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# Invariant natural killer T cells generated from human adult hematopoietic stem-progenitor cells are poly-functional

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ABSTRACT

Invariant natural killer T (iNKT) cells constitute an important subset of T cells that can both directly and indirectly mediate anti-tumor immunity. However, cancer patients have a reduction in both iNKT cell number and function, and these deficits limit the potential clinical application of iNKT cells for cancer therapy. To overcome the problem of limited iNKT cell numbers, we investigated whether iNKT cells can be generated *in vitro* from bone marrow-derived adult hematopoietic stem-progenitor cells (HSPC). Our data demonstrate that co-culture of HSPC with OP9-DL1 stromal cells, results in a functional $CD3^+$ T cell population. These T cells can be further differentiated into iNKT cells by secondary culture with CD1d-Ig-based artificial antigen-presenting cells (aAPC). Importantly, these *in vitro*-generated iNKT cells are functional, as demonstrated by their ability to proliferate and secrete IFN-γ and GM-CSF following stimulation.



## 1. Introduction

Adoptive immunotherapy is one strategy used to enhance anti-tumor immune responses in cancer patients, and it can restore early post-transplantation immune competence in allogeneic hematopoietic stem cell (HSC) transplant recipients [1,2]. Adoptive immunotherapy involves stimulation of lymphocytes such as T cells, *ex vivo*, followed by transfer of expanded numbers of activated autologous T cells back into patients. Studies by Rosenberg and colleagues demonstrated that autologous tumor-specific cells were able to directly induce tumor shrinkage *in vivo* [3,4]. A limitation of this approach is that patients must have preexisting tumor-reactive cells, and these are difficult to identify in non-melanoma malignancies. To overcome this limitation, T cell receptor (TCR) gene transfer [5] or chimeric antigen receptors [6] can be utilized. It has been hypothesized that the effectiveness of TCR gene transfer of T cells is reduced, at least in part due to the pairing of the newly introduced TCR α and β chains with the endogenous TCR proteins in mice. Thus, two groups have reported that transducing TCR genes into HSC, which can be differentiated into functional T cells, results in generation of large numbers of mature, antigen-specific T cells from undifferentiated hematopoietic progenitors [5,7].

While these antigen-specific TCR transduction strategies can induce tumor-specific responses, the utilization of adoptive immunotherapy with invariant natural killer T (iNKT) cells would be advantageous because one could potentially enhance both anti-tumor and anti-viral immune responses, due to high levels of cytokines produced by iNKT cells. Unlike MHC-restricted T cells, iNKT cells acquire their effector functions during development, and their activation, following recognition of antigen presented in the context of CD1d molecules, results in the rapid production of large amounts of cytokines [8]. iNKT cell-mediated cytokine production leads to induction of both the innate and adaptive immune responses.

Despite the importance of iNKT cells in regulating immune responses, their low frequency significantly restricts their potential

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for clinical application. OP9 stromal cells transduced with Notch ligand delta-like 1 (OP9-DL1) can be used for the directed differentiation of embryonic stem cells into T-lineage cells [9,10]. Induction of Notch signals directs stem cells to differentiate into immature double-positive T cells and inhibits B cell development, demonstrating that Notch signaling is required as a proximal event in T cell commitment from progenitors [11,12]. While the OP9-DL1 system has been shown to generate functionally mature human CD4, CD8, regulatory T cells [13–17] and murine iNKT cells from transduced embryonic stem cells or fetal liver hematopoietic progenitors [18,19], it is unclear whether this system could be utilized to generate functional human iNKT cells from adult stem cells.

In this study, we sought to determine whether iNKT cells could be generated from stem progenitor cells of Hodgkin's and non-Hodgkin's lymphoma (NHL) patients. NHLs are of significant interest because they are part of the rare group of human cancers that have actually been increasing in incidence over the past thirty years. NHL are a large heterogeneous group of which >80% are derived from B cells. Diffuse large B-cell lymphoma (DLBCL), is the most common subtype of lymphoid neoplasm, is characterized as an aggressive lymphoma with heterogeneous clinical behaviors. DLBCL accounts for 25–30% of NHL among adults in the US, and it is even more prevalent in developing countries. In contrast, mantle cell lymphoma (MCL) comprises ~6% of NHL, but the outcome for MCL is poor with a median survival of only 5–7 years [20,21]. Thus novel approaches for the treatment of NHL are essential. Immunodeficiency is one of the strongest risk factors of NHL. iNKT cells are primed cells that are large reservoirs of cytokines such as IFN-γ which can, if appropriately activated, lead to the development of a robust anti-tumor immune response [22], thus we hypothesize that iNKT cell based adoptive immunotherapy could be an important treatment modality for NHL patients. Here we demonstrate that polyfunctional human iNKT cells can be generated *in vitro* from HSPC from healthy donors and lymphoma patients.

## 2. Materials and methods

### 2.1. Peripheral blood mononuclear cells (PBMC) and bone marrow

Circulating iNKT cells numbers and percentages were determined in the PMBC and bone marrow of healthy donors and patients by flow cytometry, with written informed consent from patients and approval by the Institutional Review Board at the University of Maryland School of Medicine. The percentages of iNKT cells were assessed in newly diagnosed patients, prior to treatment and patients diagnosed with leukemia were excluded from this study, given that the percentage of iNKT cells may be directly correlated with active disease. Patients diagnosed with Hodgkin's lymphoma, marginal zone lymphoma, diffuse large B cell lymphoma, mantle cell lymphoma, or follicular lymphoma were included in this study. Healthy donor bone marrow specimens were purchased from All Cells, LLC (Alameda, CA). PBMCs from healthy donors were purchased through commercial vendors, Biological Specialty and the New York Blood Center. PBMCs were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotek, Uppsala, Sweden) density gradient centrifugation or with BD Vacutainer PPT Tubes for Molecular Diagnostics (20-959-51D; Fisher Scientific, Suwanee, GA).

### 2.2. Cell cultures

Mouse OP9 stromal cells engineered to express the green fluorescent protein (GFP) alone (OP9-GFP) or GFP and the mouse Delta-like 1 gene (OP9-DL1) were kindly provided by Dr. Zúñiga-Pflücker (University of Toronto, Sunnybrook, Ontario, Canada) and maintained as previously described [9]. Bone marrow derived human hematopoietic stem cells ($CD34^+$) were purchased from Stem Cell Technologies Inc. (Vancouver, British Columbia, Canada). $CD34^+$ cells were also isolated from patient bone marrow specimens using a human CD34 cell enrichment kit from Miltenyi Biotech (San Diego, CA) as per the manufacturer's instructions.

### 2.3. In vitro T cell expansion

Human hematopoietic stem cells and OP9-DL1 cell cocultures were maintained as previously described [23], with few modifications as described herein. Purified human HSC ($3–6 \times 10^4$) were plated in 10 cm Petri dishes containing either OP9-DL1 cells or OP9-GFP control cells. Cells were maintained in α-minimal essential medium (Invitrogen) supplemented with 20% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 5 ng/ml IL-7 and 5 ng/ml Fms-like tyrosine kinase-3 ligand (eBioscience, San Diego, CA). Every 4 days, co-cultures were disaggregated by vigorous pipetting and passage through a 40 μm cell strainer to eliminate contaminating OP9 cells. The harvested cells were washed with PBS and re-plated. After three weeks, the expanded population was stained for iNKT cells (aGC-tet$^+$CD3$^+$ or iNKT$^+$CD3$^+$), and the remaining cells were stimulated biweekly with aAPC at a 1:1 (T cell: aAPC) ratio and cultured in RPMI 1640 with 10% FBS, 100 mM sodium pyruvate, 10 mM non-essential amino acid solution, 1× vitamin solution and 50 μM 2-mercaptoethanol.

### 2.4. Treatment of iNKT cells with sera

The serum was heat-inactivated 30 min at 56 °C. The iNKT cells were subsequently washed, and cocultured with complete medium containing serum from HD or MCL patients for the indicated time periods at 37 °C. Cytokine released (IFN-γ) was measured as an indication of iNKT cell activation by using standard sandwich ELISA. The complete medium was composed of RPMI supplemented with 2 mM L-glutamine (BioWhittaker), and ciprofloxacin (Serological Proteins) in 96 well U bottom plates, until the indicated time-periods, the cultured supernatant and cells were harvested, centrifuged to remove cellular debris and used for further experiments.

### 2.5. Antibodies and flow cytometry

Data were acquired with a BD LSR II Flow Cytometer (BD Biosciences) and analyzed with FCS Express V3 (De Novo Software, Los Angeles, CA). Doublets were excluded with FSC-A and FSC-H linearity. Human antibodies were as follows: anti-TCR Vα24-Jα18 (referred to as iNKT; clone 6B11), anti-CD3 (clone UCHT1), anti-CD4 (clone RPA-T4), anti-CD8 (clone RPA-T8), anti-CD34 (clone 561), anti-CD38 (clone HIT2, Biolegend) and anti-TCR γ/δ (clone B1) from Biolegend, anti-TCR Vβ11 (Beckman Coulter), anti-iNKT (BD Biosciences), CD1d tetramers loaded with PBS57, an analog of α-GalCer (National Institutes of Health Tetramer Core Facility, Atlanta, GA).

### 2.6. Intracellular cytokine staining

HSPC derived iNKT cells were activated with PMA (50 ng/ml) and ionomycin (1 μM) in the presence of GolgiStop (BD Pharmingen) for 4 h. Cells were stained with anti-CD3-PerCp, CD1d tetramers-APC, fixed, permeabilized (PermWash, BD), and stained intracellularly for IFN-γ and IL-4.