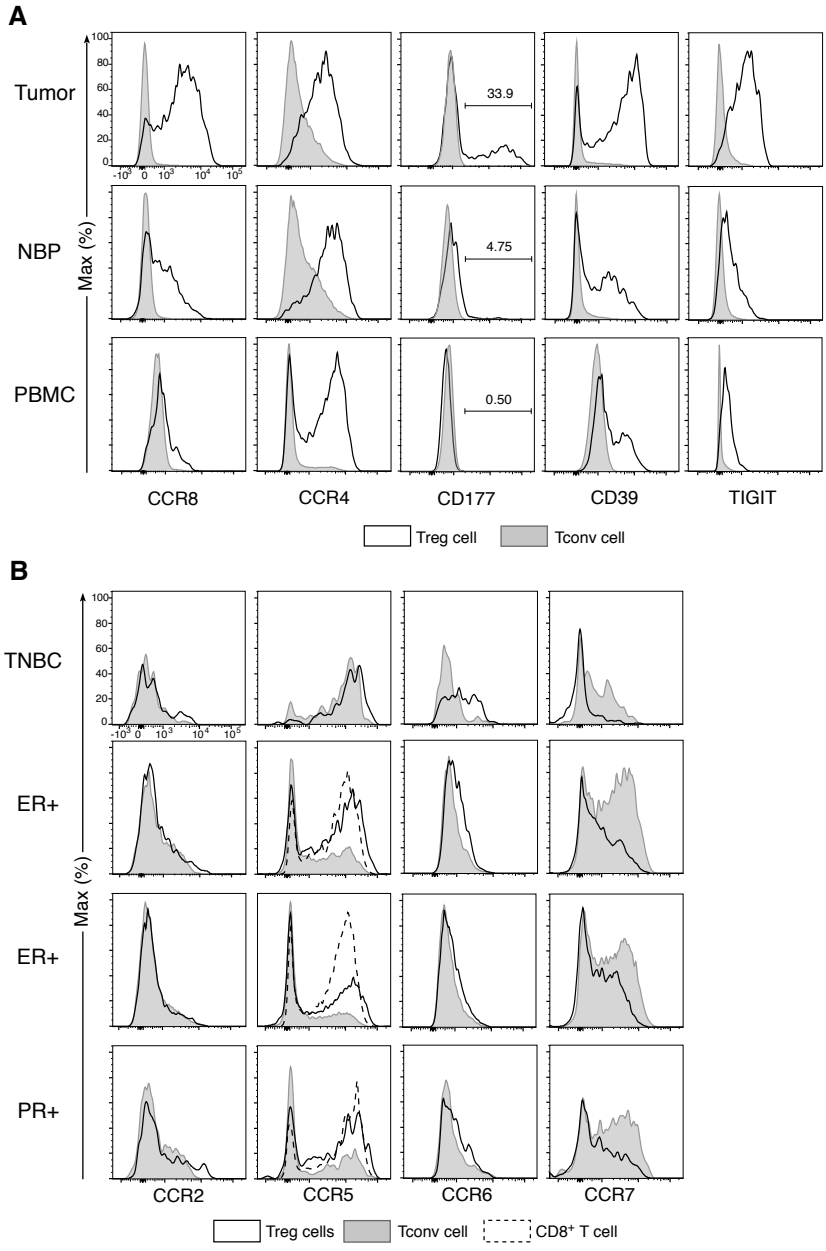
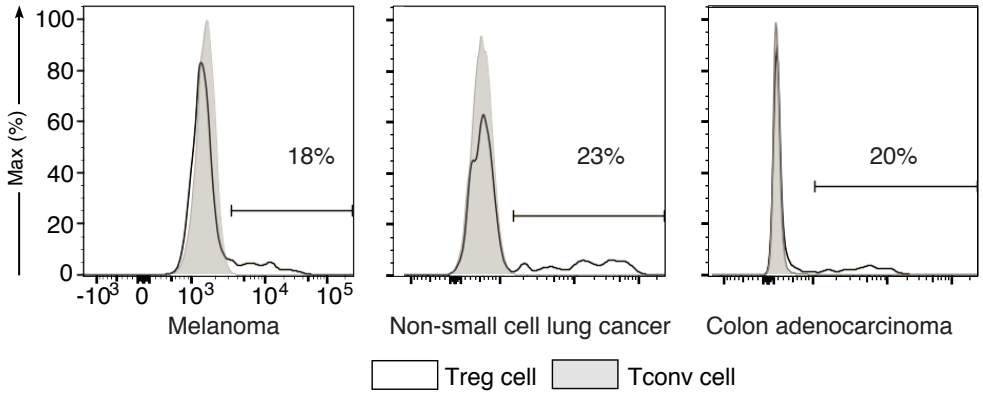
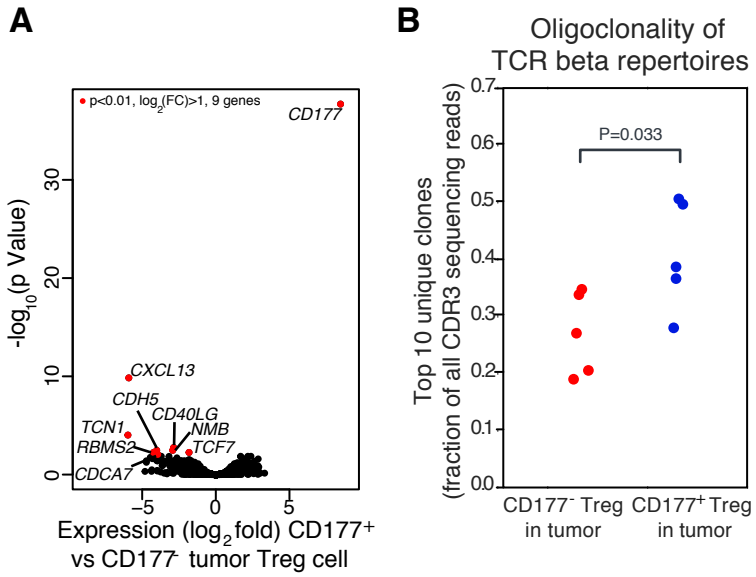
Supplemental Figures



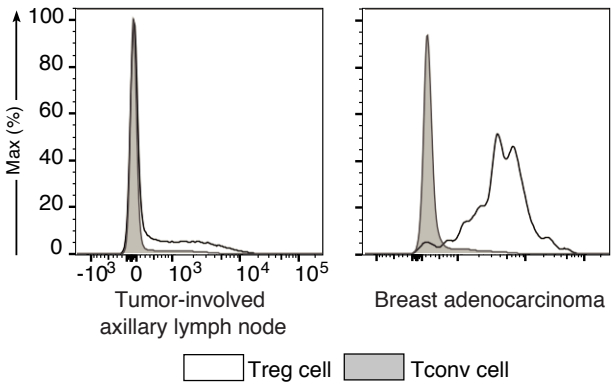
Supplemental Figure 1, related to Figure 4. Tumor Treg specific genes identified by RNA-seq are highly expressed by tumor Treg cells at the protein level.
(A) Expression of several genes found to be differentially expressed in tumor Treg cells by RNA-seq analysis was measured at the protein level by flow cytometry. MFI of genes gated on Treg cells (open histograms) and Tconv cells (shaded histograms) in breast tumor, NBP and peripheral blood. Plots represent data from individual specimens.
(B) Expression of chemokine receptors found to be differentially expressed in tumor Treg or Tconv cells relative to peripheral blood T cells by RNA-seq analysis was measured at the protein level by flow cytometry. MFI of staining for indicated chemokine receptors gated on Treg cells (open histograms), Tconv cells (shaded histograms) and $CD8^+$ T cells (dashed histograms) in ER+, PR+ and TNBC breast tumors.



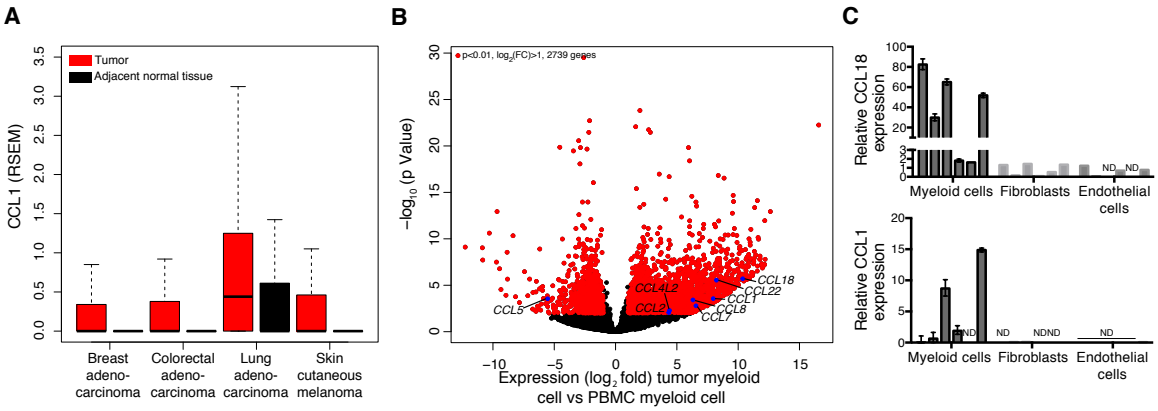
Supplemental Figure 2, related to Figure 4. CD177 is highly expressed by a subset of tumor Treg cells in several types of cancer. Flow cytometric analysis of CD177 expression by intratumoral T cells. The data are shown as MFI of CD177 staining of single cell suspension dissociated gated on Treg cells (open histograms) and Tconv cells (shaded histograms) in colorectal adenocarcinoma, melanoma and non-small cell lung cancer.



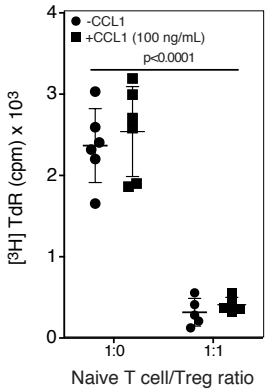
Supplemental Figure 3. related to Figure 5. Analysis of gene expression and TCR oligoclonality of intratumoral CD177$^+$ and CD177$^-$ Treg cells.
(A) Volcano plot comparing the p-value versus fold-change for gene expression in patient matched CD177$^+$ versus CD177$^-$ tumor resident Treg cells (n=5). Genes labeled in red are significantly differentially expressed (p<0.01).
(B) Frequency of the TCR beta CDR3 repertoire occupied by the 10 most abundant clones in CD177$^+$ and CD177$^-$ intratumoral Treg cells. P-value was calculated using a paired two-tailed Student's *t* test.



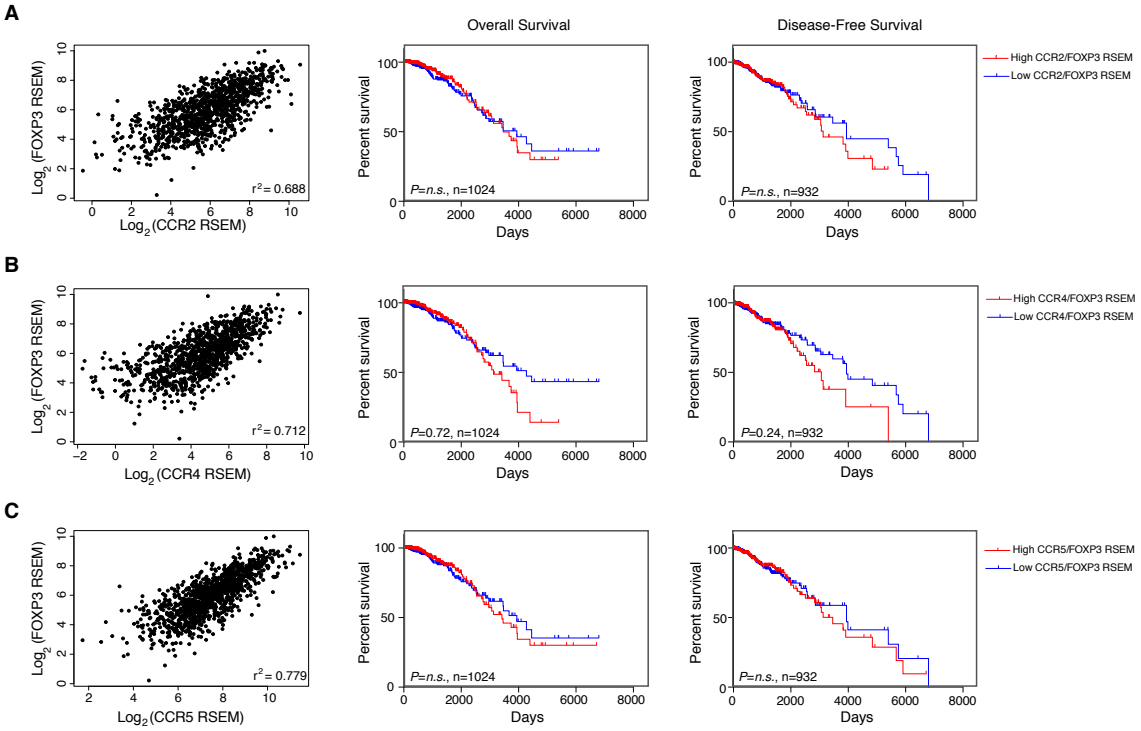
Supplemental Figure 4, related to Figure 6. CCR8 is expressed by a minor subset of Treg cells in axillary lymph nodes of stage III breast cancer patients.
Representative flow cytometric analysis of CCR8 expression by Treg cells in an involved axillary lymph node from a breast cancer patient. MFI of CCR8 gated on Treg cells (open histograms) and Tconv cells (shaded histograms). The results represent one of three independent experiments (three patients total).



Supplemental Figure 5, related to Figure 6. The CCR8 ligand CCL1 is expressed by CD11b$^+$CD14$^+$ myeloid cells in breast tumors.
(A) Boxplots comparing CCL1 normalized mRNA level in breast, colorectal and lung adenocarcinomas or melanomas versus adjacent normal tissues.
(B) Volcano plot comparing the p-value versus fold-change for genes from intratumoral CD11b$^+$CD14$^+$ myeloid cells relative to CD11b$^+$CD14$^+$ myeloid cells isolated from peripheral blood of healthy donors (n=7, n=4, respectively). Genes labeled in red are significantly differentially expressed (p<0.01).
(C) CD11b$^+$CD14$^+$ myeloid cells, CD90$^+$CD31$^-$ fibroblasts and CD31$^+$ endothelial cells were FACS purified from breast tumors and CCL1 and CCL18 mRNA levels were quantified by qPCR with RP2 as a reference gene and calibrated to expression in peripheral blood T cells.



Supplemental Figure 6, related to Figure 6. CCR8 signaling does not affect Treg cell suppressor function *in vitro*.
Tumor resident $CD4^{+}CD25^{hi}$ Treg cells were FACS isolated and their capacity to suppress *in vitro* proliferation of naïve Tconv cells induced by plate-bound CD3 and CD28 antibodies in presence of recombinant CCL1 *in vitro* was assessed by ^{3}H-thymidine incorporation on day 4 (n=3, plot is representative of two independent experiments).



Supplemental Figure 7, related to Figure 7. Ratio of chemokine receptor mRNA to Foxp3 mRNA does not correlate with survival outcome in the TCGA dataset.
Analysis of breast cancer TCGA data for relative expression of indicated genes normalized against Foxp3 mRNA expression. Spearman correlation (n=1024), overall survival (n=1024, Mantel-Cox test) and disease-free survival (n=932, Mantel-Cox test) of breast cancer patients in the TCGA dataset stratified by median tumor (A) CCR2/FOXP3, (B) CCR4/FOXP3, (C) CCR5/FOXP3 normalized mRNA ratios.

Supplemental Tables

Variable		N=105
Age (median)		50 (24-88)
Tumor Stage	T1 T2 T3	52 (49.5%) 50 (47.6%) 3 (2.9%)
Nodal Stage	N0 N1 N2 N3	71 (67.6%) 28 (26.7%) 2 (1.9%) 4 (3.8%)
Subtype	ER+ Her2+ TNBC	78 (74.3%) 10 (9.5%) 19 (18.1%)
Tumor Grade	1 2 3	8 (7.6%) 38 (36.2%) 59 (56.2%)

Supplemental Table 1, related to Figure 1. Clinical characteristics of the patients whose tumor specimens were used in this study. Description of patient age, tumor stage, nodal stage, cancer subtype and tumor grade for each patient.

Supplemental Table 2, related to Figure 4. Differential gene expression analysis of T cells in breast tumor and peripheral blood. Differential gene expression tables comparing tumor-associated Treg cells, tumor-associated Tconv cells, activated Treg cells from PBMC and activated Tconv cells from PBMC.

Supplemental Table 3, related to Figure 4. Differential gene expression analysis of T cells in breast tumor and normal breast parenchyma. Differential gene expression tables comparing tumor-associated Treg cells, tumor-associated Tconv cells, NBP-associated Treg cells and NBP-associated Tconv cells.

Supplemental Table 4, related to Figure S3. Differential gene expression analysis of CD177 expressing Treg cells. Differential gene expression table comparing tumor-associated $CD177^+$ Treg cells to tumor-associated $CD177^-$ Treg cells.

Supplemental Table 5, related to Figure S5. Differential gene expression analysis of breast tumor-associated myeloid cells. Differential gene expression table comparing tumor-associated $CD11b^+CD14^+$ myeloid cells to cells to $CD11b^+CD14^+$ myeloid cells from PBMC.

Supplemental Experimental Procedures

In vitro assays

For in vitro induction assays, $CD4^+CD25^{hi}$ Treg cells were isolated from PBMC and cultured in 24-well flat-bottom plates in presence of CD3 and CD28 antibody-coated beads at a 1:1 bead:cell ratio in RPMI1640 supplemented as above. For induction of CCR8 by tumor and NBP slices, 0.4 μM Transwell inserts were placed in each well and thin tumor or NBP slices were laid onto the Transwell surface (Corning). After 120 hr, CCR8 expression was assessed by qPCR as described in Supplemental Experimental Procedures. For induction of CCR8 cells were cultured

in 96-well U bottom plates without or with 1000U/mL recombinant human IFNα (R&D Systems) and CCR8 expression was assessed by flow cytometry.

Quantitative RT-PCR

RNA was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. The isolated RNA was used to synthesize cDNA with the qScript cDNA SuperMix (Quanta-bio). SYBR Green PCR Master Mix (Life Technologies) was used for quantitative RT-PCR. PCRs were performed in triplicate. The following primers were used: CCR8 forward, 5′-GGGCTTGAGAAGATATCAGGG-3′ and reverse, 5′-TCCAGAACAAAGGCTGTCACT-3′; CCL-1 forward, 5′-TTAAGCCCTCATTGGAGCAG-3′ and reverse, 5′-AGATGTGGACAGCAAGAGCA-3′; CCL-18 forward, 5′- GTGGAATCTGCCAGGAGGTA-3′ and reverse, 5′-TCCTTGTCCTCGTCTGCAC-3′; RP2 forward, 5′-CCCATTAAACTCCAAGGCAA-3′ and reverse, 5′-AAGCTGAGGATGCTCAAAGG-3′; ADAR forward, 5′-GGTGGCCTTTTGGAGTACG-3′ and reverse, 5′-CCAACTTTTGCTTGGTAAACG-3′.

RNA Sequencing

Total RNA was extracted and assessed for nucleic acid quantity and quality on the Agilent BioAnalyzer. For the first set of tumor Treg, Tconv and PBMC Treg and Tconv samples, after SMARTer amplification, total RNA was used for poly(A) selection and was used to create Ion Torrent compatible libraries using the Ion ChIP-Seq Kit starting with the end-repair process (Life Technologies), with 12 to 16 cycles of PCR. The resulting barcoded samples were loaded onto template-positive Ion PI™ Ion Sphere™ Particles (ISPs) using the Ion One Touch system II and Ion PI™Template OT2 200kit v2 Kit (Life Technologies). Enriched particles were sequenced on a Proton sequencing system using the 200bp version 2 chemistry. On average of 70 to 80 million single-end reads were generated per sample. For the second set of tumor Treg, Tconv and PBMC Treg and Tconv samples, SMARTer amplification with 12-16 cycles of PCR was followed by Illumina TruSeq paired-end library preparation following manufacturer's protocols. Samples were sequenced on the Illumina HiSeq 2500 to an average depth of 20 million 50-bp read pairs per sample.

Read alignment and processing followed the method described by Anders *et al* (Anders et al., 2013). Briefly, raw reads were trimmed using Trimmomatic v0.32 with standard settings to remove low-quality reads and adaptor contamination (Bolger et al., 2014). The trimmed reads were then aligned to the human genome (Ensembl assembly GRCh38) using TopHat2 v2.0.11 implementing Bowtie2 v2.2.2 with default settings (Kim et al., 2013; Langmead and Salzberg,

2012). Read alignments were sorted with SAMtools v0.1.19 before being counted to genomic features using HTSeq v0.6.1p1 (Anders et al., 2013; Li et al., 2009).

Bioinformatic Analyses

Differential gene expression was analyzed using DESeq2 1.12.3 in R version 3.3.1 (Love et al., 2014). Significantly up- and down-regulated genes in all comparisons were defined as expressed genes (threshold of 50 normalized reads) with FDR-adjusted *p*-value ≤ 0.01 and fold change of at least 2x.

To determine transcriptional signatures, the GOrilla tool for discovery of enriched GO terms in ranked gene lists was used (Eden et al., 2009). The target gene set was defined as significantly up-regulated expressed genes with FDR adjusted *p*-value ≤ 0.05 and was compared against a background set of genes defined as genes with average expression across all samples of at least 20 normalized reads.

TCR alpha and beta CDR3 repertoires were extracted from RNA-Seq data using MiXCR software (Bolotin et al. 2015) in RNA-Seq mode. Comparative post-analysis of TCR repertoires was performed using VDJtools software (Shugay et al., 2015).

Supplemental References

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