Supplementary Information for

Hobit confers tissue dependent programs to type 1 innate lymphoid cells

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***SI Appendix, Material and Methods*.**

Generation of *Zfp683* reporter mice. *Zfp683* reporter mice were generated by homologous gene targeting in C57BL/6 ES cells. The plasmid containing rabbit β-globin gene partial exon 2-3, tdTomato, IRES, humanized Cre recombinase, bovine growth hormone poly A and a loxP-flanked neomycin cassette was kindly provided by Richard Locksley. Homologous arms were amplified using C57BL/6 genomic DNA. Clones with successful targeting were confirmed by Southern blot analysis and the neomycin cassette was removed by inter-crossing with CMV-Cre mice purchased from the Jackson Laboratory.

Generation of *Zfp683*$^{-/-}$ mice. Three JM8A3.N1 (C57BL/6N-A^{tm1Brd}) ES clones (Zfp683_A06, Zfp683_A07, Zfp683_H06) carrying the targeted allele *Zfp683*$^{tm1a(KOMP)Wtsi}$ were purchased from the European Conditional Mouse Mutagenesis Consortium (EUCOMM). Targeted integration of each clone was confirmed by Southern blot analysis. The FRT-flanked neomycin cassette was removed crossing to B6.129S4-*Gt(34)26Sor*$^{tm1(FLP1)Dym}$/RainJ mice purchased from the Jackson Laboratory.

Mice. *Ncr1*-Cre mice were kindly provided by Eric Vivier and *Rorc*^{Cre} mice were kindly provided by Gerard Eberl. B6.129X1-*Gt(34)26Sor*$^{tm1(EYFP)Cos}$/J (YFP expression is prevented by the loxP-flanked stop cassette and this construct is inserted to locus encoding *Rosa26*) and B6 Cg-Gt(34)26Sor $^{m14(CAG-tdTomato)Hze}$/J (CAG promoter driven tdTomato expression is prevented by loxP-flanked stop cassette and this construct is inserted to locus encoding *Rosa26*) were purchased from the Jackson Laboratory. All

animal studies were approved by the Washington University Institutional Animal Care and Uses Committee.

Tissue processing. Mice were perfused with 1X PBS by cardiac puncture prior to harvesting tissues. Single-cell suspension of spleen and liver were obtained by pushing tissues through 70μm filters. Salivary glands were first finely minced, then digested with collagenase D for 15 minutes at 37C with shaking. Digested contents were filtered through 70μm filters and subjected to density gradient using 40% and 70% Percoll. Lamina propria lymphocytes were isolated as previously described (1). Briefly, small intestines were flushed and Peyer's patches were removed. Then, small intestines were sliced and agitated in HBSS, HEPES, EDTA and FCS followed by collagenase IV digestion. Digested contents were filtered through 70μm filters and subjected to density gradient using 40% and 70% Percoll. Intestine intraepithelial lymphocytes were isolated as previously described (1).

Flow cytometry and antibodies. Single cell suspensions were incubated with Fc block for 15 minutes then stained with antibodies. For intracellular cytokine staining, BD Bioscience Fixation/Permeabilization Solution kit was used per manufacture instruction. For transcription factor staining, True-Nuclear Transcription factor staining kit was used per manufacture instruction. When *Zfp683*-tdTomato cells were fixed and permeabilized for transcription factor staining, tdTomato fluorescence was lost. Therefore, tdTomato was stained with anti-RFP followed by PE-anti Rabbit IgG. Data were acquired on BD FACSSymphony and analyzed using FlowJo.

The following antibodies were used: anti-CD3e (145-2C11, Biolegend), anti-CD19 (6D5, Biolegend), anti-NK1.1 (PK146, Biolegend), anti-CD127 (A7R34, Biolegend), anti-NKp46 (29A1.4, Biolegend), anti-CD49b (DX5, Biolegend), anti-CD49a (HMa1, Biolegend), anti-IFNγ (XMG1.2, Biolegend), anti-granzyme B (GB11 BG/IL18RA), anti-Eomes (Dan11mag, eBioscience), anti-TCF7 (C63D9, Cell Signaling Technology), anti-RFP (rabbit polyclonal, ROCKLAND), anti-rabbit IgG (Donkey Polyclonal Ig, Biolegend).

MCMV infection. Mice were infected with salivary gland stock MCMV (5 x 10^3 plaque-forming units) intraperitoneally (i.p).

DNA analysis

DNA from mouse tissue was prepared using the QIAamp DNA Mini Kit (Qiagen) per manufacturer recommendations. DNA was adjusted to ~200ng/mL for qPCR analysis. qPCR was performed using TaqMan Universal PCR Master Mix (Life Technologies) and primers for MCMV IE1 (FW: 5'-TGGTGCTCTTTTCCCGTG-3', 5'-FAM, 3'Iowa Black, ZEN quencher Probe: 5'-TCTCTTGCCCCGTCCTGAAAACC-3', Rev: 5'CCCTCTCCTAACTCTCCCTT-3') and beta-actin (FW: 5'GGTGGGAATGGGTCAGAAG-3', 5'-FAM, 3'Iowa Black, ZEN quencher Probe: 5'TTCAGGGTCAGGATACCTCTCTTGCT-3', Rev: 5'-AGCTCATTGTAGAAGGTGTGG-3'). qPCR was completed on the AppliedBiosystems StepOne Plus qPCR instrument (Life Technologies). Reactions were performed in technical duplicate and quantified using a standard curve generated for IE1 and beta-actin, then used to calculate the ratio of IE1 DNA copies/beta-actin DNA copies.

NK cell stimulation. Isolated lymphocytes from spleen, liver, IELs were made into single-cell suspensions, followed by the addition of IL-12 (10 ng/ml) and IL-18 (10 ng/ml). After 1 hour, Brefeldin A (BD Golgi-Plug) was added to each well per manufacture's instruction. Stimulation was allowed for total 6 hours prior to staining for flow cytometry.

NK cell *in vitro* culture. NK cells were isolated from *Zfp683*^{Red} mice using EasySep Mouse NK Cell Isolation Kit (STEMCELL) per manufacture's instruction. On average, more than 90% of purity were obtained. Isolated NK cells were cultured in complete RPMI medium with IL-2 (100 ng/ml), IL-15 (50 ng/ml), IL-12 (10 ng/ml), IFNα (1000 units/ml) or TGFβ (10 ng/ml) for 72 hours. When culturing with IL-12, IFNα or TGFβ, either IL-2 or IL-15 were added.

Single-cell RNA-sequencing library preparation and analysis. Lineage negative NK1.1^{+} cells were sorted, then cDNA was prepared after the GEM generation and barcoding, followed by the GEM-RT reaction and bead cleanup steps. Purified cDNA was amplified for 11-13 cycles before being cleaned up using SPRIselect beads. Samples were then run on a Bioanalyzer to determine the cDNA concentration. GEX libraries were prepared as recommended by the 10x Genomics Chromium Single Cell 3' Reagent Kits User Guide (v3.1 Chemistry Dual Index) with appropriate modifications to the PCR cycles based on the calculated cDNA concentration. For sample preparation on the 10x Genomics platform, the Chromium Next GEM Single Cell 3' Kit v3.1, 16 rxns (PN-1000268), Chromium Next GEM

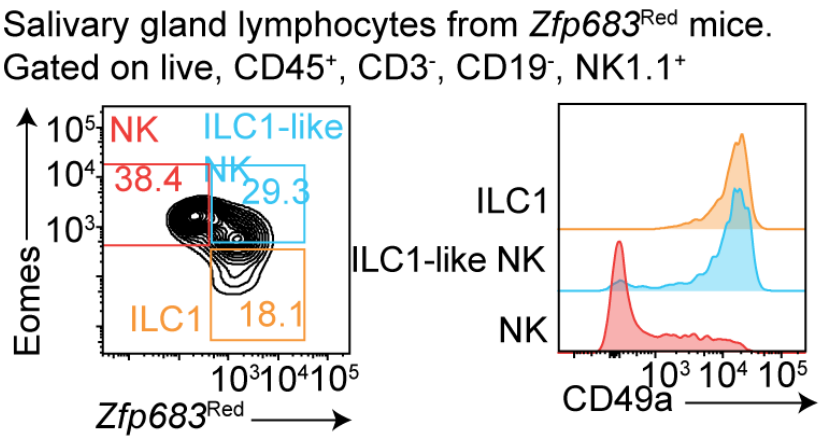
Chip G Single Cell Kit, 48 rxns (PN-1000120), and Dual Index Kit TT Set A, 96 rxns (PN-1000215) were used. The concentration of each library was accurately determined through qPCR utilizing the KAPA library Quantification Kit according to the manufacturer's protocol (KAPA Biosystems/Roche) to produce cluster counts appropriate for the Illumina NovaSeq6000 instrument. Normalized libraries were sequenced on a NovaSeq6000 S4 Flow Cell using the XP workflow and a 28x10x10x150 sequencing recipe according to manufacturer protocol. A median sequencing depth of 50,000 reads/cell was targeted for each Gene Expression Library.

Cell ranger was used to align samples to the reference mm10 genome and generate gene-cell expression matrices. Downstream analysis including normalization, scaling, and clustering was performed using Seurat package (v.4.0.3). Cells with fewer than 200 or more than 5000 detected genes, or cells with the percentage of mitochondrial reads greater than 5% were excluded from the analysis. Filtered data were scaled by library size, then log transformed. Highly variable genes were selected using the FindVariableFeatures, and clustering was performed using FindClusters function. UMAP was used to project cells into two dimensions using 20 first principal components. Contaminating small clusters, representing T cells, B cells, and myeloid cells, were excluded, and remaining NK cells/ILC1 were selected and re-clustered.

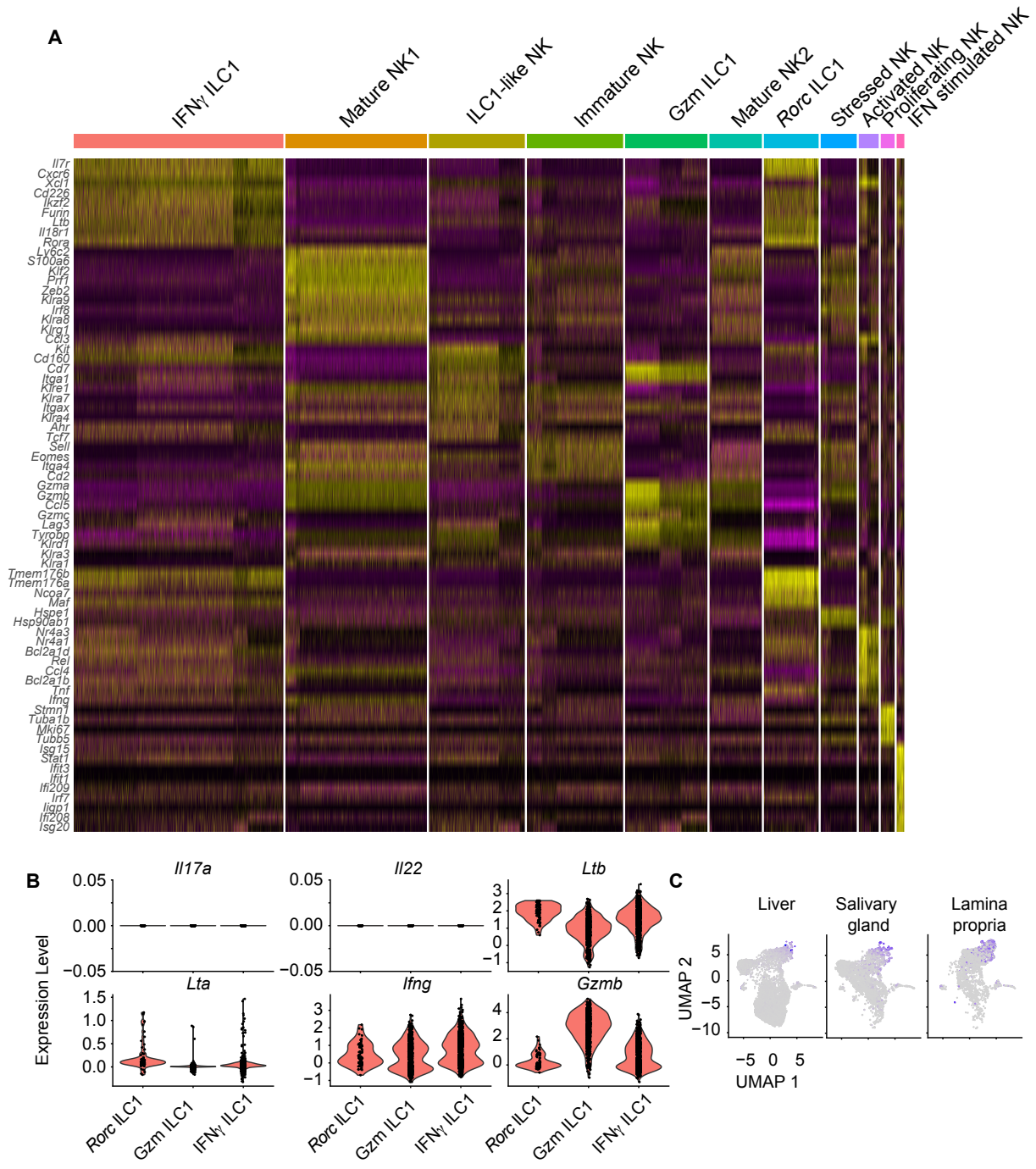
Statistical analysis. Statistical analysis was performed using Prism 9 (GraphPad Software). All data were analyzed with an unpaired Student's *t*-test. Levels of significance were expressed as *P*-value.

References for SI Appendix Material and Methods

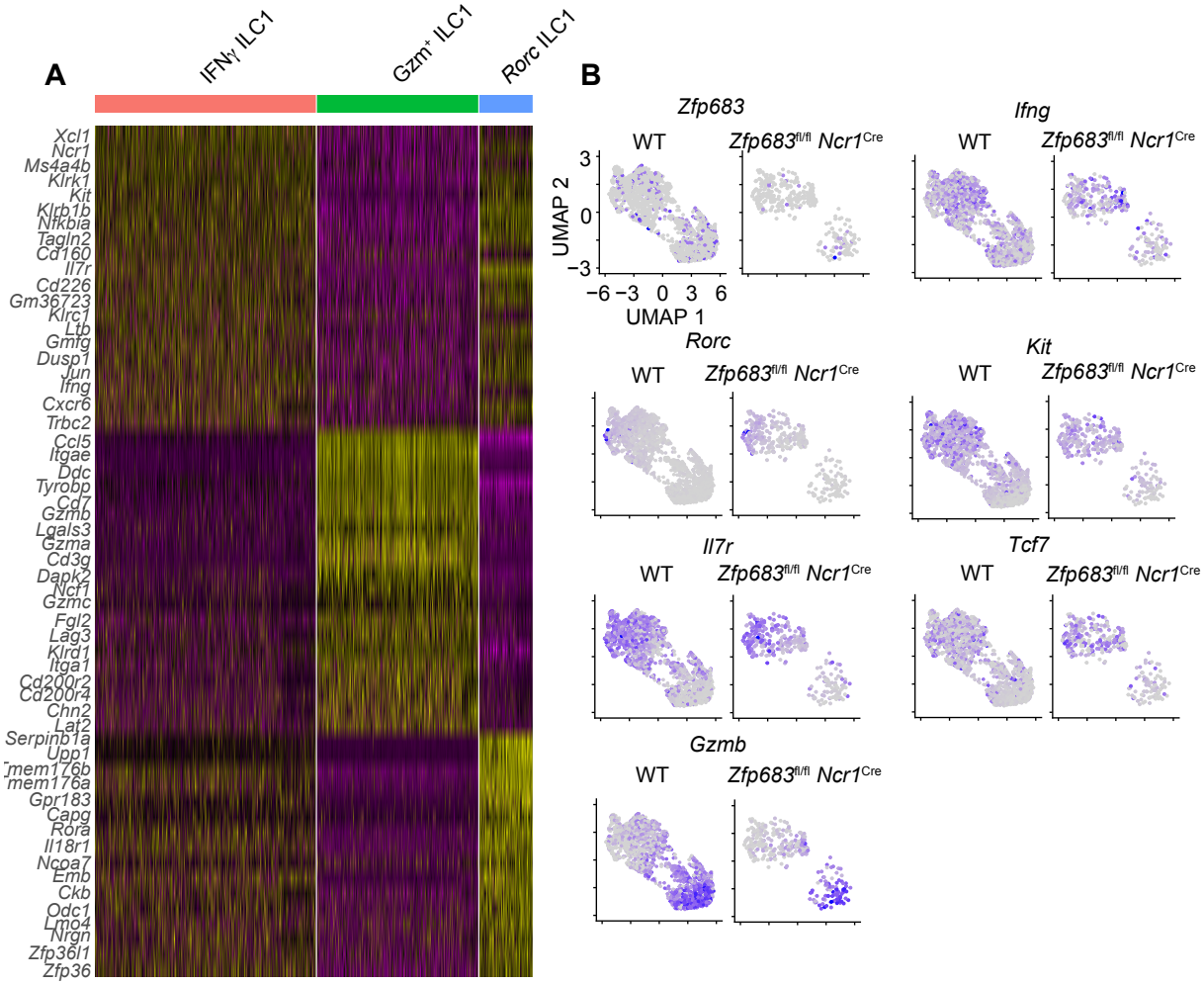
1. J. K. Bando*, et al.*, The Tumor Necrosis Factor Superfamily Member RANKL Suppresses Effector Cytokine Production in Group 3 Innate Lymphoid Cells. *Immunity* **48**, 1208-1219 (2018).



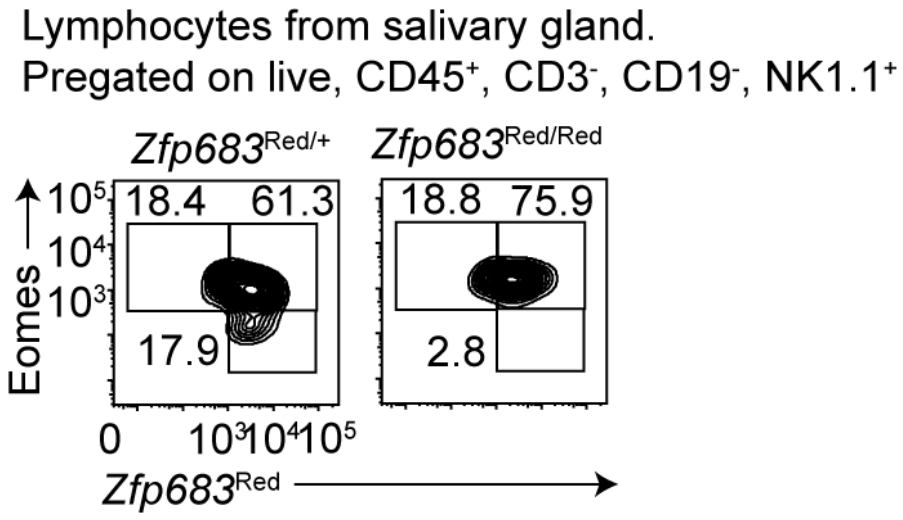
***SI Appendix,* Fig. S1.** Salivary gland CD3$^-$NK1.1$^+$ cells contain a large ILC1-like NK cell population. Flow cytometric plots showing expression of *Zfp683*^{Red} and Eomes in salivary gland CD3$^-$NK1.1$^+$ cells from heterozygous *Zfp683*^{Red} mice. Plots were pre-gated with the indicated markers.



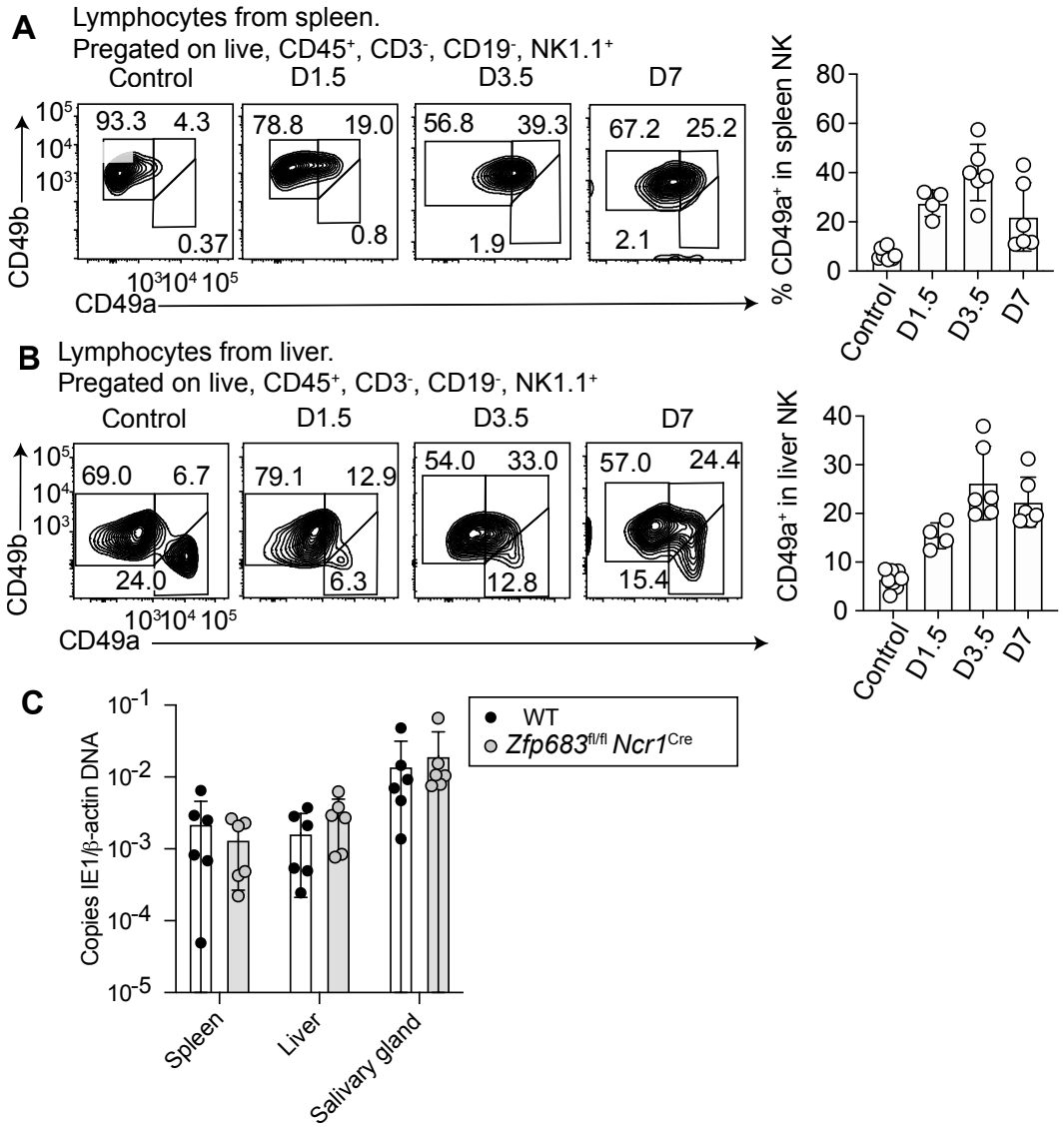
***SI Appendix,* Fig. S2.** Distinct transcriptomic profiles of NK cells and ILC1 subsets. (A) Heat map displaying the differentially expressed genes in each cluster. (B) Violin plots of selected genes among ILC1 populations. (C) UMAP plots of *Rorc* expression



***SI Appendix,* Fig. S3.** (A) Heatmap displaying the differentially expressed genes in each cluster. (B) UMAP plots of representative selected genes related to the identified clusters.



***SI Appendix,* Fig. S4.** Hobit deficiency profoundly reduces the Hobit$^+$ Eomes$^-$ population from salivary gland CD3$^-$NK1.1$^+$ cells**.** Flow cytometric plot showing expression of *Zfp683*^{Red} and Eomes in salivary gland CD3$^-$NK1.1$^+$ cells from heterozygous *Zfp683*$^{Red/+}$ or homozygous *Zfp683*$^{Red/Red}$ (Hobit KO) mice. Plots were pre-gated with the indicated markers. Plots are representative of two independent experiments with similar results.



***SI Appendix,* Fig. S5.** MCMV infection induces CD49a in spleen and liver NK cells. (A, B) Representative flow cytometric plots and quantification showing expression of CD49a in spleen (A) or liver (B) $CD3^{-}NK1.1^{+}$ cells in mice infected with MCMV at the indicated time points. Plots were pre-gated with the indicated markers. Plots are representative of two independent experiments with similar results. (*n*= 5-6). (C) MCMV viral titers were measured from indicated organs at day 7 post infection.

Supplemental Table 1
Specimen, source and cell data for scRNA-seq

Organs	Sample ID	Number of mice pooled	Cell numbers
Liver	Wild type	2	10825
	Zfp683$^{fl/fl}$ *Ncr1*^{Cre}	2	5912
Salivary gland	Wild type	2	2765
	Zfp683$^{fl/fl}$ *Ncr1*^{Cre}	2	3441
Small intestine lamina propria	Wild type	3	4070
	Zfp683$^{fl/fl}$ *Ncr1*^{Cre}	3	2556