Peripheral Blood Invariant Natural Killer T Cells of Pig-Tailed Macaques

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Abstract

In humans, invariant natural killer T (iNKT) cells represent a small but significant population of peripheral blood mononuclear cells (PBMCs) with a high degree of variability. In this study, pursuant to our goal of identifying an appropriate non-human primate model suitable for pre-clinical glycolipid testing, we evaluated the percentage and function of iNKT cells in the peripheral blood of pig-tailed macaques. First, using a human CD1d-tetramer loaded with α-GalCer (α-GalCer-CD1d-Tet), we found that α-GalCer-CD1d-Tet+ CD3+ iNKT cells make up 0.13% to 0.4% of pig-tailed macaque PBMCs, which are comparable to the percentage of iNKT cells found in human PBMCs. Second, we observed that a large proportion of Vv24+CD3+ cells are α-GalCer-CD1d-Tet+CD3+ iNKT cells, which primarily consist of either the CD4+ or CD8+ subpopulation. Third, we found that pig-tailed macaque iNKT cells produce IFN-γ in response to α-GalCer, as shown by ELISpot assay and intracellular cytokine staining (ICS), as well as TNF-α, as shown by ICS, indicating that these iNKT cells are fully functional. Interestingly, the majority of pig-tailed macaque iNKT cells that secrete IFN-γ are CD8+ iNKT cells. Based on these findings, we conclude that the pig-tailed macaques exhibit potential as a non-human animal model for the pre-clinical testing of iNKT-stimulating glycolipids.

Introduction

Natural killer T (NKT) cells are a unique subset of lymphoid cells that express both a T cell antigen receptor (TCR) and NK1.1 (NKR-P1 or CD161c), a C-lectin-type NK receptor [1,2]. A significant proportion of NKT cells express semi-invariant TCRs encoded by Vα24 and Jα18 gene segments in humans and Vα24 and Jα18 gene segments in mice, and these cells have been designated invariant NKT (iNKT) cells [3]. In humans, NKT cells represent a small but significant proportion (0.01%–0.5%) of PBMCs with a high degree of variability [4,5]. Upon activation, iNKT cells rapidly secrete both Th1 and Th2 cytokines in vivo and induce a series of cellular activation events leading to the activation of innate immune cells, such as NK cells and dendritic cells (DCs), as well as the stimulation of adaptive immune cells, such as B and T cells [6–12]. In addition, upon stimulation, iNKT cells, like NK cells, display cytotoxic activity mediated by Fas, perforin, granzyme A/B, and granulysin [13,14]. iNKT cells have also been shown to display anti-tumor activity [15,16], mediate therapeutic effects against autoimmune diseases [17–20], and promote protection against certain infectious agents [21–24].

CD1d molecules and iNKT cells are conserved between mice and humans [25]. Accordingly, mouse models have been extensively used to study the biological activity of CD1d-binding, iNKT cell-stimulating glycolipids, and the phenotypes and functions of iNKT cells [1,26]. However, these studies have indicated substantial differences in the specificity, frequency, and function of CD1d and iNKT cells between the two species. Because of this, some studies have investigated the frequency, phenotype, and function of NKT cells derived from non-human primates, including pig-tailed macaques, and found similar percentages and high variability of NKT cells between monkeys and humans [27–30]. These studies have also indicated that the phenotypes and functions of monkey iNKT cells are significantly different among different macaque species [27–30]. Pig-tailed macaques have been used as animal models to study a number of human diseases, such as Chlamydia trachomatis [31–33] and HIV-1 infection [34,35]. In this study we sought to characterize in greater detail the base line frequency, specificity, and function of iNKT cells in pig-tailed macaques and address whether pig-tailed macaques could be used as an animal model for the pre-clinical testing of various iNKT-stimulating ligands.

Materials and Methods

Animals

Pig-tailed macaques (M. nemestrina) were used in this study. All animals were negative for simian immunodeficiency virus (SIV) and simian T-cell lymphotropic virus type 1 (STLV-1) by serology as well as simian type D retrovirus by serology and polymerase chain reaction (PCR). Peripheral blood was collected by venipuncture under anesthesia. All animals used in this study
were housed and cared for according to the Guide for the Care and Use of Laboratory Animals at the Washington National Primate Research Center (WaNPRC), an institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal quarters are maintained at 75–78°C with controlled air humidity and quality. Commercial monkey chow was fed to the animals once daily, and drinking water was available at all times. Daily examinations and any medical care were provided by the WaNPRC veterinary staff in consultation with the clinical veterinarian. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington and conducted in compliance with the Public Health Services Policy on Humane Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/references/PHSPolicyLabAnimals.pdf). The animals were kept under deep sedation with ketamine HCl at a dose of 10–15 mg/kg intramuscularly to alleviate any pain and discomfort during blood draws. An animal technician or veterinary technologist monitored the animals while under sedation.

Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated from buffy coats by Ficoll-Hypaque density gradient separation. Erythrocytes were removed by osmotic lysis in ACK lysing buffer (Life Technologies, Grand Island, NY), and the remaining nucleated cells were washed twice with RPMI supplemented with 10% fetal calf serum (FCS).

Antibodies, Glycolipid, and CD1d-tetramer

Anti-human antibodies known to cross-react with macaques were selected for this study. For flow cytometric analysis, we used anti-Vα24-PE (C15; Immunotech, Quebec, Canada), anti-Vβ11-FITC (C21; Beckman Coulter, Brea, CA), anti-6B11-FITC (6B11; BioLegend, San Diego, CA), anti-CD3-perCp (SP34-2; BD Biosciences, San Jose, CA), anti-CD4-APC (SK3, BD Biosciences), anti-CD8-FTTC (SK1, BD Biosciences), anti-CD8α-perCp (SK1, BD Biosciences), anti-6B11-FITC (6B11; BioLegend), anti-CD3-perCp (SP34-2; BD Biosciences), anti-CD8β-APC (2ST8.5H7, BD Biosciences), anti-CD16a-FITC (H12F1, BD Biosciences), anti-CD8α-FITC (H12F1, BD Biosciences), anti-CD8β-FITC (2ST8.5H7, BD Biosciences), anti-CD8α-APC (SK1, BD Biosciences), anti-CD8β-APC (SK1, BD Biosciences), anti-CD8α-FITC (2ST8.5H7, BD Biosciences), anti-IFN-γ-APC (4S.B3, Abcam, Cambridge, MA), and anti-41Tα antibody-PE-Cy7 (MAb11, BioLegend). For ELISpot assay, we used anti-IFN-γ clone: GZ-4, Mabtech, Mariemont, OH) and biotin-labeled anti-IFN-γ clone: 7-B6-1, Mabtech). Lyophilized α-GalCer (Avanti Polar Lipid, Alabaster, AL) was reconstituted at 1 mg/ml with 100% DMSO then stored at −20°C. The α-GalCer-loaded human CD1d-tetramer conjugated to PE (α-GalCer-CD1d-Tet) was purchased from Proimmune Inc. (Sarasota, FL).

Flow Cytometric Analysis

For cell surface staining, 1 × 10⁶ PBMCs were incubated for 20 min at 4°C in FACS staining buffer in the presence of the antibody of interest. After washing twice, labeled cells were subjected to multicolor FACS data analysis on a BD LSRII (Becton Dickinson, Franklin Lakes, NJ) using forward and side-scatter characteristics to exclude dead cells. Anti-mouse-Ig or anti-rat compensation particle sets were used for compensation purposes (BD Biosciences). The data were analyzed using FlowJo software (Tree Star, Ashland, OR).

PBMCs Stimulation by α-GalCer

PBMCs were cultured in a 96-well U-bottom plate at 1 × 10⁶ cells/well in the presence of 5 μg/ml or 0.1 μg/ml of α-GalCer for 6 hours at 37°C followed by the addition of Brefeldin A (BioLegend) at 5 μg/ml for the last 4 hours of incubation. In a
negative control group, cells were stimulated with medium containing 0.1% of DMSO vehicle.

**Intracellular Cytokine Staining**

For intracellular IFN-γ and TNF-α staining, the PBMCs were stimulated with α-GalCer, as described above. After stimulation, the cells were incubated with anti-CD3, anti-CD4, and anti-CD8 antibodies, as well as the human CD1d tetramer loaded with α-GalCer for 20 min. The cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) following the manufacturer’s instructions. The permeabilized cells were stained with PE-Cy7-labeled anti-TNF-α and APC-labeled anti-IFN-γ antibodies for 30 min on ice in the dark. After washing twice, the cells were resuspended in staining buffer and analyzed by flow cytometric analysis. Acquisition and analysis were carried out by first gating for live cells by forward scatter (FSC) and side scatter (SSC) then subsequently gating for iNKT cells by positivity to CD3⁺ and α-GalCer-CD1d-Tet⁺CD3⁺ among the live cells. IFN-γ⁺ and TNF-α⁺ cells were then further gated from the iNKT cells.

**IFN-γ ELISpot Assay**

The total IFN-γ producing cells among the α-GalCer stimulated PBMC cells were determined by an ELISpot assay using the monkey IFN-γ ELISpot kit (Mabtech). Briefly, the Multiscreen HA ELISpot plate (Millipore, Billerica, MA) was first coated with anti-IFN-γ antibodies. Next, the PBMC cells from pig-tailed macaques were added at 5 × 10⁵ cells/well and stimulated with α-GalCer at 0.1 µg/ml or 1 µg/ml for 24 hours at 37°C. In the negative control groups, the cells were cultured with 0.1% DMSO. After washing five times, the plate was incubated with biotin-labeled anti-IFN-γ antibodies for 1 hour followed by incubation with avidin-HRP. Finally, the spots were developed with an AEC ELISpot substrate kit (BD Biosciences).

**Results and Discussion**

Here, we aimed to characterize the properties of iNKT cells derived from pig-tailed macaques to determine whether the cells in this species exhibit similar properties to human iNKT cells. The goal of the study was to determine whether pig-tailed macaques represent an appropriate animal species for pre-clinical testing. We first determined the frequency of iNKT cells among PBMCs...
collected from pig-tailed macaques. To accomplish this, we identified NKT cells by staining PBMCs with an α-GalCer-loaded human CD1d-tetramer (α-GalCer-CD1d-Tet). As shown in Fig. 1, we detected a distinct population of PBMCs that react with α-GalCer-CD1d-Tet, but not with the unloaded human CD1d-tetramer. The percentage of these α-GalCer-CD1d-Tet+ cells ranged from 0.13% to 0.4% of the total PBMCs (Fig. 2B). These results confirm those from a previously published study

Figure 3. Vβ11 and 6B11 phenotypes of Vα24+ cells among PBMCs from pig-tailed macaques. One million PBMCs were incubated with anti-Vα24-PE together with either anti-Vβ11-FITC or 6B11-FITC followed by flow cytometric analysis, as described in Fig. 1.

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Figure 4. CD4/CD8 phenotype of α-GalCer-CD1d-Tet+CD3+ NKT cells derived from pig-tailed macaques. (A) One million PBMCs were first incubated with α-GalCer-CD1d-Tet-PE and anti-CD3-PerCP. Cells were also stained with anti-CD4-APC and anti-CD8-FITC then subjected to flow cytometric analysis, as described in Fig. 1. Data represent one of two similar experiments. (B) The percentage of α-GalCer-CD1d-Tet+CD3+ NKT cells of each pig-tailed macaque are listed. (C) One million PBMCs were first incubated with anti-Vα24-PE then stained with CD8α-perCP anti-CD8β-APC and subjected to flow cytometric analysis, as described in Fig. 1.

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[30], and indicate that the percentage of iNKT cells in the peripheral blood of pig-tailed macaques is comparable to what has been observed in the peripheral blood of humans. To determine the correlation between α-GalCer-CD1d-Tet+ cells and Vα24+ cells, we co-stained pig-tailed macaque PBMCs with α-GalCer-CD1d-Tet and anti-Vα24 antibodies, as shown in Fig. 2A. We found that approximately two-thirds of the Vα24+ cells were α-GalCer-CD1d-Tet+ cells (Fig. 2B), and there was a strong positive linear correlation ($p = 0.0187; R^2 = 0.9599$) between the percentages of the two subpopulations (Fig. 2C).

In humans, the majority of α-GalCer-CD1d-Tet+ iNKT cells have been shown to “co-express” an invariant Vα24-Jα18 chain and a semi-invariant Vβ11 chain [36–38]. Therefore, we sought to determine whether α-GalCer-CD1d-Tet+ iNKT cells derived from pig-tailed macaques also co-express Vα24 and Vβ11. Unfortunately, the anti-human Vβ11 antibody failed to cross-react with pig-tailed macaque iNKT cells, as has been shown with iNKT cells derived from other monkey species (Fig. 3) [27–29].

We next determined the CD4 and CD8 phenotypes of pig-tailed macaque iNKT cells (Fig. 4A) and found that iNKT cells primarily consisted of the CD4+ and CD8+ subpopulations. The few remaining cells were double negative (DN) (Fig. 4B). All CD8+ iNKT cells were also found to be CD8αβ+ (Fig. 4C). These results confirmed an earlier study showing that pig-tailed macaque iNKT cells consist of a significant percentage of a CD4− subpopulation [30]. Furthermore, the CD4/CD8 distribution is somewhat different from iNKT cells derived from other monkey species, which are largely made up of CD8+ cells [27–29]. More
In this study, we first measured the percentage of CD8+ NKT cells among the total IFN-γ-secreting NKT cells derived from the PBMCs of five pig-tailed macaques. As shown in Fig. 5, a significant percentage of CD8+ NKT cells among total IFN-γ-secreting NKT cells was much higher than IFN-γ-secreting CD4+ NKT cells (Fig. 6). Furthermore, the IFN-γ ELISpot assay showed that 100–400 per million PBMCs secreted IFN-γ in response to 1 μg/ml of α-GalCer (Fig. 6C). This result corroborates our ICCS assay and indicates that a significant number of NKT cells among PBMCs secrete IFN-γ upon α-GalCer stimulation.

To investigate the function of pig-tailed macaque NKT cells in this study, we first measured the percentage of α-GalCer-activated NKT cells secreting IFN-γ, TNF-α, and IL-10, using an ICCS assay. As shown in Fig. 5, a significant percentage of pig-tailed macaque NKT cells secreting TNF-α and/or IFN-γ, whereas they failed to secrete a significant amount of IL-10 (data not shown). We then analyzed the percentages of CD4+ and CD8+ NKT cell subpopulations among the total IFN-γ-secreting NKT cells. Although both CD4+ and CD8+ NKT cells produced IFN-γ after stimulation with α-GalCer, the percentage of IFN-γ-secreting CD8+ NKT cells was much higher than IFN-γ-secreting CD4+ NKT cells (Fig. 6).

Regarding the functionality of NKT cells, human NKT cells activated by α-GalCer are known to secrete a myriad of cytokines, with CD8α NKT cells biased toward a Th1 phenotype, CD4+ NKT cells predominantly secreting Th2 cytokines, and DN NKT cells exhibiting an intermediate Th1/Th2 phenotype [40]. Non-human primate NKT cells have been shown to display a similar function to human NKT cells, but there are some differences among different species. For example, rhesus macaque NKT cells secrete large amounts of TGF-β, IL-6, and IL-13, and modest levels of IFN-γ, whereas IL-10 secretion was negligible and no detectable IL-4 was observed [41]. However, sooty mangabey NKT cells have been shown to secrete virtually all cytokines tested, including IFN-γ, TNF-α, IL-2, IL-13, and IL-10 [29]. In addition, their CD8α NKT subpopulation produced a high amount of IFN-γ and expressed significantly higher levels of granzyme B and perforin [42].

To investigate the function of pig-tailed macaque NKT cells in this study, we first measured the percentage of α-GalCer-activated NKT cells secreting IFN-γ, TNF-α and IL-10, using an ICCS assay. As shown in Fig. 5, a significant percentage of pig-tailed macaque NKT cells secreting TNF-α and/or IFN-γ, whereas they failed to secrete a significant amount of IL-10 (data not shown). We next analyzed the percentages of CD4+ and CD8+ NKT cell subpopulations among the total IFN-γ-secreting NKT cells. Although both CD4+ and CD8+ NKT cells produced IFN-γ after stimulation with α-GalCer, the percentage of IFN-γ-secreting CD8+ NKT cells was much higher than IFN-γ-secreting CD4+ NKT cells (Fig. 6). Furthermore, the IFN-γ ELISpot assay showed that 100–400 per million PBMCs secreted IFN-γ in response to 1 μg/ml of α-GalCer (Fig. 6C). This result corroborates our ICCS assay and indicates that a significant number of NKT cells among PBMCs secrete IFN-γ upon α-GalCer stimulation.

We then performed various correlation analyses and found a marginal correlation between the relative number of α-GalCer-activated cells secreting IFN-γ among PBMCs, as determined by ELISpot assay, and the percentage of total NKT cells among PBMCs, as determined by FACS analysis (R2 = 0.7847, p = 0.0455) (Fig. 7A). However, the correlation became much stronger when we performed a correlation analysis between the relative number of α-GalCer-activated cells secreting IFN-γ among PBMCs and the percentage of CD8α NKT cells among PBMCs (R2 = 0.9576, p = 0.0135) (Fig. 7A). Interestingly, when we compared the relative number of IFN-γ-secreting cells among PBMCs and the percentages of IFN-γ-secreting CD8α and CD4+ NKT cells by ICCS assay, we found a strong correlation for the relative number of IFN-γ-secreting cells among PBMCs with IFN-γ-secreting CD8α NKT cells (R2 = 0.8965, p = 0.0146), but not with IFN-γ+ CD4+ NKT cells (R2 = 0.2559, p = 0.3866) (Fig. 7B). Thus, our current functional study demonstrates that the majority of pig-tailed macaque NKT cells that secrete IFN-γ consist of CD8α NKT cells, although CD4+ NKT cells can also produce Th1 cytokines, including IFN-γ and TNF-α.

We would like to emphasize that due to the lack of available antibodies that cross-react with pig-tailed macaque cells, we could...
only perform a limited study of the phenotype and function of pig-tailed macaque NKT cells. Despite this difficulty, however, our current study demonstrates that the percentage of NKT cells present in the peripheral blood of pig-tailed macaques is comparable to the NKT cells found in human peripheral blood. Furthermore, similar to humans, a large proportion of \( V\alpha^{24} \) CD3\( ^{+} \) cells are \( \alpha\)-GalCer-CD1d-Tet\( ^{+} \) NKT cells, and almost half of these express CD4 molecules.

Together, these results highlight the properties of pig-tailed macaque NKT cells, which resemble human cells to some degree. In light of previous successful research studies using pig-tailed macaques for certain human diseases [31–35], our study provides further evidence supporting the use of pig-tailed macaques in the pre-clinical testing of various NKT cell-stimulating ligands. In particular, they may be useful for evaluating therapeutic and prophylactic measures across a myriad of human diseases in the future.

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Author Contributions

Conceived and designed the experiments: MT SLH XL RGN. Performed the experiments: XL RGN PP. Analyzed the data: XL MT. Contributed reagents/materials/analysis tools: MT XL. Wrote the paper: MT SLH XL.

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