

Supporting Information

Shimizu et al. 10.1073/pnas.1406240111

SI Materials and Methods

Mice and Cell Lines. Pathogen-free, 6- to 8-wk-old C57BL/6 mice and CD45.1 congenic mice were purchased from CLEA Japan. B6 mice, $\text{J}\alpha 18^{-/-}$ mice, and iNKT-cloned mice (1) and $\text{IFN-}\gamma^{-/-}$ mice (2) were maintained under specific pathogen-free conditions and treated in compliance with RIKEN institutional guidelines. B16 melanoma and NIH 3T3 cell lines were purchased from the American Type Culture Collection. B16 (CD1d-B16), NIH 3T3 (CD1d-NIH 3T3), and HEK293 (CD1d-293) cells expressing high levels of CD1d were generated by retrovirus transduction, as previously described (3–5).

Reagents. The following mAbs were purchased from BD Biosciences, e-Bioscience, R&D Systems, or BioLegend: anti-mouse -CD3 (145-2C11), -CD19 (6D5), -CD27 (LG.3A10), -CD28(37.51), CD43(S7), -CD44 (IM7), -CD49d (R1-2), -CD62L (MEL-14), -CD69 (H1.2F3), -CD122 (TM-b1), -NK1.1 (PK136), NKG2D (CX5), Ly6C(AL-21), -Klrg1(2F1), -IFN- γ (XMG1.2). CD1d-dimerix and TCR V β screening panel were purchased from BD. Anti-granzyme A(3G8.5) was purchased from Santa Cruz. For analysis, a FACSCalibur or Canto II instrument and CELLQuest, Diva (BD Biosciences), or FlowJo (Tree Star) software were used. α -Galactosylceramide (α -GalCer) was synthesized and provided by Y.I. at RIKEN. Isoglobotrihexosylceramide (iGB3) was purchased from Alexis Biochemicals. Glycosphingolipid (GSL) of *Sphingobium yanoikuyae* was purified by us as previously described (6). For depletion in vivo, an anti-NK1.1 Ab was prepared in the laboratory of Y.S. and K.S. from a hybridoma (PK136; ATCC).

Cell Preparation. Bone marrow-derived dendritic cells (DCs) were generated in the presence of GM-CSF and pulsed with 100 ng/mL α -GalCer for 48 h from day 6 and matured by LPS as previously described (7). In some experiments, other natural killer T cell (NKT cell) ligands, 10 μ g/mL iGB3, or GSL was used for pulsing instead of α -GalCer. Mononuclear cells (MNCs) from spleen, lung, and liver were isolated as previously described (8). In brief, splenocytes were obtained by pressing the spleen through a 70- μ m cell strainer, and erythrocytes were lysed with ammonium-chloride-potassium lysing buffer (Gibco) followed by two washes in RPMI. For isolation of lung and liver MNCs, the tissues were digested with collagenase D (Roche) and then layered on Percoll gradients (40–60%) (Amersham Pharmacia Biotech) and centrifuged for 20 min at 900 \times g.

ELISPOT Assay. ELISPOT assays for IFN- γ -secreting cells were performed by culturing with or without 100 ng/mL α -GalCer for 16 h as previously described (7). The number of ligand-dependent IFN- γ spots was counted microscopically.

Cytokine Secretion Assays and Intracellular Staining. IFN- γ release from invariant NKT cells (iNKT cells) was determined using a cytokine secretion detection kit according to the manufacturer's instructions (Miltenyi Biotec Inc.). Briefly, cells were incubated on ice for 5 min with IFN- γ -capture reagent and then diluted with warm RPMI with 5% FCS following incubation for 45 min at 37 $^{\circ}$ C. After two washes, cells were resuspended in cold PBS-containing 0.5% BSA and 2-mM EDTA and incubated with phycoerythrin-coupled IFN- γ -detection reagent for 10 min at 4 $^{\circ}$ C, followed by staining of other cell-surface markers. For intracellular cytokine staining of iNKT cells for analysis by flow cytometry, isolated lung MNCs were stimulated with 10 μ g/mL immobilized anti-CD3 Ab and 2 μ g/mL

soluble anti-CD28 in the presence of Golgi Plug (BD Bioscience) for 2 h and then were preincubated with anti-CD16/32 Ab to block nonspecific binding of antibodies to Fc γ R, washed, and incubated with mAbs to the indicated cell-surface markers. Cells then were permeabilized in Cytotfix-Cytoperm Plus (BD Bioscience) and stained with anti-IFN- γ mAbs.

Cytotoxicity Assay. The cytotoxic activity of lung MNCs against B16 melanoma cells was analyzed using the LDH assay kit (Takara Bio Company) according to the manufacturer's instructions. In brief, 1×10^4 B16 melanoma cells were cultured with pooled lung MNCs from naive or DC/Gal-immunized mice at various effector/target (E/T) ratios for 16 h. The culture supernatant was incubated with freshly prepared reaction mixture containing the tetrazolium salt, and absorbance was measured at 490 nm. Data reported are mean \pm SD of triplicate wells from three independent experiments. After the background control value was subtracted, cytotoxicity values (%) were calculated as $\text{Cytotoxicity (\%)} = [(\text{effector:target cell mix} - \text{effector cell control}) - \text{spontaneous target cell control}] / (\text{maximum target control} - \text{spontaneous target cell control}) \times 100$.

Microarray Analysis. Total RNA extracted from samples of isolated iNKT cells was used for microarray analysis with a GeneChip Mouse Genome 430 2.0 Array. All procedures were performed according to the manufacturer's instructions (Affymetrix). Data were analyzed by GeneSpring software (Agilent Technologies).

In Vivo Tumor Experiments. Mice were immunized with or without 1×10^6 DC/Gal. Four months later, the mice were injected i.v. with 2×10^5 B16 melanoma cells. Fourteen days later, mice were killed, and the numbers of lung metastases were analyzed. In some experiments, mice were treated with anti-NK1.1 Ab (300 μ g per mouse) two times per week beginning 2 d before inoculation with B16 cells.

Quantitative PCR Assay. To evaluate gene expression by iNKT cells, FACS-sorted iNKT cells were directly subjected to cDNA synthesis and preamplification, without purifying RNA, using a CellsDirect One-Step qRT-PCR Kit (Invitrogen) with a mixture of pooled gene-specific primers (0.2 μ M each; Table S1). After 18 cycles of preamplification (each cycle: 95 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 4 min), an aliquot was used as template for quantitative PCR using FastStart Universal Probe Master (Roche), a gene-specific forward and reverse primer pair (Table S1), and the corresponding FAM-labeled hydrolysis probe (Universal Probe Library Set; Roche). Quantitative PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Gene expression was measured by the $\Delta\Delta C_T$ method in which hypoxanthine phosphoribosyltransferase 1 (HPRT1) expression was used as the internal control.

Luminex and ELISA. Sorted KLRG1 $^{+}$ (Killer cell lectin-like receptor subfamily G, member 1-positive) or KLRG1 $^{-}$ iNKT cells (1×10^5) were stimulated with 10 μ g/mL immobilized CD3 Ab plus 10 μ g/mL soluble CD28 Ab for 24 h. The culture supernatants were analyzed for IFN- γ production by ELISA (BD Biosciences) and for IL-4, IL-17A, CCL3, and CCL4 production by Luminex (Bio-Rad).

T-Cell Receptor V β Repertoire Assay. Each iNKT cell subset was isolated to high purity. Total RNA was extracted and reverse transcribed for synthesis of first-strand cDNA using a SMARTer RCAE cDNA amplification kit (Clontech). Both universal mix primer and primers specific for the T-cell receptor β (TCR β) constant region sequence were used for second-strand amplifications, resulting in TCR β PCR products of high purity which

were then submitted for high-throughput DNA sequencing of long reads using a Roche 454-GS Junior system.

All reads of the TCR β repertoire sequence were analyzed using Perl scripts based on the USEARCH algorithm (<http://drive5.com/usearch/>). V-region consensus sequences in each cluster were

searched on the ImMunoGeneTics (IMGT) sites (www.imgt.org/IMGT_vquest/share/textes/).

Statistical Analysis. Differences were analyzed using the Mann-Whitney u test. $P < 0.05$ was considered statistically significant.

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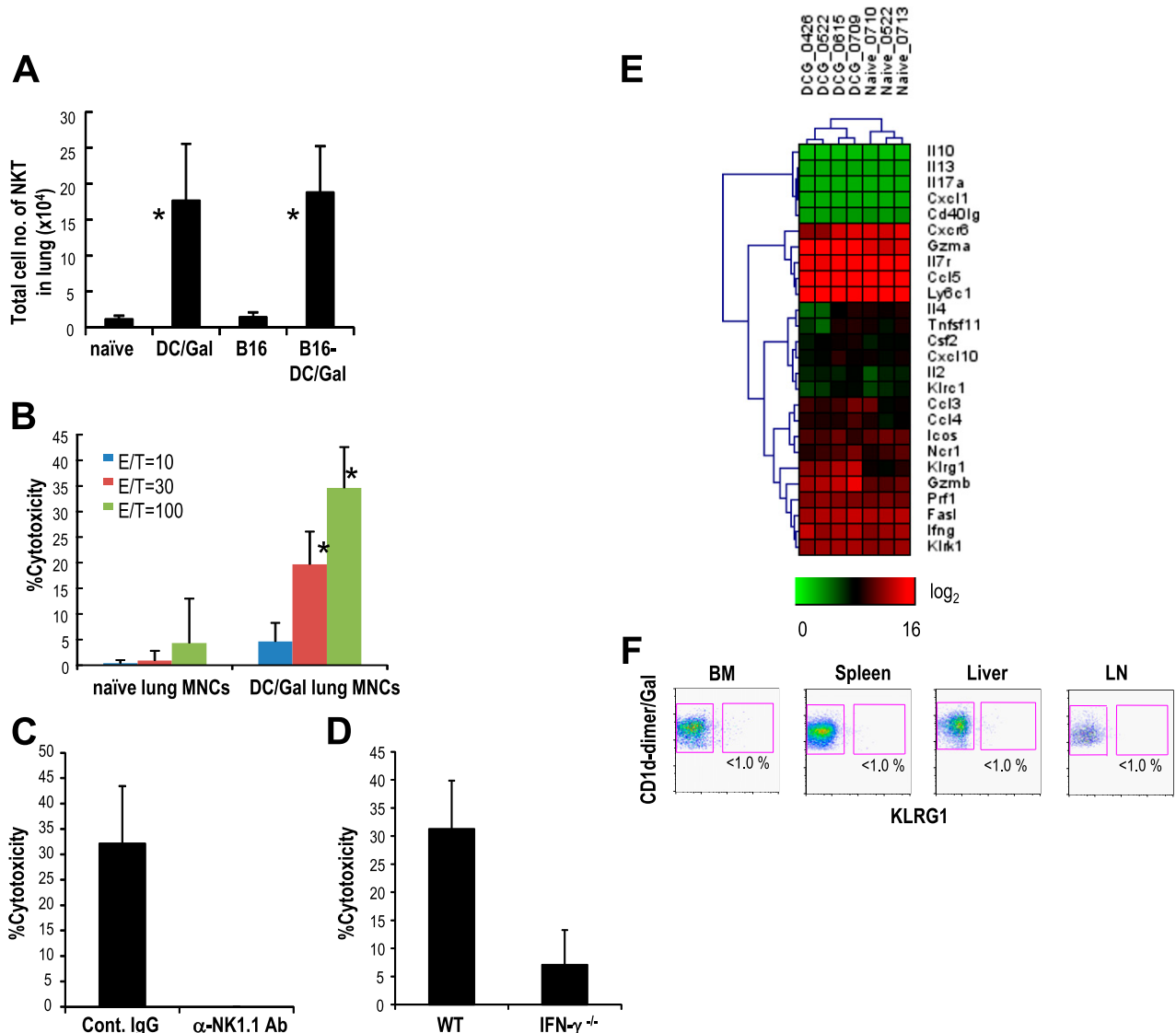


Fig. S1. Analyses of iNKT cells in the lung following the administration of DC/Gal. B16 melanoma cells (2×10^5 cells per mouse) were administered i.v. to C57BL/6 mice. On day 7, B16-bearing or naïve mice were immunized with 1×10^6 DC/Gal. (A) The number of iNKT cells in lung was assessed in the four groups of mice on day 14. $*P < 0.05$ naïve or B16 versus DC/Gal or B16-DC/Gal ($n = 4$; data are shown as mean \pm SEM). (B) Cytotoxicity assay against B16 melanoma was assessed 1 wk after treatment with DC/Gal. Tumor cells as targets were mixed with pooled lung MNCs from three to five naïve or DC/Gal-injected mice at various E:T ratios for 16 h. $*P < 0.05$ naïve versus DC/Gal ($n = 4$; data are shown as mean \pm SEM). (C and D) As in B, cytotoxicity against B16 was assessed 1 wk after injection of DC/Gal by comparing lung MNCs from DC/Gal-administered NK1.1 $^+$ cell-depleted (C) or IFN- γ $^{-/-}$ mice (D) with those from WT mice at E:T ratio = 100 ($n = 3$; data are shown as mean \pm SEM). (E) Microarray analysis of splenic iNKT cells was evaluated in naïve ($n = 3$) and DC/Gal-immunized mice ($n = 4$). (F) KLRG1 expression on naïve iNKT in each organ was assessed by gating on CD19 $^+$ CD1d-dimer/Gal $^+$ cells ($n = 5$).

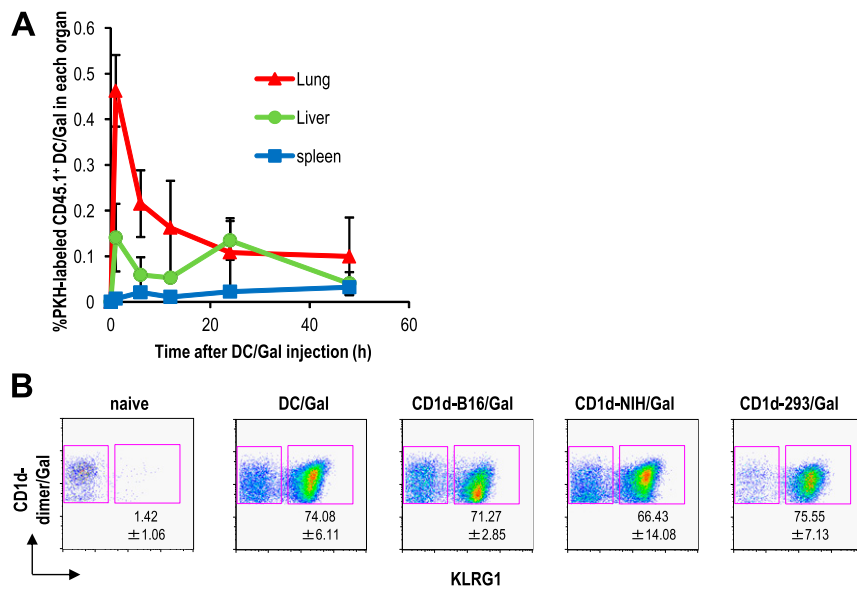


Fig. S2. The kinetics of migration of BM-DC/Gal and generation of KLRG1⁺ iNKT cells in the lung by CD1d⁺ cells loaded with α -GalCer. (A) One million PKH26-labeled CD45.1 BM-DC/Gal cells were administered i.v. to CD45.2 C57BL/6 mice. At the indicated time points, the frequency of transferred DC/Gal (CD45.1⁺ PKH26⁺) in each organ was analyzed by flow cytometry ($n = 4$; data are shown as mean \pm SEM). (B) C57BL/6 mice were immunized with 5×10^5 α -GalCer-loaded syngeneic CD1d-transfected B16 melanoma cells (CD1d-B16/Gal), CD1d-transfected NIH 3T3 (CD1d-NIH 3T3/Gal) fibroblasts, or CD1d-transfected HEK293 cells (CD1d-293/Gal). One week later, the expression of KLRG1 by iNKT cells was verified by gating on CD19⁻CD1d-dimer/Gal⁺ cells.

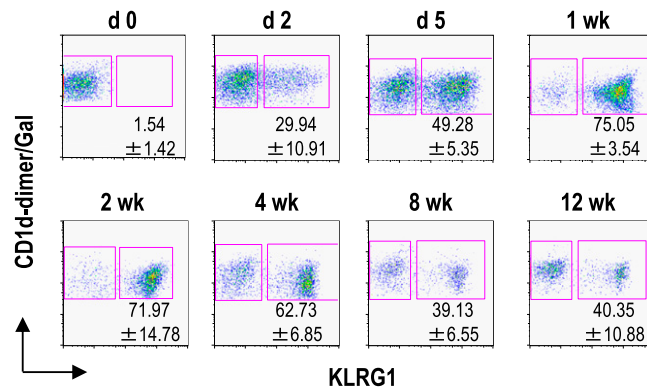


Fig. S3. Frequency of KLRG1⁺ iNKT cells for long periods in DC/Gal-injected mice. The frequency of KLRG1⁺ iNKT cells in the lung was analyzed from day 2 to 12 wk after immunization with DC/Gal ($n = 4-6$, data are shown as mean \pm SEM).

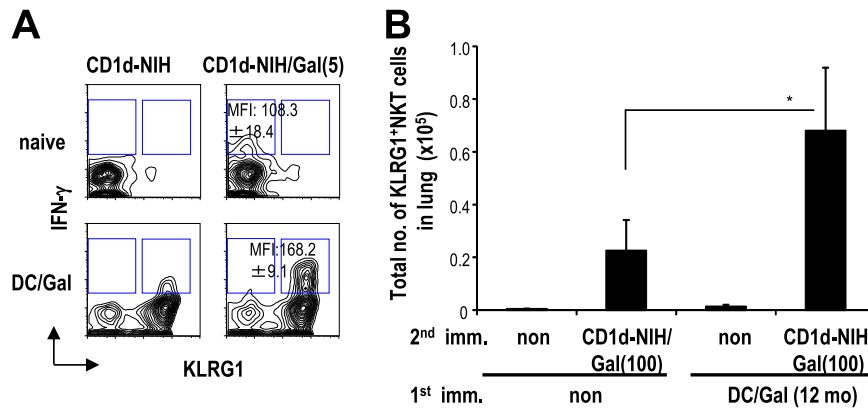


Fig. 54. The recall response of the KLRG1⁺ iNKT cells. Mice were immunized with or without DC/Gal (100) as the first immunization. (A) As in Fig. 3D, lung MNCs were cocultured with 5 ng/mL α -GalCer-loaded CD1d-NIH 3T3 [CD1d-NIH/Gal(5)] for 3 h in the presence of brefeldin A and then were assessed for intracellular staining of IFN- γ . Analysis gates were set on CD19⁻CD1d-dimer/Gal⁺ cells. Data shown are means obtained from two independent experiments ($n = 4$ mice per group). (B) The mice that had been immunized with DC/Gal(100) were left untreated or were administered CD1d-NIH/Gal(100) 12 mo later as a second immunization. One week later, the number of KLRG1⁺ iNKT cells in the lung was analyzed by flow cytometry after gating on CD19⁻CD1d-dimer/Gal⁺ cells. Data are representative of two separate experiments ($n = 4$ –6 per group; data are shown as mean \pm SEM). * $P < 0.05$.

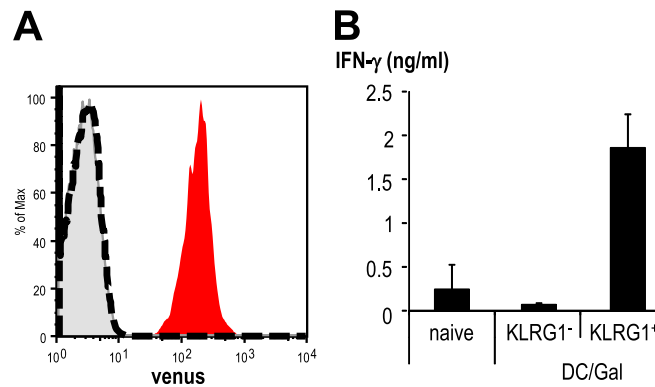


Fig. 55. IFN- γ -producing capacity of naive and memory-like iNKT cells from V α 14NT mice. V α 14NT mice were immunized with DC/Gal 1 mo previously. IFN- γ production by KLRG1⁺ and KLRG1⁻ iNKT cells in the lung of immunized mice and by naive iNKT cells from nonimmunized mice was assessed. (A) iNKT cells from V α 14NT mice were recognized as venus⁺ cells (red), and iNKT cells from WT mice were detected as dots. (B) The supernatants from the cultures with plate-bound anti-CD3 Ab plus anti-CD28 Ab for 24 h were analyzed at the protein level for IFN- γ by ELISA ($n = 4$; data are shown as mean \pm SEM).

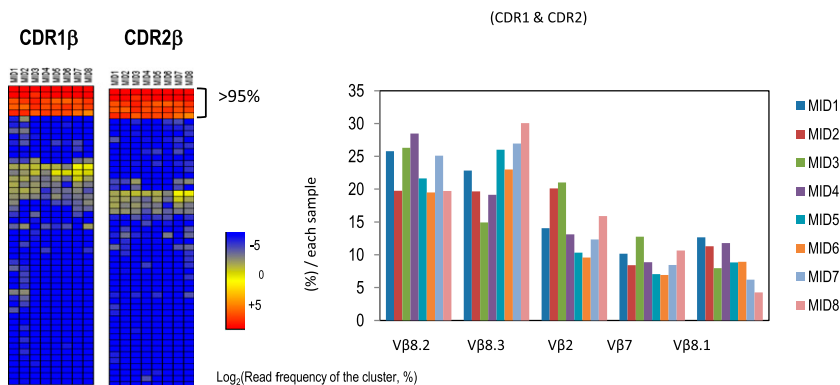


Fig. 56. RNA sequence of complementary-determining region1 β (CDR1 β) and CDR2 β of effector memory-like KLRG1⁺ iNKT cells. The naive iNKT cells and KLRG1⁺ iNKT cells were sorted and tested for TCR RNA-seq analysis. In brief, pooled naive or KLRG1⁺ iNKT cells were prepared from five or six naive, DC/Gal-immunized mice (at day 7 or day 28). In some experiments, DC/Gal-injected mice were boosted with DC/Gal 28 d after the first immunization (DC/Gal-DC/Gal). CDR1 β and CDR2 β of V β in naive (MD1,2), DC/Gal-injected mice [day 7 (MD3,4); day 28 (MD5,6)] or DC/Gal-DC/Gal-boosted mice (MD7,8) were analyzed respectively in two independent experiments at each stage (Left). The cluster of reads of CDR3 by RNA sequence was analyzed, and the number of reads in each V β family was calculated (Right).

Table S1. Specific primer sequences used for real-time PCR

Gene	5'-primer	3'-primer
<i>IL-4</i>	catcggcattttgaacgag	cgagctcactctctgtggtg
<i>IFN-γ</i>	atctggaggaactggcaaaa	ttcaagacttcaaagagtctgagga
<i>CCL3</i>	tgcccttgctgttcttctct	gtggaatcttcggctgtag
<i>CCL4</i>	gccctctctctctctcttgct	gagggtcagagcccattg
<i>Gzma</i>	ggccatctcttgctactctcc	cgtgtctctccaatgattct
<i>FasL</i>	accggtggtatTTTTcatgg	aggctttggttggtgaactc
<i>Eomes</i>	accggcaccaaaactgaga	aagctcaagaaaggaaacatgc
<i>Runx3</i>	ttcaacgaccttcgattcgt	ttggtgaacacggtgattgt
<i>Tbx21</i>	caaccagcaccagacagaga	acaaacatcctgtaatggcttg
<i>HPRT1</i>	tcctcctcagaccgctttt	cctggttcatcatcgtaatc