KLRG$^+$ invariant natural killer T cells are long-lived effectors

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Immunological memory has been regarded as a unique feature of the adaptive immune response mediated in an antigen-specific manner by T and B lymphocytes. However, natural killer (NK) cells and γδ T cells, which traditionally are classified as innate immune cells, have been shown in recent studies to have hallmark features of memory cells. Invariant NKT cells (iNKT cells) are able to recognize and respond to an antigen in the context of CD1d and can persist for a long period, proliferate well, and participate in a recall response. KLRG1 also has been identified as a surface marker on mature NK cells and memory NK cells (10). Memory NK cells express KLRG1hi and have potential implications for future iNKT-cell-based therapies.

**Results**

Prolonged Activation of iNKT Cells in the Lung by an Injection of α-GalCer-Loaded DCs. We previously reported that α-GalCer-loaded DCs (DC/Gal) induced an expansion of the IFN-γ-producing iNKT cell population for 2–4 d in the mouse spleen and that

**Significance**

Both natural killer (NK) cells and γδ T cells, classified as innate immune cells, recently have been shown to have features of memory cells. However, after activation, a memory fate of invariant NK T cells (iNKT cells) has not been identified. Here we show the presence of effector memory-like KLRG1+ (Killer cell lectin-like receptor subfamily G, member 1-positive) iNKT cells in the lung. The KLRG1+ iNKT cells are able to recognize and respond to an antigen in the context of CD1d and can persist for a long time and then mount a potent secondary response upon encountering with the same antigen months later. In addition, we suggest that the KLRG1+ iNKT cells could contribute extensively to immune surveillance, especially in preparation for a possible encounter with tumor diseases.
the longevity of transferred DC/Gal in vivo, we injected mice i.v. with DC/Gal in WT or tumor-bearing mice (Fig. 1A and B and Fig. S1A). Also, lung mononuclear cells (MNCs) in the DC/Gal-injected mice (Fig. S1B), but not in the DC/Gal-injected NK1.1+ cell-depleted or IFN-γ− mice (Fig. S1C and D), displayed the cytotoxic activity against tumor cells. Thus, DC/Gal treatment induced a prolonged antitumor effect in the lung mediated by IFN-γ−producing NKT cells. Initially, to search for a specific marker of these iNKT cells, we decided to examine phenotypical changes in the iNKT cells in the spleen soon after return to the baseline level. The gene expression of iNKT cells in mice 1 wk before and 1 wk after immunization with DC/Gal was analyzed and compared by gene array (Fig. S1E). KLRG1 expression was detected in iNKT cells in lung from DC/Gal-injected mice (Fig. 1C) but not from naive mice (Fig. S1F).

Long-Term Persistence of Effector Memory-Like iNKT Cells. To assess the longevity of transferred DC/Gal in vivo, we injected mice i.v. with the PKH-labeled DCs and observed the labeled DC/Gal in the lung for up to 48 h in vivo (Fig. S2A). Next, we detected an increased number of KLRG1+ iNKT cells after injection of DC/Gal and also CD1d-transfected B16 melanoma cells loaded with α-GalCer (CD1d-B16/Gal), CD1d-transfected NIH 3T3 cells loaded with α-GalCer (CD1d-NIH 3T3/Gal), or CD1d-HEK293/Gal (Fig. S2B). However, we could not detect these cells in mice given DC or CD1d-NIHC cells. Thus, T-cell results suggested that α-GalCer-loaded CD1d cells play an important role in this study. Because an injection of CD1d-B16/Gal could simultaneously induce both antigen-specific T-cell and the iNKT-cell response (5, 17), thus complicating the interpretation of our experiments, we decided to use DC/Gal as the main immunization regimen in this study.

The KLRG1+ iNKT cells could be distinguished from naive iNKT cells based on the expression of several other surface markers. The KLRG1+ iNKT cells expressed more CD43, CD49d, Ly6C, NK1.1, and NKGD2 and less CD27 and CD69 than the naive iNKT cells (Fig. 2A). These phenotypic characteristics of KLRG1+ iNKT cells may be partly similar to those of effector memory T cells and memory NK cells. These three types of cells commonly express KLRG1+Ly6C+, CD62Llow, and CD27low (18). In addition, the KLRG1+ iNKT cells express NKGD2+ and CD43−, which are shared with memory NK cells (10).

Next, we sought to investigate the kinetics of the proliferative ability of KLRG1+ iNKT cells, which began to be detected 2 d after DC/Gal administration (Fig. S3). The absolute number of total iNKT cells increased and then returned to the baseline level (Fig. 2B). Interestingly, although the expansion and contraction of KLRG1+ iNKT cells was completed by 30 d after DC/Gal administration in spleen, the frequency of KLRG1+ iNKT cells in the lung and liver was maintained for 90 d after the contraction phase (Fig. 2C). Furthermore, we evaluated the persistence of KLRG1+ iNKT cells in the lung at later phases and found that they persisted for up to 8 to 9 mo after an administration of DC/Gal in Fig. 2D.

Characterization of KLRG1+ iNKT Cells. The phenotypes of long-lived KLRG1+ iNKT cells were determined by quantitative real-time RT-PCR for the expression levels of cytokine, chemokine, and cytotoxic molecules. The KLRG1+ iNKT cells expressed specific transcripts compared with naive iNKT cells, such as ccl3, ccl4, and granzyme A and expressed higher levels of IFN-γ and fasL transcripts than naive iNKT or KLRG1− iNKT cells (Fig. 3A).

Several transcription factors, such as T-bet (Th21) and Eomesoderm (eomes) have been studied for their roles in the development of iNKT cells in the thymus (19) and also have been reported as factors generating memory T cells (20). In contrast, there has been no study evaluating these transcription factors.
Fig. 3. Characterization of KLRG1+ iNKT cells in DC/Gal-injected mice. C57BL/6 mice were immunized with DC/Gal. One month later, KLRG1+ and KLRG1− iNKT cells in the lung were purified by FACS Aria. As a control, iNKT cells in the lung of naive mice were shown as naive iNKT cells. (A and B) Quantitative analyses of gene expression were performed by quantitative real-time PCR using the primer sets shown in Table S1 (n = 4; data are shown as mean ± SEM). (C and D) To examine the functional features of KLRG1+ iNKT cells, the expression of granzyme A (C) and the production of IFN-γ (D) in iNKT cells in the lung were analyzed. For intracellular staining of IFN-γ, lung MNCs were stimulated with anti-CD3 Ab plus anti-CD28 Ab for 2 h in the presence of brefeldin A. Analysis gates were set on CD19+ CD1d-dimer/Gal+ cells (n = 4–6; data are shown as mean ± SEM). *(P < 0.05 naive iNKT or KLRG1+ iNKT cells versus naive iNKT cells.) (E and F) The expression of granzyme A and CD49d of KLRG1+ iNKT cells in the lung was analyzed 6 mo after immunization with DC/Gal (n = 4; data are shown as mean ± SEM). (G and H) Mice that had been immunized with DC/Gal were challenged with B16 melanoma cells 4 mo later. In some experiments, the mice were treated with control rat IgG or anti-NK1.1 Ab. IFN-γ secretion assay for B16 reactive iNKT cells was performed 12 h later in mice vaccinated with or without DC/Gal (G). Antitumor effects were evaluated 2 wk later by counting the number of metastases in the lungs (H) (n = 4–6; data are shown as mean ± SEM). **P < 0.01 naive versus DC/Gal, anti-NK1.1 Ab treatment (DC/Gal) versus control rat IgG treatment (DC/Gal).

We found that the expression levels of Eomes, tbx21, and runx3 were higher in KLRG1+ iNKT cells than in naive iNKT cells (Fig. 3B). We verified at the protein level that the KLRG1+ iNKT cells from DC/Gal-injected mice, but not from naive mice, expressed a high level of granzyme A (Fig. 3C). We then sought to determine the function of KLRG1+ iNKT cells by assessing the response of KLRG1+ iNKT cells compared with that of naive iNKT cells. When the level of IFN-γ produced by iNKT cells from DC/Gal-injected or naive mice was assessed 2 h after the stimulation with anti-CD3 and anti-CD28 in vitro, the IFN-γ mean fluorescence intensity (MFI) of KLRG1+ iNKT cells was much higher than that of naive iNKT cells (Fig. 3D). Also, KLRG1+ iNKT cells produced higher amounts of IFN-γ, CCL3, and CCL4 than naive iNKT cells, while producing lower IL-4 (Fig. 3E). To verify the ligand dependency, we used CD1d-NIH 3T3/Gal cells instead of DCs, because they do not express costimulatory molecules and IL-12. After the stimulation with CD1d-NIH 3T3/Gal in vitro, KLRG1+ iNKT cells showed an IFN-γ MFI higher than that of naive iNKT cells (Fig. S4A).

Thus, based on the characterization of transcription factors and cytokine production, the KLRG1+ iNKT cells belong to Th1-type polarized iNKT cells. These KLRG1+ iNKT cells also showed more potent and rapid response than naive iNKT cells to α-GalCer (Fig. S4A) and to anti-CD3Ab (Fig. 3D and E) a prominent function that distinguishes them from naive iNKT cells.

In addition to the findings described above, we verified the long persistence of KLRG1+ iNKT cells in the lung; these cells coexpressed granzyme A and CD49d 6 mo after immunization (Fig. 3F). To determine the antitumor activity of KLRG1+ iNKT cells, we performed a study in which DC/Gal-treated mice were challenged with the B16 melanoma i.v. 4 mo later. We first determined that the frequency of IFN-γ-producing KLRG1+ iNKT cells in lung was increased 12 h after B16 challenge (Fig. 3G). Then, for the tumor challenge, we counted the number of tumor metastases in the lung 2 wk after B16 melanoma challenge. We found that fewer metastases developed in mice given DC/Gal (Fig. 3H). Furthermore, to determine if NK1.1+ cells were responsible for the observed protection against B16, we treated mice with anti-NK1.1 Ab just before challenge with B16 at 4 mo after DC/Gal immunization. The number of B16 metastases in the lung was increased, suggesting that the antitumor effects are mediated by KLRG1+ iNKT cells and also to some extent by NK cells.
cells into Jα14+ iNKT cells can persist in vivo, we adoptively transferred KLRG1− iNKT cells from Vα14+ mice, which we had established previously (21). The use of naive Vα14+ iNKT cells from Vα14NT mice was analyzed 1 wk later (n = 4 per group). (B and C) A total of 1 × 10^5 naive iNKT cells from Vα14NT mice was transferred into C57BL/6 mice, and the mice were immunized with DC/Gal on the same day. Twelve weeks later, the mice were rechallenged with or without DC/Gal. (B) The total number of Vα14+ iNKT cells in the lung in all three groups was assessed 1 wk later. (C) The frequency of Vα14+ iNKT cells (10^4 per group). *P < 0.05.

The iNKT-Cell Recall Response. To monitor how long KLRG1− iNKT cells can persist in vivo, we adoptively transferred KLRG1− iNKT cells into Jα18−/− mice. The KLRG1− iNKT cell population was sorted from DC/Gal-injected Vα14+ iNKT cloned (Vα14NT) mice, which we had established previously (21). The use of KLRG1− iNKT cells derived from DC/Gal-injected Vα14NT mice permitted us to track the fate of transferred cells as CD1d-dimer/GalCer+ cells in recipient mice (Fig. S5A). Before conducting this study, we verified that naive iNKT cells in Vα14NT mice did not express KLRG1. The level of expression of KLRG1 in DC/Gal-injected Vα14NT mice was similar to that in DC/Gal-injected WT mice, and KLRG1− Vα14+ iNKT cells from immunized mice produced more IFN-γ than naive Vα14+ iNKT cells (Fig. S5B). The KLRG1− Vα14+ iNKT cells that had been transferred into Jα18−/− mice were expanded when they re-encountered DC/Gal (Fig. 44). This result suggests that KLRG1− iNKT cells are capable of eliciting a recall antigen response.

Next, to reflect the physiological condition better, a small number of naive Vα14+ iNKT cells were transferred into C57BL/6 mice. Adoptive transfer of naive Vα14+ iNKT cells from Vα14NT mice into WT mice allowed us to distinguish between antigen-experienced iNKT cells and iNKT cells newly developed from the thymus. WT mice were transferred with naive Vα14+ iNKT cells, followed by DC/Gal immunization on the same day, and the frequency and the number of KLRG1− Vα14+ iNKT cells were assessed 12 wk later. The Vα14+ iNKT cells were almost undetectable in WT mice that received those cells without being immunized with DC/Gal. In contrast, Vα14+ iNKT cells could be detected even 12 wk later in DC/Gal-immunized mice that had received Vxα14+ Venus+ iNKT cells (Fig. 4B). The number of Vxα14+ Venus+ iNKT cells was increased by rechallenging with DC/Gal 12 wk later (Fig. 4B) while maintaining the expression of KLRG1 phenotype (Fig. 4C). These results suggest that KLRG1− iNKT cells can be long-lived in the periphery and participate in a recall antigen response.

Next, we sought to determine whether an antigen-specific secondary response could occur in DC/Gal-immunized WT mice without receiving iNKT cell transfer. The KLRG1− iNKT cells in the lung were boosted efficiently with DC/Gal 2 mo after the first immunization. The total number of KLRG1− iNKT cells in mice after the boost with DC pulsed with a low dose (10 ng/mL) of α-GalCer was almost 10-fold higher than in mice primed with the same DC pulsed with a low dose of α-GalCer but that did not receive the DC/Gal boost. As expected, KLRG1− iNKT cells failed to expand in mice that received DC alone (Fig. 5A). It is noteworthy that when DC/Gal-immunized mice were boosted with DC pulsed with isoglobotrihexosylceramide (iGB3) or glycosphingolipid (GSL), known endogenous ligands, we were not...
able to detect a secondary boosting effect. That is, the KLRG1+ iNKT cells in the lung were increased, but the level was similar to the primary response in mice upon administration of DC/Gal or DC/GSL (Fig. 5A). Interestingly, when the mice that had been immunized with DC/Gal 1 y previously were boosted with CD1d-NIH 3T3/Gal, the number of KLRG1+ iNKT cells in the lung was greater than the number induced during the primary response to the administration of CD1d-NIH/Gal cells (Fig. S4B).

Collectively, these results show that KLRG1+ iNKT cells in the lung are able to recognize and respond specifically to cognate antigen and that KLRG1+ iNKT cells are long-lived and can mount a potent secondary response.

Analysis of the TCR Repertoire of KLRG1+ iNKT Cells. It is well known that the α chain of the iNKT cell TCR is invariant; however, there is more variability in the β chain, although it is restricted mainly to Vβ7, Vβ8, and Vβ2 (22). We next used flow cytometry to evaluate the TCRVβ repertoire of iNKT cells in naive mice, DC/Gal-injected mice, and DC/Gal-DC/Gal-injected mice. We did not find any accumulation of a specific Vβ repertoire in KLRG1+ iNKT cells of DC/Gal-injected or DC/Gal-DC/Gal–injected mice compared with naive iNKT cells. However, there was an increase in TCRVβ1.1/8.2+ iNKT cells, accompanied by a decrease of other TCRVβ+ iNKT cells in DC/GSL or DC/Gal-DC/GSL–boosted mice, whereas TCRVβ7+ iNKT cells increased in both DC/Gal3B3-injected and DC/Gal-DC/Gal–boosted mice (Fig. 5B). In accordance with previous reports (23), iGB3 is recognized predominantly by Vβ7+ iNKT cells. GSL was found to be recognized predominantly by Vβ8+ iNKT cells (Fig. 5B). These data indicate that the recall antigen response of KLRG1+ NKT cells depends on the ligand rather than on the DCs alone.

Next, we used massively parallel RNA sequencing from a group of pooled iNKT cells to analyze the Vβ complementary-determining regions1β (CDR1β), CDR2β, and CDR3β in detail. To look carefully for evidence of antigen selection of the effector memory-like KLRG1+ iNKT cells, we prepared iNKT cells from four types of mice, i.e., naive NKT cells from naive mice, KLRG1+ iNKT cells from mice that had been administered DC/Gal 7 d or 28 d previously, and KLRG1+ iNKT cells harvested from mice that had been injected with DC/Gal and boosted with DC/Gal 28 d later (DC/Gal-DC/Gal) (Fig. 6). When we analyzed the RNA sequences in detail after clustering of CDR1 and CDR2, the CDR1β and CDR2β of the KLRG1+ iNKT cells matched those of naive iNKT cells almost completely (Fig. S6).

Analysis of CDR3β of KLRG1+ iNKT Cells by RNA Sequencing. We then analyzed the CDR3β sequences of KLRG1+ iNKT cells, because the TCR Vβ repertoire in the iNKT cells became more skewed in the effector or memory phases. Both the CDR3 composition of the Vβ chains and the association with different Jβ segments make the junctional diversity of CDR3β quite heterogeneous in naive mice. We found that even after clustering the common CDR1 and CDR2, the CDR3βs of KLRG1+ iNKT cells were somewhat heterogeneous in DC/Gal-injected mice; however, the CDR3β sequences were distinctly different from those of naive iNKT cells (Fig. 6A). The pattern of CDR3 regions would be clonal in some cases. In addition, such clones accumulated in clusters and became more prominent after boosting (Fig. 6A). Interestingly, further analysis of the clusters of CDR3 reads...
demonstrated that naive iNKT cells are comprised of many different small read clusters (<1%) and very few large read clusters (>1%), whereas activated KLRG1+ iNKT cells at day 7, effector memory-like KLRG1+ iNKT cells at day 28, and boosted effector memory-like KLRG1+ iNKT cells were all selected to make specific large clusters in all the Vj families (Fig. 6B), possibly leading to memory-like patterns in a CDR3β-dependent manner.

Next, we performed principal component analysis of whole CDR3 clusters of KLRG1+ iNKT cells and successfully identified such CDR3 clusters (Fig. 6 C, i). Group 1 contained CDR3 clusters in which any reads in CDR3β could be detected in the effector and the boosting phase (Fig. 6 C, ii), whereas the group 2 was composed of CDR3 clusters in which some reads in CDR3β could be detected in the effector phase, but more could be detected in the memory and the boosting phase (Fig. 6 C, iii). The TCR clone reads in the groups 1 and 2 were enriched in infection with pathogens.

Discussion

One of the key findings in our current study is that lung-resident KLRG1+ iNKT cells may be negatively selected by activation-induced cell death and that some of them survive with resting, naive iNKT cells. They have distinct phenotypes, such as CD49d and granzyme A, and up-regulated expression of CD43 and NKG2D molecules and are long-lived in the periphery. In addition, the effector memory-like iNKT cells respond rapidly as a secondary response and show a prominent function that distinguishes them from naive iNKT cells. Last, the TCRs of KLRG1+ iNKT cells accumulate some particular CDR3β, most likely as the result of clonal expansion.

Another key finding is that KLRG1+ iNKT cells have biological properties similar to those of memory NK and T cells (12, 14, 18, 24). Specifically, KLRG1+ iNKT cells are able to exhibit a potent secondary response upon encountering the cognate iNKT-cell ligand even after several weeks or months; these responses resemble KLRG1+ memory NK (11) and T-cell responses (12–14, 18, 24). However, the kinetics of KLRG1+ iNKT cells (i.e., expansion fold) resemble the kinetics of memory CD4+ T cells rather than those of memory CD8+ T cells (25, 26).

The evidence in this study suggests a previously unidentified role of KLRG1+ iNKT cells, which are able to persist locally in the lung and patrol and conduct immune surveillance in preparation for a possible encounter with and fight against tumor and infections. The strategy for the selective expansion of KLRG1+ iNKT cells could be explored in the future. Recently, we have successfully generated induced pluripotent stem cell (iPS)-reprogrammed iNKT cells that showed potential antitumor activity (27). Therefore, establishing a strategy for the effective generation of KLRG1+ iNKT cells by selectively expanding iNKT cells or by using the iPS-reprogrammed iNKT approach ultimately would allow us to engage in novel therapeutic interventions against human infections and cancer.

Materials and Methods

DCs of mice were generated from bone marrow as described previously (6). In antitumor studies, mice were immunized with or without 1 × 10^6 DC/Gal. Four months later, the mice were injected with 2 × 10^6 B16 melanoma i.v. Fourteen days later, mice were sacrificed and the number of lung metastases were analyzed (4). Detailed information on materials and methods used in this study is provided in SI Materials and Methods.

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Supporting Information

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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, Jα18−/− mice, and iNKT-cloned mice (1) and IFN-γ−/− 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), -CD8287,37.51), -CD43,47, -CD44 (IM7), -CD49d (R1-2), -CD62L, -CD69 (H1.2F3), -CD122 (TM-b1), -NK1.1 (PK136), -NK2D (CX5), Ly6C(AL-21), -KlrG1(2F1), -IFN-γ (XMG1.2). CD1d-dimeric and TCR Vβ screening panel were purchased from BD. Anti-granzyme A (3G8.5) was purchased from Santa Cruz. 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) ligands, 10 μg/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-μm 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 × 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 described previously (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°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°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 μg/mL immobilized anti-CD3 Ab and 2 μg/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 FcγR, washed, and incubated with mAbs to the indicated cell-surface markers. Cells then were permeabilized in Cytofix-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 × 105 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 ± SD of triplicate wells from three independent experiments. After the background control value was subtracted, cytotoxicity values (%) were calculated as Cytotoxicity (%) = [(effector:target cell mix – effector cell control) – spontaneous target cell control] / (maximum target control – spontaneous target cell control) × 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 × 106 DC/Gal. Four months later, the mice were injected i.v. with 2 × 106 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 μg 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 μM each; Table S1). After 18 cycles of preamplification (each cycle: 95°C for 30 s, 60°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 ∆∆CT 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 × 105) were stimulated with 10 μg/mL immobilized CD3 Ab plus 10 μg/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 Vβ 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 RACE cDNA amplification kit (Clontech). Both universal mix primer and primers specific for the T-cell receptor β (TCRβ) constant region sequence were used for second-strand amplifications, resulting in TCRβ 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.

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 ± 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 ± SEM).
Fig. S4. 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 ± SEM). *P < 0.05.

Fig. S5. IFN-γ–producing capacity of naive and memory-like iNKT cells from Vα14NT mice. Vα14NT 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 Vα14NT 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 ± SEM).

Fig. S6. RNA sequence of complementary-determining region1β (CDR1β) and CDR2β 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). CDR1β and CDR2β of Vβ 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 Vβ family was calculated (Right).
### Table S1. Specific primer sequences used for real-time PCR

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