Supporting Information

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Materials and Methods

Mice and Materials. C57BL/6J (B6) mice were purchased from the Jackson Laboratory. Six- to ten-week-old females were used for all experiments unless otherwise indicated. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

α-Galactosylceramide (α-GalCer, KRN7000) was purchased from Avanti Polar Lipids; lipopolysaccharides (LPS) and 5-fluorouracil (5-FU) from Sigma; recombinant murine IL-3, IL-6 and stem cell factor (SCF) from PeproTech; and polybrene from Millipore. Fluorochrome-conjugated mCD1d/PBS-57 tetramer reagents were provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Fixable Viability Dye eFluor455UV was purchased from affymetrix eBioscience.

Antibodies and Flow Cytometry. Fluorochrome-conjugated antibodies specific for mouse CD3, CD4, CD8, CD19, CD11b, CD24, CD62L, CD44, DX5, F4/80, Gr-1, TCRβ, TCR Vβ7, TCR Vβ8, and TCR Vα8.3 were purchased from BioLegend; for mouse NK1.1, IFN-γ, IL-4, TCR Vα2, TCR Vα3.2, TCR Vβ3, TCR Vβ4, TCR Vβ5, TCR Vβ6, TCR Vβ11, and TCR Vβ13, from BD Biosciences. Fc Block (anti-mouse CD16/32) was purchased from BD Biosciences. Cells were stained as previously described (1) and analyzed using an LSRFortessa flow cytometer (BD Biosciences). FlowJo software was used to analyze the data.

ELISA. The ELISAs for detecting mouse cytokines were performed following a standard protocol from BD Biosciences. The capture and biotinylated antibody pairs for detecting mouse IFN-γ and IL-4 were also purchased from BD Biosciences. The streptavidin–HRP conjugate and mouse IFN-γ and IL-4 Single-Use ELISA Ready-Set-Go (RSG) Standards were purchased from affymetrix eBioscience. The 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was purchased from KPL. The samples were analyzed for absorbance at 450 nm using an Infinite M1000 microplate reader (Tecan).

Single-Cell iNKT TCR Cloning. The single-cell iNKT TCR RT-PCR was performed based on an established protocol (2), with certain modifications. iNKT cells were sorted from mouse spleen cells based on a stringent forum of surface markers ($CD3^{lo}$mCD1d/PBS-57^{+}TCR $V\beta8^{+}NK1.1^{hi}$) using a FACSAria II flow cytometer (BD Biosciences) (lo, low; hi, high). Single cells were sorted directly into PCR plates containing cell lysis buffer. The plates were then immediately flash frozen and stored at −80 °C until use. Upon thawing, the cell lysate from each cell was split in half on the same PCR plate and processed directly into iNKT TCR cloning for both α and β chain genes using a OneStep RT-PCR kit (QIAGEN), following the manufacturer's instructions and using the iNKT TCR gene-specific primers. These primers were designed to amplify the ∼200 bps spanning the CDR3 regions of the iNKT TCR α and β chain cDNAs and were customer-synthesized by Integrated DNA Technologies (IDT): for TCRα (FW primer: 5′-GGG AGA TAC TCA GCA ACT CTG GAT AAA GAT GC -3′; BW primer: 5′- CCA GAT TCC ATG GTT TTC GGC ACA TTG -3′) and for TCRβ (FW: 5′- GGA GAT ATC CCT GAT GGA TAC AAG GCC TCC -3′; BW: 5′-GGG TAG CCT TTT GTT TGT TTG CAA TCT CTG -3′). Verified sequences (productive germline Vα14-Jα18-Cα assembly for TCRα and Vβ8-D/J/*N*-Cβ assembly for TCRβ) were used to construct the complete cDNA sequences encoding the TCR α and β chains from a single cell, based on information about murine TCR genomic segments [the international ImMunoGeneTics information system (IMGT), www.imgt.org]. The selected iNKT TCR α and β pair cDNAs were then synthesized as a single bicistonic gene, with codon optimization and a F2A sequence linking the TCRα and TCRβ cDNAs to enable their coexpression (GenScript).

The 293.T/mCD3 Stable Cell Line. HEK293.T human embryonic kidney epithelial cells (ATCC) were stably transduced with a lentiviral vector (3) coexpressing all four chains of mouse CD3 complex (CD3γ, CD3δ, CD3ε, and CD3ζ), through linking the four cDNAs with three different 2A sequences (F2A, foot-and-mouth disease virus 2A; P2A, porcine teschovirus-1 2A; and T2A, Thosea asigna virus 2A). The transduced cells were then transiently transfected with an MOT1 vector encoding a mouse CD8 TCR, using a standard calcium precipitation procedure (1). Single cells supporting the high surface expression of OT1 TCRs (gated as $CD3^{+}$TCR $V\beta5^{+}$) were sorted out using flow cytometry and grown into single-cell clones. A stable, single-cell clone that lost OT1 TCR expression, but retained the capacity to support mouse TCR surface expression, was selected and designated as the 293.T/mCD3 stable cell line.

Mock and miNKT Retroviruses. Mock (MIG) retroviral vector was reported previously (1). miNKT retroviral vector was constructed by inserting the synthetic bicistronic gene (iNKT TCRα-F2A-TCRβ) into the MIG vector, replacing the IRES-EGFP segment. Retroviruses were made using HEK293.T cells, following a standard calcium precipitation protocol as previously described (1).

HSC Isolation, Transduction, Adoptive Transfer, and Secondary Bone Marrow Transfer. The procedures were reported previously (1). In brief, B6 mice were treated with 5-fluorouracil (250 μg per gram body weight). Five days later, bone marrow (BM) cells were harvested and cultured for 4 d in BM cell culture medium containing recombinant murine IL-3 (20 ng/mL), IL-6 (50 ng/mL), and SCF (50 ng/mL). On days 2 and 3, BM cells were spin-infected with retroviruses supplemented with 8 μg/mL of polybrene, at 770 × g, 30 °C for 90 min. On day 4, BM cells were collected and i.v. injected into B6 recipients that had received 1,200 rads of total body irradiation (∼1–2 × 10^6 transduced BM cells per recipient). For secondary BM transfer, fresh total BM cells harvested from the primary BM recipients were i.v. injected into secondary B6 recipient mice that had received 1,200 rads of total body irradiation (∼10 × 10^6 total BM cells per recipient). The BM recipient mice were maintained on the combined antibiotics sulfmethoxazole and trimethoprim oral suspension (Septra; Hi-Tech Pharmacal) in a sterile environment for 6–8 wk until analysis or use for further experiments.

Bone Marrow-Derived Dendritic Cell Generation, Antigen Loading, and Mouse Immunization. B6 mouse BMDCs were generated from BM cell cultures and matured with LPS as described previously (1). The LPS-matured BMDCs were then cultured at 37 °C in a 6-well plate at 10 × 10^6 cells/well/2 mL BMDC culture medium containing 5 μg/mL of α-GalCer for 2 h, with gentle shaking every 30 min. The α-GalCer–loaded BMDCs were then washed twice with PBS and used to immunize mice through i.v. injection (∼1 × 10^6 BMDCs/mouse).

In vitro iNKT Cell Functional Assays. Spleen cells containing iNKT cells were cultured in vitro in a 24-well plate at 2 × 10^6 cells per well in regular mouse lymphocyte culture medium, with or

without the addition of α-GalCer (100 ng/mL), for 5 d. On days 3 and 5, cells were collected and assayed for iNKT cell expansion using flow cytometry, and the cell culture supernatants were collected and assayed for effector cytokine (IFN-γ and IL-4) production by ELISA. On day 3, some cells were also treated with 4 μL/6 mL BD GolgiStop for 4–6 h and then assayed for intracellular cytokine production using flow cytometry via intracellular staining using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences).

In vivo iNKT Cell Functional Assay. Mice were immunized with α-GalCer–loaded BMDCs through i.v. injection (~1×10^6 BMDCs per mouse) and then periodically bled to monitor the in vivo iNKT cell responses using flow cytometry.

B16 Melanoma Lung Metastasis Mouse Model. Mice that received i.v. injection of $0.5–1 \times 10^6$ B16.F10 melanoma cells were allowed to develop lung metastasis over the course of 2 wk (4). On day 3 post tumor challenge, the experimental mice received i.v. injection of 1×10^6 BMDCs that were either unloaded or loaded with α-GalCer. On day 14, mice were euthanized, and their lungs were harvested and analyzed for melanoma metastasis by counting tumor nodules under a Zeiss Stemi 2000-CS microscope (Carl Zeiss AG) at 10× magnification. Representative lungs were also analyzed by immunohistology.

Immunohistology. Lung tissues collected from the experimental mice were fixed in 10% neutral-buffered formalin and embedded in paraffin for sectioning (5 μm thickness), followed by hematoxylin and eosin staining using standard procedures (UCLA Translational Pathology Core Laboratory, Los Angeles, CA). The sections were imaged using an Olympus BX51 upright microscope equipped with an Optronics Macrofire CCD camera (AU Optronics) at 40× and 100× magnifications. The images were analyzed using Optronics PictureFrame software (AU Optronics).

Statistical analysis. Student's two-tailed *t* test was used for paired comparisons. Data are presented as mean ± SEM, unless otherwise indicated. $P < 0.01$ was considered significant.

1. Yang L, Baltimore D (2005) Long-term in vivo provision of antigen-specific T cell immunity by programming hematopoietic stem cells. *Proc Natl Acad Sci USA* 102(12): 4518–4523.
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3. Yang L, et al. (2008) Engineered lentivector targeting of dendritic cells for in vivo immunization. *Nat Biotechnol* 26(3):326–334.
4. Fujii S, Shimizu K, Kronenberg M, Steinman RM (2002) Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 3(9):867–874.

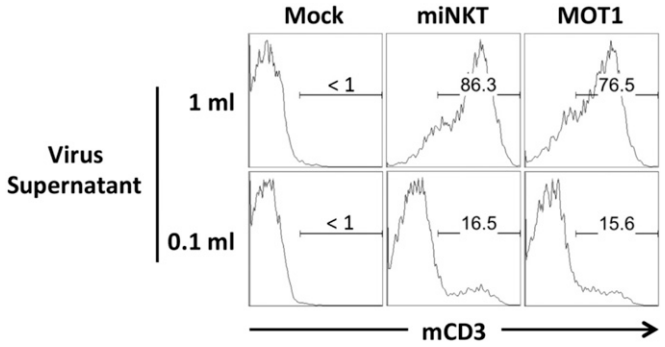

Fig. S1. Titration of the miNKT retroviral vector. The 293.T/mCD3 cells were transduced with the titrated volume of indicated virus supernatants. Three days later, virus-mediated expression of mouse TCRs was measured using flow cytometry. Representative FACS plots showing the detection of mouse TCRs on cell surface are presented. Note mouse CD3 (mCD3) molecules only display on cell surface in complex with the transgenic mouse TCRs, therefore, they can be used as an indicator of transgenic TCR expression. The results show comparable titers of the miNKT and MOT1 retroviral vectors, estimated as ~$0.5–1 \times 10^6$ IFU/mL (infectious units per milliliter). Mock, the control retroviral vector encoding an EGFP reporter gene; miNKT, the retroviral vector encoding a selected pair of mouse iNKT TCR α and β chain genes; MOT1, the retroviral vector encoding the α and β chain genes of OT1 TCR, a mouse conventional αβ TCR specific for chicken ovalbumin (1).

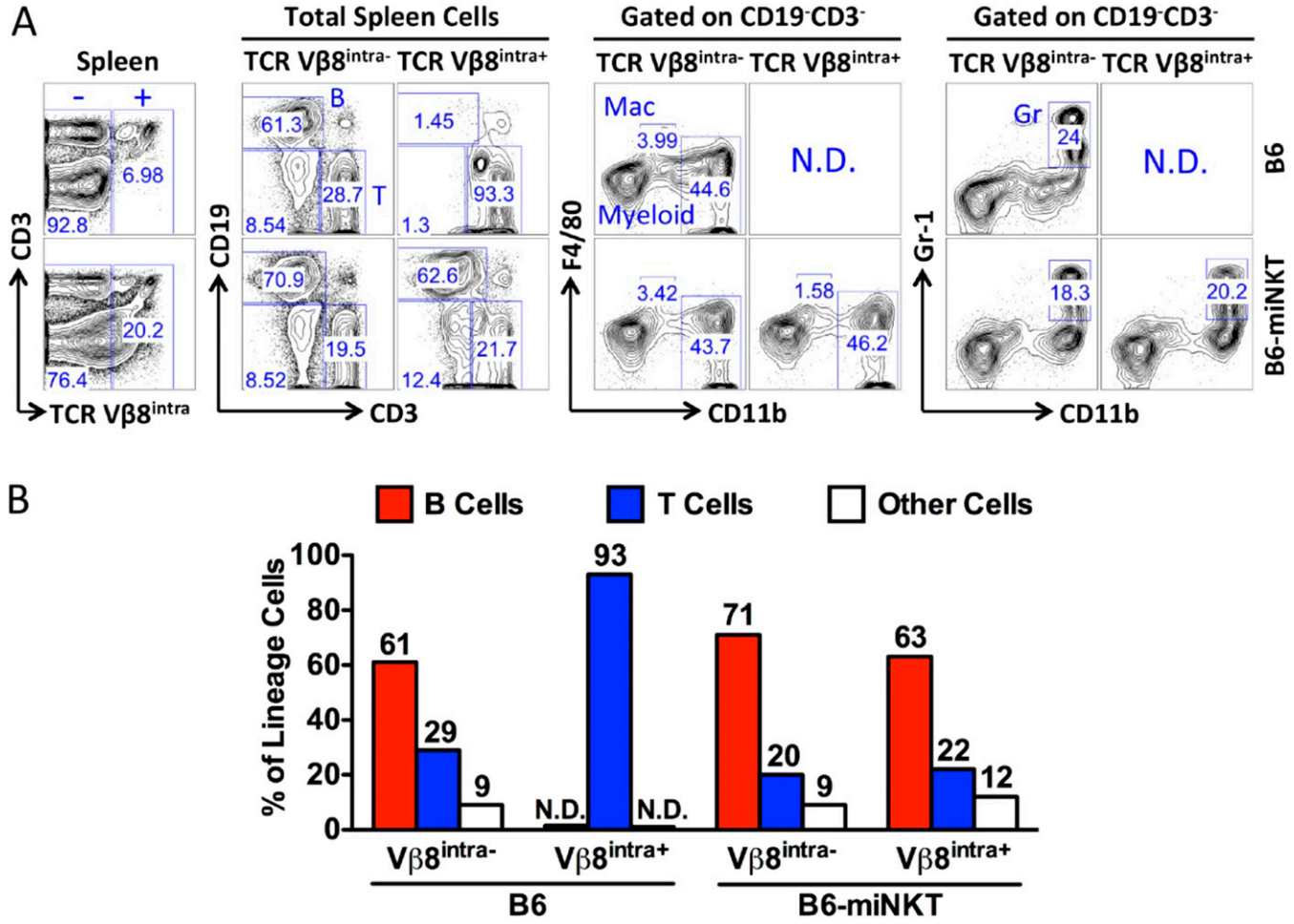

Fig. S2. Lineage differentiation of iNKT TCR-engineered HSCs. B6-miNKT and control B6-Mock mice were analyzed for the presence of iNKT TCR-engineered cells at 6–8 wk post HSC transfer. The experiments were repeated at least three times, and representative FACS plots (*A*) and bar graphs (*B*) are shown. Engineered cells were detected by intracellular staining of the transgenic TCRβ chain (gated as TCR $V\beta8^{intra+}$). Comparison analysis of the spleen cells of B6-miNKT and B6 control mice is presented. N.D., not detected.