Supporting Information

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SI Methods

Mice. WT C57BL/6 mice (B6), $\beta 2m^{-/-}$ mice (B6.129P2-*B2m*^{tm1Unc}; backcrossed 11 times to B6 background), and *CD1d*$^{-/-}$ mice (B6.129S6-*Cd1d1*/*Cd1d2*^{tm1Spb}/J; backcrossed 12 times to B6 background) were obtained from Jackson Laboratories. *MHC class II*$^{-/-}$ (B6.129-*H2-Ab1*^{tm1Gru}; backcrossed 12 times to B6 background), *Rag2*$^{-/-}\gamma c^{-/-}$ (B10;B6-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl}; mixed background B6/B10), and nude (B6.Cg/NTac-*Foxn1*^{nu} NE10; backcrossed 10 times to B6 background) mice were purchased from Taconic. *Rag2*$^{-/-}$ mice were obtained from breeding *Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl} with the B6 strain. Class II$^{-/-}$ $\beta 2m^{-/-}$ mice (B6.129-*H2-Ab1*^{tm1Gru} *B2m*^{tm1Jae} N17; backcrossed 17 times to B6 background) were donated from the National Institute of Allergy and Infectious Diseases Taconic's repository. All mice were maintained in a specific pathogen-free barrier unit at the University of Michigan. All experiments followed the guidelines of the University of Michigan Animal Care and Use Committee. Approval for the use of mice was obtained from the University of Michigan according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH) (1).

Cell Preparation. Single-cell suspensions of spleen, thymus, and bone marrow were prepared in RPMI supplemented with 5% (vol/vol) FCS. Red blood cells were lysed, and cells were passed through a nylon mesh and then counted and stained. Liver was prepared by perfusion and then crushed through a nylon mesh. Cells were then subjected to a 40:70 percoll gradient and centrifuged at 600 × *g* for 20 min at room temperature. Cells at the interface were collected and then counted.

Flow Cytometry. All cell suspensions were treated with 2.4G2 and then surface stained with the following fluorochrome-conjugated antibodies: CD49b (DX5), NK1.1 (PK136), CD122 (TM-β1), CD3 (145-2C11), CD1d tetramer (NIH Tetramer Core Facility), TCRβ (H57-597), CD4 (RM-45), CD8 (53-6.7), NKp46 (29A1.4), Ly49H (3D10), Ly49D (4E5), Ly49A (YE1/48.10.6), Ly49C/I (5E6), CD94 (18d3), CD244 [m2B4 (B6)458.1], NKG2D (CX5), NKG2A (20d5), CD62L (MEL-14), CD44 (IM7), CD11b (M1/70), and CD43 (S7). For intracellular cytokine staining, cells were first incubated for 4 h at 37 °C in the presence of protein transporter inhibitor Golgi stop (BD Bioscience). Subsequently, cells were surface stained, fixed, and permeabilized using a CytoFix/CytoPerm kit (BD Biosciences) according to the manufacturer's procedure, then stained for intracellular cytokines using fluorochrome-conjugated antibodies against IFNγ (XMG1.2) or granzyme B (GB11). All antibodies were purchased from BD Biosciences, eBioscience, or BioLegend. Cells were acquired on FACSCanto or LSRII flow cytometers (BD Bioscience) using FACSDiVa software, and data were analyzed using FlowJo software (Tree Star). When indicated, cells were sorted on a FACS Aria at the flow cytometry core facility at the University of Michigan.

All FACS analyses were performed after excluding contamination of doublets using a commonly used strategy. Briefly, samples were first gated by comparing forward scatter width (FSC-W) versus forward scatter height to exclude doublets; events with a high FSC-W profile were gated out of the total sample population. The remaining population was then examined based on side scatter width (SSC-W) versus side scatter height profile, and any SSC-W^{high} events were similarly excluded.

Gene Chip Analysis. Splenocytes were first enriched by removing B cells using a mouse biotin selection kit (EasySep; Stem Cell Technologies) and then sorted on a FACS Aria at the flow cytometry core facility (University of Michigan). RNA from sorted cells was extracted using RNeasy Micro Kit (Qiagen), and contaminated DNA was removed using a DNA-free kit (Ambion). cDNA was prepared from 10 ng total RNA using the NuGen WT-Pico V2 kit (Ovation PicoSL WTA System V2 P/N 3312). Biotinylated single-stranded cDNA was then prepared from 3 μg cDNA (Encore Biotin Module; catalog nos. 4200-12, 4200-60, and 4200-A01). Single-stranded cDNA was then fragmented, and 3.7 μg cDNA was hybridized for 20 h at 48 °C on Mouse Gene ST 1.1 strip arrays using the Affymetrix Gene Atlas System (Version 1.0.4.267). Arrays were scanned using the same system. Expression values were calculated using a robust multi-array average (RMA) (2). RMA was calculated using the oligo package of bioconductor in R (Version 2.15.1.; www.R-project.org). Gene chip data of thymic precursors and various T-cell subsets in Fig. 4 were downloaded from the Immunological Genome Project website (www.immgen.org), and data of sorted CD1d-independent natural killer T (CD1d^{ind}NKT) cells and NK cells were added to the analysis for comparison. All heat maps and plots were generated in R software by the University of Michigan Computational Medicine and Bioinformatics core facility.

Stimulation with Cytokines. For IFNγ response, total splenocytes were cultured in complete RPMI supplemented with recombinant mouse IL-12 (5 ng/mL; PeproTech) and mouse IL-18 (25 ng/mL; R&D Systems). Cells were collected at indicated time points and incubated for 4 h at 37 °C in the presence of protein transporter inhibitor Golgi stop (BD Biosciences). Cells were first surface stained, fixed/permeabilized using CytoFix/CytoPerm kit (BD Biosciences) according to the manufacturer's procedure, and then stained for intracellular cytokines using fluorochrome-conjugated IFNγ (XMG1.2). For cell survival, total splenocytes were cultured at 37 °C in complete RPMI supplemented with different combinations of recombinant cytokines [mouse IL-2 (250 ng/mL; eBioscience), mouse IL-7 (20 ng/mL; eBioscience), and human IL-15 (15 ng/mL; R&D Systems)] for a total of 14 d. Fresh media supplemented with these cytokines were replenished every 3 d and cells were collected on days 7 and 14 and examined for cell frequency and numbers.

Stimulation with Immobilized Anti-CD3. Total splenocytes or sorted cell populations were incubated in 96-well, flat-bottomed plates (Fisher) coated with anti-CD3 (145-2C1; eBioscience) and anti-CD28 (37.51; eBiosceince) antibodies for 48 h at 37 °C. Cells were restimulated with 50 ng/mL PMA (Roche) and 500 ng/mL Ionomycin (Roche) for 4 h at 37 °C in the presence of protein transporter inhibitor Golgi stop (BD Biosciences). Cells were first surface stained, fixed/permeabilized using CytoFix/CytoPerm kit (BD Biosciences) according to the manufacturer's procedure, and then stained for intracellular cytokines using fluorochrome-conjugated IFNγ (XMG1.2).

Treatment with Poly:IC. Poly:IC (Fisher) was injected i.p. at 3.75 mg/kg body weight, and treated mice were killed the next day (after ~16 h). Splenocytes were harvested and then incubated for 4 h at 37 °C in the presence of protein transporter inhibitor Golgi stop (BD Biosciences). Cells were first surface stained, fixed/permeabilized using CytoFix/CytoPerm kit (BD Biosciences) according to the manufacturer's procedure, and then stained for intracellular cytokines using fluorochrome-conjugated granzyme B (GB11).

Statistics. All statistical analyses were performed in Prism (GraphPad Software). Means between two groups were compared with two-tailed *t* test. Means among three or more groups were compared with one-way ANOVA with Bonferroni's post-test.

1. Committee on Care and Use of Laboratory Animals (1985) *Guide for the Care and Use of Laboratory Animals* (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.

2. Irizarry RA, et al. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4(2):249–264.

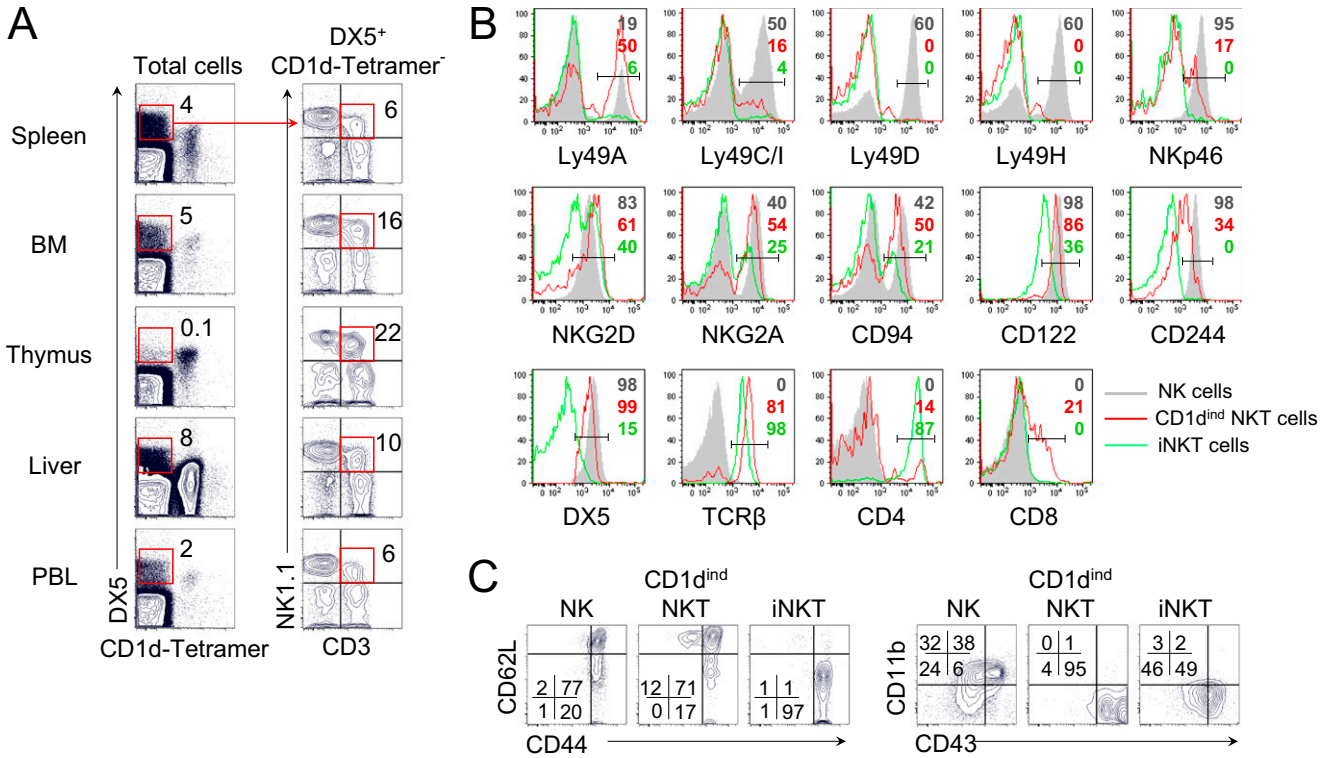

Fig. S1. Distribution of NK cell markers in CD1d^{ind}NKT cells. (*A*) Distribution of DX5 versus CD1d tetramer in the spleen, bone marrow (BM), thymus, liver, and blood [peripheral blood lymphocytes (PBL)]. Gated DX5$^+$CD1d tetramer$^-$ cells were analyzed for the expression of CD3 and NK1.1 markers. (*B*) Splenic NK (NK1.1$^+$DX5$^+$CD3$^-$CD1d tetramer$^-$), CD1d^{ind}NKT (NK1.1$^+$DX5$^+$CD3$^+$CD1d teramer$^-$), and iNKT (NK1.1$^+$DX5$^-$CD3$^+$CD1d tetramer$^+$) cells were compared for the expression of Ly49A, Ly49C/I, Ly49D, Ly49H, NKp46, NKG2D, NKG2A, CD94, CD122, CD244 (2B4), DX5, TCRb, CD4, and CD8. Plots in (*C*) show the distribution of CD62L versus CD44 (T cell activation markers) and CD11b versus CD43 (NK cell maturation markers). Data *A*–*C* are representative of three independent experiments with and n = 3–5 mice per experiment.

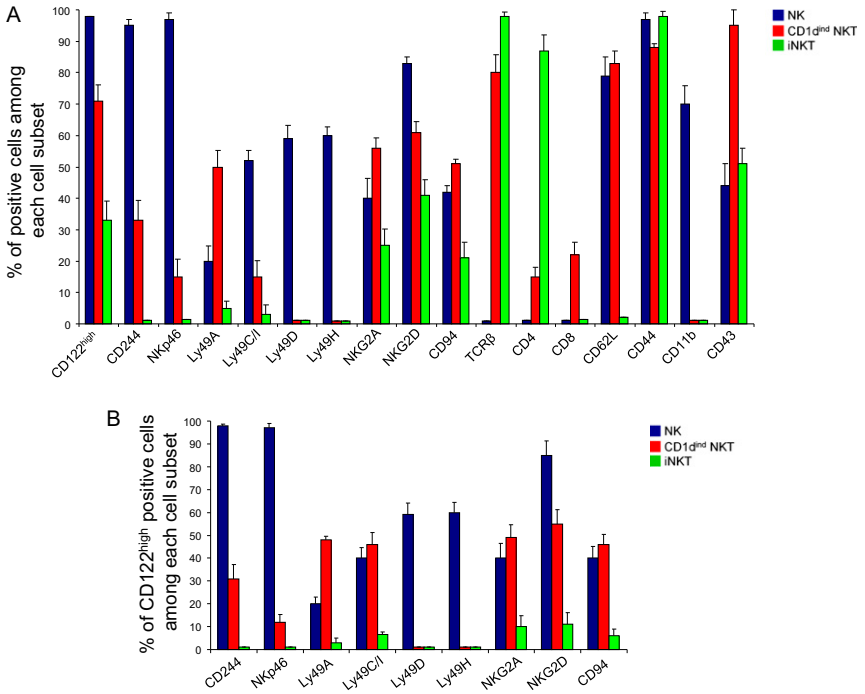

Fig. S2. Phenotypic characterization of CD1d^{ind}NKT cells. Spleen NK (NK1.1$^+$DX5$^+$CD3$^-$CD1d tetramer$^-$), CD1d^{ind}NKT (NK1.1$^+$DX5$^+$CD3$^+$CD1d tetramer$^-$), and invariant NKT (iNKT) (NK1.1$^+$DX5$^-$CD3$^+$CD1d tetramer$^+$) cells from C57BL/6 mice were compared for their expression of CD122, CD244 (2B4), NKp46, Ly49A, Ly49C/I, Ly49D, Ly49H, NKG2A, NKG2D, CD94, TCRβ, CD4, CD8, CD62L, CD44, CD11b, and CD43. (*A*) Graph summarizing the frequency of marker-positive cells among total NK (blue), CD1d^{ind}NKT (red), and iNKT (green) cell subsets. (*B*) Graph shows the frequency of CD122^{high} marker-positive cells among NK (blue), CD1d^{ind}NKT (red), and iNKT (green) cell subsets. Data are representative of three independent experiments with n = 3–5 mice per experiment. Mean ± SEM in *A* and *B*.

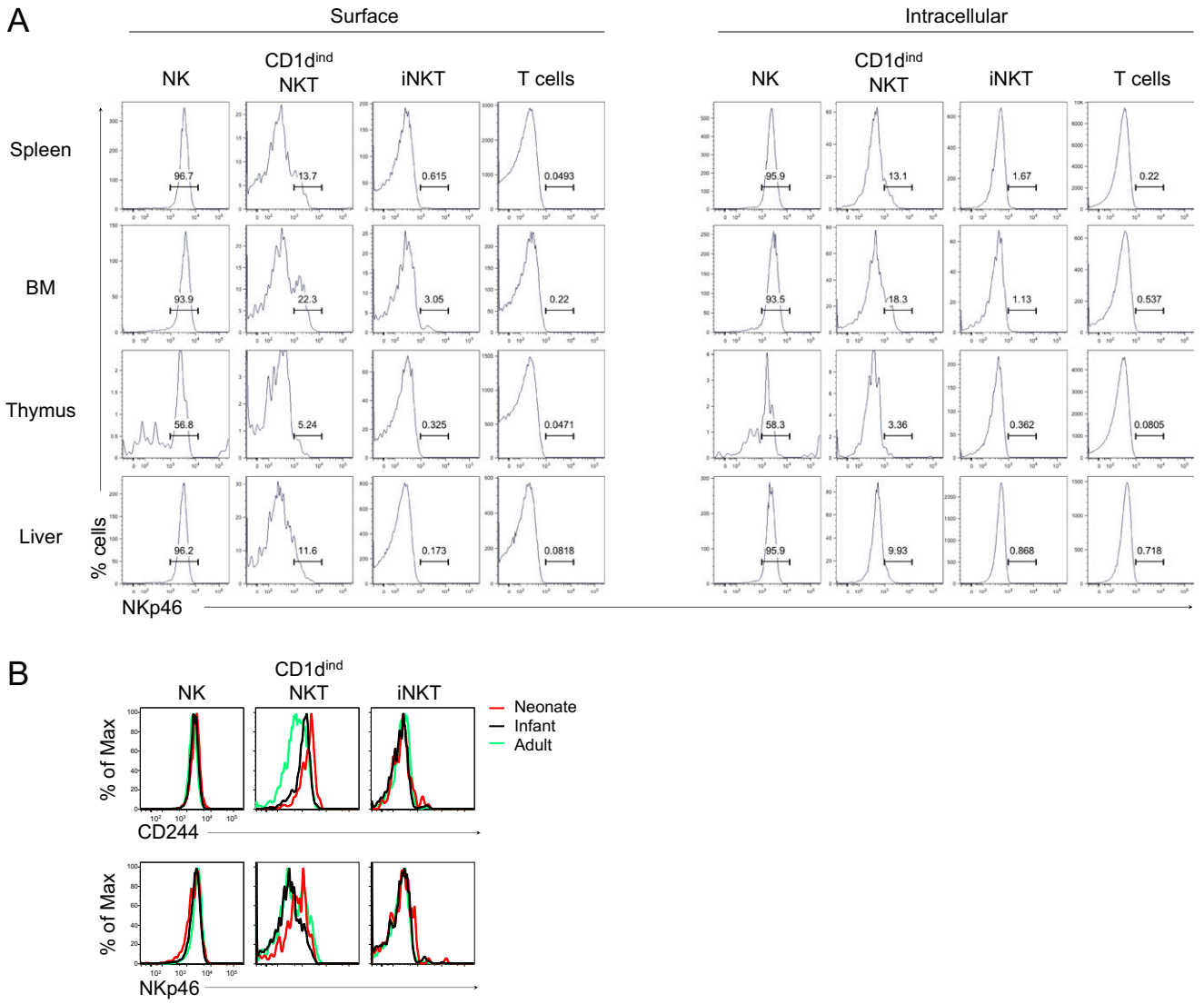

Fig. S3. (*A*) CD1d^{ind}NKT cells do not express intracellular NKp46. (*Left*) Cells were surface stained with a mixture containing NK1.1, DX5, CD3, and NKP6 antibodies and CD1d tetratmer. (*Right*) Cells were surface stained as described above. Subsequently, cells were fixed and permeabilized using CytoFix/CytoPerm kit (BD Biosciences) according to the manufacturer's procedure and then stained for intracellular NKp46. Histograms show surface (*Left*) and intracellular (*Right*) expression of NKp46 among gated NK (NK1.1$^+$DX5$^+$CD3$^-$CD1d tetramer$^-$), CD1d^{ind}NKT (NK1.1$^+$DX5$^+$CD3$^+$CD1d tetramer$^-$), iNKT (NK1.1$^+$DX5$^-$CD3$^+$CD1d tetramer$^+$), and conventional T (NK1.1$^-$DX5$^-$CD3$^+$CD1d tetramer$^-$) cells in spleen, bone marrow (BM), thymus, and liver. Numbers indicate frequency of NKp46-positive cells. Data are representative of two independent experiments with $n = 3$ mice per experiment. (*B*) CD1d^{ind}NKT but not iNKT cells express NKp46 early in life. Splenocytes were isolated from C57BL/6 mice at different ages, including neonate (10 d old), infant (20 d old), and adult (60 d old) ages. Histograms show surface expression of 2B4 (CD244) and NKp46 from gated NK (NK1.1$^+$DX5$^+$CD3$^-$CD1d tetramer$^-$), CD1d^{ind}NKT (NK1.1$^+$DX5$^+$CD3$^+$CD1d tetramer$^-$), and iNKT (NK1.1$^+$DX5$^-$CD3$^+$CD1d-Teramer$^+$) cell subsets. Results show overlays from neonate (red), infant (blue), and adult (green) mice. Data are representative of three independent experiments with $n = 3$–4 per experiment.

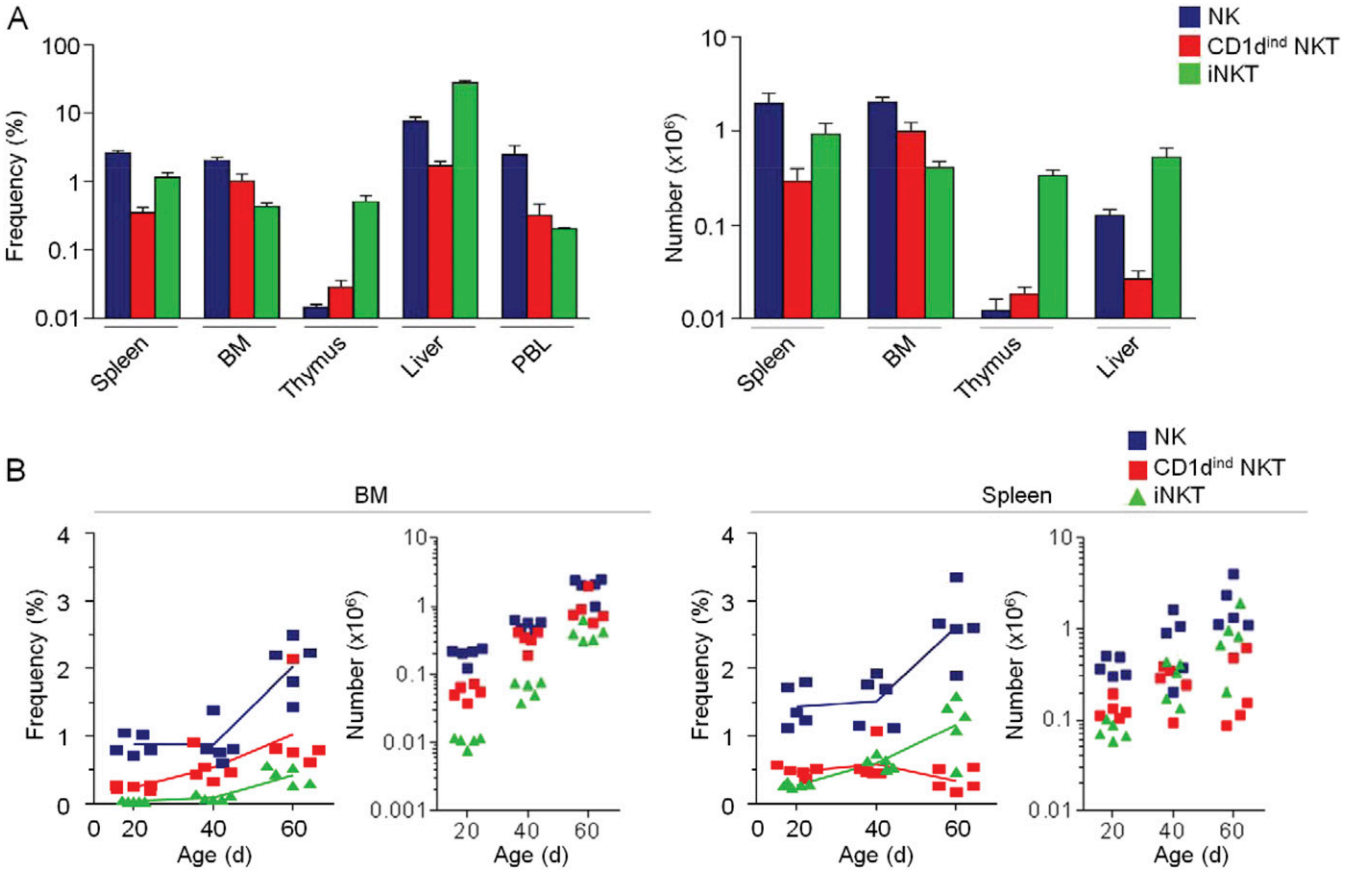

Fig. S4. Distribution and ontogeny of CD1d^{ind}NKT cells. (*A*) Graphs summarize the frequency and numbers of NK (NK1.1$^+$DX5$^+$CD3$^-$CD1d tetramer$^-$; blue), CD1d^{ind}NKT (NK1.1$^+$DX5$^+$CD3$^+$CD1d tetramer$^-$; red), and iNKT (NK1.1$^+$DX5$^-$CD3$^+$CD1d tetramer$^+$; green) cells in spleen, BM, thymus, liver, and blood [peripheral blood lymphocytes (PBL)] of C57BL/6 mice. (*B*) Graphs show the frequency and number of NK (blue), CD1d^{ind}NKT (red), and iNKT (green) cell subsets in the BM (*Left*) and spleen (*Right*) at 20, 40, and 60 d of age. Data in *A* are representative of three independent experiments with $n = 3$–5 mice per experiment with mean ± SEM. Data in *B* are from two independent experiments with a total of $n = 5$ mice per age group.

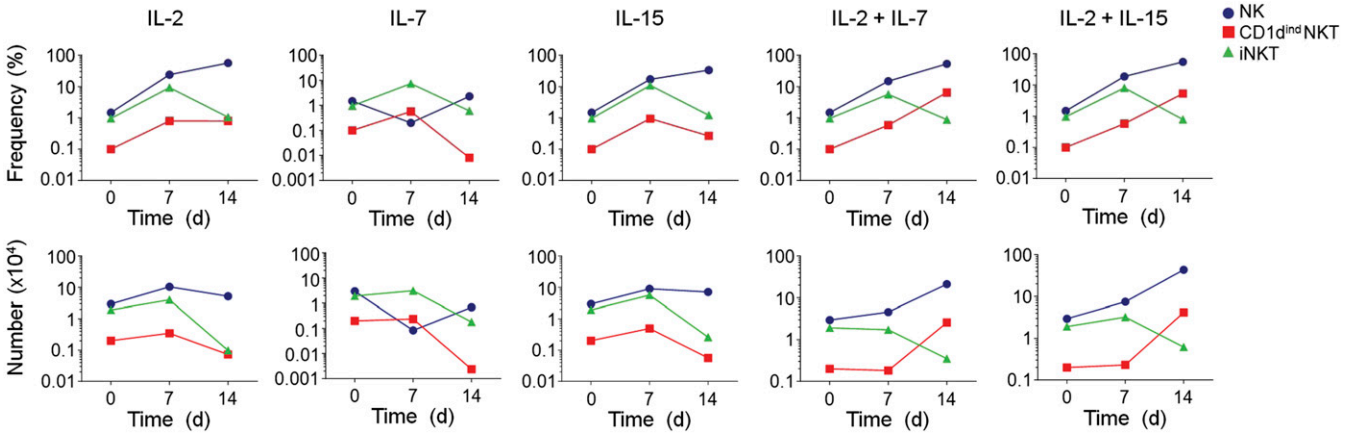

Fig. S5. Survival of CD1d^{ind}NKT cells in response to γc cytokines. Total splenocytes from C57BL/6 mice were cultured with different cytokine combinations IL-2 (250 ng/mL), IL-7 (20 ng/mL), and IL-15 (15 ng/mL), as indicated. On days 7 and 14, cells were harvested, counted, and stained. Graphs summarize the frequency and number of NK (blue), CD1d^{ind}NKT (red), and iNKT (green) cells as a function of culture time. Data are representative of two independent experiments with $n = 3$ mice per experiment.

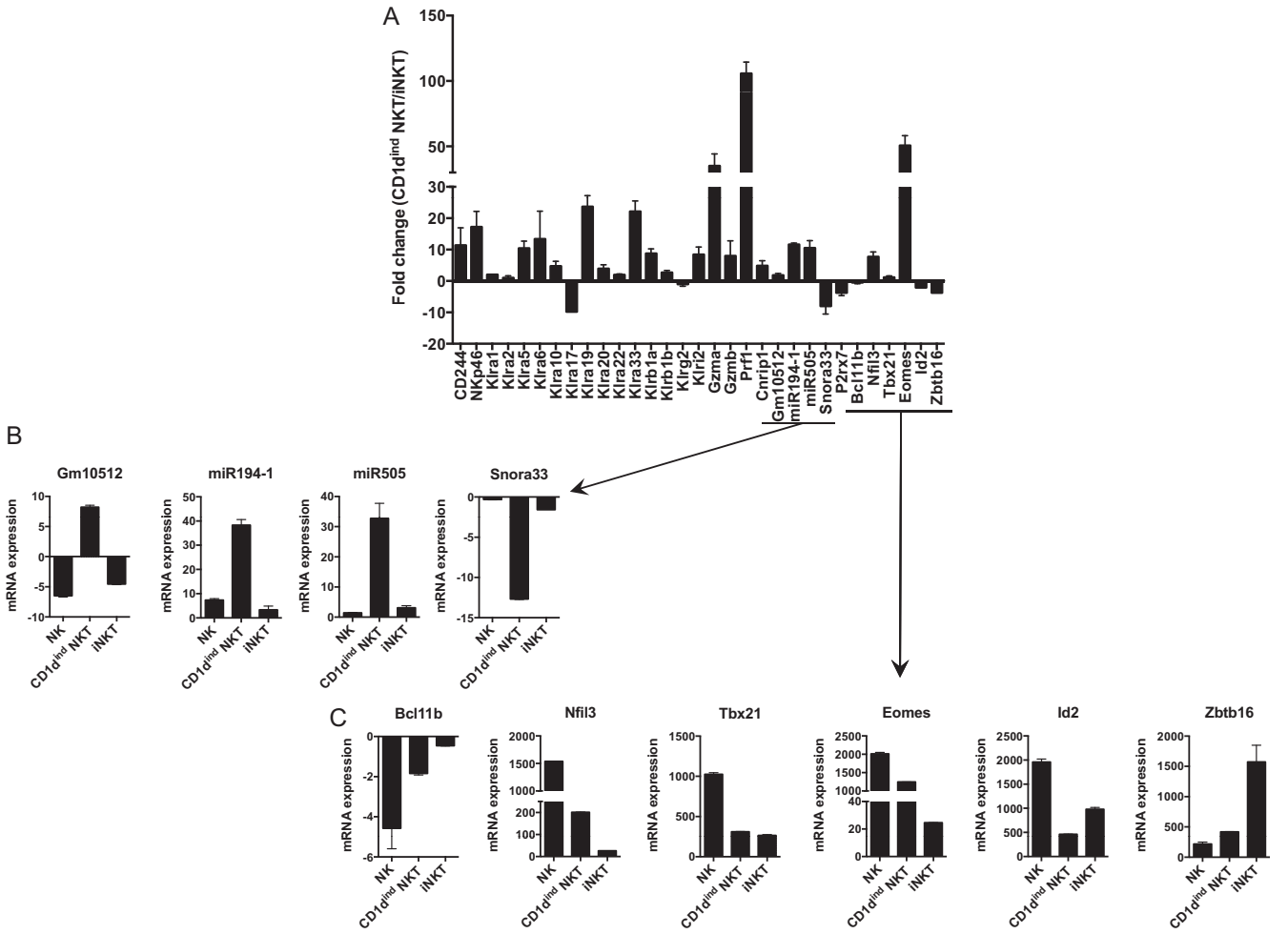

Fig. S6. Gene expression profile of CD1d^{ind}NKT cells. Splenic NK (NK1.1$^+$DX5$^+$CD3$^-$CD1d tetramer$^-$), CD1d^{ind}NKT (NK1.1$^+$DX5$^+$CD3$^+$CD1d tetramer$^-$), iNKT (NK1.1$^+$DX5$^-$CD3$^+$CD1d tetramer$^+$), and conventional T (NK1.1$^-$DX5$^-$CD3$^+$CD1d tetramer$^-$) cells were sorted and total RNA was isolated with TRIzol reagent (Invitrogen) according to standard protocol. RNA was treated using a DNA-free kit (Ambion) to eliminate all traces of genomic DNA. cDNA was obtained using the standard protocol of reverse transcription, and quantitative gene expression analysis was conducted on an ABI Prism 7900 instrument (Applied Biosystems) using SYBR (Life Technologies) Green quantitative PCR. Primer sequence sets were obtained from Invitrogen. Sequences were obtained from National Center for Biotechnology Information GenBank (see list below). Data were analyzed using the $2^{-\Delta\Delta Ct}$ (cycle threshold) method, and results are expressed as the fold change in CD1d^{ind}NKT versus iNKT cells (*A*). Selected genes from *A* are also presented in *B* and *C* as the fold change in NK versus T cells, CD1d^{ind}NKT versus T cells, and iNKT versus T cells. Results are representative of three independent experiments with a total of $n = 8$ mice. Mean ± SEM.

	Primer sequences	
Genes	Forward (5′ to 3′)	Reverse (5′ to 3′)
CD244	ACAGGCGTGTTTCTGAGGTG	TGTGGAATCAGAAGGCTTGC
NKp46	TACTGTGCCTTGGGCTATGTC	CCAGATGATGGGTTTCGGGA
Klra1	CAAGGGTGTGACTGGAAGGT	AGTGTTGTGGGTCTCGGGAA
Klra2	ATGGCCAACTTGTCCCTCAG	TGGCCTCAAGTGATTTCTGTGT
Klra5	GGGATATAGACCAGAAAACGCCA	CACTCATCGTGGGAGTGCAT
Klra6	CCCTCACCAGAATCACTCCG	AGTAAGTGACCTCCGGCTCA
Klra10	GATTCCTCACGGGACACAGG	TGCTGGCAGTTCGCTTTACA
Klra17	CCCCCATAACTGCAGGATCT	AGAGTGTGTTCTCTGTGTCTCT
Klra19	CGTCCGAGAAAACACCAACT	ACCTCTGGCTCACTCATTGTG
Klra20	GAACATACTTCATACATCACTGCC	GTTCTCACTAGTTTCTGCAACCTT
Klra22	AACCCACCCTTTTCTCACCC	AGTTCAAATGTTGTCTTGAGTGGT
Klra33	ACGTGGTTCCTTCAGACAGT	TGTGTTCAAGGCAAGTTTAGATGG
Klrb1a	TGAGTGTTTTAGTGCGAGTCCT	AGTCTTGTGGGCACTCTAGC
Klrb1b	GGAAGTGGATAAATGGCACAGC	TTTTCTCCTGAGATGGCGGC
Klrg2	CTGGGTCTGTGCCAGAGAAA	CCATGGGGACAACTCCAGAC

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Genes	Primer sequences	
	Forward (5′ to 3′)	Reverse (5′ to 3′)
Klri2	GAATATTCCACCCCCAGCCC	CCATGTTGTCAGCCAGGGAT
Gzma	CTCCGTGGTGGAAAGGACTC	AGAGGTGATGCCTCGCAAAA
Gzmb	GACAACACTCTTGACGCTGG	CGATGATCTCCCCTGCCTTT
Prf1	GGTGGGACTTCAGCTTTCCA	GAAAAGGCCCAGGAGGAACA
Cnrip1	GACACAGAAGGTGTAGCCCC	CACACTGTCTCGAAGGTCCC
Gm10512	TATAGCCCAGTCAAGCCCCT	TGTCAGATACCAGCCAGGGA
miR194-1	ATCGGGTGTAACAGCAACTC	CTCCACTGGAATCCGAGCAC
miR505	GGGGGAGCCAGGAAGTATTG	AAACCAGCAAGTGTTGACGC
Snora33	AATGCCATGGTACGAGCCTT	TGTAGCCACTTTCAGGGACC
P2rx7	CACCGTGCTTACAGGTGCTA	GTCTTGGGGAACTCCTTCCG
Bcl11b	CGGGCGATGCCAGAATAGAT	CTCCACATGGTCAGCCTCTG
Nfil3	GGTTACAGCCGCCCTTTCTT	GTCCGGCACAGGGTAAATCT
Tbx21	CTGGAGCCCACAAGCCATTA	TTTCCACACTGCACCCACTT
Eomes	CTCCCACGGATTCCCCTAGA	GGGCTCCGGGAAGAAGTTTT
Id2	GGACTCGCATCCCACTATCG	TGATGTCCGTGTTCAGGGTG
Zbtb16	TTGGGGGTCAGCTAGAAAGC	GTGCCCGGAGAAGAAAGGTT

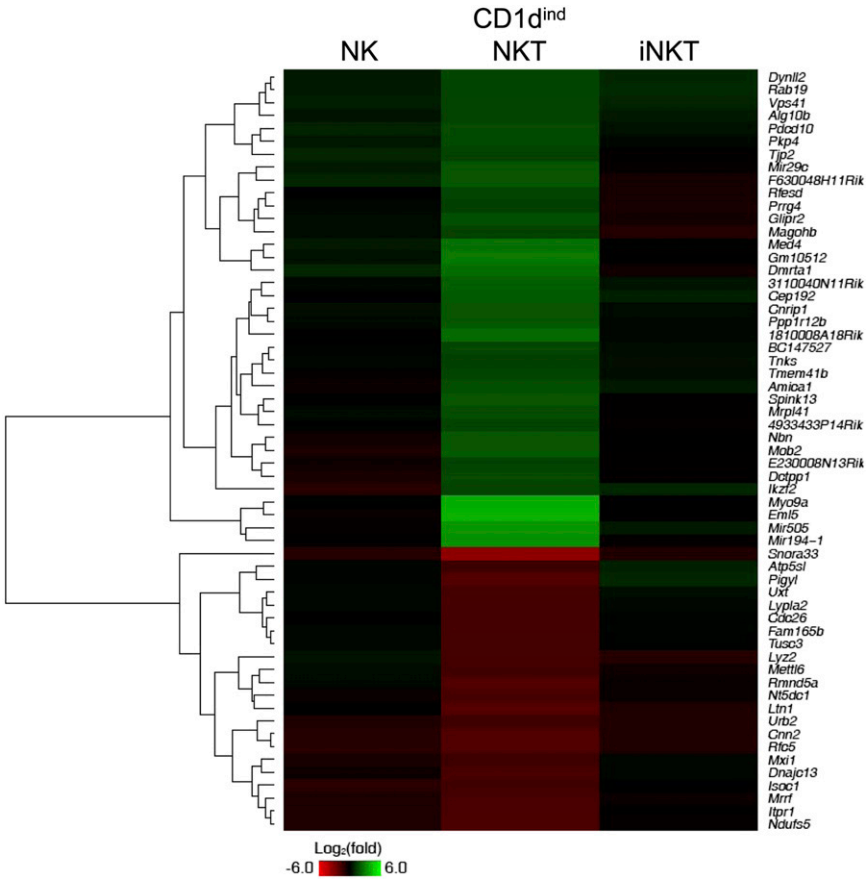

Fig. S7. Distinct gene expression profile of CD1d^{ind}NKT cells. Heat map shows genes with expression change higher than twofold in CD1d^{ind}NKT cells but lower than twofold in NK and iNKT cells relative to T cells. There are 287 genes that passed these criteria. To restrict the number of genes so that their names are readable, the heat map shows only the genes whose $\log_2$-transformed expression change is larger than 1.5 in CD1d^{ind}NKT versus T cells. Data are from cell subsets sorted from $n = 7$ mice.

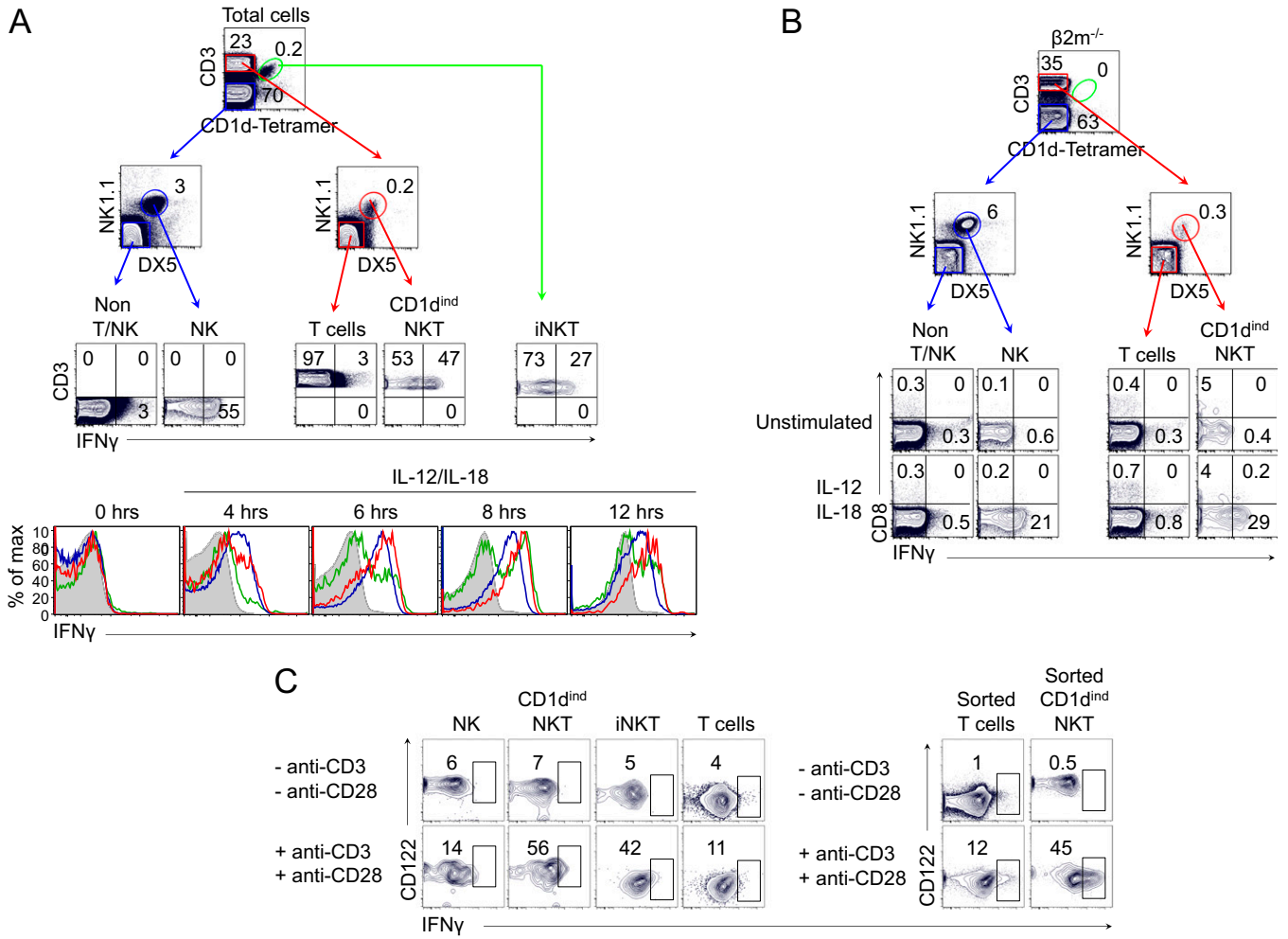

Fig. S8. CD1d^{ind}NKT cell production of IFNγ in response to cytokine signals and TCR engagement. Splenocytes from WT (*A*) and *β2m*$^{-/-}$ (*B*) mice were stimulated with IL-12 (5 ng/mL) and IL-18 (25 ng/mL) for 4 to 12 hours. Plots show the intracellular expression of IFNγ among non-T/NK cell gate (CD3^{-}CD1d tetramer^{-}NK1.1^{-}DX5^{-}), NK cells (CD3^{-}CD1d tetramer^{-}NK1.1^{+}DX5^{+}), T cells (CD3^{+}CD1d tetramer^{-}NK1.1^{-}DX5^{-}), CD1d^{ind}NKT cells (CD3^{+}CD1d tetramer^{-}NK1.1^{+}DX5^{+}), and iNKT cells (CD3^{+}CD1d tetramer^{+}) after 4 (*B*) and 6 (*A*) hours of stimulation. Histograms show overlay of the intracellular expression of IFNγ from gated NK (blue), CD1d^{ind}NKT (red), iNKT (green), and T (grey) cells as a function of time. Data are representative of three (*A*) and two (*B*) independent experiments with n = 3–5 mice per experiment. (*C*) Total splenocytes (*Left*) or sorted cell subsets (*Right*) were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 48 hours. Plots show the distribution of CD122 versus IFNγ among gated NK, CD1d^{ind}NKT, iNKT, and T cells (*Left*) or sorted CD1d^{ind}NKT and T cells (*Right*). Data are representative of three independent experiments with n = 2–3 mice per experiment. Results from sorted cells are from n = 6 pooled spleens per experiment.

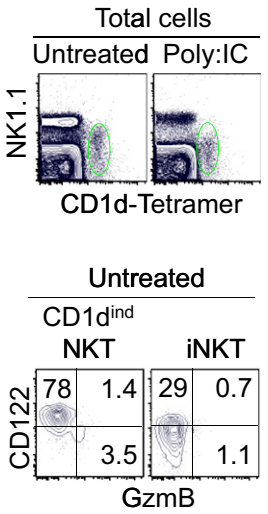

Fig. S9. Steady state expression of Granzyme B in CD1d^{ind}NKT cells. Upper plots show the distribution of NK1.1 versus CD1d tetramer in the spleen of untreated versus Poly:IC-treated wild type mice. Lower panels show the steady state expression of GzmB in both CD1d^{ind}NKT and iNKT cells in untreated mice. Data are representative of three independent experiments with $n = 4$ Poly:IC-treated mice and $n = 2$ untreated mice per experiment.

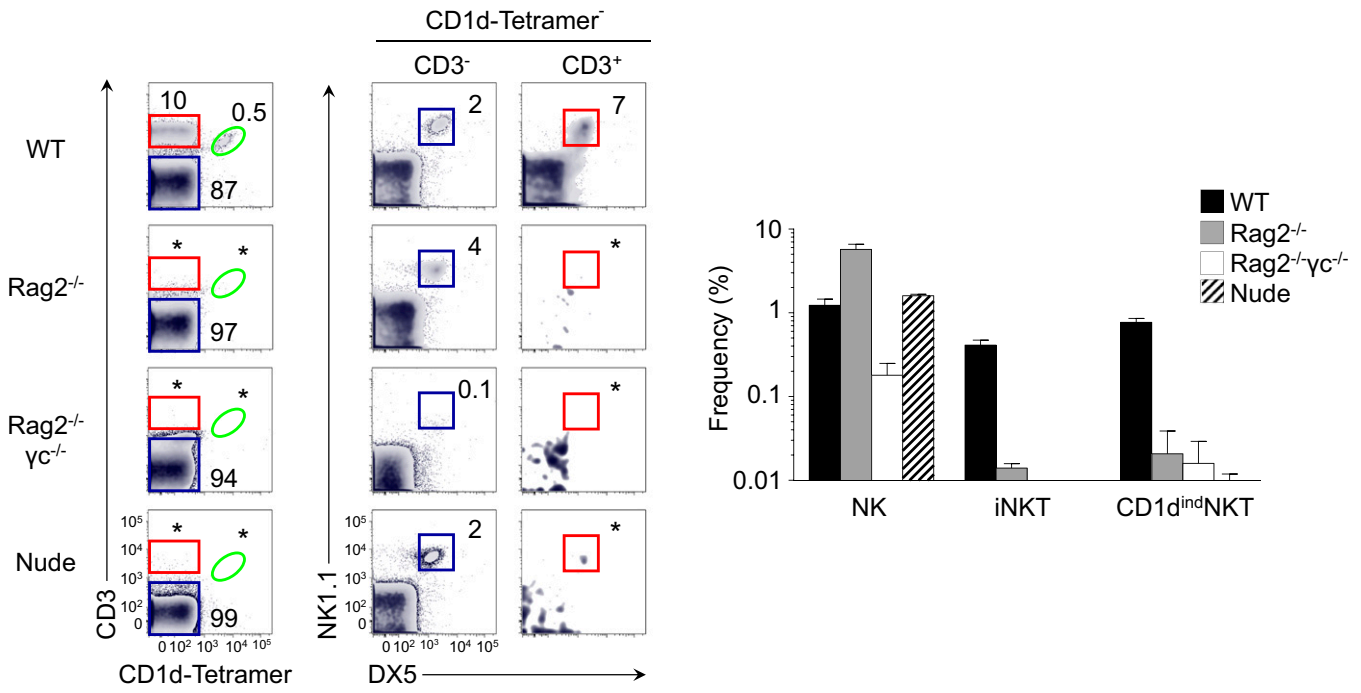

Fig. S10. Outcomes of CD1d^{ind}NKT cells in T cell-deficient mice. Flow cytometry of bone marrow cells isolated from WT, *Rag2*$^{-/-}$, *Rag2*$^{-/-}\gamma c^{-/-}$, and athymic nude mice. Distribution of CD3 versus CD1d tetramer is from total bone marrow cells and distribution of NK1.1 versus DX5 is shown among gated CD3$^-$CD1d tetramer$^-$ and CD3$^+$CD1d tetramer$^-$ cells. Numbers indicate percentages of cells in gates, and the asterisk indicates frequency below 0.1%. Graphs summarize the frequency of NK (CD3$^-$CD1d tetramer$^-$NK1.1$^+$DX5$^+$), iNKT (CD3$^+$CD1d tetramer$^+$), and CD1d^{ind}NKT cells (CD3$^+$CD1d tetramer$^-$NK1.1$^+$DX5$^+$) in the bone marrow of WT B6, *Rag2*$^{-/-}$, *Rag2*$^{-/-}\gamma c^{-/-}$, and nude mice. Data are representative of three independent experiments with $n = 2$–4 mice per experiment. Mean ± SEM.