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

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Abstract

In humans, invariant natural killer T (*i*NKT) 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 *i*NKT 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$^+$ *i*NKT cells make up 0.13% to 0.4% of pig-tailed macaque PBMCs, which are comparable to the percentage of *i*NKT cells found in human PBMCs. Second, we observed that a large proportion of Vα24$^+$CD3$^+$ cells are α-GalCer-CD1d-Tet$^+$CD3$^+$ *i*NKT cells, which primarily consist of either the CD4$^+$ or CD8$^+$ subpopulation. Third, we found that pig-tailed macaque *i*NKT cells produce IFN-γ in response to α-GalCer, as shown by ELISpot assay and intracellular cytokine staining (ICCS), as well as TNF-α, as shown by ICCS, indicating that these *i*NKT cells are fully functional. Interestingly, the majority of pig-tailed macaque *i*NKT cells that secrete IFN-γ are CD8$^+$ *i*NKT 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 *i*NKT-stimulating glycolipids.

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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 Vα14 and Jα18 gene segments in mice, and these cells have been designated invariant NKT (*i*NKT) cells [3]. In humans, *i*NKT cells represent a small but significant proportion (0.01%–0.5%) of PBMCs with a high degree of variability [4,5]. Upon activation, *i*NKT 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, *i*NKT cells, like NK cells, display cytotoxic activity mediated by Fas, perforin, granzyme A/B, and granulysin [13,14]. *i*NKT 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 *i*NKT cells are conserved between mice and humans [25]. Accordingly, mouse models have been extensively used to study the biological activity of CD1d-binding, *i*NKT cell-stimulating glycolipids, and the phenotypes and functions of *i*NKT cells [1,26]. However, these studies have indicated substantial differences in the specificity, frequency, and function of CD1d and *i*NKT cells between the two species. Because of this, some studies have investigated the frequency, phenotype, and function of *i*NKT cells derived from non-human primates, including pig-tailed macaques, and found similar percentages and high variability of *i*NKT cells between monkeys and humans [27–30]. These studies have also indicated that the phenotypes and functions of monkey *i*NKT 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 *i*NKT 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 *i*NKT cell-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

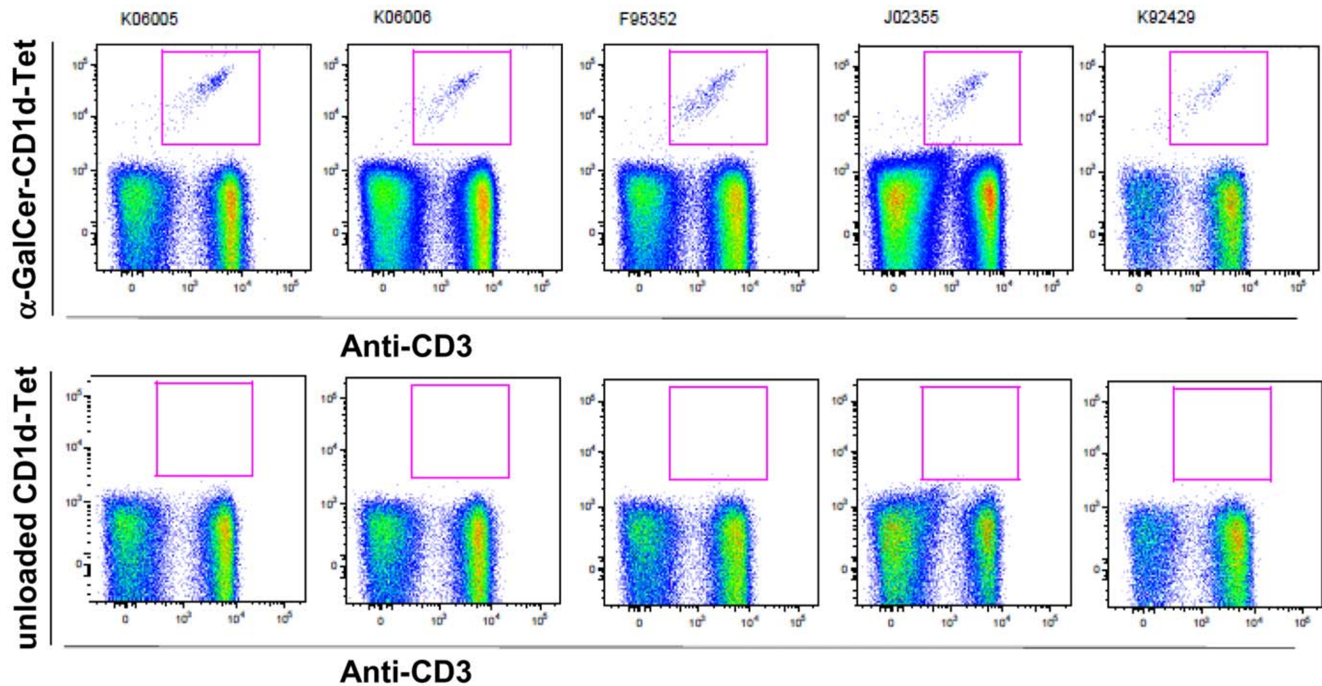

Figure 1. α-GalCer-CD1d-Tet+CD3+ cells among PBMCs from pig-tailed macaques. Peripheral blood mononuclear cells (PBMCs) obtained from each pig-tailed macaque were incubated with anti-CD3-PerCP together with α-GalCer-loaded human CD1d-tetramer-PE (α-GalCer-CD1d-Tet-PE) in upper panel or unloaded human CD1d-tetramer-PE as a negative control in lower panelData were analyzed using Flowjo software (Tree Star). For all figures, the data represent one of three similar experiments.
doi:10.1371/journal.pone.0048166.g001

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°F 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-Vα24-FITC (C15; Immunotech), 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-FITC (SK1, BD Biosciences), anti-CD8α-perCp (SK1, BD Biosciences), anti-CD8β-APC (2ST8.5H7, BD Biosciences), anti-IFN-γ-APC (4S.B3, Abcam, Cambridge, MA), and anti-TNF-α 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, 1×10^6 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 FACScan flow cytometry 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 1×10^6 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

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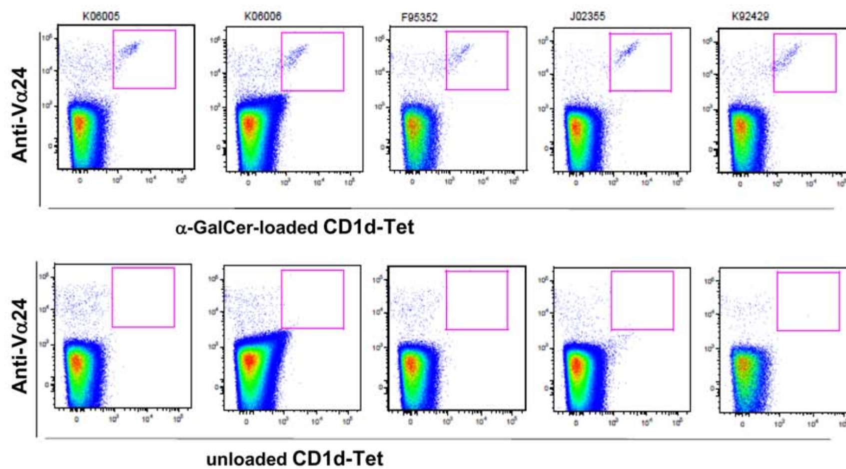

B

Monkey ID	Valpha24+CD3	CD1dTet+CD3+
K06005	0.55	0.32
K06006	0.29	0.19
F95352	0.49	0.33
J02355	0.23	0.1
K92429	0.68	0.4

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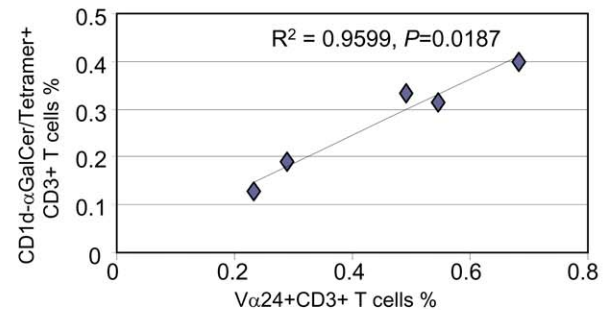

Figure 2. Correlation between Vα24+CD3+ cells and α-GalCer-CD1d-Tet+CD3+ cells among PBMCs from pig-tailed macaques. (A) One million PBMCs were incubated with anti-Vα24-FITC together with α-GalCer-CD1d-Tet-PE or unloaded CD1d-Tet-PE as a negative control then subjected to flow cytometric analysis, as described in Fig. 1. (B) The percentages of Vα24+CD3+ cells and α-GalCer-CD1d-Tet^{+}CD3^{+} cells among PBMCs from each pig-tailed macaque are listed. (C) The percentage of Vα24^{+}CD3^{+} cells and the percentage of α-GalCer-CD1d-Tet^{+}CD3^{+} cells among PBMCs from each pig-tailed macaque are scatter-plotted to evaluate the correlation between these variables, and a linear regression analysis was applied.
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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 *i*NKT cells by positivity to CD3^{+} and α-GalCer-loaded CD1d tetramer^{+} among the live cells. IFN-γ^{+} and TNF-α^{+} cells were then further gated from the *i*NKT 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 5×10^{5} 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 *i*NKT cells derived from pig-tailed macaques to determine whether the cells in this species exhibit similar properties to human *i*NKT 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 *i*NKT cells among PBMCs

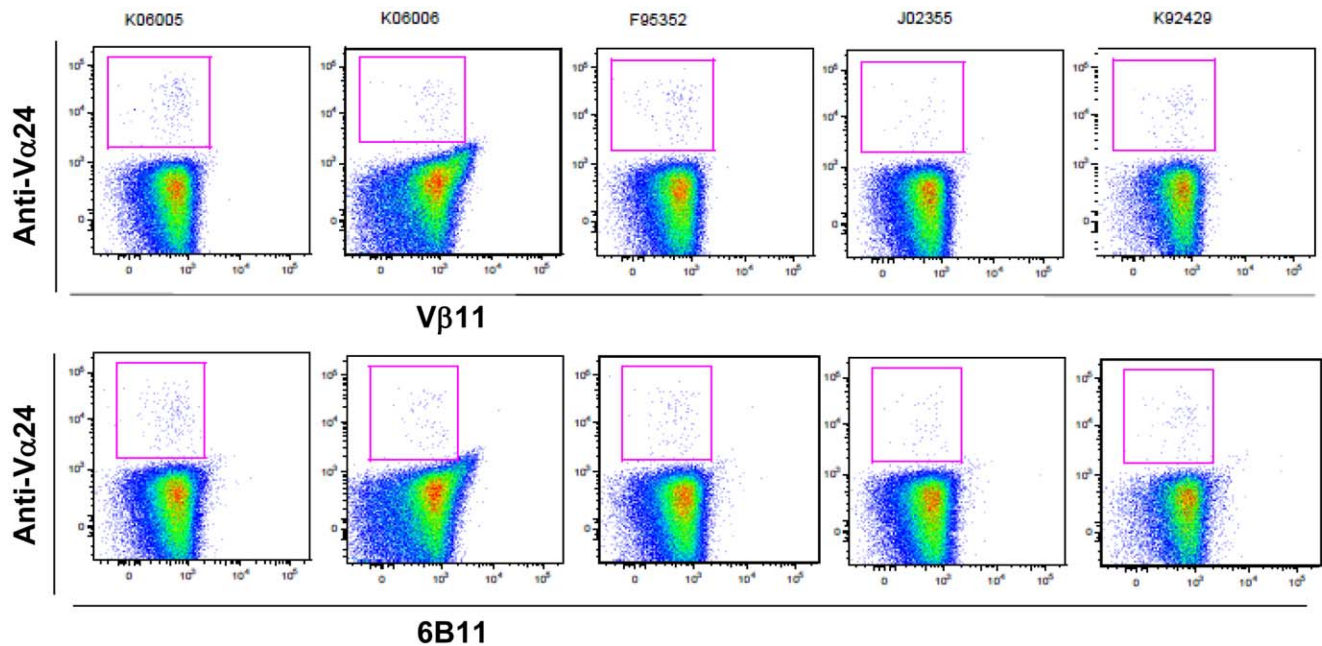

Figure 3. Vβ11 and 6B11 phenotypes of Vα24+ cells among PBMCs from pig-tailed macaques. One million PBMCs were incubated with anti-Vα24-PE together with either anti-Vβ11-FITC or 6B11-FITC followed by flow cytometric analysis, as described in Fig. 1.
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collected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

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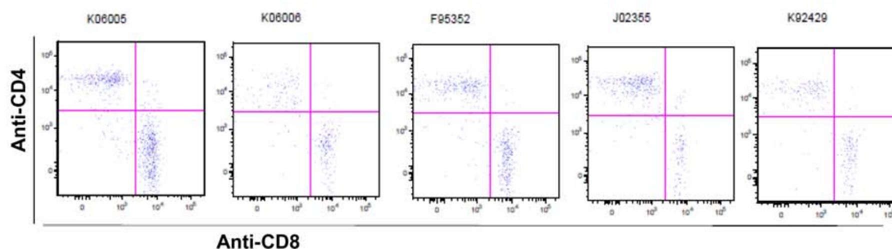

B

Quantitative analysis of Pig Tailed NKT cell subsets			
	% of lymphocytes	% of total NKT cells	
Monkey ID	iNKT (αGalCer-CD1d Tet+ CD3+)	NKT-CD4+	NKT-CD8+
K06005	0.32	37.7	54.4
K06006	0.19	31.7	61.4
F95352	0.33	45.4	49
J02335	0.13	70.7	21.1
K92429	0.4	48.7	45.7

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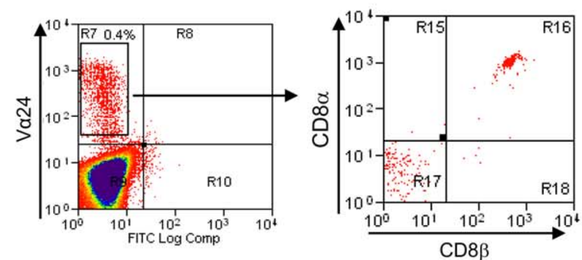

Figure 4. CD4/CD8 phenotype of α-GalCer-CD1d-Tet+CD3+ *i*NKT cells derived from pig-tailed macaques. (A) One million PBMCs were first incubated with α-GalCer-CD1d-Tet-PE and anti-CD3-PerCP. Cells were also stained with anti-CD4-APC and anti-CD8-FITC then subjected to flow cytometric analysis, as described in Fig. 1. Data represent one of two similar experiments. (B) The percentage of α-GalCer-CD1d-Tet^{+}CD3^{+} cells among PBMCs and the percentage of CD4^{+} or CD8^{+} cells among α-GalCer-CD1d-Tet^{+}CD3^{+} *i*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.
doi:10.1371/journal.pone.0048166.g004

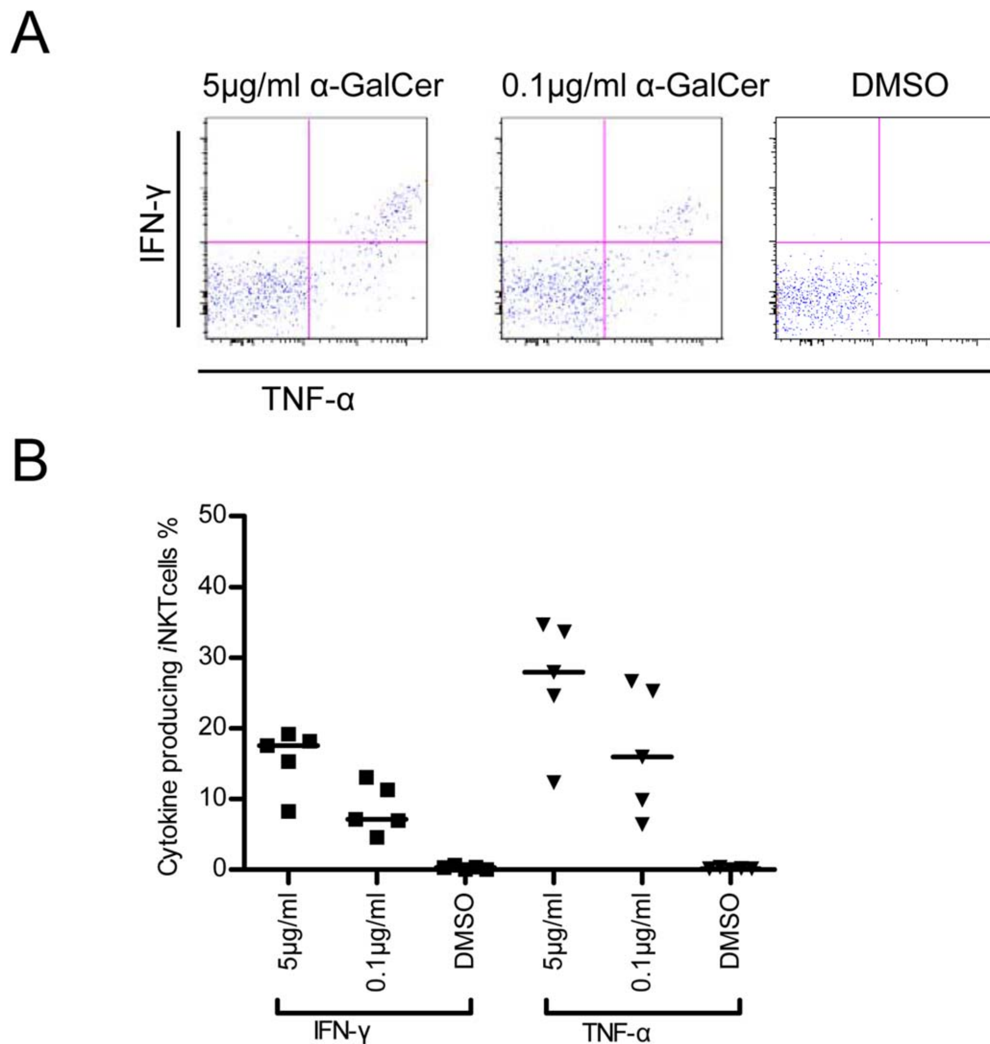

Figure 5. Secretion of IFN-γ and TNF-α by pig-tailed macaque *i*NKT cells upon stimulation with α-GalCer. One million PBMC cells were stimulated with 0.1 µg/ml or 5 µg/ml of α-GalCer followed by the addition of 5 µg/ml Brefeldin A for the last 4 hours of incubation. Invariant natural killer T (*i*NKT) cells were then gated with α-GalCer-CD1d-Tet+ and CD3+ followed by flow cytometric analysis. (A) Flow cytometric figure shows the pattern of IFN-γ and TNF-α expression by α-GalCer-activated, IFN-γ-secreting *i*NKT cells gated from PBMCs from one representative pig-tailed macaque. (B) The graph shows the percentages of *i*NKT cells secreting the respective cytokines among total *i*NKT cells derived from the PBMCs of five pig-tailed macaques.
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[30], and indicate that the percentage of *i*NKT 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+ *i*NKT 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+ *i*NKT 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 *i*NKT cells, as has been shown with *i*NKT cells derived from other monkey species (Fig. 3) [27–29]. Furthermore, the 6B11 antibody, which is known to react with the CDR3 region of the Vα24-Jα18 chain, failed to react with Vα24+ cells derived from pig-tailed macaques (Fig. 3). Although the CDR3 region between the human and rhesus Vγ24 chain is almost identical (98% homology) [39], it is possible that the amino-acid sequence of the pig-tailed macaque Vα24 chain varies enough from the human Vα24 chain to lack the 6B11 epitope. This issue requires clarification and will be resolved in a future study.

We next determined the CD4 and CD8 phenotypes of pig-tailed macaque *i*NKT cells (Fig. 4A) and found that *i*NKT cells primarily consisted of the CD4+ and CD8+ subpopulations. The few remaining cells were double negative (DN)(Fig. 4B). All CD8+ *i*NKT cells were also found to be CD8αβ+ (Fig. 4C). These results confirmed an earlier study showing that pig-tailed macaque *i*NKT cells consist of a significant percentage of a CD4+ subpopulation [30]. Furthermore, the CD4/CD8 distribution is somewhat different from *i*NKT cells derived from other monkey species, which are largely made up of CD8+ cells [27–29]. More

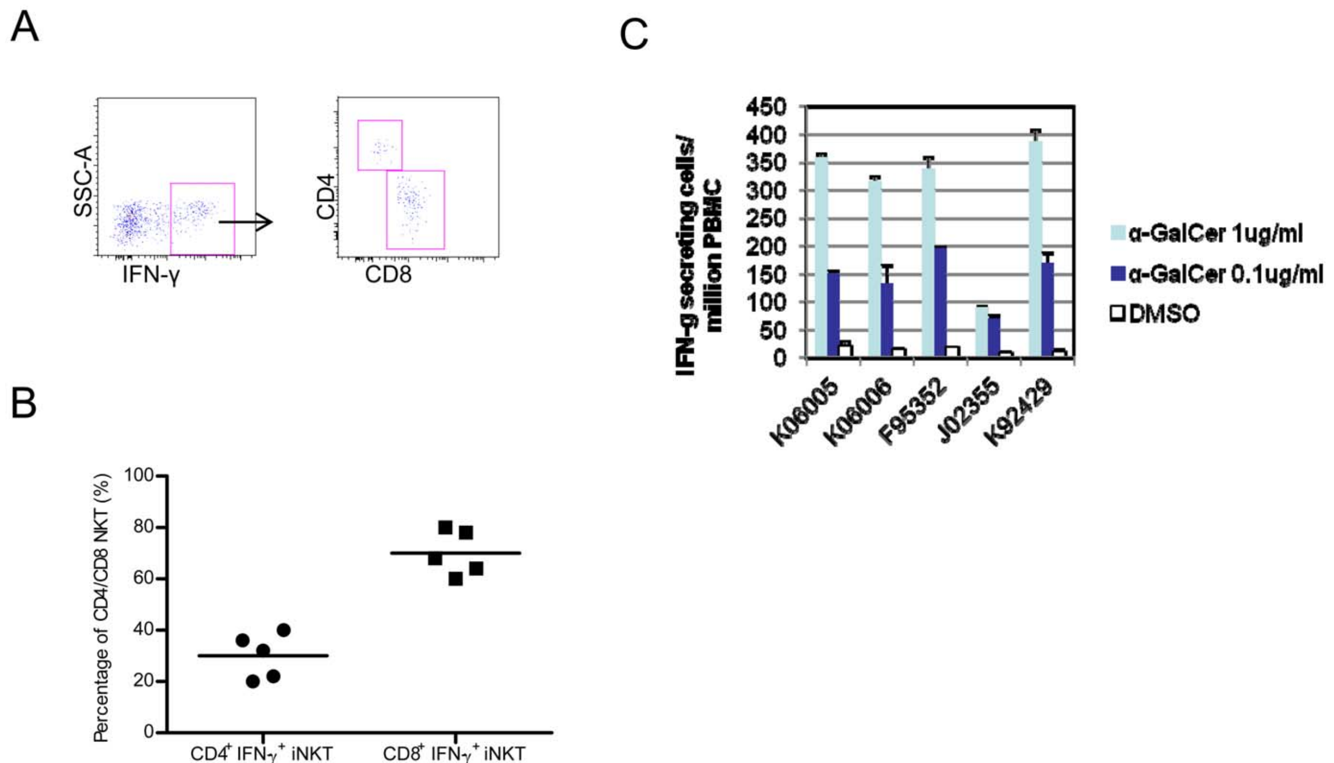

Figure 6. IFN-γ secreting pig-tailed macaque *i*NKT cells, as determined by intracellular cytokine staining and ELISpot assay. (A) Flow cytometric figure shows the pattern of CD4+ versus CD8+ expression by α-GalCer-activated, IFN-γ-secreting *i*NKT cells gated from the PBMCs from one representative pig-tailed macaque. v(B) The graph shows the percentages of CD4+ or CD8+ *i*NKT cells among total IFN-γ secreting *i*NKT cells derived from the PBMCs of five pig-tailed macaques. v(C) The relative number of α-GalCer-activated, IFN-γ-secreting *i*NKT cells among PBMCs. In this assay, 5×10^5 pig-tailed macaque PBMC cells were stimulated with 0.1 μg/ml or 1 μg/ml of α-GalCer, and the relative numbers of IFN-γ-secreting cells were determined by an ELISpot assay. The results are expressed as the mean ± SD of triplicated wells.
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importantly, this distribution pattern resembles human *i*NKT cells, except DN *i*NKT cells are more abundant in humans [4,5].

Regarding the functionality of *i*NKT cells, human *i*NKT cells activated by α-GalCer are known to secrete a myriad of cytokines, with $CD8^+$ *i*NKT cells biased toward a Th1 phenotype, $CD4^+$ *i*NKT cells predominantly secreting Th2 cytokines, and DN *i*NKT cells exhibiting an intermediate Th1/Th2 phenotype [40]. Non-human primate *i*NKT cells have been shown to display a similar function to human *i*NKT cells, but there are some differences among different species. For example, rhesus macaque *i*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 *i*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 $CD8^+$ 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 *i*NKT cells in this study, we first measured the percentage of α-GalCer-activated *i*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 *i*NKT cells secreted 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 $CD8^+$ *i*NKT cell subpopulations among the total IFN-γ-secreting *i*NKT cells. Although both $CD4^+$ and $CD8^+$ *i*NKT cells produced IFN-γ after stimulation with α-GalCer, the percentage of IFN-γ-secreting $CD8^+$ *i*NKT cells was much higher than IFN-γ-secreting $CD4^+$ *i*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 *i*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 *i*NKT cells among PBMCs, as determined by FACS analysis ($R^2 = 0.7847$, $p = 0.0455$) (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 $CD8^+$ *i*NKT cells among PBMCs ($R^2 = 0.9576$, $p = 0.0135$) (Fig. 7A). Interestingly, when we compared the relative number of IFN-γ-secreting cells among PBMCs and the percentages of IFN-γ-secreting $CD8^+$ and $CD4^+$ *i*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 $CD8^+$ *i*NKT cells ($R^2 = 0.8965$, $p = 0.0146$), but not with IFN-γ^+ $CD4^+$ *i*NKT cells ($R^2 = 0.2559$, $p = 0.3866$) (Fig. 7B). Thus, our current functional study demonstrates that the majority of pig-tailed macaque *i*NKT cells that secrete IFN-γ consist of $CD8^+$ *i*NKT cells, although $CD4^+$ *i*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

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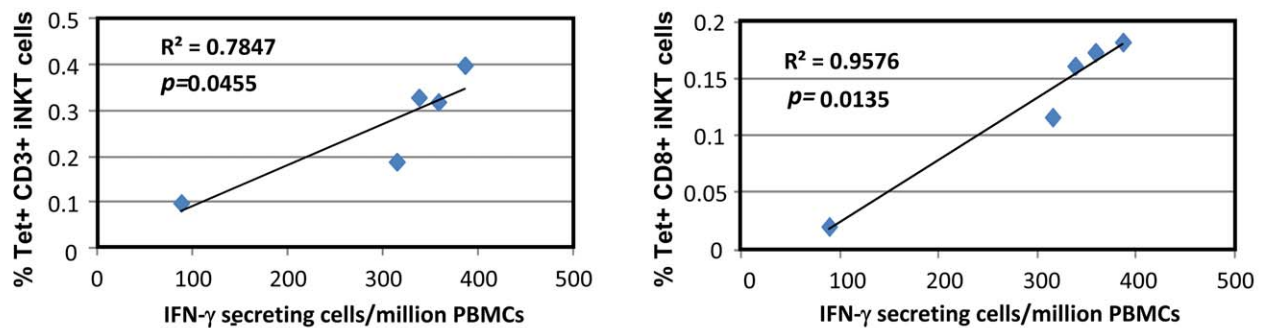

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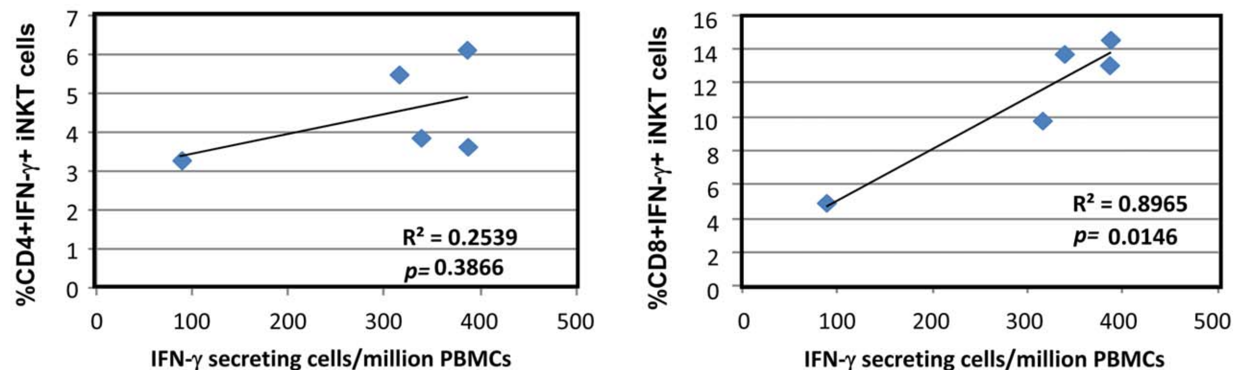

Figure 7. Correlation analysis of the surface phenotypes of pig-tailed macaque *i*NKT cells that secrete IFN-γ. (A) The relative numbers of IFN-γ secreting cells among PBMCs (ELISpot assay) and the percentage of α-GalCer-CD1d-Tet$^+$CD3$^+$ *i*NKT cells or CD8+ *i*NKT cells among PBMCs from each pig-tailed macaque (FACS analysis) are scatter-plotted to evaluate the correlation between these variables, and linear regression analysis was applied. (B) The relative numbers of IFN-γ secreting cells among PBMCs (ELISpot assay) and the percentage of CD4+ IFN-γ-secreting *i*NKT cells or CD8+ IFN-γ-secreting *i*NKT cells among PBMCs from each pig-tailed macaque (ICCS assay) are scatter-plotted to evaluate the correlation between these variables, and linear regression analysis was applied.
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only perform a limited study of the phenotype and function of pig-tailed macaque *i*NKT cells. Despite this difficulty, however, our current study demonstrates that the percentage of *i*NKT cells present in the peripheral blood of pig-tailed macaques is comparable to the *i*NKT cells found in human peripheral blood. Furthermore, similar to humans, a large proportion of Vα24$^+$CD3$^+$ cells are α-GalCer-CD1d-Tet$^+$CD3$^+$ *i*NKT cells, and almost half of these express CD4 molecules.

Together, these results highlight the properties of pig-tailed macaque *i*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 *i*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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