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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.

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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 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 antitumor 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 Biotech, 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 StemCell 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 Biotec (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 ($3\text{--}6 \times 10^4$) were plated in 10cm 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 30min 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 γδ(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 hours. Cells were stained with anti-CD3-PerCp, CD1d tetramers-APC, fixed, permeabilized (PermWash, BD), and stained intracellularly for IFN-γ and IL-4.

2.7. iNKT cell assays

To assess cytokine production by HSPC-derived iNKT cells, 1×10^4 iNKT cells were incubated in 200 µl of RPMI containing 10% FBS in the presence of PMA/ ionomycin or anti-CD3/ CD28 microbeads for 48–72 h in a 96-well, U-bottom plate (Costar, Corning, NY). The supernatants were harvested, and cytokine production was measured by ELISA, as previously described [24].

2.8. Preparation of aAPC

CD1d-based aAPC were prepared as previously described [25, 26]. In brief, to conjugate 0.5ml of epoxy beads (Dynabeads, M-450, Epoxy, 4×10^8 beads/ml) in sterile 0.1M Borate buffer, pH 7.0–7.4, in the presence of anti-CD28 mAb (Biolegend). The bead protein combination was mixed with rotation and incubated for 24h at 4°C. The beads were subsequently washed and the hCD1d molecules were loaded with 40× molar excess lipid antigen (α -GalCer, Enzo) in PBS.

2.9. Statistical analysis

Two-tailed student's t test, one-way analysis of variance (ANOVA) or two-way ANOVA were used as appropriate, p value less than 0.05 was considered significant. All analyses were performed using Prism 5.02 by GraphPad (La Jolla, CA). *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. The error bars in the graphs represent the SEM.

3. Results

3.1. iNKT cells are reduced in Cancer Patients

We first examined the percentage of iNKT cells in healthy donors and newly diagnosed lymphoma patients prior to treatment or surgery (Fig 1A). As shown in Fig 1B, the percentage of iNKT cells was 0.08 ± 0.01 in lymphoma patients (N=55) compared to 0.24 ± 0.05 in healthy donors (N=42). Analysis of the frequency of classical T cell subsets in the total PBMCs showed a high degree of variability, but no consistent differences were noted.

We next evaluated iNKT cells in the bone marrow and peripheral blood of lymphoma patients. It has been reported that tumors can shed factors that inhibit iNKT cell activation into the tumor microenvironment [27, 28]. Thus we hypothesized that in patients without bone marrow involvement; the marrow may provide an additional source and contain a higher relative percentage of iNKT cells, compared to the peripheral blood. As shown in Figure 1C, in each of ten non-Hodgkin lymphoma (NHL) patients, the percentage of iNKT cells was lower in the peripheral blood (0.10 ± 0.04) than in the bone marrow (0.59 ± 0.31). These data substantiate the idea that circulating iNKT cells are reduced in patients with systemic malignancies. Furthermore, while the levels of iNKT cells are higher in the bone marrow than in the peripheral blood, their absolute numbers remain inadequate for clinical application (less than 10^4 iNKT cells/ million lymphocytes).

3.2. *Ex vivo* iNKT cell expansion is impaired in MCL patients

A potential mechanism for reduced number of iNKT cells in MCL patients is the direct effect of circulating malignant MCL cells, which could interfere with iNKT cell activity. In order to examine the function of iNKT cells in absence of malignant cells, we attempted to expand iNKT cells from MCL patients *ex vivo*. CD1d-Ig based artificial antigen presenting cells (aAPC) were generated by coupling CD1d-Ig onto magnetic beads in combination with anti-CD28 mAb [25] (schematic, Fig 2A). Enriched T cells were stimulated with α -GalCer-loaded aAPC biweekly for one month. Starting with equivalent T cell numbers in each group, iNKT cells isolated from the peripheral blood of MCL patients did not expand, in contrast to iNKT cells isolated from healthy donors and other cancer patients (Fig 2B and

Table 1). To ascertain whether the loss in iNKT cell proliferation and function was cancer type-specific, enriched T cells from patients with diffuse large B cell lymphoma (DLBCL) and melanoma were stimulated with α -GalCer-loaded aAPC (Table 1). As shown in Fig 2C, not only was iNKT cell proliferation impaired, the total cultured T cell population was also non-functional and could not be activated by stimulation with anti-CD3/CD28 or PMA/ionomycin. These data suggest that MCL, and possibly other types of lymphomas, produce a factor that results in enduring suppression of the iNKT cell population and an inhibition of the overall T-cell population.

3.3. Cancer sera chronically stimulate iNKT cells

To investigate whether soluble inhibitory factors present in the serum of MCL patients could directly modulate iNKT cell function, healthy donor iNKT cells were cultured in medium containing 10% heat-inactivated serum collected from MCL patients or healthy individuals. iNKT cells were strongly activated when cultured in MCL serum, in the absence of any exogenous stimuli. This activation decreased over time, but remained significantly higher than iNKT cells cultured in “normal” serum (in which IFN- γ was undetectable; Fig 3A). To determine if this stimulation was specific to MCL patient derived serum, iNKT cells were cultured with sera from patients with other types of cancer (Fig 3B). Interestingly, we found that compared to cultures with healthy donor serum, treatment with cancer patient derived serum induced iNKT cell activation.

Next we performed time course studies with MCL-derived serum to investigate the potential long-term effects of chronic stimulation on iNKT cell function. iNKT cells cultured in MCL serum were strongly activated as indicated by high levels of IFN- γ production, which decreased over time (Fig 3C). These iNKT cells had a higher level of proliferation on day 4, which steadily declined, compared to the control, which peaked on day 7 and then slowly decreased (Fig 3D). In order to determine whether the decrease in proliferation was due to activation induced cell death (AICD) or exhaustion secondary to chronic stimulation, we assessed the expression of the cell surface protein, Programmed Death-1 (Fig 3E). On day 4, the PD-1⁺CD44⁺ iNKT cell percentage and absolute number were much higher in the MCL serum cultured population compared to the control. However, this population was drastically reduced at day 10 (Fig. 3F), in contrast to the control in which PD-1⁺CD44⁺ iNKT cell levels remained relatively stable. We initially posited that the decrease in the PD-1⁺ population in the MCL serum treated group was due to apoptosis, however, it was found that the “normal” serum treated samples had fewer viable cells at days 10 & 14 compared those cultured in the MCL patient sera. Thus, our data suggest that the MCL serum contains soluble factors that non-specifically stimulate iNKT cells, leading to a decrease in their proliferative potential and resulting in an exhausted or anergic phenotype.

3.4. Generation of mature human T cells using OP9-DL1 cultures

Based on the low absolute numbers of circulating iNKT cells and our inability to expand from the peripheral blood of some patients, we sought to develop a bridge to the clinical use of these cells by differentiating iNKT cells from adult progenitor stem cells. Human CD34⁺ HPC were enriched using magnetic cell sorting and seeded on the OP9-DL1 feeder layer (Fig 4A). We also measured cellular expansion (Fig 4B). Cellular proliferation and

differentiation were studied using CD34, CD38, V α 24, V β 11, CD4, CD8, CD3, and TCR $\gamma\delta$ as post-selection markers (see schematic in Fig 4C). Phenotypically mature T cells could be easily discerned in the cultures because, as time progressed, the cells lost expression of CD34 and CD38, transitioned to double-positive lymphocytes, and then primarily to CD4⁺CD3⁺ T cells. CD34 expression decreased as the cells became CD3⁺ in the presence of Notch signaling (expression of DL1) in the OP9 stromal cells. Notably, when the HSPC were cultured with the OP9-DL1, there was much greater differentiation than with the control- OP9-GFP (data not shown) [13].

3.5. Expansion of Primary iNKT cells using artificial antigen presenting cells (aAPC)

Similar to previous studies [15], we found that initially the expanded T cells were largely V α 24⁺; but we have been able to use bead based-CD1d-expressing artificial antigen presenting cells (aAPC) to expand human iNKT cells *in vitro*. Thus, OP9-DL1-generated primary T cells were co-cultured with α -GalCer-loaded aAPC (Fig 5A). We found that these cells produced IFN- γ following stimulation (Fig 5B). Given the above phenotypic and functional characteristics, we posit that adult human bone marrow stem cells can be used to generate functionally mature iNKT cells.

To further optimize our system, we sought to increase the number and percentage of iNKT cells. In these studies, T cells were generated from CD34⁺ cells isolated from the bone marrow from newly diagnosed patients with Hodgkin's lymphoma. After incubating the HSPC derived T cell population with aAPC for one week, the cells were stained with an iNKT cell specific mAb, clone 6B11. Then anti-mouse IgG1 microbeads were used to separate the iNKT cells. Although the initial iNKT cell population (V α 24⁺V β 11⁺) was still relatively low (>1%), after two weeks of stimulation with aAPC the percentage was approximately 60–90% of 2–3 million cells, that represents a 9400 fold expansion based on percentages and over a 18,800 fold expansion based on iNKT cell numbers (Fig 5C). Thus, the initial manipulation to enrich the iNKT population was helpful in generating an enhanced post-stimulation specific population.

In order to confirm that the HSPC-aAPC derived iNKT cells from patients with Hodgkin's lymphoma were mature and fully functional, we next sought to determine whether there were distinct populations producing specific cytokines or if the same population of cells produced both IFN- γ and IL-4. HSPC derived iNKT cells were cultured were stimulated with PMA and ionomycin and cytokine production was assessed (Fig 6A). Assessment of cytokine production by intracellular cytokine staining revealed that these tetramer positive iNKT cells were capable of producing both IFN- γ and IL-4. In addition, it was found that these patient derived cells were also capable of producing GM-SCF (Fig 6B). Therefore, these data indicate that HSPC-derived iNKT cells are polyfunctional and can produce multiple types of cytokines.

4. Discussion

Adoptive immunotherapy involves the isolation, *ex vivo* expansion and infusion of lymphocytes [29]. These infused lymphocytes can play an important role in enhancing immune function in transplant recipients. Patients that have received stem cell transplants

are severely immunocompromised after transplantation, and are at an increased risk for infection and the infusion of large numbers of hematopoietic progenitors is insufficient to completely restore immune competence [30, 31]. Recent studies have investigated the potential mechanisms that can be used to enhance immune reconstitution after stem cell transplant in order to help restore the reservoir of “immunological memory” that is lost after transplantation. Many studies have shown that T cell survival in the periphery is dependent on the continuous delivery of survival signals [32]. Consequently, studies focused on T cells have examined endogenous telomerase activity, central versus effector memory subsets, and the addition of cytokines or co-stimulatory signals to enhance proliferative potential and increase survival [33–36]. iNKT cells can help prevent graft-versus-host disease and can mediate graft-versus-leukemia. In addition, the rapid production of cytokines by iNKT cells can help support the activation of other immune cells. In this study, we demonstrate that OP9-DL1 cultures in combination with aAPC support the differentiation of functionally mature iNKT cells from human stem progenitor cells. This is highly significant because the propagation of human iNKT cells to date has been technically challenging due to the low frequency of human circulating iNKT cells and there are few studies demonstrating the generation of human iNKT cells from adult progenitor stem cells. While Woo et al, generated NKT cells from cord blood in the absence of stromal cells [37], we are the first to successfully generate NKT cells from lymphoma patient stem cells. We previously developed CD1d-Ig based aAPC [24, 25, 38], which can be used for the activation and expansion of primary iNKT cells, and herein we have taken the next step and demonstrated its use for the propagation of bone marrow stem progenitor cell-derived iNKT cells.

Other groups have used soluble forms of CD1d molecules and have shown that when loaded with lipid antigen, these molecules are directly able to target iNKT cells *in vitro* [39–41]. The physical interaction of NK cells with CD1d [42] has been examined using a bead system. Work from Tsuji’s group using functional recombinant human CD1 dimeric proteins, show that these CD1d molecules can be used to stimulate and expanded human invariant iNKT cells as efficiently as autologous immature DCs. Given that the engagement of the T cell receptor (TCR) by the CD1d-antigen complexes is a fundamental requirement of iNKT cell activation, once T cells are generated using the OP9-DL1 stromal culture system, antigen:CD1d-Ig complexes in combination with the appropriate costimulatory signaling, offer a reliable method to activate and expand polyfunctional iNKT cell populations. Importantly, our system allows us to expand the iNKT cell population from the bone marrow of lymphoma patients. Thus, our studies could have wide ranging applications because our system can be further modified to meet clinical standards so that the expanded cells can be given back to patients in the setting of bone marrow transplantation.

Our study is significant because we have found, as have others, that many cancer patients have ~10 fold lower number of circulating iNKT cells (see Figs 1 & 2), compared to healthy donors. In fact in a recent study, examining the *in vitro* expansion of 25 healthy donors, it was found that growth rates were highly variable did not correlate with age, gender, basal circulating levels or CD1d- ligand used [43]. Although iNKT cell numbers are highly variable between individuals, their significant reduction in cancer patients, suggest a critical role for iNKT cells in cancer immune surveillance. Thus, we hypothesize that infusing iNKT

cells following stem cell transplantation will enhance immunoreconstitution and help control opportunistic infections and tumor recurrence following transplantation. In good agreement with our hypothesis, studies by de Lalla *et al* have shown that reconstitution of the peripheral iNKT cell compartment following HLA-haploidentical hematopoietic stem cell transplantation is correlated with leukemia control in children [44, 45].

We have several ongoing studies focused on investigating this significant decrease in the circulating iNKT cell population in cancer patients. We have found that in certain gynecological malignancies, tumor shed inhibitory lipids which block iNKT cell activation. Thus, it is possible that the low frequency of iNKT cells in some cancer patients may be caused by a lack of stimulation. Conversely, we have found that in certain hematological malignancies, tumor shed factors that appear to induce iNKT cell exhaustion. Taken together, our data suggest that autologous DC from these patients may not offer the optimal level of stimulation that is available when utilizing the aAPC system. The lack of stimulation provided by cancer patient DC may explain why previous studies have found the absence of a significant *in vivo* response following either direct α -GalCer injections or adoptive transfer of autologous α -GalCer pulsed DC, as has been previously suggested [46].

In previous studies we have shown that CD1d-Ig based aAPC can be used as a tool to examine the effect of specific costimulatory signals on iNKT cell activation. For example, in primary murine liver iNKT cells we found that anti-CD28 had strong co-stimulatory effect on primary liver iNKT cells, compared to stimulation with aAPC expressing anti-CD44 and anti-CD69. In humans we have found that co-stimulation is important for iNKT cell proliferation. Importantly, we have found that iNKT cell specific co-stimulatory molecules, such as CD44 and CD161, are important for cytokine production and cytotoxicity. Further studies will be conducted to determine if other costimulatory molecules such as 41BB or OX40 can enhance the proliferative potential or effector functions of stem progenitor derived iNKT cells.

In a recent review by Fujii, et al. several novel iNKT cell-based immunotherapeutic strategies are discussed [47]. First, they described a method using artificial adjuvant vector cells containing tumor mRNA and α -GalCer/CD1d, which could be used to stimulate endogenous iNKT cells [48]. In addition, they discuss their studies using induced pluripotent stem cells (iPSC) to generate large numbers of iNKT cells with adjuvant activity [49]. In contrast to their murine studies, we were unable to generate mouse iNKT cells from mouse HSPC using aAPC or bone marrow derived dendritic cells (data not shown). Similar to their work, we believe that in the context of adoptive immunotherapy, the generation of iNKT cells from HSC could be utilized in combination with other tumor specific T cell types, as well as in the setting of transplantation in order to boost host immune responses. Moreover, the availability of technologies and clinical grade- reagents required for the selective expansion of iNKT cells coupled with the cost-effectiveness and versatility of the bead based- aAPC system highlights the feasibility of this therapeutic strategy.

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Highlights

- iNKT cells can be generated from CD34⁺ stem progenitor cells using the OP9-DL1 system in combination with aAPC.
- iNKT cells can be generated from bone marrow HSPC from lymphoma patients.
- HSPC-derived NKT cells produce IFN- γ , IL-4 and GM-CSF.

Figure 1.

iNKT cells are decreased in cancer patients. (A) PBMC were isolated from healthy donors and PBMC and bone marrow were collected cancer patients and analyzed for Vα24⁺Vβ11⁺ iNKT cells by flow cytometry. The data are representative of 42 healthy donors and 55 newly-diagnosed lymphoma patients. The PBMC and BM data from lymphoma patients (LP) are matched samples obtained from the same donor. (B) The percentages from all of the healthy donors and cancer patients in this study were averaged. ***p=0.0004. (C) iNKT cells are higher in the bone marrow compared to the peripheral blood. MNC were isolated from the bone marrow and peripheral of lymphoma patients and stained for flow cytometry. Data were collected from ten NHL patients, and the data are shown as mean ± SEM. Data were acquired using an LSR II and analyses were performed using FCS Express software.

Figure 2.

iNKT cells from MCL patients are non-functional. (A) Schematic diagram of CD1d:Ig-based aAPC. In this system, CD1d-Ig is used to provide the cognate antigen-specific signal through the TCR and anti-CD28 Abs provide the costimulatory signal. (B) CD3⁺ cells were isolated from the blood of healthy donors and MCL patients. iNKT cell% (α GC-tet⁺CD3⁺) were determined by FACS and the cells were stimulated biweekly with aAPC. Data shown are after 2 rounds of stimulation. The top number indicates the iNKT cell % and the bottom number indicates the total number of iNKT cells. (C) CD1d-aAPC expanded iNKT cells were stimulated with anti-CD3/CD28 microbeads or PMA and ionomycin. T cell activation was assessed by IFN- γ production. N.D.-not detected. Data are representative of three independent experiments.

Figure 3. iNKT cell function is decreased following long term exposure to MCL patient serum. (A) Pretreatment with sera from MCL patients stimulates iNKT cells in the absence of exogenous antigen. Primary HD iNKT cells were incubated with heat-inactivated serum from MCL patients or pooled AB serum for 48h. Culture supernatants were harvested and standard sandwich ELISA was used to measure IFN- γ production, as an indication of iNKT cell activation. (B). Activation of iNKT cells following culture in cancer patient derived sera. iNKT cells cultured for the indicated time in the presence of 10% serum from healthy donors or the cancer patients and irradiated feeder cells. In the absence of exogenous antigen or cytokines, iNKT cells produce IFN- γ when cultured in MCL serum. (C) Long term exposure to MCL patient sera results in a loss of iNKT cell function. iNKT cell culture supernatants were harvested at the indicated time points post treatment, and standard sandwich ELISA was used to measure IFN- γ production. (D) The proliferative capacity of

iNKT cells is decreased after long term exposure to MCL patient sera. WST-1 was used to assess iNKT cell proliferation. The optical density (O.D.) is shown as an indication of cellular proliferation. (E) Pretreatment with sera from MCL patients results in increased iNKT cell surface expression of PD-1. iNKT cells were stained for PD-1 and CD44 cell surface expression following culture in MCL patient sera. Cells are gated on CD3⁺/α-GalCer tetramer, and percentage of PD-1⁺CD44⁺ was determined using FCS Express software. Data are representative of two independent experiments. Data was analyzed by one-way ANOVA. (F) Total iNKT cell numbers after incubation with sera from MCL patients or healthy donors. MCL indicates mantle cell lymphoma; DLBCL indicates diffused large B cell lymphoma and Mel indicates melanoma. The patient ID corresponds with the data shown in Table 1.

Figure 5.

Expansion of iNKT cells from HSPC using aAPC. (A) iNKT cell expansion following aAPC stimulation. T cells were generated from HSPC using the OP9-DL1 co-culture system. The T cells were then expanded using CD1d-based aAPC. The expanded population was stained for iNKT cells (α -GalCer-tet⁺CD3⁺), and the remaining cells were stimulated biweekly with aAPC. Data shown are after a single round of stimulation. (B) Cultured iNKT cells are functional. HSPC-generated T cells co-cultured with CD1d-aAPC were stimulated with anti-CD3/CD28 microbeads or PMA and ionomycin for 48 hr. Culture supernatants were harvested and iNKT cell activation was assessed by IFN- γ production, which was measured by standard sandwich ELISA. Data from healthy donor is shown and is representative data obtained from five different donors. (C) Enrichment of iNKT cells increases antigen-specific proliferation. iNKT cells were generated from HSPC using the OP9-DL1/aAPC co-culture system, then enriched using magnetic bead separation in combination with mAb specific for the iNKT TCR (clone 6B11). The sorted cells were stimulated biweekly with α -GalCer loaded-aAPC. The initial population and the expanded

population were stained for canonical iNKT cells (CD3 and iNKT). Data shown are after 2 weeks of stimulation post enrichment.

Figure 6.

Human HSPC derived iNKT cells are polyfunctional. Human bone marrow CD34⁺ cells were isolated from a patient with Hodgkin's Lymphoma. iNKT cells were generated using the OP9-DL1 system in combination with αAPC. (A) The iNKT cells were stained with either unloaded tetramer or α-GalCer loaded tetramer. The CD1d-tetramer⁺CD3⁺ population was gated following stimulation with PMA and ionomycin and induction of IL-4 and IFN-γ by iNKT cells was assessed by flow cytometry. (B) Primary human iNKT cells (10⁴) were co-cultured with anti-CD28/CD3 microbeads (10⁵) or PMA and ionomycin for 48 hours. Medium served as a negative control. Culture supernatants were harvested and standard sandwich ELISA was used to measure cytokine production. One experiment representative of data obtained from two lymphoma patients is shown.

Table 1*Ex vivo* expansion of iNKT cells

		Initial % iNKT cells	% Post aAPC expansion	Fold Expansion
Healthy Controls	HD1 *	1.34	82.10	61
	HD2	0.14	95.56	682
	HD3	0.12	38.21	318
	HD4	0.03	67.37	2245
Cancer patients	DLBCL47**	0.2	5.21	26
	DLBCL49	0.04	6.91	172
	Melanoma	0.26	61	234
	MCL1 ***	0.03	0.03	1
	MCL2	0.02	0.0	0
	MCL46	0.02	0.0	0
	MCL48	0.13	0.0	0

* HD-Healthy Donor

** DLBCL- Diffuse Large B cell Lymphoma

*** MCL-Mantle Cell Lymphoma