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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 autoimmune disorders. 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+CD1d+/PBS-57+ TCR Vβ8+NK1.1+ (Fig. 1A) (15). mCD1d/PBS-57 is the tetramer reagent that specifically identifies iNKT T-cell receptor (TCR) transgenic mice (5, 6) and iNKT induced pluripotent stem (iPS) cell-derived transgenic mice (7) provide valuable tools to study iNKT cell biology in mice, but these methods are both costly and time-consuming. In addition, approaches using transgenic mice have no direct clinical application. As an alternative, a TCR-engineered HSC adoptive transfer strategy could overcome these limitations and become clinically applicable. Since its demonstration in mice in the early 2000s, this HSC-engineered T-cell strategy has been widely used to successfully generate both mouse and human antigen-specific conventional αβ T cells in multiple mouse and humanized mouse models (8–13). Human clinical trials testing this strategy for treating melanoma are also ongoing (14). Based on these previous works and the scientific principle that iNKT cells follow a “TCR instruction” development path similar to that of conventional αβ T cells (15), we hypothesized that HSCs could be engineered to express iNKT TCR genes and be programmed to develop into clonal iNKT cells. In the present report, we demonstrated the feasibility of this new HSC-engineered iNKT cell approach in mice and provided evidence to support its therapeutic potential in a mouse melanoma lung metastasis model.
TCRs (16). We included TCR Vβ8 staining to focus on the dominant Vβ8+ 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 × 10^6] infectious units (IFU)/mL; Fig. S1] and achieved high efficiencies of HSC transduction (routinely 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 CD8−CD4− cell signature (CD62LloCD44hi) (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 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 postimmunization for conventional αβ T cells (18). This speedy in vivo response is a signature of iNKT cells (17). These results indicate that the HSC-engineered iNKT cells are fully functional.

**Development of the HSC-Engineered iNKT Cells.** Next, we analyzed the development of the HSC-engineered iNKT cells. iNKT cell progenitors gated as TCRγδ⁺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. 3A) (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 TCRβ+CD1d/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 TCRβ+CD1d/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.
αβ has been shown to induce allelic exclusion and block the rear-control B6-Mock mice (Fig. 3 mus, similar to that observed for endogenous iNKT cells in the acquire the expression of NK1.1 (Control Point 2) (15). In B6- need to undergo an additional maturation step in the periphery to genic TCR (V

Overexpression of prerearranged αβ TCR genes in HSCs has been shown to induce allelic exclusion and block the rearrangements of endogenous TCR genes in the resulting conventional αβ T cells (13). Study of the iNKT cells generated in the B6-miNKT mice revealed that these cells expressed the transgenic TCR (Vβ8'), but not the other TCR Vβ chains analyzed in our experiment (Fig. 3 D and E). In particular, these HSC-engineered iNKT cells did not express the TCR Vβ7 used by ~10% of endogenous iNKT cells (Fig. 3 D and E). Analysis of TCR α chain expression also showed an exclusion of other TCR Vα expression on the engineered iNKT cells (Fig. 3D). These results suggest that the iNKT TCR-engineered HSCs give rise to clonal iNKT cells that only express the transgenic TCRs, likely through an allelic exclusion mechanism during iNKT cell development in thymus.

We also studied the lineage differentiation of iNKT TCR-engineered HSCs. By detecting intracellular expression of transgenic iNKT TCRs (gated as Vβ intras), 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).

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 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. 4 C–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 speculate that this may be due to a “saturation” of the antitumor capacity of iNKT cell-induced effector cells like NK cells and tumor-specific conventional αβ T cells that were limiting in mice. Total clearance of tumor metastasis likely requires combination therapy such as combining with adoptive transfer of additional
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. By increasing the iNKT cells to a suitable level, their development and function in health and disease conditions can be easily monitored. In particular, this method allows us to generate large numbers of clonal iNKT cells, thus enabling the investigation of similarities and differences between individual iNKT cell clones.
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 and strong responses, and the clinical availability of a potent stimulatory reagent of iNKT cells make iNKT cells attractive therapeutic targets (4). 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 $\alpha \beta$ 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**

The full description of materials and methods is provided in SI Materials and Methods.

**Single-Cell iNKT TCR Cloning.** The cloning method was performed based on an established protocol (24), with several modifications. Details are provided in SI Materials and Methods.

**HSC Isolation, Transduction, Adoptive Transfer, and Secondary Bone Marrow Transfer.** The procedures were reported previously (10) and are provided in SI Materials and Methods.

**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.

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