Genetic engineering of hematopoietic stem cells to generate invariant natural killer T cells

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Invariant natural killer T (iNKT) cells comprise a small population of αβ T lymphocytes. They bridge the innate and adaptive immune systems and mediate strong and rapid responses to many diseases, including cancer, infections, allergies, and autoimmunity. However, the study of iNKT cell biology and the therapeutic applications of these cells are greatly limited by their small numbers in vivo (~0.01–1% in mouse and human blood). Here, we report a new method to generate large numbers of iNKT cells in mice through T-cell receptor (TCR) gene engineering of hematopoietic stem cells (HSCs). We showed that iNKT TCR-engineered HSCs could generate a clonal population of iNKT cells. These HSC-engineered iNKT cells displayed the typical iNKT cell phenotype and functionality. They followed a two-stage developmental path, first in thymus and then in the periphery, resembling that of endogenous iNKT cells. When tested in a mouse melanoma lung metastasis model, the HSC-engineered iNKT cells effectively protected mice from tumor metastasis. This method provides a powerful and high-throughput tool to investigate the in vivo development and functionality of clonal iNKT cells in mice. More importantly, this method takes advantage of the self-renewal and longevity of HSCs to generate a long-term supply of engineered iNKT cells, thus opening up a new avenue for iNKT cell-based immunotherapy.

Results

Cloning of iNKT TCR Genes and Construction of Retroviral Delivery Vectors. We used a robust and high-throughput single-cell TCR cloning technology recently established in our laboratories to obtain iNKT TCR genes (Materials and Methods). Single iNKT cells were sorted from mouse spleen cells using flow cytometry based on a stringent collection of surface markers gated as CD3\textsuperscript{+}CD1d\textsuperscript{-}/PBS-57\textsuperscript{-} TCR V\textsuperscript{α}8\textsuperscript{-}/NK1.1\textsuperscript{-} (Fig. 1A) (15). mCD1d/ PBS-57 is the tetramer reagent that specifically identifies iNKT cells

Significance

This article describes a new method for generating large numbers of invariant natural killer T (iNKT) cells in mice through genetic engineering of blood stem cells. iNKT cells are potent immune cells that regulate many human diseases, including cancer, infections, allergies, and autoimmunity. However, both the study of iNKT cell biology and the clinical application of iNKT cells have been greatly hindered by their small numbers (~0.01–1% in mouse and human blood). The method reported here provides a powerful new tool to study iNKT cell biology in a mouse model. It can also be applied to humans, opening a new avenue for iNKT cell-based immunotherapy that has the potential to provide patients with therapeutic levels of iNKT cells throughout life.

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TCRs (16). We included TCR Vβ8 staining to focus on the dominant Vβ8¹ population of mouse iNKT cells (1, 2). The sorted single iNKT cells were then subjected to TCR cloning (Fig. 1B). Several verified iNKT TCR α and β pairs were inserted into the murine stem cell virus (MSCV)-based retroviral vector to yield TCR gene delivery vectors (Fig. 1 C and D). Their vector-mediated expressions were then tested in 293.T/mCD3, a stable cell line that has been engineered to express mouse CD3 molecules that are required to support the surface display of mouse TCRs (Fig. 1E). One vector that mediated high expression of a high-affinity iNKT TCR was selected for the follow-up studies and was denoted as the miNKT vector (Fig. 1 D and F). The control MIG vector that encodes an EGFP reporter gene was denoted as the Mock vector (Fig. 1 D and F) (10).

**Generation of Clonal iNKT Cells Through Genetic Engineering of HSCs.** Following an established protocol (10), we performed miNKT-transduced bone marrow transfer in B6 mice to generate the recipient mice denoted as B6-miNKT (Fig. 2A). In brief, HSC-enriched bone marrow cells harvested from donor B6 mice were cultured in vitro, transduced with either Mock or miNKT retroviral vectors, then separately transferred into irradiated recipient B6 mice. The recipient mice were allowed to reconstitute their immune system over the course of 6–8 wk, followed by analysis to determine the presence of iNKT cells. Similar to the previously reported conventional αβ TCR engineering approach (10), we obtained desirable titers of the newly constructed miNKT retroviral vector ~0.5–1 × 10⁶ infectious units (IFU)/mL; Fig. S1] and achieved high efficiencies of HSC transduction (typically over 50% of the cultured bone marrow cells). Compared with the Mock-engineered recipient mice, denoted as B6-Mock, we observed a significant increase of iNKT cells in the B6-miNKT mice from thymus to peripheral tissues, suggesting the successful generation of HSC-engineered iNKT cells (Fig. 2 B and C). Through titrating the miNKT vector-transduced HSCs used for bone marrow transfer, we were able to control the increase of the iNKT cells from as high as 50% of the total αβ T cells, down to a desired level in the B6-miNKT mice (Fig. 2 D and E). The ability to regulate the number of engineered iNKT cells can be valuable for clinical applications of this HSC-engineered iNKT cell strategy. Study of the iNKT cells from the B6-miNKT mice revealed that these iNKT cells displayed a typical phenotype of mouse iNKT cells in that they exhibited high expression of the NK1.1 marker, as well as a memory T-cell signature (CD62LloCD44hi) and a CD4 SP or CD8 SP phenotype pattern (Fig. 2F) (17). Almost all of these iNKT cells showed positive staining for TCR Vβ8, indicating that they expressed the transgenic clonal iNKT TCR and suggesting that they were derived from the miNKT-engineered HSCs (Fig. 2F). The production of high levels of iNKT cells in the B6-miNKT mice persisted for up to 6 mo following the initial bone marrow transfer and also post secondary bone marrow transfer, highlighting the long-term effectiveness of this HSC-engineered iNKT cell strategy (Fig. 2 G and H).

**Functionality of the HSC-Engineered iNKT Cells.** We then analyzed the functionality of the HSC-engineered iNKT cells. When stimulated with the agonist glycolipid α-GalCer (α-GalCer) in vitro, the engineered iNKT cells proliferated vigorously by over 20-fold in 5 d and produced large amounts of the effector cytokines IFN-γ and IL-4 (Fig. 2 I–K). When B6-miNKT mice were immunized with bone marrow-derived dendritic cells (BMDCs) loaded with α-GalCer, the engineered iNKT cells mounted a strong and rapid response in vivo, expanding close to 20-fold in 3 d (Fig. 2L). Notably, the in vivo expansion of these cells peaked at day 3 postimmunization, compared with 7 d for the miNKT-TCR transgenic receptor expression pattern (Fig. 2F) (17). Almost all of these iNKT cells showed positive staining for TCR Vβ8, indicating that they expressed the transgenic clonal iNKT TCR and suggesting that they were derived from the miNKT-engineered HSCs (Fig. 2F). The production of high levels of iNKT cells in the B6-miNKT mice persisted for up to 6 mo following the initial bone marrow transfer and also post secondary bone marrow transfer, highlighting the long-term effectiveness of this HSC-engineered iNKT cell strategy (Fig. 2 G and H).

**Development of the HSC-Engineered iNKT Cells.** Next, we analyzed the development of the HSC-engineered iNKT cells. iNKT cell progenitors gated as TCRβ⁺mCD1d/PBS-57⁺ were detected in the thymus of the B6-miNKT mice and were found to follow a classic developmental path similar to that observed for endogenous iNKT progenitor cells in the control B6-Mock mice (Fig. 3) (15). These progenitor cells appeared as CD4⁺CD8⁻ (DN), CD4⁺CD8⁺ (DP), and CD4⁺CD8⁻ (CD4 SP), corresponding with an iNKT development from DN to DP, then to CD4 SP or back to DN cells (Fig. 3A). The expression of CD24, CD44, and DX5 markers on iNKT progenitor cells further defined their
Fig. 2. Generation of functional iNKT cells through TCR gene engineering of hematopoietic stem cells (HSCs). B6 mice receiving adoptive transfer of HSCs transduced with either the Mock retroviral vector (denoted as B6-Mock mice) or miNKT retroviral vector (denoted as B6-miNKT mice) were allowed to reconstitute their immune system in a duration of 6–8 wk, followed by analysis. The experiments were repeated at least three times, and representative results are presented. iNKT cells were detected as TCRβlomCD1d/PBS-57+ using flow cytometry. (A) Schematic representation of the experimental design to generate HSC-engineered iNKT cells in mice. (B and C) Increase of iNKT cells in B6-miNKT mice compared with that in the control B6-Mock mice. (B) FACS plots showing the detection of iNKT cells in various tissues. (C) Bar graphs showing the fold increase of percent iNKT cells in the indicated tissues. (D and E) Control of iNKT cell numbers in B6-miNKT mice through titrating the miNKT vector-transduced HSCs used for adoptive transfer. (D) FACS plots showing the detection of iNKT cells in the spleens of various B6-miNKT recipient mice. Tc indicates the conventional αβ T cells (gated as TCRβmCD1d/PBS-57−). (E) Bar graphs showing the percent iNKT of total αβ T cells in spleen. (F) Phenotype of the HSC-engineered iNKT cells. FACS plots are presented showing the surface markers of iNKT cells detected in the liver of B6-miNKT mice. (G and H) Long-term production of HSC-engineered iNKT cells. FACS plots are presented showing the detection of iNKT cells in the spleen of B6-miNKT mice for up to 6 mo after initial HSC adoptive transfer (G) and at 2 mo after secondary bone marrow transfer (BMT) (H). (I–K) Functionality of the HSC-engineered iNKT cells tested in vitro. Spleen cells of B6-miNKT mice were cultured in vitro in the presence of α-GalCer (100 ng/mL). (I) FACS plots showing the time-course proliferation of iNKT cells. (J) FACS plots showing the cytokine production in iNKT cells on day 3, as measured by intracellular cytokine staining. (K) ELISA analysis of cytokine production in the cell culture medium at day 3. Data are presented as mean of duplicate cultures ± SEM, *P < 0.01 (B6-miNKT samples compared with the corresponding B6-Mock controls). (L) Functionality of the HSC-engineered iNKT cells tested in vivo. B6-Mock or B6-miNKT mice were given i.v. injection of 1 × 10⁶ bone marrow-derived dendritic cells (BMDCs) loaded with α-GalCer (denoted as BMDC/α-GalCer) and then periodically bled to monitor iNKT cell responses. FACS plots are presented showing the change of iNKT cell frequencies in blood.
transgenic iNKT TCRs (gated as Vαβ-engineered HSCs. By detecting intracellular expression of development in thymus. clonal iNKT cells that only express the transgenic iNKT TCRs, results suggest that the iNKT TCR-engineered HSCs give rise to Vαβ-mock mice (Fig. 3C), and similar to that observed for endogenous iNKT cells in the NK1.1 expression compared with iNKT cells detected in the thy-
mus, similar to that observed for endogenous iNKT cells in the control B6-mock mice. In B6-miNKT mice, iNKT cells detected in the periphery did up-regulate acquire the expression of NK1.1 (Control Point 2) (15). In B6−
need to undergo an additional maturation step in the periphery to iNKT cells also differ from conventional developmental path from Stages 1̸(Control Point 1), αβ
αβ T cells from the liver of B6-Mock or B6-miNKT mice are shown. Pan-TCR Vα panel includes Vα2, Vα3.2, and Vα8.3, whereas pan-TCR Vβ panel includes Vβ13, Vβ4, Vβ5, Vβ6, Vβ11, and Vβ13. N.D., not detected.

Fig. 3. Development of the HSC-engineered iNKT cells. B6-miNKT and control B6-Mock mice were analyzed for iNKT cell development at 6–8 wk post HSC transfer. The experiments were repeated at least three times, and representative results are presented. iNKT cells were detected as TCRβ⁺mCD1d/PBS-57⁺ using flow cytometry. (A and B) FACS plots showing the characteristic development of iNKT cells in thymus. (C) FACS plots showing the maturation of iNKT cells in the periphery measured by the up-regulation of the NK1.1 marker. Comparisons of iNKT cells from thymus and periphery (liver) are shown. (D and E) FACS plots and bar graphs showing the exclusion of nontransgenic TCR expression on the HSC-engineered iNKT cells. Comparisons of iNKT and conventional αβ T (Tc) cells from the liver of B6-Mock or B6-miNKT mice are shown. Pan-TCR Vα panel includes Vα1, Vα4, Vα5, Vα6, Vα11, and Vα13.

Antitumor Capacity of the HSC-Engineered iNKT Cells. Finally, we studied the cancer therapy potential of the HSC-engineered iNKT cells. B6-miNKT mice and control B6-Mock mice were challenged with B16.F10 melanoma cells through i.v. injections and analyzed for lung metastasis 2 wk later (Fig. 4A). Experimental mice received immunization with either unloaded or α-GalCer−loaded BMDCs (denoted as BMDC/no treatment or BMDC/α-GalCer, respectively) on day 3 post tumor challenge to boost iNKT cell activities and to mimic a therapeutic vaccination treatment (Fig. 4A). Monitoring of the HSC-engineered iNKT cells in the B6-miNKT mice showed that these cells actively responded to tumor challenge, evidenced by their expansion from ∼1.5% to ∼7% in blood (Fig. 4B). In comparison, endogenous iNKT cells in the control B6-Mock mice also responded to tumor challenge, but their limiting starting number (∼0.2%) only allowed them to reach ∼1.7% in blood (Fig. 4B). We observed a significant protection from lung metastasis in the B6-miNKT mice compared with that in the control B6-Mock mice, as evidenced by the reduction of both the number and size of tumor nodules (Fig. 4C−E). Inclusion of a BMDC/α-GalCer immunization further expanded the HSC-engineered iNKT cells (up to ∼30% in blood; Fig. 4B). However, no significant further reduction of lung tumor nodules was observed (Fig. 4C).

We also studied the lineage differentiation of iNKT TCR-engineered HSCs. By detecting intracellular expression of transgenic iNKT TCRs (gated as Vβintratex), TCR-engineered HSCs and their progeny cells could be tracked (Fig. S2). Notably, because only T cells express the CD3 molecules that are required to support the surface display of TCRs and their signaling, the other cells that lack CD3 molecules can only express the transgenic iNKT TCRs intracellularly, and these TCRs are not functional. In addition to generating iNKT cells, our results show that TCR-engineered HSCs can also differentiate into all other blood cell lineages analyzed, including B cells (gated as CD19⁺), macrophages (gated as CD3⁻CD19⁻F4/80⁺), myeloid cells (gated as CD3⁻CD19⁻CD11b⁺), and granulocytes (gated as CD3⁻CD19⁻Gr-1⁺) (Fig. S2).
effector cells. Notably, depigmentation of tumor nodules was observed in high numbers in the B6-iNKT mice (Fig. 4). Key molecules in the pigment synthesis pathway are a major class of tumor antigens for melanoma, and mutating or down-regulating these molecules are common strategies by which melanoma tumor cells escape immune attack, often leading to depigmentation (19). The presence of a large fraction of depigmented tumor nodules in the B6-miNKT mice therefore suggests a strong immune response against these tumors, presumably induced by the HSC-engineered iNKT cells through activation of antitumor NK and conventional αβ T cells (Fig. 4) (3, 4).

Discussion
In this report, we describe a new method of generating large numbers of iNKT cells in mice through iNKT TCR gene engineering of HSCs. Compared with existing iNKT TCR transgenic mouse technology and iNKT iPS cell-derived transgenic mouse technology, this new method is cost-effective and high-throughput. It is easy to implement through a standard retrovirus-transduced bone marrow transfer and has a fast turnover to generate iNKT cells within as few as 6 wk (Fig. 2). Most importantly, unlike transgenic mouse technologies, this method can be applied to humans through gene-modified CD34+ cell transfer and therefore has direct translational potential (20).

In our study, we showed that the HSC-engineered mouse iNKT cells followed a classical iNKT cell development path, Check Point 1 in the thymus to gain iNKT TCR expression and Check Point 2 in the periphery to gain NK1.1 expression (Fig. 3). They also displayed a typical iNKT cell phenotype (TCRβlomCD1d/PBS-57hiNK1.1hiCD62LloCD44hiCD4−/−CD8−) and exhibited full iNKT cell functionality with potent and fast response to antigen stimulation, both in vitro and in vivo (Fig. 2). These findings confirm the new HSC-engineered iNKT cell method as a powerful tool to study mouse iNKT cell biology.
For example, by studying the antigen recognition and functional differentiation of single iNKT cell clones, critical clues might be revealed to increase understanding of the origins of various iNKT cell subsets with distinct functions, such as those iNKT cell subsets biased to produce Th1, Th2, or Th17 effector cytokines (3). The flexibility of this method also allows the convenient generation of iNKT cells of different genomic backgrounds at a fast pace and an affordable cost, allowing examination of the functions of designated genes for regulating iNKT cell biology (8).

The therapeutic potential of this HSC-engineered iNKT cell approach is also promising. A broad range of applications, fast and strong responses, and the clinical availability of a potent stimulatory reagent α-GalCer make iNKT cells attractive therapeutic targets (4). In the past 2 decades, a series of iNKT cell-based clinical trials have been conducted, mainly targeting cancer (4, 21). A recent trial reported encouraging antitumor immunity in patients with head and neck squamous cell carcinoma, attesting to the potential of iNKT cell-based immunotherapies (22). However, most trials yielded unsatisfactory results (4, 21). Overall, these trials all worked through the direct stimulation or ex vivo expansion of patients’ endogenous iNKT cells, thus yielding only short-term, limited clinical benefits to a small number of patients. The low frequency and high variability of iNKT cells in humans (~0.01–1% in blood), as well as the rapid depletion of these cells poststimulation, are considered to be the major stumbling blocks limiting the success of these trials. However, if successfully applied to humans, the reported new HSC-engineered iNKT cell approach has the potential to provide patients with a lifelong supply of therapeutic levels of iNKT cells, taking advantage of the longevity and self-renewal of HSCs (23), thus eliminating a key barrier against current iNKT cell-based immunotherapies. It is worthy to note that simply engineering conventional αβ T cells with iNKT TCR genes will not convert these cells into iNKT cells. The unique functions of iNKT cells can only be acquired during iNKT cell development, leaving HSC engineering the sole approach to produce functional engineered iNKT cells.

Materials and Methods


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, CD11b, CD24, CD62L, CD44, DX5, F4/80, Gr-1, TCRβ, TCRVβ, 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. FluoroBlock (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 biotinated 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′,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 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+ mCD1d/ PBS-57+ TCR Vβ8 NK1.1) 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 CD3R3 regions of the iNKT TCR α and β chain cDNAs and were subsequently synthesized by Integrated DNA Technologies (IDT): for TCRα (FW primer: 5′-GGG AGA AGA TCA TCA GCA ACT CTG GAT AAA GAT GC -3′; BW primer: 5′- CCA GAT TCC ATG GTT TCC TTC 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 Vra14-Ix18-α assembly for TCRαs and Vβ8-18-β assembly for TCRβs) were provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Fixable Viability Dye eFluor455UV was purchased from affymetrix eBioscience.

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 asigina virus 2A). The transduced cells were then transiently transfected with an MOI vector encoding a mouse CD8 TCR, using a standard calcium precipitation procedure (1). Single cells supporting the high surface expression of OTI TCRs (gated as CD3+ TCR Vβ5+) were sorted out using flow cytometry and grown into single-cell clones. A stable, single-cell clone that lost OTI 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 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 × 10^8 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 (~1×10^6 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 Cytotox/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 × 10⁶ 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 × 10⁶ 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 × 10⁶ 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 10x 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 40x and 100x 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.


**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 × 10⁶ 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 Vβ8^+^). Comparison analysis of the spleen cells of B6-miNKT and B6 control mice is presented. N.D., not detected.