Supporting Information

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SI Materials and Methods

Animals. BALB/c, BALB/c *nu*/*nu*, and C57BL/6 mice were purchased from Clea. T-cell receptor (TCR)-βδ KO mice and IL-2 KO mice were purchased from Jackson Laboratory. Anti-ovalbumin (OVA)-specific TCR-αβ (DO11.10) transgenic (Tg) mice were provided by Dennis Loh (Washington University, St. Louis). CD1d-deficient mice were provided by Luc Van Kaer (Vanderbilt University, Nashville, TN). Jα18-deficient mice were generated as described previously (1). The OT-I Tg, OT-II Tg, and IL-4 KO mice used in this study were 6–7 wk of age. A total of three independent experiments were performed for all in vivo experiments ($n = 5$ per group). Animal care was conducted in accordance with the guidelines of Chiba University. All animal experiments were approved by the Chiba University Review Board for Animal Care.

Antibodies. For flow cytometry analysis, 1 million cells were incubated with anti-CD16/CD32 (24G2) mAbs and stained with the indicated staining reagents in accordance with standard methods. Anti-cγ (TUGm2), anti-CD4 (RM4-5), anti–IL-2Rα (3C7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti–IL-2Rβ (TM-β1), and anti-DO11.10 TCR (KJ1-26) mAbs were purchased from BD Pharmingen. Anti–IL-21R (4A9) mAb was purchased from Biolegend. For in vitro neutralization of cytokines, anti–IL-2 (JES6-1A12) and anti–IL-4 (11B11) mAbs were purchased from Biolegend, anti–IL-7 mAb was purchased from R&D Systems, and anti–IL-15 (AIO.3) and IL-21 (FFA21) mAbs were purchased from eBioscience.

Proliferation Assays. For the analysis of memory T helper (Th) Th1- or Th2-cell proliferation in response to cytokines, [^{3}H]-thymidine (37 kBq per well) was added to the culture for the last 16 h, and the incorporated radioactivity was measured on a β plate. For the analysis of proliferation of keyhole limpet hemocyanin (KLH)-specific CD4 T cells, CD44^{hi} and CD44^{lo} CD4 T cells were purified from the spleen of mice immunized with KLH and complete Freund's adjuvant by cell sorting. The cells were stimulated with KLH and irradiated allophycocyanin for 48 h in vitro. ^{3}H-thymidine was added to the culture for the last 16 h. Cells that incorporated BrdU were detected with a BrdU flow kit (BD Biosciences) in accordance with the manufacturer's protocol. BrdU solution (1 mg/mL) was added to the culture at 45 min before harvest. Staining of BrdU was performed according to the manufacturer's instructions (BD Biosciences).

Generation of Bone Marrow-Derived Dendritic Cells. Bone marrow-derived dendritic cells (BMDCs) from BALB/c mice were seeded in culture medium containing 200 U/mL of murine GM-CSF (Peprotech). On day 6, adherent cells were collected and pulsed with α-galactosylceramide (α-GalCer; 100 ng/mL), α-glucuronsylceramide (GSL-1′; 40 μg/mL), or sulfatide (10 μg/mL) for 24 h. GSL-1′ was prepared as described previously (2); sulfatide was purchased from Matrea.

Measurement of Airway Hyperreactivity and Airway Inflammation. Airway inflammation was induced by exposure to a 1% solution of OVA (grade V; Sigma-Aldrich) in PBS, aerosolized using a nebulizer (Omron) for 30 min. Airway hyperreactivity was assessed by methacholine-induced (Sigma-Aldrich) airflow obstruction at 24 h after the last antigen challenge, a computer-controlled small animal ventilator (SCIREQ). To examine airway inflammation, bronchoalveolar lavage was performed at 24 h after the last OVA challenge. For histological analysis of asthmatic lungs, the mice were killed by asphyxiation at 48 h after the last OVA challenge.

ELISA. The concentrations of IL-4, IL-5, IL-13, and IFN-γ in supernatants were measured by ELISA, as described previously (3).

Intracellular Staining. For the detection of phosphorylated STAT5 and STAT3, cells were fixed using Phosflow Lyse/Fix Buffer (BD Biosciences) and permeabilized with Phosflow Perm Buffer III (BD Biosciences). Cells were then incubated with anti-STAT5 (pY694) mAbs or anti-STAT3 (pY705) mAbs (BD Biosciences). Phosphorylation of STAT5 or STAT3 was assessed by flow cytometry.

Data Analysis. Values are presented as mean ± SEM. Statistical analysis was performed with GraphPad Prism. Differences were determined using the two-tailed Student *t* test or one-way ANOVA with Dunnett's multiple-comparison test. A P value <0.05 was considered statistically significant.

Quantitative PCR Analysis. Total RNA was isolated from the liver (three mice per group) using TRIzol reagent (Sigma-Aldrich). Reverse transcription was carried out using SuperScript II reverse transcriptase (Invitrogen). Samples were then analyzed by quantitative PCR analysis with an ABI Prism 7300 Sequence Detection System (Applied Biosystems) under standard conditions. The probes for the detection of cytokines were purchased from Roche Diagnostics. Primers for the probes ware as follows: IL-2: forward, gctgttgatggacctacagga; reverse, ttcaattctgtggcctgctt; IL-4: forward, cctgctcttctttctcgaatgt; reverse, cacatccatctccgtgcat; IL-6: forward, tgatggatgctaccaaactgg; reverse, ttcatgtactccaggtagctatgg; IL-7: forward, ttcctccactgatccttgttc; reverse, tcagttcctgtcattttgtcca; IL-9: forward, gcctctgttttgctcttcagtt; reverse, gcattttgacggtggatca; IL-10: forward, cagagccacatgctcctaga; reverse, tgtccagctggtcctttgtt; p35: forward, ccatcagcagatcattctagacaa; reverse, cgccattatgattcagagactg; IL-15: forward, cagctcagagaggtcaggaaa; reverse, catgaagaggcagtgctttg; IL-21: forward, gacattcatcattgacctcgtg; reverse, tcacaggaagggcatttagc; IFN-β: forward, ggaaagattgacgtgggaga; reverse, ccaggcgtagctgttgtactt; IFN-γ: forward, atctggaggaactggcaaaa; reverse, ttcaagacttcaaagagtctgagg; HPRT: forward, tcctcctcagaccgctttt; reverse, cctggttcatcatcgctaatc. mRNA expression was normalized using the HPRT signal.

1. Cui J, et al. (1997) Requirement for Valpha14 NKT cells in IL-12–mediated rejection of tumors. *Science* 278:1623–1626.
2. Kinjo Y, et al. (2005) Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 434:520–525.
3. Kimura M, et al. (2001) Regulation of Th2 cell differentiation by mel-18, a mammalian polycomb group gene. *Immunity* 15:275–287.

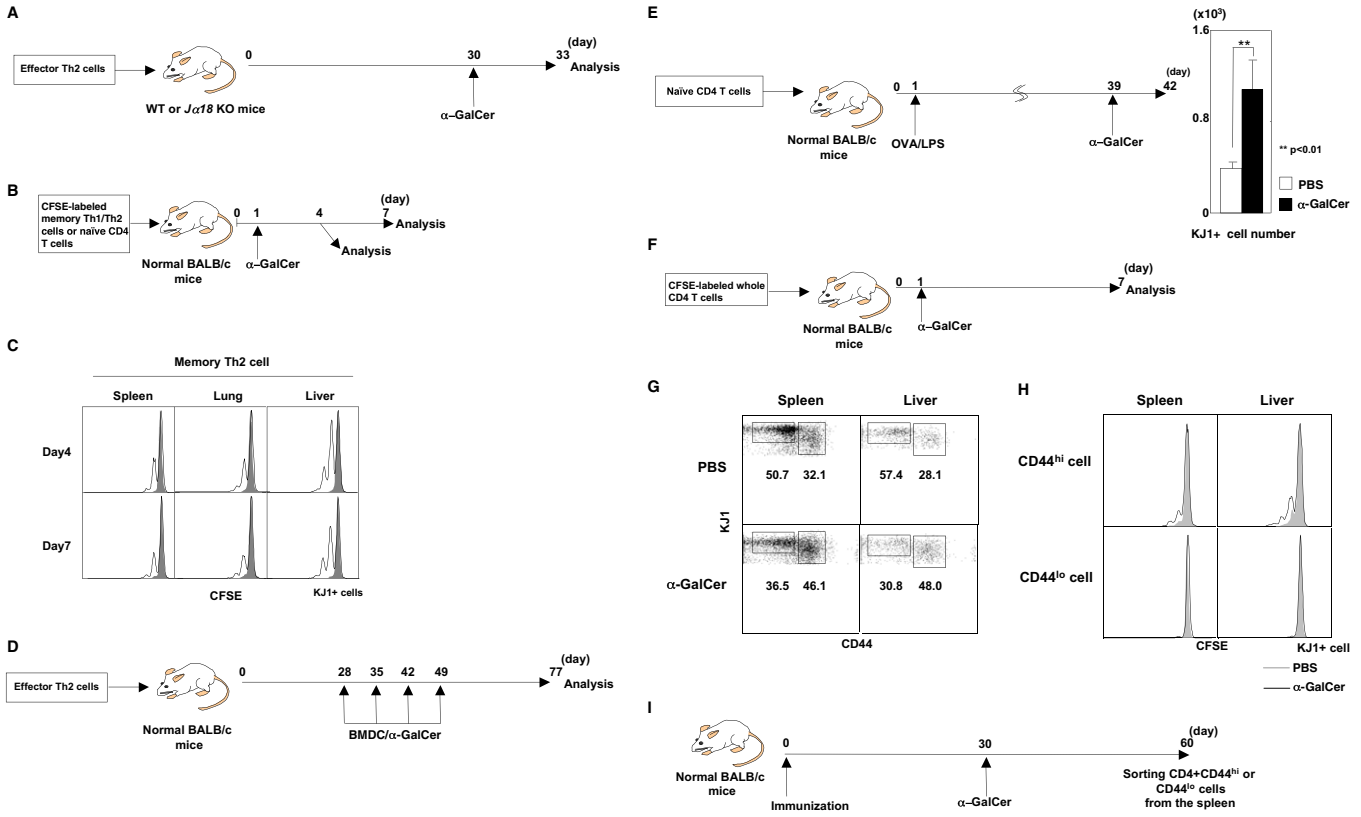

Fig. S1. Activation of invariant natural killer T (iNKT) cells induced the proliferation of memory CD4 T cells in vivo. (*A*) Experimental protocol of memory Th2-cell generation and activation of iNKT cells. Effector Th2 cells (3×10^7) generated from CD4 T cells in DO11.10 Tg mice were transferred into syngeneic WT or Jα18 KO mice. Thirty days later, α-GalCer was injected. (*B*) Experimental protocol of carboxyfluorescein succinimidyl ester (CFSE) analysis of memory Th2 cells. Memory Th2 cells (5×10^6) purified from Th2 memory mice and $CD44^{lo}CD62L^{hi}$ naïve CD4 T cells purified from normal DO11.10 Tg mice were labeled with CFSE and then transferred into syngenic mice. One day later α-GalCer was injected. On day 7, cell division of transferred cells in the spleen, lung, and liver were assessed by flow cytometry. (*C*) Cell division of CFSE-labeled memory Th2 cells in the spleen, lung, and liver at 3 d and 6 d (on days 4 and 7, respectively) after α-GalCer administration. (*D*) Experimental protocol for activation of iNKT cells with α-GalCer–pulsed BMDC transfer. Effector Th2 cells (3×10^7) generated from CD4 T cells in DO11.10 Tg mice were transferred into syngeneic WT mice. Four weeks later, α-GalCer–pulsed BMDCs (4×10^5) were transferred into the mice once a week for 4 wk. (*E*) Experimental protocol of memory CD4 T-cell generation from naïve CD4 T cells and activation of iNKT cells in vivo. Splenic CD4 T cells (1×10^7) from DO11.10 Tg mice were transferred into normal BALB/c mice, and these mice were immunized with OVA (100 μg) and LPS (100 μg). On day 39, α-GalCer was injected. The numbers of $KJ1^+$ memory CD4 T cells in the liver are shown. Values are mean ± SEM ($n = 5$). $^*P < 0.05$; $^{**}P < 0.01$. (*F*) Freshly prepared whole CD4 T cells from the spleens of DO11.10 Tg mice were labeled with CFSE (1×10^7) and transferred into normal BALB/c mice. (*G*) Percentages of naïve CD4 T cells ($CD44^{lo}$) and naturally occurring $CD44^{hi}$ memory CD4 T cells were assessed at 6 d after α-GalCer administration. (*H*) Cell division of $CD44^{hi}$ and $CD44^{lo}$ CD4 T cells. (*I*) Assessment of endogenous memory CD4 T-cell number and response to antigen. Normal WT mice were immunized with KLH (100 μg) and complete Freund's adjuvant, and then injected with α-GalCer 30 d later. On day 60, $CD44^{hi}$ and $CD44^{lo}$ CD4 T cells in the spleen were assessed.

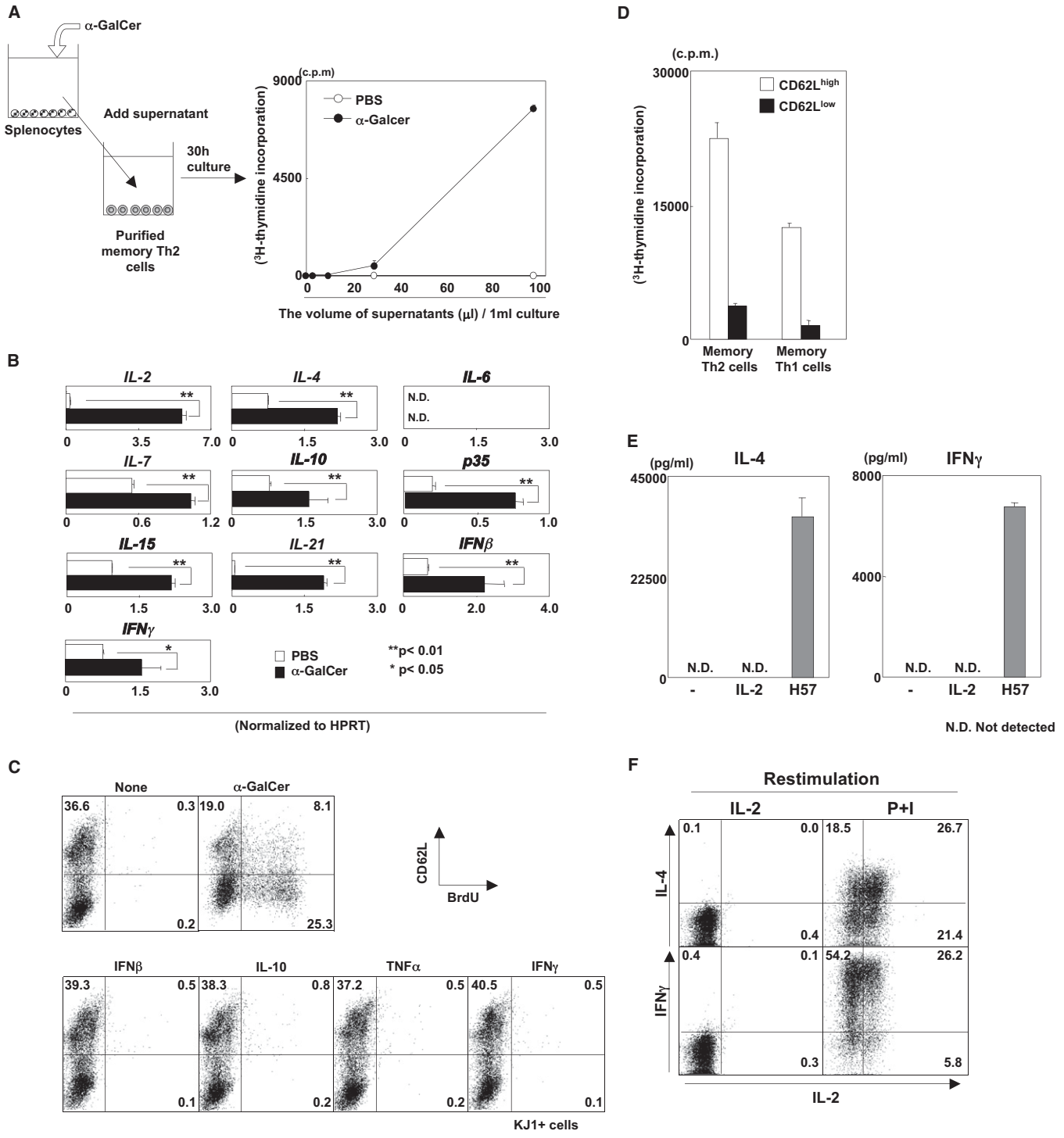

Fig. S2. IL-2 produced by activated iNKT cells induced the proliferation of memory Th2 cells. (*A*) Culture supernatant from splenocytes stimulated with α-GalCer were transferred to another culture including purified memory Th2 cells (2×10^5) and incubated for a further 40 h. Transferred volumes of culture supernatant are indicated. [^{3}H]-thymidine was added to the culture for the last 16 h, and incorporated radioactivity was measured. (*B*) Quantitative RT-PCR analysis of cytokines in the liver of BALB/c mice at 3 d after treatment with α-GalCer (100 μg/kg). (*C*) Memory Th2 cells (2×10^5) purified from the spleens of memory Th2 mice were cocultured with splenocytes (2×10^6) from BALB/c mice in the presence of α-GalCer (100 ng/mL), IFN-β (100 U/mL), IL-10 (10 ng/mL), TNF-α (50 ng/mL), or IFN-γ (100 U/mL). Three days later, BrdU incorporation by memory Th2 cells was assessed by flow cytometry. (*D*) Purified CD62L^{hi} and CD62L^{lo} memory Th1 and Th2 cells (2×10^5) were stimulated with IL-2 (25 U/mL) for 40 h, after which the incorporation of [^{3}H]-thymidine was assessed. (*E*) Memory Th2 cells were prepared from splenocytes of memory Th2 mice and stimulated with IL-2 (25 U/mL) or plate-bound anti–TCR-β mAbs for 72 h. IL-4 and IFN-γ levels in culture supernatant were measured by ELISA. (*F*) After stimulation of memory Th2 cells with IL-2 for 72 h, the cells were restimulated with IL-2 (25 U/mL) or phorbol myristate acetate (50 ng/mL) plus ionomycin (500 nM) for 4 h. Intracellular staining profiles of IL-2, IL-4, and IFN-γ are shown with the percentages of cells in each area. Memory Th2 cells used in this experiment were generated in BALB/c background mice.

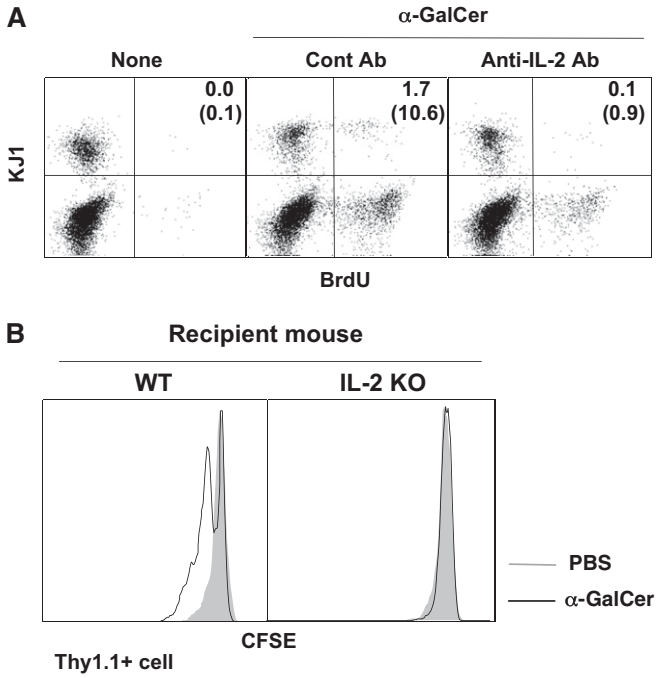

Fig. S3. IL-2 produced by activated iNKT cells induced the proliferation of memory Th1 cells. (*A*) BrdU incorporation by memory Th1 cells in vitro. Memory Th1 cells (2×10^5) prepared from memory Th1 mice (BALB/c background) were cocultured with splenocytes (2×10^6) from BALB/c mice and then stimulated with α-GalCer (100 ng/mL) in the presence or absence of the indicated neutralizing anti–IL-2 mAbs (10 μg/mL) against cytokines. (*B*) Cell division of memory Th1 cells induced by α-GalCer stimulation was assessed in WT and IL-2 KO mice. CFSE-labeled memory Th1 cells (Thy1.1$^+$; C57BL/6 background) (5×10^6) were transferred into WT or IL-2 KO mice (Thy1.2$^+$; C57BL/6 background). The next day, α-GalCer was injected in these mice. Six days later, division of memory Th1 cells was assessed by flow cytometry.

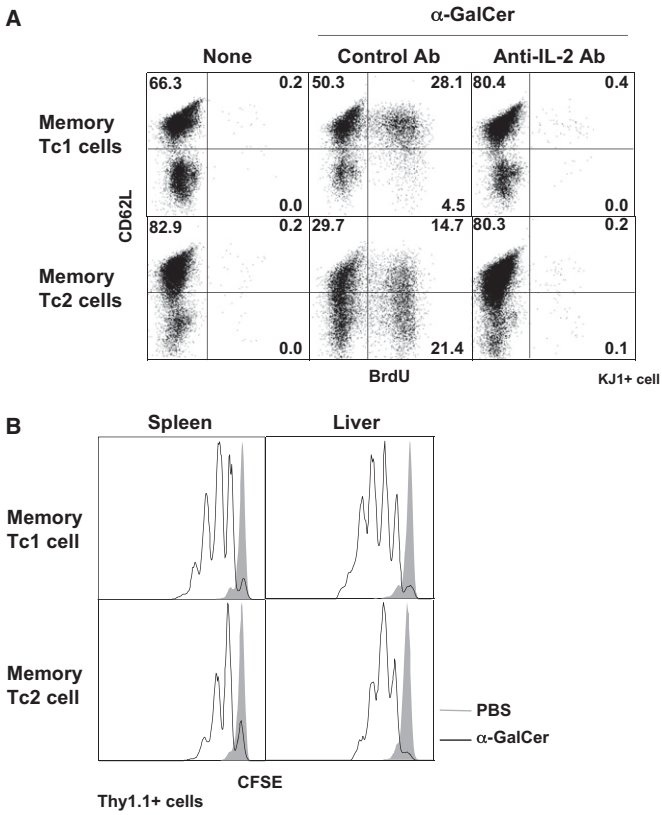

Fig. S4. Activation of iNKT cells induced proliferation of memory CD8 T cells with IL-2. Memory Tc1 or Tc2 cells were generated by transferring effector Tc1 or Tc2 cells into TCR-βδ KO mice. Effector Tc1 or Tc2 cells were induced from splenic CD8 T cells from OT-I Tg (Thy1.1$^+$) mice using the same culture conditions as described for effector Th1 and Th2 cells. (*A*) After purification of memory Tc1 or Tc2 cells from the spleens of memory Tc1 or Tc2 mice by fluorescence activated cell sorting, memory CD8 T cells (2×10^5) were cultured with splenocytes (2×10^6) in the presence of α-GalCer (100 ng/mL) and anti–IL-2 mAbs (10 μg/mL) for 72 h. Incorporated BrdU was detected by a flow cytometer. (*B*) CFSE-labeled memory Tc1 or Tc2 cells (Thy1.1$^+$) (5×10^6) were transferred into naïve syngeneic mice (Thy1.2+). The next day, α-GalCer was injected into the mice (100 μg/kg). Six days later, cell division of memory Tc1 or Tc2 cells in the liver and spleen were assessed by flow cytometry. The donor cells and recipient mice used in this experiment were C57BL/6 background.

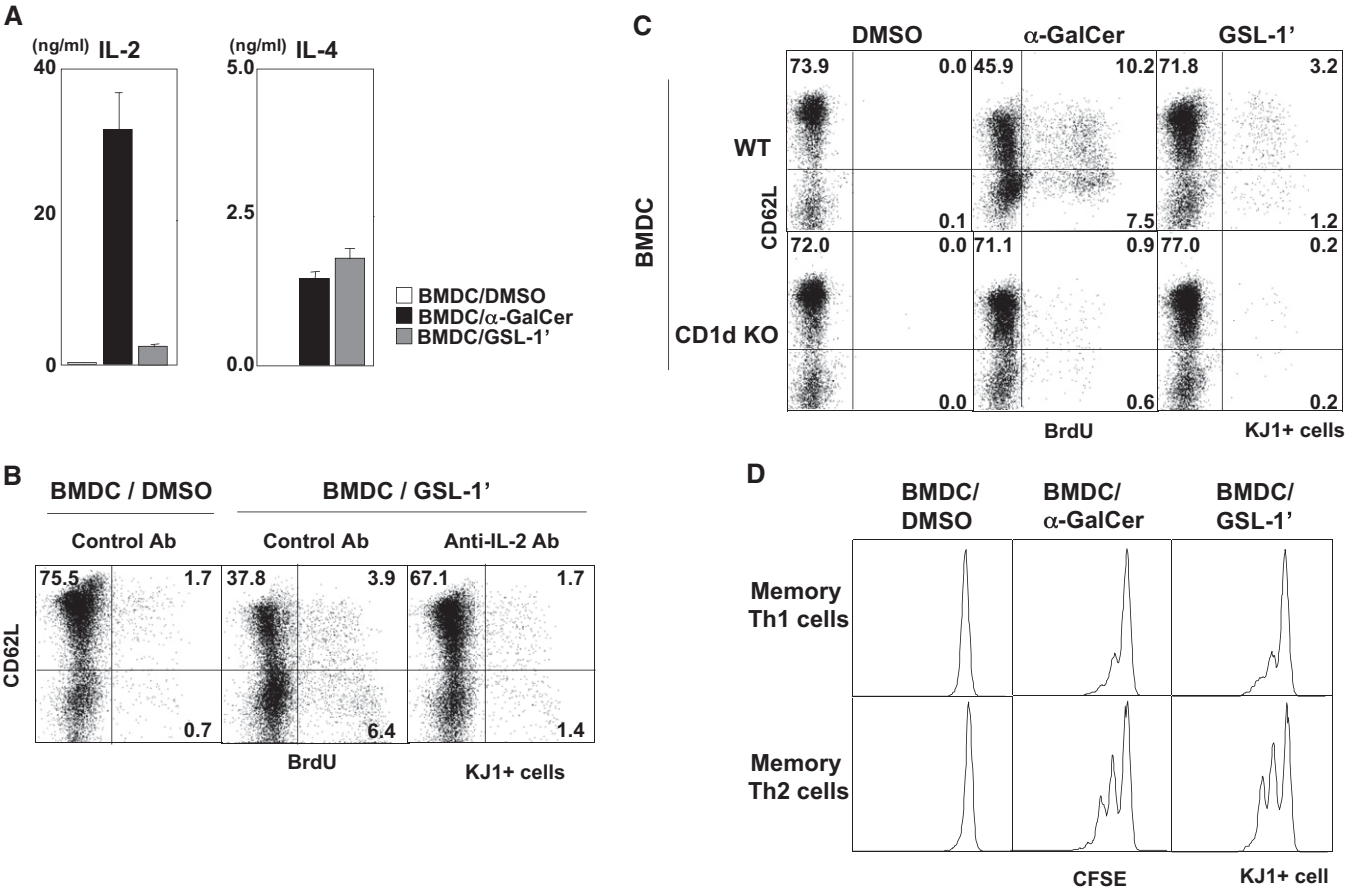

Fig. S5. A ligand for iNKT cells in the bacterial cell wall induced proliferation of memory Th1 and Th2 cells. (*A*) Splenocytes (2×10^6) from BALB/c mice were stimulated with GSL-1′– or α-GalCer–pulsed BMDCs (4×10^5) for 48 h. Cytokine concentrations in culture supernatants were determined by ELISA. (*B*) BrdU incorporation by memory Th2 cells stimulated with splenocytes and GSL-1′–pulsed WT BMDCs was assessed in the presence of anti–IL-2 mAbs (10 μg/mL). (*C*) BrdU incorporation in memory Th2 cells stimulated with GSL-1′–pulsed WT or CD1d KO BMDCs. Th2 cells (2×10^5) prepared from memory Th2 mice, BALB/c splenocytes (2×10^6), and GSL-1′– or α-GalCer–pulsed BMDCs (4×10^5) were cocultured for 72 h. BrdU incorporation in memory Th2 cells was assessed by flow cytometry. (*D*) One day after cell transfer of CFSE-labeled memory Th1 or memory Th2 cells (5×10^6), GSL-1′–pulsed BMDCs (5×10^5) were administered i.v. Six days later, cell division of memory Th1 and Th2 cells was assessed. The memory Th1 and Th2 cells used in this experiment were generated in BALB/c background mice.

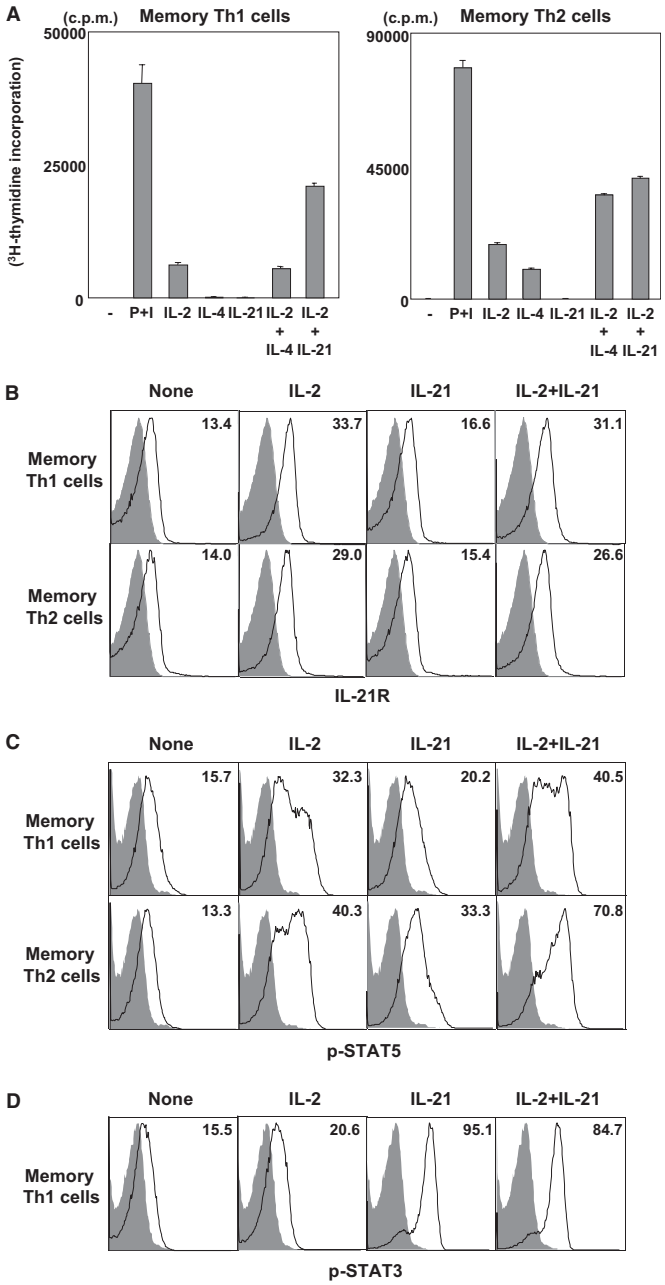

Fig. S6. IL-21 enhanced IL-2–induced memory CD4 T-cell proliferation by amplifying STAT5 activation. (*A*) Proliferation of memory Th2 cells (2×10^5) stimulated with IL-2 (25 U/mL), IL-4 (100 U/mL), IL-21 (100 ng/mL), IL-2 (25 U/mL) + IL-4 (100 U/mL), or IL-2 (25 U/mL) + IL-21 (100 ng/mL) was determined by incorporation of [^{3}H]-thymidine. (*B*) IL-21 receptor expression on memory Th1 and Th2 cells stimulated with cytokines for 3 d. (*C*) Phosphorylation of STAT5 in memory Th2 cells (1×10^6) stimulated with IL-2, IL-21, or both for 30 min. In *B* and *C*, values represent mean fluorescence intensity. (*D*) The phosphorylation of STAT3 in memory Th2 cells (1×10^6) stimulated with IL-2, IL-21, or both for 30 min. Values are mean fluorescence intensity. The memory Th2 cells used in this experiment were generated in BALB/c background mice.

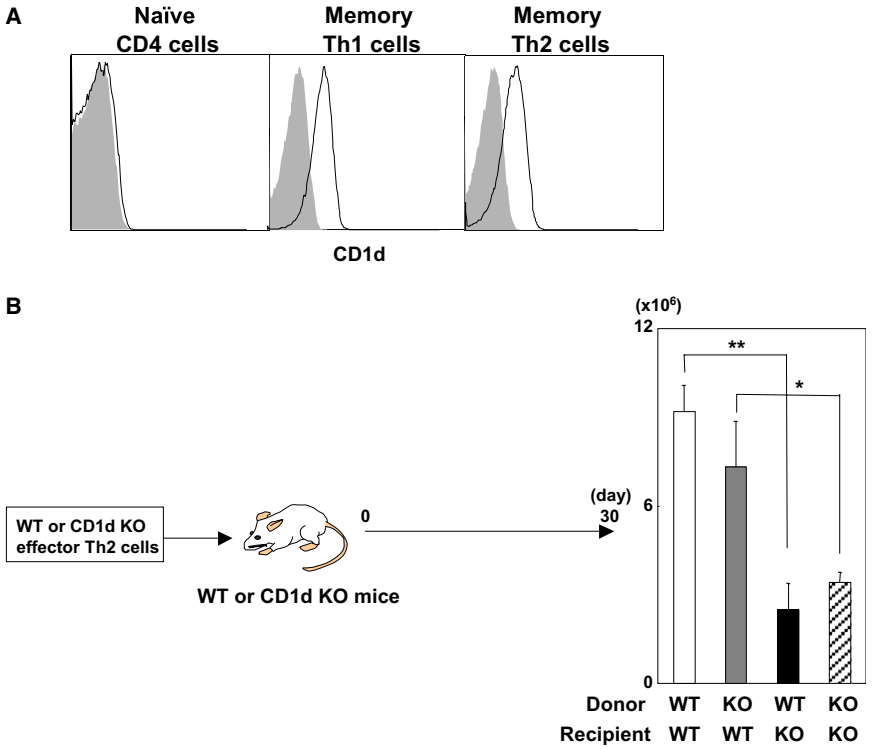

Fig. S7. Generation of CD1d KO memory Th2 cells in WT and CD1d KO mice. (*A*) CD1d expression on naïve CD4 T cells, memory Th1 cells, and memory Th2 cells. (*B*) WT or CD1d KO effector Th2 cells (3×10^7) were transferred into WT or CD1d KO mice. Thirty days after cell transfer, the absolute numbers of memory Th2 cells in the spleen were assessed. The donor cells and recipient mice used in these experiments were BALB/c background. Values are mean ± SEM ($n = 5$). $^*P < 0.05$; $^{**}P < 0.01$.

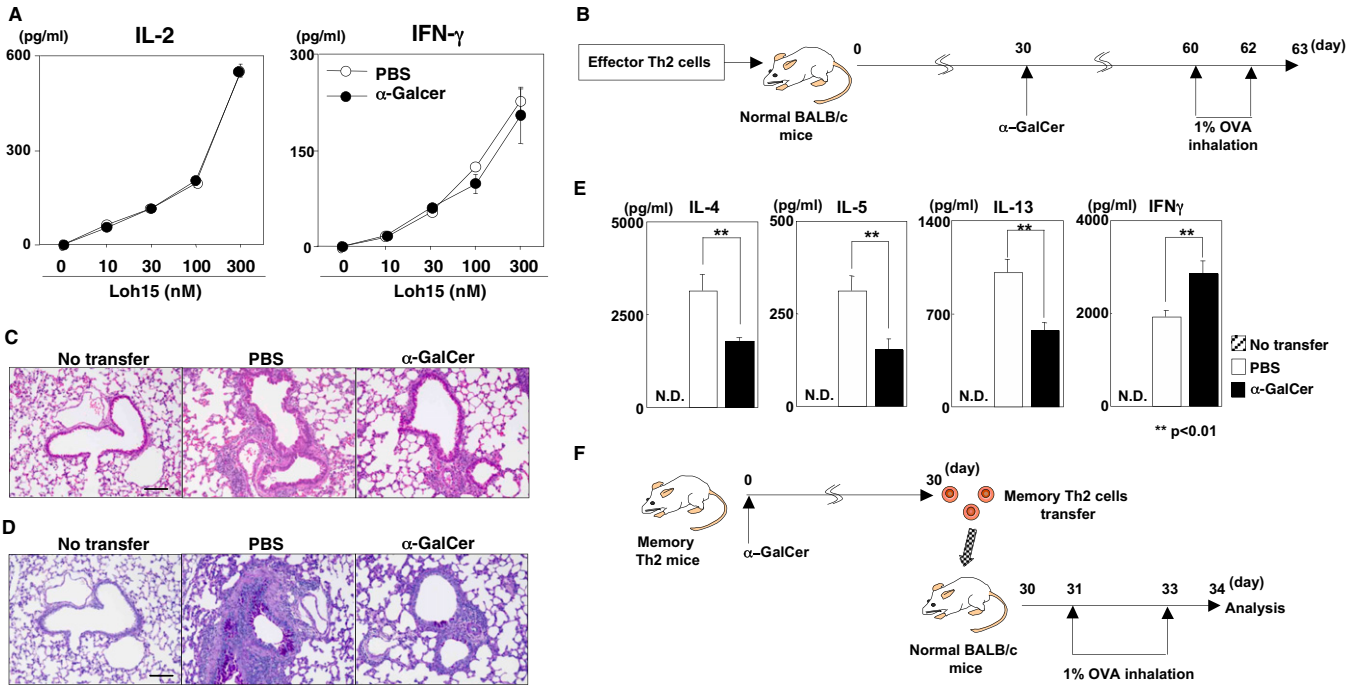

Fig. S8. Activation of iNKT cells altered the function of memory Th2 cells, but not of memory Th1 cells. (*A*) Memory Th1 cells were prepared from spleens of memory Th1 mice treated with α-GalCer at 30 d before sacrifice. After stimulation of memory Th1 cells with OVA peptide and irradiated allophycocyanin for 72 h, cytokine levels in the supernatants were measured by ELISA. (*B*) Experimental protocol for memory Th2 cell-dependent allergic airway inflammation. Thirty days after α-GalCer administration, memory Th2 mice were exposed to OVA aerosol to induce airway inflammation. (*C*) Antigen-induced leukocyte infiltration into the lung was evaluated by H&E staining. (*D*) Antigen-induced goblet cell hyperplasia was evaluated by periodic acid-Schiff staining. Representative photographic views of lung section from memory Th2 mice treated with or without α-GalCer are shown. (Scale bar: 100 μm.) (*E*) Cytokine levels in the bronchoalveolar lavage fluid of memory Th2 mice treated with α-GalCer. Bronchoalveolar lavage fluid was collected at 12 h after the last challenge. Values are mean ± SEM ($n = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$. (*F*) Memory Th2 cells (1×10^6) were prepared from the spleens of memory Th2 mice treated with α-GalCer and then transferred into normal BALB/c mice. Allergic airway inflammation was induced in these mice via two OVA challenges. The donor cells and recipient mice used in these experiments were BALB/c background.