Supplemental Experimental Procedures

Parabiosis

Parabiotic mice were generated as reported (Franklin et al., 2014) using 5- to 6-week-old age- and weight-matched $CD45.1^+$, $CD45.1^+CD45.2^+$ or $CD45.2^+$ PyMT female mice and maintained for 2 weeks post-surgery. Briefly, matching skin incisions were made from the elbow to the knee of each mouse. Forelimb and hindlimb connections were made with sutures and skin incisions were closed using woundclips. Tissues were isolated for analysis from the unconnected side of each parabiotic partner.

Immune cell isolation

Mammary gland-, prostate- or tumor-associated lymphocytes were isolated as previously described (Franklin et al., 2014). Mammary glands, prostate tissues and tumors were prepared by mechanical disruption using a razor blade followed by treatment with 280 U/ml Collagenase Type 3 (Worthington Biochemical) and 4 µg/ml DNase I (Sigma) at 37°C for 1 h with periodic short vortexing. Digested tissues were mashed through 70-µm filters, and collected by centrifugation. Cell pellet was resuspended in 44% Percoll, layered on top of 66% Percoll (Sigma), and centrifuged at 1,900 *g* for 30 min without brake. Cells at the Percoll interface were collected, stained with antibodies and analyzed by flow cytometry. Single-cell suspensions of spleen and lymph nodes were obtained by homogenization between the frosted ends of two histological slides and filtered through 70-µm filters.

Flow cytometry

For flow cytometry experiments, cells were pre-incubated with 2.4G2 mAb to block FcγR binding, and were stained with panels of antibodies. Fluorochrome-conjugated or biotinylated antibodies against CD28 (37.51), CD45.1 (A20), CD5 (53-7.3), CD103 (2E7), CD127 (A7R34), CD3 (17A2), CD49b (DX5), Eomes (Dan11mag), ICOS (C398.4A), Ly49E/F (CM4), Ly49G2 (4D11), Ly49H (3D10), NK1.1 (PK136), NKG2A/C/E (20d5), NKG2D (CX5), Nkp46 (29A1.4), PD-1 (RMP1-30), T-bet (4B10), TCR-β (H57-597), TCR-δ (GL3), TRAIL (N2B2) and TNF-α (TN3-19) were purchased from eBioscience. Antibodies against CD122 (TM-b1), CD45 (30-F11), CD49a (Ha31/8), Ki67 (B56), Ly49C/I (5E6), Vα2 (B20.1), Vα3.2 (RR3-16), Vα8.3 (B21.14), Vα11.2 (RR8-1), Vβ2 ((B20.6), Vβ3 (KJ25), Vβ4 (KT4), Vβ5 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8 (F23.1), Vβ9 (MR10-2), Vβ10.b (B21.5), Vβ11 (RR3-15), Vβ12 (MR11-1), Vβ13 (MR12-3), Vβ14 (14-2) and Vβ17 (KJ23) were purchased from BD Pharmingen. Antibodies against CD4 (GK1.5), CD8α (53-7.3), CD45.2 (104) and IFN-γ (XMG1.2) were purchased from Tonbo. Antibodies against CD244 (m2B4), Ly49A (YEI/48.10.6) and Ly49D (4E5) were purchased from BioLegend. Antibody against GzmB (GB11) was purchased from Invitrogen. Antibody against Ly49F (HBF-F19) was purchased from Miltenyi Biotec. All antibodies were tested with their respective isotype controls. Fluorochrome-conjugated CD1d/PBS-57 tetramer was supplied by the NIH Tetramer Core Facility. Cells were incubated with specific antibodies and a LIVE/DEAD kit (Invitrogen) for 30 min on ice in the presence of 2.4G2 mAb to block FcγR binding. To determine IFN-γ and TNF-α expression, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma), 1 µM ionomycin (Sigma) and GolgiStop (BD Biosciences) for 4 h and stained with cell-surface markers after stimulation. GzmB, IFN-γ and TNF-α staining were carried out using the intracellular cytokine staining cytofix/cytoperm kit from BD Pharmingen. Ki67, T-bet and Eomes staining were carried out using the intracellular transcription factor buffer set from BD Pharmingen. Incorporation of EdU was measured using the Click-iT EdU flow cytometry assay kit according to the manufacturer's instructions (Invitrogen). Mice were injected *i.p.* with 50 µg per g of body weight of EdU (resuspended in PBS) and sacrificed 20 h after injection. All samples were acquired with a LSRII flow cytometer (Becton Dickinson) and analyzed with FlowJo software version 9.6.2 (Tree Star).

Cell sorting

After gating on morphology and singulets, $CD45^+$ cells were gated as follow: $TCR\alpha\beta^+CD8\alpha^+PD\text{-}1^+$ ($TCR\beta^+TCR\delta^-CD1d/PBS\text{-}57^-CD4^-CD8\alpha^+PD\text{-}1^+NK1.1^-$), $TCR\alpha\beta^+CD8\alpha^+PD\text{-}1^-NK1.1^-$ ($TCR\beta^+TCR\delta^-CD1d/PBS\text{-}57^-CD4^-CD8\alpha^+PD\text{-}1^-NK1.1^-$), $TCR\alpha\beta^+CD8\alpha^+NK1.1^+$ ($TCR\beta^+TCR\delta^-CD1d/PBS\text{-}57^-CD4^-CD8\alpha^+PD\text{-}1^-NK1.1^+$), $TCR\alpha\beta^+CD8\alpha^-NK1.1^+$ ($TCR\beta^+TCR\delta^-CD1d/PBS\text{-}57^-CD4^-CD8\alpha^-NK1.1^+$), $TCR^-NK1.1^+$, ILC1l ($TCR^-NK1.1^+CD49a^{hi}$), cNK ($TCR^-NK1.1^+CD49a^-$), $TCR\alpha\beta^+$ ILTC1 ($TCR\beta^+TCR\delta^-CD1d/PBS\text{-}57^-NK1.1^+CD49a^{hi}$), $TCR\alpha\beta^+CD8\alpha^+NK1.1^-$ ($TCR\beta^+TCR\delta^-CD1d/PBS\text{-}57^-CD4^-CD8\alpha^+NK1.1^-$), $TCR\gamma\delta^+$ ILTC1 ($TCR\beta^-TCR\delta^+CD1d/PBS\text{-}57^-NK1.1^+CD49a^{hi}$), $TCR\gamma\delta^+NK1.1^-$ ($TCR\beta^-$

TCRδ^{+}CD1d/PBS-57^{-}NK1.1^{-}) cells were isolated from pooled tumors of 20- to 24-week-old PyMT mice or Perforin$^{-/-}$ PyMT mice and sorted using FACS Aria-II machines (Becton Dickinson) at the Flow Cytometry Core Facility at MSKCC.

Cytospin
FACS sorted cells were loaded on Superfrost Plus slides (Fisher Scientific) using the Cytospin 4 Cytocentrifuge (ThermoScientific) and air dried for 24 h. Dried slides were stained with Hematoxylin and Eosin. Images were captured at an original magnification of 60x using an oil objective with Zeiss Axioplan 2 widefield microscope.

Tumor measurement
Tumors of PyMT mice were measured weekly using a caliper, beginning when a single tumor diameter reached approximately 3-4 mm. Tumor volume was calculated using the equation [(L x W^2) x (π/6)] where "L"=length and "W"=width. Individual tumor volumes were added together to calculate total tumor burden per mouse. For Tramp mice, whole mice bodies were weighed after sacrifice, the urogenital tract (UG) were weighed after the bladder had been emptied, and UG/Body x100 was used to score the tumor burden.

Pathology scoring
Mammary glands from euthanized animals were fixed in Safefix II (Protocol) and embedded in paraffin. 5-mm sections were stained with haematoxylin and eosin. Tumor stages were blindly scored by a pathologist at MSKCC Pathology Core Facility.

Single cell killing assay
Sorted effector cell populations were cultured for 3-4 days to recover from stress associated with cell sorting in T cell medium [RPMI supplemented with 10% FBS, 1 mM sodium pyruvate, non-essential amino acids (Gibco), 10 mM Hepes, 55 μM 2-Mercaptoethanol, 100 U/ml Penicillin G and 0.1 mg/ml Streptomycin]. For ILC1l, cNK, TCRαβ^{+} ILTC1 and TCRγδ^{+} ILTC1, T cell medium was supplemented with human IL-2 (1,000 U/ml) and human IL-15/IL-15Rα complex (75 ng/ml). For TCRαβ^{+}CD8α^{+}NK1.1^{-} and TCRγδ^{+} NK1.1^{-} T cells, T cell medium was supplemented with human IL-2 (200 U/ml) and anti-CD3/CD28/4-1BB beads (Invitrogen). Polydimethylsiloxane grids containing 50x50x50 μm^3 wells were submerged in imaging medium (T cell medium without phenol red) supplemented with 100 U/ml recombinant human IL-2, and were seeded with AT3 cells (Stewart and Abrams, 2007) labeled with the Cell-trace Violet dye (Invitrogen) to facilitate their identification. In general, individual well contained 1 to 3 AT3 cells. 1 μg/ml propidium iodide (PI) was added to the medium to enable real-time labeling of dead cells. Effector cells were added, and were imaged using a 20x objective lens (Carl Zeiss) at a 10-min interval for 10 to 11 hours. Brightfield, cell trace violet and PI images were collected at each time point. Killing efficiency was defined by number of wells with PI signal over total number of wells, normalized to background cell death scored in wells containing AT3 cells only. Quantification was restricted to wells containing one effector cell and AT3 cell(s) in the same well. Only a single killing event per well was scored.

Cytotoxicity assay
For killing of RMA-s cells, the target cells were labeled with the membrane dye PKH26 (Sigma-Aldrich) and mixed in 96-well plates with effector lymphocyte populations at effector to target ratios at 10:1, 3:1 and 1:1. The cells were incubated at 37 ºC for 6 h. 2.5 mM EDTA was added to disrupt conjugate formation. Specific lysis of PKH26^{+} target cells was assessed by flow cytometry as previously described (Purbhoo et al., 2004).

Quantitative real-time PCR
Cells were sorted directly in Tri-Reagent (Sigma). RNA was prepared using RNeasy Mini Kit, including on-column DNase digestion with the RNase-Free DNase Set according to the manufacturer's instructions (Qiagen). Complementary DNA (cDNA) was synthesized using SuperScript First-Strand Synthesis kit (Invitrogen) and qPCR was carried out with the QuantiTect SYBR Green PCR Kit (Qiagen) using the Mx3005P qPCR machine (Stratagene). The mRNA levels of *Klf2, S1pr1* and *Gapdh* were determined by qPCR using the following primers (Lee et al., 2015): *Klf2*: forward 5'-ACCAACTGCGGCAAGACCTA-3', and reverse 5'-CATCCTTCCCAGTTGCAATGA-3'; *S1pr1*: forward 5'-

GTGTAGACCCAGAGTCCTGCG-3', and reverse 5'-AGCTTTTCCTTGGCTGGAGAG-3'; *Gapdh*: forward 5'-TGGCCTACATGGCCTCCA-3' and reverse 5'-TCCCTAGGCCCCTCCTGTTAT-3'. Relative expression was determined using the delta Ct method.

RNAseq and transcriptome analysis

Cells were sorted directly in Tri-Reagent (Sigma). RNA was prepared, complementary DNA (cDNA) libraries were amplified using the SMARTer RACE Amplification Kit (Clontech), and were sequenced in replicate for 40 million reads using 50 bp paired-end with Ion Proton Instrument at the Integrated Genomics Operation Core Facility at MSKCC. Two RNA-seq data sets corresponding to different cell sorting strategies (Table S1, Table S2 and Table S3) were processed separately in the same manner. The raw output BAM files were converted to FASTQ using PICARD Sam2Fastq. Reads were then trimmed using fastq_quality_trimmer with default settings. The trimmed reads were first mapped to the mouse genome using rnaStar (Dobin et al., 2013) (version 2.3.0e). The genome assembly mm9 was used with junctions from ENSEMBL (Mus_musculus.NCBIM37.67) and a read overhang of 49. Then any unmapped reads were mapped to mm9 using BWA MEM (Li and Durbin, 2009) (version 0.7.5a). The two resulting BAM files were merged and gene level counts were computed using htseq-count (Anders et al., 2015) (options -s y -m intersection-strict) and the same gene models (Mus_musculus.NCBIM37.67). Within each batch, a gene was filtered out from further analysis of this batch if the mean of its RPKM (reads per kilobase per million) values across all samples was less than 2 or its TPM (tags per million) value was less than 2 in at least 5/6 of the samples. This resulted in 10,262 expressed genes for [$TCR\alpha\beta^{+}CD8\alpha^{-}NK1.1^{+}$, $TCR\alpha\beta^{+}CD8\alpha^{+}NK1.1^{+}$, $TCR\alpha\beta^{+}CD8\alpha^{+}PD\text{-}1^{-}NK1.1^{-}$, $TCR\alpha\beta^{+}CD8\alpha^{+}PD\text{-}1^{+}$, and $TCR^{-}NK1.1^{+}$ cell populations; (set 1)] presented in Table S1; and 10,702 expressed genes for [ILC1l, cNK, $TCR\alpha\beta^{+}$ ILTC1, $TCR\alpha\beta^{+}CD8\alpha^{+}NK1.1^{-}$, $TCR\gamma\delta^{+}$ ILTC1 and $TCR\gamma\delta^{+}NK1.1^{-}$ cell populations (set 2)] presented in Table S2 and Table S3 that went into further analysis. To perform a multi-factor differential gene expression analysis, samples were annotated based on markers used in FACS (See tables below). Variables corresponding to markers (NK1.1, TCRβ and PD-1 for gene expression analysis of set 1, Table S1; and NK1.1, TCRβ, TCRγδ, CD49a for gene expression analysis of set 2, Table S3 were assigned binary values across samples according to the sorting strategy in order to define factors for the analysis. DESeq2 (Love et al., 2014) (version 1.6.3) was then used to fit a full generalized linear model within each set (~ NK1.1 + TCRβ + PD-1 for set 1 and ~ NK1.1 + TCRβ + TCRγδ + CD49a for set 2). Then for each factor within the model, genes differentially expressed with respect to this factor (absolute $\log_2$ fold change > 1 and Benjamini-Hochberg adjusted $P < 0.001$), as estimated using a Wald test implemented in DESeq2, were extracted. Differentially expressed genes obtained in this manner were used for further analysis and visualization.

Heatmaps were generated using regularized log (rlog) transformed counts with the DESeq2 (Love et al., 2014) package and pheatmap (Kolde), for differentially expressed genes obtained for factors shown on the left side of the heatmap. Unsupervised hierarchical clustering was performed using hclust with Euclidean distance (method = "complete") as implemented in pheatmap (Kolde). Clusters were then extracted using the "cutree" function and used for Gene Ontology (GO) enrichment analysis. The GOstats (Falcon and Gentleman, 2007) (version 2.32.0) package was used for GO analysis using all genes expressed in the batch (as described above) as the background, and significantly enriched terms were reported based on FDR-adjusted $P < 0.01$. Principal component analysis (PCA) was performed on rlog transformed counts of all differentially expressed genes (for any of the factors of the model) within a batch using the prcomp package (with parameters center = TRUE, scale. = TRUE). All heatmaps and PCA plots were plotted using the ggplot2 package (version 1.0.1). All analysis after count table generation was conducted in the R statistical environment (Team, 2008) (version 3.1.3). Differential expression analysis between $TCR^{-}NK1.1^{+}CD49a^{hi}$ and $TCR^{-}NK1.1^{+}CD49a^{-}$ samples in RNA-seq set 2 was performed on expressed genes in this set using DESeq2 with default settings. The log2-transformed gene expression changes from this analysis were plotted for the core innate lymphoid cell class 1 (ILC1) and conventional natural killer cell (cNK) signature genes defined by Robinette et al. (Robinette et al., 2015) (as obtained from their Supplementary Table 5).

Multi-factor differential gene expression analysis matrix used to analyze the set 1 RNAseq sata shown in Figure S2G and Table S1

	Sample	Condition	TCRβ	NK1.1	PD-1
1	$TCR^{-}NK1.1^{+}\_1$	$TCR^{-}NK1.1^{+}$	0	1	0
2	$TCR\beta^{+}CD8\alpha^{-}NK1.1^{+}\_1$	$TCR\beta^{+}CD8\alpha^{-}NK1.1^{+}$	1	1	0
3	$TCR\beta^{+}CD8\alpha^{+}NK1.1^{+}\_1$	$TCR\beta^{+}CD8\alpha^{+}NK1.1^{+}$	1	1	0
4	$TCR\beta^{+}CD8\alpha^{+}PD1^{+}\_1$	$TCR\beta^{+}CD8\alpha^{+}PD1^{+}$	1	0	1
5	$TCR\beta^{+}CD8\alpha^{+}PD1^{-}NK1.1^{-}\_1$	$TCR\beta^{+}CD8\alpha^{+}PD1^{-}NK1.1^{-}$	1	0	0
6	$TCR^{-}NK1.1^{+}\_2$	$TCR^{-}NK1.1^{+}$	0	1	0
7	$TCR\beta^{+}CD8\alpha^{-}NK1.1^{+}\_2$	$TCR\beta^{+}CD8\alpha^{-}NK1.1^{+}$	1	1	0
8	$TCR\beta^{+}CD8\alpha^{+}NK1.1^{+}\_2$	$TCR\beta^{+}CD8\alpha^{+}NK1.1^{+}$	1	1	0
9	$TCR\beta^{+}CD8\alpha^{+}PD1^{+}\_2$	$TCR\beta^{+}CD8\alpha^{+}PD1^{+}$	1	0	1
10	$TCR\beta^{+}CD8\alpha^{+}PD1^{-}NK1.1^{-}\_2$	$TCR\beta^{+}CD8\alpha^{+}PD1^{-}NK1.1^{-}$	1	0	0

Multi-factor differential gene expression analysis matrix used to analyze the set 2 RNAseq sata shown in Figure S4B and Table S3

	Sample	Condition	NK1.1	TCRβ	TCRγδ	CD49a
1	ILC1l_1	ILC1l	1	0	0	1
2	cNK_1	cNK	1	0	0	0
3	$TCR\gamma\delta^{+}$ILTC1_1	$TCR\gamma\delta^{+}$ILTC1	1	0	1	1
4	$TCR\alpha\beta^{+}$ILTC1_1	$TCR\alpha\beta^{+}$ILTC1	1	1	0	1
5	$TCR\gamma\delta^{+}NK1.1^{-}\_1$	$TCR\gamma\delta^{+}NK1.1^{-}$	0	0	1	0
6	$TCR\beta^{+}CD8\alpha^{+}NK1.1^{-}\_1$	$TCR\beta^{+}CD8\alpha^{+}NK1.1^{-}$	0	1	0	0
7	ILC1l_2	ILC1l	1	0	0	1
8	cNK_2	cNK	1	0	0	0
9	$TCR\gamma\delta^{+}$ILTC1_2	$TCR\gamma\delta^{+}$ILTC1	1	0	1	1
10	$TCR\alpha\beta^{+}$ILTC1_2	$TCR\alpha\beta^{+}$ILTC1	1	1	0	1
11	$TCR\gamma\delta^{+}NK1.1^{-}\_2$	$TCR\gamma\delta^{+}NK1.1^{-}$	0	0	1	0
12	$TCR\beta^{+}CD8\alpha^{+}NK1.1^{-}\_2$	$TCR\beta^{+}CD8\alpha^{+}NK1.1^{-}$	0	1	0	0

Supplemental References

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