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Rapid *ex vivo* expansion of highly enriched human invariant natural killer T cells via single antigenic stimulation for cell therapy to prevent graft-versus-host disease

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Abstract

Background aims. CD1d-restricted invariant natural killer (iNK) T cells are rare regulatory T cells that may contribute to the immune-regulation in allogeneic stem cell transplantation (ASCT). Here, we sought to develop an effective strategy to expand human iNK T cells for use in cell therapy to prevent graft-versus-host disease (GVHD) in ASCT. *Methods.* Human iNK T cells were first enriched from peripheral blood mononuclear cells (PBMCs) using magnetic-activated cell sorting separation, then co-cultured with dendritic cells in the presence of agonist glycolipids, alpha-galactosylceramide, for 2 weeks. *Results.* The single antigenic stimulation reliably expanded iNK T cells to an average of 2.8×10^7 per 5×10^8 PBMCs in an average purity of 98.8% in 2 weeks (N = 24). The expanded iNK T cells contained a significantly higher level of $CD4^+$ and central memory phenotype ($CD45RA^-CD62L^+$) compared with freshly isolated iNK T cells, and maintained their ability to produce both Th-1 (interferon [IFN]γ and tumor necrosis factor [TNF]α) and Th-2 type cytokines (interleukin [IL]-4, IL-5 and IL-13) upon antigenic stimulation or stimulation with Phorbol 12-myristate 13-acetate/ionomycin. Interestingly, expanded iNK T cells were highly autoreactive and produced a Th-2 polarized cytokine production profile after being co-cultured with dendritic cells alone without exogenous agonist glycolipid antigen. Lastly, expanded iNK T cells suppressed conventional T-cell proliferation and ameliorated xenograft GVHD (hazard ratio, 0.1266; $P < 0.0001$). *Conclusion.* We have demonstrated a feasible approach for obtaining *ex vivo* expanded, highly enriched human iNK T cells for use in adoptive cell therapy to prevent GVHD in ASCT.

Key Words: *cell therapy*, ex vivo *expansion, graft-versus-host disease, human iNK T cells*

Introduction

Allogeneic hematopoietic stem cell transplantation (ASCT) remains the only curative immunotherapy for several hematologic malignancies through in part varying degrees of graft-versus-leukemia (GVL) effects [1,2]. While relapse of the disease due to insufficient GVL effects is the leading cause for post-transplantation mortality, graft-versus-host disease (GVHD) is the most common post-transplantation complication occurring in approximately 50% of patients following ASCT. GVHD is often fatal without aggressive and timely treatment [3]. The mainstay of treatments for acute GVHD is corticosteroids and intensification of immunosuppressants. However, these treatments may delay the engraftment of stem cells, increase the risk of life-threatening infections and blunt GVL effects leading to the early relapse of leukemia [3]. Thus, novel strategies to maintain an optimal balance between GVHD and GVL by donor lymphocytes are needed to improve the clinical outcome of ASCT.

CD1d-restricted invariant natural killer (iNK) T cells are rare but powerful regulatory T cells that influence adaptive immune responses through their ability to produce a varying degree of both Th-1 and Th-2 type cytokines upon activation [4]. The iNK T cells are thought to play a role in preventing GVHD

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in ASCT [5–9]. For example, adoptive transfer of murine $CD4^+$ iNK T cells has been shown to suppress acute and chronic GVHD through the expansion of conventional regulatory T cells [10–12], and activation of donor iNK T cells using Th-2 polarizing agonist glycolipid antigen or liposomal αGalCer can ameliorate GVHD in murine models [9,13]. In addition, a handful of correlative pre-clinical studies demonstrated that the higher dose of $CD4^-$ iNK T cells in the allograft or early reconstitution of iNK T cells post-ASCT is associated with lower incidence of acute GVHD [5,14–16]. Unlike conventional regulatory T cells, iNK T cells may have additional GVL effects through intrinsic NK-like properties or by promoting GVL by donor lymphocytes [17–19]. Therefore, iNK T-cell–based immunotherapy is a novel approach to potentially balance the GVHD and GVL effects of donor lymphocytes in ASCT. In this study, we explored a strategy to expand highly pure human iNK T cells from adult donors, and assessed their immunoregulatory function to prevent xenogenic GVHD in ASCT.

Materials and Methods

Materials

This study was performed in accordance with the research protocol approved by The University of Texas M.D. Anderson Institutional Review Committee. Informed written consent from all study subjects was waived because all leukoPaks from adult donors were purchased through the MDACC Blood Bank. T-cell media (TCM) was used for cell culture, and contained Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum, 55 μmol/L 2-mercaptoethanol, 10 μg/mL gentamicin, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 1x non-essential amino acid and essential amino acid (Invitrogen). Anti-iNKT microbeads (6B11) were purchased from Miltenyi Biotech, and the following antibodies were purchased from BioLegend or BD Bioscience: iNK T-cell receptor (TCR) (6B11), CD4 (RPA-T4), CD8α (SK11), interferon (IFN)γ (B27), tumor necrosis factor (TNF)α (MAB11), interleukin (IL)-4 (8D4-8), IL-13 (JES10-5A2) and CD3 (OKT3). The following cytokines used for cell culture were purchased from BioLegend or PeproTech: IL-2, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-7. The agonist glycolipid, αGalCer, was synthesized as previously described [20,21].

Ex vivo *expansion of iNK T cells*

Monocyte-derived dendritic cells (DCs) were generated as previously reported [22]. Briefly, peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll-Plaque density gradient centrifugation protocol. Monocytes were isolated via plastic adherence, and cultured in TCM containing IL-4 (100 ng/mL) and GM-CSF (200 IU/mL) for 5 days. After irradiation (5000 cGy), DCs were cryopreserved until further use. DCs from a single donor were used to expand iNK T cells from up to 4–5 allogeneic donors. The iNK T cells were first enriched from 2×10^8 to 1×10^9 PBMCs prepared from the entire leukoPak using anti-iNKT-MicroBeads and Magnetic Activated Cell Sorting (MACS) separation according to the manufacturer's instructions (Miltenyi Biotech). Subsequently, they were co-cultured with 2×10^5 DCs per well in 1–3 wells of 24-well tissue culture plate in TCM in the presence of αGalCer (100 nmol/L) and IL-2 (200 IU/mL) for 10–14 days. Growth factor and TCM was replenished every 2–3 days, but αGalCer was not.

Flow cytometry

Freshly isolated or expanded iNK T cells were subjected to multi-parameter flow cytometric analysis for the following surface markers: CD3, CD4, CD8α, iNK invariant TCRα chain (clone: 6B11), CD45RA and CD62L. Dead cells were excluded using Fixable Viable Stain 620 (BD Bioscience). For intracellular cytokine analysis, iNK T cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 30 ng/mL) and ionomycin (1 μg/mL) in the presence of monensin (1 μmol/L) for 3 h. After staining of surface antigens, cells were fixed and permeabilized using BD Cytofix//Cytoperm (BD Bioscience), and stained for intracellular cytokines prior to multi-parameter flow cytometric analysis. An LSRFortessa Cell Analyzer (BD Bioscience) was used to acquire samples and FlowJo version 10.3 software (Tree Star) was used for analysis.

The iNK T-cell functional assay

Fifty thousand iNK T cells were stimulated by 25,000 DCs pulsed with or without αGalCer (100 nmol/L) in triplicates in a 96-well plate for 48 h, and culture supernatants were assessed for the presence of IL-4, IL-5, IL-13, IFNγ, TNFα and GM-CSF using DuoSet Elisa Development Systems (R&D Systems) according to the manufacturer's instruction. BD OptEIA (BD Bioscience) was used as 3,3',5,5'-Tetramethylbenzidine (TMB) substrate reagent, and Cytation 5 (BioTek) was used to measure absorbance at 450 nm.

For human Th-1/Th-2/Th-17 cytokine array (Raybiotech), 2×10^5 iNK T cells and 1×10^5 irradiated DCs were co-cultured with and without