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Alteration of the relative levels of iNKT cell subsets is associated with chronic mycobacterial infections

Jin S. Im¹, Tae-Jin Kang^{4,5}, Seong-Beom Lee⁴, Chi-Hong Kim⁴, Sang-Haak Lee⁴, Manjunatha M Venkataswamy¹, Evan Serfass¹, Bing Chen^{1,3}, Petr A. Illarionov⁶, Gurdial S. Besra⁶, William R. Jacobs Jr^{1,3}, Gue-Tae Chae⁴, and Steven A. Porcelli^{1,27}

¹Department of Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, NY10461

²Department of Medicine, Albert Einstein College of Medicine, Bronx, NY10461

³Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, NY10461

⁴Institute of Hansen's Disease, Catholic University, Seoul, 137-701, South Korea.

⁶School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Abstract

CD1d-restricted invariant natural killer T cells (iNKT cells) have been identified as an important type of effector and regulatory T cell, but their roles in the chronic infectious diseases caused by *M. tuberculosis* and *M. leprae* remain poorly defined. Here, we studied circulating human iNKT cells in blood samples from tuberculosis (TB) and leprosy patients. We found that the percentages of iNKT cells among total circulating T cells in TB and leprosy patients were not significantly different from those in normal controls. However, both TB and leprosy patients showed a selective reduction of the proinflammatory CD4⁺CD8^β[−] (DN) iNKT cells with a proportionate increase in the CD4⁺ iNKT cells. Similar phenotypic alterations in circulating iNKT cells were observed in a mouse model of *M. tuberculosis* infection. Taken together, these findings indicate that the selective reduction of circulating DN iNKT cells is associated with chronic infections caused by *M. tuberculosis* and *M. leprae*.

Keywords

Invariant natural killer T cells; CD1d; glycolipid; α -galactosylceramide; mycobacteria; tuberculosis; leprosy

Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) and *Mycobacterium leprae* (*M. leprae*) are the causative pathogens of two important chronic infectious diseases – tuberculosis (TB) and leprosy [1]. For the great majority of immunocompetent hosts, the immune response is sufficient to control initial infection with these pathogens and prevent the development of overt clinical disease. However, these microbes remain latent for long periods of time in infected individuals and subsequently reactivate to cause disease if the host immune system weakens

⁷Correspondence should be addressed to S.A.P.

⁵current address: Department of Pharmacy, Samyook University, Seoul 130-650, South Korea

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[2]. In the case of *M. tuberculosis*, only 10–15% of people are estimated to develop active TB within 2 years of initial infection, and the remainder develop latent infection that carries a variable risk of reactivation for the remainder of the life of the infected host [2]. The reactivation of dormant *M. tuberculosis* and *M. leprae* is closely related to diminished host immunity due to aging, malnutrition, immunosuppressive therapy or coexisting active disease processes such as HIV infection [3–6]. The successful elimination of persistent microbes requires a complex interplay between innate and acquired immune responses, including interactions between infected macrophages and effector T cells within the network of cytokines, chemokines, and corresponding receptors on the cells. Orchestrating all the elements of innate and acquired immunity at the right time and location is critical to maximize the control of microbial infections, as well as to minimize immune-mediated tissue damage caused by overly active immune responses. Thus, regulatory leukocytes such as CD4⁺CD25⁺ T cells, plasmacytoid dendritic cells, and invariant natural killer T cells (iNKT cells) may play crucial roles in coordinating the specific functions of various immune effector involved in controlling or eradicating *M. tuberculosis* and *M. leprae* [7,8].

CD1d-restricted iNKT cells are a conserved subset of T cells that express an invariant T cell receptor (TCR) α chain (V α 24-J α 18 in humans, and V α 14-J α 18 in mice) paired with TCR β chains encoded by one or a few V β gene segments (V β 11 in humans, and predominantly V β 2, 7 and 8 in mice). These cells are phenotypically and functionally diverse [9]. Many iNKT cells are CD4⁺, and these have been associated with the production of predominantly T_H2-associated cytokines such as IL-4, IL-5, IL-13. This subset is believed to play a prominent role in suppression of autoimmune or chronic inflammatory diseases, and in promoting allergic conditions such as asthma. Relatively few iNKT cells are CD8⁺, and most of those that are express only the CD8 α subunit, which means that they likely express only CD8 $\alpha\alpha$ homodimers and not the CD8 $\alpha\beta$ heterodimers found on most conventional CD8⁺ T cells (Fig. 1D). In addition, a substantial fraction of iNKT cells are negative for both CD4 and CD8 (i.e., so-called double negative or DN T cells). These have been found to produce predominantly IFN γ and other TH1-associated proinflammatory cytokines [10,11]. All iNKT cells are known to recognize synthetic or natural glycolipid antigens presented by the MHC class I-like CD1d protein, and provide a potent link between innate and acquired immunity via their rapid cytokine production and their ability to activate a variety of other cell types such as NK cells and dendritic cells [12]. The immunoregulatory functions of iNKT cells and their ability to favor either a predominantly T_H1 or T_H2 response have been shown to have important effects in mouse models of allergic, autoimmune, inflammatory, neoplastic and infectious diseases [9,13–15].

Previous studies in mouse models have suggested that iNKT cells may play a protective role in *M. tuberculosis* infection. Although knockout mice that lack iNKT cells have not been found to have significantly reduced host immunity to *M. tuberculosis* [16–20], this may reflect the critical role that conventional CD4⁺ T cells play in this model. It is noteworthy that iNKT cells have been found in the lungs of both normal and *M. tuberculosis* infected mice, and infection leads to an increase in the cell surface expression of CD1d on macrophages and dendritic cells [21]. Furthermore, the pharmacologic activation of iNKT cells using their synthetic CD1d-presented glycolipid ligand α -galactosylceramide (α GalCer) significantly slowed the growth of *M. tuberculosis* in infected animals and extended their survival [22]. Studies of human iNKT cells have shown that these have the ability to kill *M. tuberculosis* organisms within infected macrophages, possibly through their production of the antimicrobial peptide granulysin [23]. A possible involvement of iNKT cells in leprosy has also been suggested by the recent observation that functional CD1d proteins are expressed on human Schwann cells, which is the major host cell type in which *M. leprae* replicates and persists. In addition, in vitro studies show that activation of human iNKT cells by Schwann cells in the presence of α GalCer stimulates a strong bias towards regulatory T_H2 cytokine production, which could potentially

contribute to the predominantly T_H2 response observed in most cases of chronic lepromatous leprosy [24].

In the current study, we evaluated the levels and phenotypic subsets of iNKT cells in human subjects with chronic tuberculosis or leprosy. To accurately quantitate and characterize human iNKT cells, we first evaluated reagents for detecting iNKT cells, established a reliable protocol to accurately assess iNKT cells from the clinical samples, and then used these methods to characterize iNKT cells of the peripheral blood samples obtained from tuberculosis and leprosy patients and from normal controls. We found significant reductions in the DN subset of circulating iNKT cells in patients with TB or leprosy, which were associated with reciprocal increases in the size of the $CD4^+$ iNKT cell subset. This alteration in the proportions of the major phenotypic iNKT cell subsets was also demonstrated in a mouse model of *M. tuberculosis* infection. Our results indicated that circulating iNKT cells in patients with these mycobacterial diseases undergo qualitative and quantitative changes as a result of infection that persist for months or years.

Material and Methods

Animals and reagents

Six week old female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in pathogen free conditions in the animal facilities of the Albert Einstein College of Medicine. The following antibodies were purchased from BD Biosciences (San Jose, CA): anti-human CD4 (RPA-T4, FITC and APC conjugated), anti-human CD3 (SK7, PerCP conjugated), anti-human iNKT cell TCR α (6B11, PE conjugated), anti-human CD8 α (RPA-T8, APC conjugated), anti-mouse CD β (53-5.8, FITC conjugated), anti-mouse CD3 ϵ (145-2C11, APC conjugated), anti-mouse CD4 (L3T4, PerCP conjugated). Anti-human CD8 β (2ST8.5H7, PE conjugated) was purchased from Beckman-Coulter (Miami, FL). Streptavidin conjugated with APC or PE was purchased from Prozyme (San Leandro, CA), and streptavidin conjugated with PE-Cy7 was purchased from BD Biosciences. Red Blood Cell Lysing Buffer Hybri-MaxTM (R7757) was purchased from Sigma-Adrich.

Subjects

A total of 80 study subjects (31 healthy controls, 22 tuberculosis patients and 27 leprosy patients) were recruited through the inpatient services or the outpatient clinics of the Hansen's Disease Center of the Catholic University Hospital, Seoul, South Korea over the period of 2 months. The tuberculosis patients comprised 8 females and 14 males, and their ages ranged from 17 to 69 years (mean $\pm$ SD = 39.52 ± 14.92). All patients were diagnosed with pulmonary tuberculosis and had been under medication for an average of 3.6 ± 3.4 months at the time of study. Leprosy patients (11 males and 16 females) ranged in age from 49 to 77 years (mean 62.92 ± 8.48). All leprosy patients had either completed or were currently receiving antimicrobial treatment. Mean duration of therapy was 6.0 ± 6.7 years (range, 4 months to 18 years). Healthy control subjects with no history of previous tuberculosis or leprosy consisted of 22 males and 8 females, with age ranging from 21 to 52 years (mean 24.00 ± 6.83 years).

Manipulation of clinical samples

Ten ml of peripheral blood from each study subject were collected by venipuncture, and mononuclear cells were isolated using ficoll-hypaque gradient centrifugation within several hours of blood collection. Purified peripheral blood mononuclear cells were frozen in duplicate aliquots in complete media containing 10% DMSO using NalgeneTM 1C cryofreezing containers and stored at -80°C until analyzed. The 80 samples were analyzed in 10 batches, with each batch containing approximately 4 to 5 patient samples and 3 to 5 normal control samples. Some of the samples from normal control subjects were analyzed twice, and in these

cases the average of the duplicate values was used to represent a data point for the corresponding individual. Peripheral blood from an additional healthy subject was processed and frozen into multiple vials, and one such sample was added to each group as a quality control for sample processing and to assess reproducibility of the FACS procedure over multiple analyses. A stepwise thawing process was adopted to increase the viability of frozen clinical samples [25]. This involved incubations of frozen cryovials at 37°C just long enough to completely thaw the contents to liquid, followed by addition of an equal part of warm complete medium every 5 minutes until a final volume of 8 ml was reached. Samples were then pelleted by centrifugation, and resuspended in fresh 37°C medium at the required cell density for staining for FACS analysis.

CD1d tetramer generation

Human CD1d (hCD1d) and mouse CD1d (mCD1d) proteins were produced, purified, and biotinylated as previously reported [26,27]. α GalCer was loaded onto monomeric CD1d proteins and tetramer assembly with SA-PE or APC was done according to the published protocol [28].

FACS analysis of human iNKT cells

All staining for FACS was performed at room temperature (RT) in phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 0.05% NaN_3 , and immediately analyzed using an LSR-II flow cytometers (BD Biosciences, San Jose, CA). 4',6-Diamidino-2-phenylindole (DAPI) was added to exclude dead cells from the analysis. Two sets of reagents were used to characterize iNKT cells. One set consisted of APC-conjugated α GalCer-loaded human CD1d tetramers (α GalCer/hCD1d tetramers) used in combination with anti-CD4-FITC, anti-CD8 β -PE, anti-CD3-PerCP and PE-Cy7 conjugated empty hCD1d tetramer. The other set consisted of anti-iNKT cell TCR mAb 6B11-PE with anti-CD4-APC, anti-CD3-PerCP and anti-CD8 α -FITC. Staining with tetramer was done at 1 $\mu\text{g/ml}$ for 1 hr at RT, and mAbs were added at 1:20 dilutions during the final 15 minutes of incubation at RT.

Mouse infection study

Eight week old C57BL/6 mice were infected intravenously with 10^6 *M. tuberculosis* organisms. For these experiments, we used the genetically modified *M. tuberculosis* strain mc²6030 which is attenuated for virulence because of deletion of the virulence-associated RD1 region and of genes for pantothenate synthesis (Δ panCD). This strain was used because it establishes a self-limited infection in mice, and is approved for use under Biosafety level 2 laboratory conditions [29]. Mice were sacrificed three weeks after infection, at which time one ml of peripheral blood was harvested by cardiac puncture. Blood samples were treated with RBC lysing buffer, and leukocytes were stained with PE conjugated α GalCer-loaded mCD1d tetramers (1 $\mu\text{g/ml}$) for 1 hr at RT and with anti-CD8 β -FITC, anti-CD3 ϵ -APC, and anti-CD4-PerCP (each at 1:100 final dilutions) for the last 15 min of staining. MAb 2.4G2 (2 $\mu\text{g/ml}$) was used to block Fc receptors during the staining. The fluorescence profiles of stained mouse leukocytes were analyzed using the LSR-II analyzer after adding DAPI for exclusion of dead cells.

Statistical Analysis

All statistical analyses were performed using Prism version 4.0 software (GraphPad, San Diego, CA). Data sets were analyzed using two way ANOVA, and p values for comparisons between groups were determined using the Tukey post-test. Student t-test was used to assess the significance of differences in parameters in the murine infection study. Pearson's correlation coefficient (r) was calculated to measure the degree of the correlation between two sets of parameters.

RESULTS

Comparison of α GalCer/hCD1d tetramer and mAb 6B11 staining for measurement of human iNKT cells

Circulating iNKT cells are a relatively rare population, ranging in frequency among total T cells from <0.01% to approximately 1% in previous studies that have used several different methods for their measurement (23–31). Both α GalCer/hCD1d tetramers and mAb 6B11 have been claimed to be highly sensitive and specific as staining reagents for human iNKT cells, although to our knowledge no direct comparison of these reagents has been previously published. We therefore initially carried out studies to compare the levels of iNKT cells identified using these two reagents in human blood samples from a group of normal controls and leprosy patients. We used a gating strategy that selected singlet events using FSC-A versus FSC-H analysis, along with exclusion of DAPI stained cells to reduce non-specific background staining (Fig. 1A). Using this approach, we found that staining with either α GalCer/hCD1d tetramers or mAb 6B11 identified a discrete population of cells within the CD3⁺ lymphocyte fraction (Fig. 1B), and that the percentages of iNKT cells identified with either reagent were highly reproducible (standard deviation of ~1% for the mean of replicates of frozen control lymphocyte samples prepared from a single donor).

Evaluation of iNKT cell levels in the group of normal control samples and leprosy patient samples using tetramer and 6B11 staining revealed a wide distribution in iNKT frequencies, but staining for normals (0.08646% versus 0.08125%; Fig. 2C, left) and for leprosy patients (0.1142% and 0.0990; Fig. 2C, middle). The iNKT cell frequencies for individuals determined by the two methods were closely correlated, as indicated by the highly significant Pearson's correlation value (Fig. 2C, right, $r=0.9920$, $p<0.0001$). Interestingly, only a minor fraction of CD8 α ⁺ iNKT cells expressed CD8 β as shown in Fig 1D, with the exception of one individual who had exceptionally high percentage of CD8 α ⁺ β ⁺ iNKT cells. Overall, these initial results indicated that the two methods were interchangeable for accurately determining the frequency of iNKT cells in peripheral blood samples. However, since the availability of APC conjugated α GalCer/hCD1d tetramers allowed us to use a broader array of available PE-conjugated mAbs for further phenotyping of iNKT cell subsets, we chose to routinely use α GalCer/hCD1d tetramer staining for further characterization of iNKT cells in clinical samples.

DN iNKT cells were selectively reduced in both tuberculosis and leprosy patients

To assess whether infection with *M. tuberculosis* or *M. leprae* was associated with changes in the overall frequency of iNKT cells in the circulation, we quantitated the total percentage of cells staining with anti-CD3 mAb and α GalCer/hCD1d tetramer in the blood samples from normal subjects and tuberculosis or leprosy patients (Fig. 2). A trend toward slightly decreased frequency of iNKT cells which was not statistically significant was observed only in tuberculosis patients (0.0575 ± 0.0974), while both leprosy patients (0.1128 ± 0.1688) and healthy volunteers (0.1160 ± 0.1934) had very similar average frequencies of iNKT cells. This is in contrast to significantly elevated frequencies of total T cells in both tuberculosis and leprosy patients compared to healthy subjects (Fig 3B). A wide distribution in iNKT cell frequencies spanning nearly two orders of magnitude was observed in all groups [30], consistent with findings of previous studies.

An interesting difference between the study groups was observed when the iNKT cell population was broken into subsets using CD4 and CD8 β as phenotypic markers (Fig. 2, Table 1a). This revealed a significant reduction in average percentage of iNKT cells expressing a CD4[−]CD8 β [−] (i.e., double negative or DN) phenotype in both tuberculosis and leprosy patients compared to healthy subjects. The decrease in the proportion of DN iNKT cells in both patient groups was associated with proportionate increases in CD4⁺ iNKT cells, which were also

statistically significant. The expression of CD8 β ⁺ or coexpression of CD4⁺ and CD8 β ⁺ on iNKT cells in all groups was extremely low, with no significant differences between the groups.

We then asked whether the alteration in composition of iNKT subsets were accompanied with similar alteration of corresponding conventional T cell subsets (Fig. 3 and Table 1b). Gating on all CD3⁺ cells, we found that tuberculosis patients displayed a significantly reduced mean percentage of CD8 β ⁺ T cells as well as DN T cells compared to controls, whereas leprosy patients had a significantly decreased average percentage of DN T cells but not CD8 β ⁺ T cells. The reduced mean percentages of DN T cells and/or CD8 β ⁺ cells were associated with significant reciprocal increases in the mean percentages of CD4⁺ T cells in both patient groups compared to healthy controls. Tuberculosis patients had the highest average percentage of CD4⁺ T cells among the study groups. Each value for percentage of conventional T cells expressing CD4 was plotted against the corresponding value for percentage of iNKT cells expressing CD4 from the same subject to see whether these values were significantly correlated within individual subjects (Fig. 4). Statistical analysis demonstrated that no significant correlations between these values were present in all three study groups, and that these were not more pronounced in the patient groups compared to the normal controls. Thus, the increases in CD4 expression among iNKT cells observed in the patients may occur independently of an increase in CD4 expression among conventional T cells.

Alteration of iNKT cell phenotypic subsets during murine *M. tuberculosis* infection

To help assess whether the changes we observed in the phenotypic distributions within the iNKT cell population were directly related to mycobacterial infection in the patient groups, we carried out a similar analysis of circulating iNKT cells in *M. tuberculosis* infected mice. Mice received either saline or an inoculum of 10⁶ organisms of *M. tuberculosis* strain mc²6030 by injection into the tail vein, and phenotypic analyses of total CD3⁺ cells and iNKT cells in peripheral blood samples of infected or uninfected mice were carried out at 3 weeks post infection (Fig 5). Infection was validated by measuring colony forming units (CFU) from homogenized lung, spleen, and liver of infected mice (data not shown). FACS analysis of peripheral blood samples showed that a trend toward decreased percentages of circulating iNKT cells expressed a CD4⁺CD8 β ⁺ DN phenotype in infected mice. This was associated with a statistically significant reciprocal increase in CD4⁺ iNKT cells in the infected animals. CD8 β ⁺ iNKT cells were extremely rare and in most cases undetectable in both infected and uninfected animals (Fig 5A). In contrast to the phenotypic alterations in the iNKT cell subsets, there were no differences in these parameters within the conventional T cells (total CD3⁺ cells) in the circulation of uninfected versus infected mice (Fig. 5B). These results mirrored our observations in human subjects with chronic *M. tuberculosis* or *M. leprosy* infections, providing support for a direct causative link between the phenotypic alterations of circulating iNKT cell subsets and mycobacterial infection in tuberculosis and leprosy patients.

DISCUSSION

Ex vivo studies to investigate role of human iNKT cells in many diseases have been hampered by the relatively low numbers and high variability in frequencies of circulating iNKT cells. For these reasons, only handful studies have been performed to demonstrate changes in the frequencies and/or functions of circulating iNKT cells from patients with various diseases such as diabetes, Crohn's disease, atopic disorders, viral infection, multiple sclerosis, and tuberculosis [30–38]. These studies have used a variety of different methods to analyze human iNKT cells such as the combinations of mAbs such as anti-V α 24 plus anti-V β 11, anti-CD56 plus anti-CD3, or 6B11 plus anti-CD3. In some cases, α GalCer-loaded murine CD1d tetramers have been used to identify human iNKT cells, since these were known to cross react with the TCRs of many but perhaps not all human iNKT cells. Although these studies have led to

accumulation of evidence pointing to a role for iNKT cells as immunoregulators or effectors in a variety of human diseases, there have been contradictory results in some cases that may reflect variations related to the methods used for identifying iNKT cells. This has been particularly true in studies of human type 1 diabetes, in which iNKT cell frequencies have been variably reported to be normal or significantly decrease [30,31,39]. Similarly to the studies of human type 1 diabetes, investigations on the frequency of iNKT cells in human tuberculosis patients has produced contradictory results. One study reported an increase in the frequency of CD56⁺CD3⁺ cells in TB, while two other independent studies showed a reduced frequency of V α 24⁺V β 11⁺ T cells or CD56⁺CD3^{dim} cells [37,40,41]. However, none of these studies characterized iNKT cells using the more definitive approach of staining with human α GalCer/hCD1d tetramers. In addition, many details regarding important aspects of experimental procedures have been missing from the published literature, such as details of clinical sample processing, numbers of events acquired and analyzed, and gating strategies for minimizing levels of nonspecific background staining. This makes it difficult to derive solid conclusions regarding possible changes in iNKT cells in tuberculosis patients.

The current study was undertaken to provide a methodologically detailed and thorough phenotypic characterization of circulating iNKT cells from tuberculosis and leprosy patients using human α GalCer/hCD1d tetramers. Before settling on the use of tetramers as our standard approach to iNKT cell identification and enumeration, we first compared this directly to staining with mAb 6B11, which is another reagent that has been claimed to be specific for CD1d-restricted iNKT cells. Four different reagents are currently available to visualize human iNKT cells for FACS analysis. The combination of anti-V α 24 and V β 11 mAbs has been widely used to detect iNKT cells because the majority of V α 24⁺V β 11⁺ T cells are iNKT cells expressing the invariant V α 24-J α 18 TCR α chain that is the molecular signature of the great majority of iNKT cells [31–34,38,42]. However, this method cannot distinguish true iNKT cells from V α 24⁺V β 11⁺ conventional T cells which are known to exist, and fails to detect a small population of recently described α GalCer/CD1d tet⁺ NKT cells that appear to express neither V α 24 nor V β 11 [43,44]. The mouse mAb 6B11, specific for the CDR3 region of the invariant TCR α chain of human iNKT cells, was developed to overcome the shortcoming of the combination of anti-V α 24 and V β 11 Abs, but is not able to detect α GalCer/CD1d tet⁺ non-V α 24, non-V β 11 NKT cells [31]. Thus, while 6B11 appears to be a highly specific reagent for identifying human iNKT cells, and may miss small populations that can be identified using CD1d tetramers. Tetramers of murine CD1d loaded with α GalCer have been used to characterize human iNKT cells in some studies, sometimes in conjunction with anti-V α 24 mAb. Whether or not murine tetramer is interchangeable with human tetramers for characterizing human iNKT cells remains controversial, and the application of murine α GalCer/mCD1d tetramers to the study of human iNKT cells has resulted in contradictory outcomes [30,31].

Based on the above considerations, we believe that human α GalCer/hCD1d tetramers may represent the best available reagent for sensitive and specific detection of human iNKT cells. In fact, our direct comparison of α GalCer/hCD1d tetramer and mAb 6B11 staining indicated that both of these reagents clearly identify iNKT cell populations of nearly identical size in human peripheral blood samples (Fig. 1). In our analysis, 6B11⁺ iNKT cells showed a trend toward slightly lower frequencies compared to tetramer staining, perhaps reflecting the small population of α GalCer/CD1d reactive NKT cells that do not express the canonical V α 24-J α 18 TCR α chain [44]. Although it is debatable whether these rare cells are functionally equivalent to classical iNKT cells, their inclusion is unlikely to significantly skew the results. This fact, together with the more convenient fluorochrome combinations available to us with the tetramer reagents, led us to focus on the use of α GalCer/hCD1d tetramers in our current study.

Our analysis found that tuberculosis patients, but not leprosy patients, showed a trend toward lower average frequency of tetramer⁺ iNKT cells, although this did not achieve statistical significance with the sample size studied. The results are supported indirectly by two previous studies which demonstrated the reduced frequencies of circulating CD3⁺CD56⁺ cells or Vα24⁺Vβ11⁺ T cells among tuberculosis patients [37,41]. Notably, both tuberculosis and leprosy patients had a significantly reduced average percentage of CD4⁺CD8β⁻ (DN) iNKT cells, with a reciprocal increase in average percentage of CD4⁺ iNKT cells. No change in average percentage of CD8β⁺ iNKT cells was observed in either patient group, indicating that this minor subpopulation of iNKT cells may not actively participate in the disease process. Similar alterations in the iNKT subsets in the peripheral blood of age and gender matched mice infected with attenuated *M. tuberculosis* supported the conclusion that the selective reduction of peripheral DN iNKT cells in tuberculosis or leprosy patients could be directly related to chronic mycobacterial infection.

DN iNKT cells differ from CD4⁺ iNKT cells functionally in several ways. Activated DN iNKT cells produce more pro-inflammatory cytokines such as IFNγ and TNFα than immunoregulatory cytokines such as IL-4 and IL-10 while CD4⁺ iNKT cells produce more regulatory cytokines and proliferate more strongly [10,11]. In addition, human DN iNKT cells, known for the potent cytolytic activity, are able to secrete granzyme which has direct cytotoxic effects on microbes such as *M. tuberculosis* [23]. Lastly and most relevant to our study, DN iNKT cells constitutively express a variety of chemokine receptors at higher levels than CD4⁺ iNKT cells, and they migrate to a greater extent in response to corresponding chemokine gradients [10,45–47]. This feature of DN iNKT cells and their prominent proinflammatory effector properties suggests that circulating peripheral DN iNKT cells in tuberculosis and leprosy patients may have migrated out of the circulation and into infected tissues in response to local chemokine gradients. Consistent with this proposed migration into infected and inflamed tissues, human iNKT cells T cells have been detected in granulomatous skin lesions of tuberculoid leprosy patients [48]. Thus, the decrease in the proportion of DN iNKT cells and reciprocal increase in percentage of CD4⁺ iNKT cells may reflect the migration of the DN subset out of the circulation and into infected tissues over a period of months in tuberculosis patients, or possibly years in the case of leprosy patients.

Our study also demonstrated that most CD8α⁺ iNKT cells are likely to express CD8αα⁺ homodimers rather than the CD8αβ⁺ heterodimers usually found on conventional T cells, and that CD8αβ⁺ iNKT cells are less than 5% of total iNKT cells (Fig 1D). Studies of CD8αα⁺ and CD8αβ⁺ iNKT clones and lines suggest that these two iNKT subsets may be functionally distinct [49,50]. Activated CD8αβ⁺ iNKT cells produce IFNγ but not regulatory T_H2 cytokines like conventional CD8β⁺ T cells, whereas CD8αα⁺ iNKT cells can produce both IFNγ and T_H2 cytokines and show potent cytolytic activity, similar to CD4⁺CD8β⁻ iNKT cells. CD8αα⁺ iNKT cells have often been included into CD4⁺CD8β⁻ iNKT cells because of the striking functional resemblance with CD4⁺CD8β⁻ iNKT cells, but also have been referred as CD8⁺ iNKT cells in other studies [51,52]. Since there exist both CD4⁺CD8β⁻ and CD8αα⁺ iNKT cells in the periphery, CD8⁺ iNKT cells should be classified as CD8αα⁺ iNKT cells or CD8αβ⁺ iNKT cells to avoid further confusion. In current study, we included CD8αα⁺ iNKT cells as CD4⁺CD8β⁻ (DN) iNKT cells and referred CD8αβ⁺ iNKT cells as CD8β⁺ iNKT cells.

Although the average percentages of DN iNKT cells were decreased with a proportionate increase in the average percentage of CD4⁺ iNKT cells in both patient groups, the large variations in percentage of iNKT cell subsets were present among individuals within study groups. Such variations may be inevitable due to the multiple genetic and environmental differences between human subjects. We attempted to confirm our findings in a model experimental system using a mouse model, which minimizes such genetic and environmental factors. This experiment showed that effects of *M. tuberculosis* infection on iNKT cell

phenotypic subsets in such a highly controlled setting were generally very similar to the overall trends that we observed in our studies of human subjects with mycobacterial diseases. This supports the validity of our findings in human subjects, and suggests that the observed effects on iNKT cells in these tuberculosis and leprosy patients were directly related to their mycobacterial infections.

In summary, we have evaluated two reagents, α GalCer/hCD1d tetramers and invariant TCR α chain specific mAb 6B11, for their accuracy and reliability in the identification and characterization of human iNKT cells in peripheral blood samples. Our results confirmed that both reagents performed well and gave comparable results. Using primarily the α GalCer/CD1d tetramer approach, we have found phenotypic alterations in iNKT cells subsets in human subjects with tuberculosis or leprosy that are suggestive of a role for these specialized T cells in the host response to these chronic mycobacterial infections.

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Figure 1. Comparison of methods for assessment of human iNKT cells in clinical samples
 (A) Gating strategy for analysis of viable lymphocytes. Cells from leukocyte gate (left) were again gated for singlets (middle). DAPI was used to exclude dead cells (right). (B) FACS analysis of human iNKT cells. Human iNKT cells from peripheral blood leukocytes gated as in (A) were visualized by their staining with α GalCer/hCD1d tetramer (left) or mAb 6B11 (right). Background staining with hCD1d tetramer that was not loaded with α GalCer (empty tetramer) or with buffer only (No Ab) is shown for comparison. (C) Comparison α GalCer/hCD1d tetramer or 6B11 staining in the analysis of peripheral iNKT frequencies. Human peripheral iNKT cells from healthy volunteers (left) or leprosy patients (middle) were enumerated by both α GalCer/hCD1d tetramer or 6B11 staining, and the frequencies determined

by two methods were subjected to correlation analysis (right). (D) Subset analysis of tetramer⁺ or 6B11⁺ iNK T cells. Anti-CD8 α or CD8 β Abs was used in combination with tetramer or 6B11, respectively. CD8 α ⁺ and CD8 β ⁺ iNK T cells from the individual were plotted. Each symbol represents data obtained from a single subject. Horizontal bar is the median value for each group. An average of 281,686 and 366,154 gated events were analyzed in the normal control and leprosy patient groups, respectively, and the absolute numbers of iNKT cells subjected to further analysis ranged from 215 to 499 on average.

Figure 2. Frequency and phenotypic analyses of peripheral iNKT cells from tuberculosis and leprosy patients

iNKT cells were visualized as α GalCer/hCD1d tetramer⁺CD3⁺ cells and frequencies of iNKT cells were expressed as percentages of total live blood mononuclear cells. The phenotype of iNKT cells (i.e. CD4⁺, CD8 β ⁺, or CD4⁻, CD8 β ⁻ (DN)) were further analyzed from tetramer⁺CD3⁺ cells. Representative FACS analyses of iNKT cells from one leprosy patient and one healthy subject are shown (A). Both groups of patients had selective reductions in DN iNKT cells with a proportionate increase in CD4⁺ iNKT cells, while tuberculosis patients showed a trend toward slightly reduced frequency of circulating iNKT cells (B). Each symbol represents data from one individual. Percentage of CD4⁺CD8 β ⁺ cells among iNKT cells was

less than 3.3 %. Horizontal bar is the median value for each group. P value was calculated by one way Anova. HS: healthy subject.

Figure 4. Correlation analysis between conventional CD4⁺ T cells and CD4⁺ iNKT cells in all study groups

Each symbol represents data from one subject. The percentage of iNKT cells (i.e., α GalCer/hCD1d tetramer⁺) that is CD4⁺ is plotted on the Y-axis, and the percentage of total T cells (i.e., CD3⁺) that is CD4⁺ is plotted on the X-axis.

Figure 5. Phenotypic alteration of murine iNKT cells in the periphery upon *M. tuberculosis* infection Age and gender matched inbred C57BL/6 mice were infected with 10^6 *M. tuberculosis* (attenuated strain mc²6020), and the phenotypes of iNKT cells (i.e., α GalCer/mCD1d tetramer⁺) and conventional T cells (i.e., CD3⁺) were analyzed. Peripheral iNKT cells from infected mice showed increased percentages of CD4⁺ iNKT cells with concomitant decreases in DN iNKT cells (A), while there were no significant changes in conventional CD4⁺, CD8β⁺ or DN CD3⁺ T cells (B).

Table 1a. Subsets in iNKT cells (mean $\pm$ standard deviation)			
	% CD4+ iNKT cells	% CD8β+ iNKT cells	% DN iNKT cells
Healthy subjects	35.25 $\pm$ 18.19	5.365 $\pm$ 6.987	57.45 $\pm$ 21.14
Tuberculosis patients	49.59 $\pm$ 18.14	5.714 $\pm$ 6.486	41.44 $\pm$ 21.51
Leprosy patients	58.08 $\pm$ 20.61	5.061 $\pm$ 5.730	35.31 $\pm$ 23.24
Table 1b. Subsets in conventional T cells (mean $\pm$ standard deviation)			
	% CD4+ T cells	% CD8β+ T cells	% DN T cells
Healthy subjects	56.34 $\pm$ 10.75	30.87 $\pm$ 8.371	11.36 $\pm$ 8.349
Tuberculosis patients	69.36 $\pm$ 13.34	23.89 $\pm$ 10.11	5.842 $\pm$ 4.870
Leprosy patients	65.68 $\pm$ 14.50	27.68 $\pm$ 12.16	7.370 $\pm$ 7.326
Table 1c. Subsets in iNKT T cells (mean $\pm$ standard deviation)			
	% CD4+ T cells	% CD8β+ T cells	% DN T cells
Control mice	47.15 $\pm$ 11.11	2.58 $\pm$ 3.78	51.07 $\pm$ 11.19
Infected mice	65.67 $\pm$ 10.44	0.00 $\pm$ 0.00	35.24 $\pm$ 8.74
Table 1d. Subsets in conventional T cells (mean $\pm$ standard deviation)			
	% CD4+ T cells	% CD8β+ T cells	% DN T cells
Control mice	61.75 $\pm$ 2.73	33.97 $\pm$ 1.83	4.29 $\pm$ 0.92
Infected mice	61.45 $\pm$ 2.48	33.57 $\pm$ 2.17	4.97 $\pm$ 0.79