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Lower numbers of natural killer T cells in HIV-1 and *Mycobacterium leprae* co-infected patients

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Summary

Natural killer T (NKT) cells are a heterogeneous population of lymphocytes that recognize antigens presented by CD1d and have attracted attention because of their potential role linking innate and adaptive immune responses. Peripheral NKT cells display a memory-activated phenotype and can rapidly secrete large amounts of pro-inflammatory cytokines upon antigenic activation. In this study, we evaluated NKT cells in the context of patients co-infected with HIV-1 and *Mycobacterium leprae*. The volunteers were enrolled into four groups: 22 healthy controls, 23 HIV-1-infected patients, 20 patients with leprosy and 17 patients with leprosy and HIV-1-infection. Flow cytometry and ELISPOT assays were performed on peripheral blood mononuclear cells. We demonstrated that patients co-infected with HIV-1 and *M. leprae* have significantly lower NKT cell frequencies [median 0.022%, interquartile range (IQR): 0.007–0.051] in the peripheral blood when compared with healthy subjects (median 0.077%, IQR: 0.032–0.405, $P < 0.01$) or HIV-1 mono-infected patients (median 0.072%, IQR: 0.030–0.160, $P < 0.05$). Also, more NKT cells from co-infected patients secreted interferon-γ after stimulation with DimerX, when compared with leprosy mono-infected patients ($P = 0.05$). These results suggest that NKT cells are decreased in frequency in HIV-1 and *M. leprae* co-infected patients compared with HIV-1 mono-infected patients alone, but are at a more activated state. Innate immunity in human subjects is strongly influenced by their spectrum of chronic infections, and in HIV-1-infected subjects, a concurrent mycobacterial infection probably hyper-activates and lowers circulating NKT cell numbers.

Keywords: co-infection; HIV-1; interferon-γ; leprosy; *Mycobacterium leprae*; natural killer T cell

Introduction

Natural killer T (NKT) cells are a specialized T-cell lineage with unique functional characteristics that distinguish them from conventional T lymphocytes.[1] Their role in immune responses that require opposite regulatory pathways has been attributed to an apparent flexibility of NKT cells with regard to their predominant cytokine profile.[2] Peripheral NKT cells display a memory-activated phenotype and can rapidly secrete large amounts of cytokines including interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4) and IL-13 upon antigenic stimulation.[3]

The NKT cells are a heterogeneous population of lymphocytes[4] that has attracted a great deal of attention because of their potential to link the innate and adaptive arms of the immune system. Characteristically, they respond very rapidly to certain stimuli, rendering them able to activate a number of immune effectors.[5] Presentation of α-galactosylceramide (α-GalCer) by CD1d-expressing antigen-presenting cells, such as dendritic cells and B cells, results in rapid activation of NKT cells. It is clear that the capacity to participate in early immune responses and to modulate both innate and adaptive immunity confers upon NKT cells the potential to mediate important activities in the control of pathogens and subsequent clearance of infections.[6]

Gansert *et al.*[7] provided evidence that α-GalCer could activate antimicrobial pathways in a CD1d-restricted

manner in humans. The protection conferred by NKT cells could be a result of the fact that the cytokines they produce are not only critical in activating early innate immune responses, but also favour the development of classical pathogen-specific T-cell responses that are ultimately responsible for clearing the infection.[8]

Leprosy is a debilitating chronic, infectious disease caused by *Mycobacterium leprae* that involves mostly the skin and peripheral nerves.[9] The majority of infected individuals do not develop clinical leprosy, but a few subjects manifest the disease depending on their immunological status.[10] A concern has been that with the increasing prevalence of HIV-1 infection in many countries where leprosy is endemic[11] HIV-1 co-infection might shift the clinical spectrum of leprosy from paucibacillary to multibacillary forms, enhancing the transmission of *M. leprae*.[12] Frommel *et al.*[13] revisited the overall rise in HIV-1 seropositivity and the increase of such co-infections. An HIV-1-positive subject infected with *M. leprae* might be expected to manifest the lepromatous form of the disease or, alternatively, to develop rapid progression from tuberculoid to lepromatous forms, as HIV-1 infection impairs the cellular immune response.[14] In this study, the frequency and *ex vivo* functions of NKT cells in healthy controls, HIV-1-positive patients and HIV-1 and *M. leprae* co-infected patients were measured, and it was shown that co-infected subjects have reduced NKT cells in the peripheral blood when compared with healthy subjects and leprosy mono-infected patients, but they secrete more IFN-γ when compared with leprosy mono-infected patients.

Materials and methods

Subjects and sample collection

Volunteers were recruited at the Federal University of Sao Paulo and the Federal University of Pará, Brazil. Written informed consent was obtained from all volunteers according to the guidelines of the Brazilian Ministry of Health, and approved by the Institutional Review Board. Leprosy patients were treated according to World Health Organization guidelines[15] and the co-infected patients were treated with the appropriate multidrug therapy for paucibacillary and multibacillary leprosy, when indicated. The initial treatment for patients with HIV-1 infection or HIV-1 and leprosy co-infection was defined using modified criteria adopted by the Brazilian Ministry of Health, which includes patients with a $CD4^+$ T-cell count < 350 cells/μl or clinical conditions related to AIDS.[16] Leprosy patients were matched for paucibacillary and multibacillary forms to the cases in the co-infected group according to World Health Organization criteria. The HIV-1 mono-infected and co-infected patients received highly active antiretroviral therapy and multidrug therapy. Patients with immune reconstitution inflammatory syndrome were not included in the study.[17]

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from volunteers and were stored in liquid nitrogen until used in the assays. The following monoclonal antibodies were used in the FACS assays: anti-HLA-DR-peridinin chlorophyll protein (PerCP) (clone L243), from BD Biosciences (San Jose, CA); CD4-phycoerythrin–cyanine-7 (PE-Cy7) (clone SK3), CD3-allophycocyanin–cyanine-7 (APC-Cy7) (clone SK7) and CD161 allophycocyanin (APC) (clone DX12), from BD PharMingen (San Jose, CA); and Vα24-PE (clone C15), Vβ11- FITC (clone C21) from Immunotech (Marseille, France). All the antibodies were used for cell-surface staining. Fluorescence minus one was used for gating strategy.

After thawing, cells were centrifuged at 300 ***g*** for 5 min and transferred into 96-well V-bottomed plates (Nunc, Roskilde, Denmark) in 100 μl staining buffer [PBS supplemented with 0.1% sodium azide (Sigma, St Louis, MO) and 1% fetal bovine serum, pH 7.4–7.6] with the surface monoclonal antibodies panel. Cells were incubated at 4° in darkness for 30 min, washed twice, and re-suspended in 100 μl fixation buffer [1% paraformaldehyde (Polysciences, Warrington, PA) in PBS, (pH 7.4–7.6)].

Samples were acquired on a FACSCanto, using FACSDiva software (BD Biosciences), and then analysed with FlowJo software version 9.2 (Tree Star, San Carlo, CA). Fluorescence voltages were determined using matched unstained cells. Compensation was carried out with CompBeads (BD Biosciences) single-stained with CD3-PerCP, CD4-FITC, CD8-APC-Cy7, CD4-PE-Cy7, CD3-PE and CD3-APC. Samples were acquired until at least 800 000 events in a lymphocyte gate.

Antigen-loaded DimerX with α-GalCer

For DX-α-GalCer stimulation, 20 μg human CD1d-immunoglobulin recombinant fusion proteins (DimerX; BD Biosciences) was mixed with 5 μg α-GalCer (AXXORA, San Diego, CA) in a final volume of 100 μl and incubated overnight at 37°. An additional 320 μl PBS was added the next day. The antigen-loaded DimerX complexes were added to culture wells at a final concentration of 15 μl/ml. PBS was used as a loading (vehicle) control for all α-GalCer stimulation assays. Titration of the DimerX reagent was performed to ensure maximum stimulation of all NKT cells in PBMC cultures.

Measurement of cytokine-producing cells by ELISPOT

To determine the amount of IFN-γ-secreting and IL-4-secreting cells, MAIP ELISPOT plates (Millipore, Billerica,

MA) were coated with either anti-IFN-γ (10 μg/ml) or anti-IL-4 (15 μg/ml) (Mabtech, Nacka Strand, Sweden), in PBS, 50 μl per well, each overnight at room temperature. After three washes with PBS, PBMC (3×10^5) were added, and incubated with or without DimerX-α-GalCer stimulation (specific for NKT cells) or PMA (50 ng/ml) plus ionomycin (500 ng/ml) as a positive control; for negative control DimerX loaded with PBS was used to establish the background level for each group of patients. The plates were incubated at 37° in 5% CO_2 for 16–20 hr. At the end of the culture period, the plates were washed twice with PBS and twice with PBS plus 0.1% Tween-20 (PBST), and the biotinylated antibodies were added to the appropriate wells: anti-IL-4 (1 μg/ml) (Mabtech) and anti-IFN-γ (1 μg/ml) (Mabtech), in PBS supplemented with 0.1% Tween and 1% BSA (PBSTB), for 30 min at room temperature. The plates were washed again three times with PBSTB, and alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA) was added (50 μl of 1 : 1000 dilution in PBSTB) and plates were incubated for 30 min at room temperature. Plates were washed twice with PBST, incubated with blue substrate (Vector Labs, # SK-5300; Burlingame, CA) until spots were clearly visible, and then rinsed with tap water. When plates were dry, spots were counted using an automated ELISPOT reader and Immunospot S5 Analyser (CTL, LLC, Shaker Heights, OH).

Statistical analyses

Groups were compared using non-parametric models; data were reported with median and 25–75% interquartile range (IQR). Correlations were performed using the Spearman non-parametric test and *P*-values were considered significant if < 0.05. Results are expressed in medians and IQR.

Results

Study volunteers and characteristics

The study subjects were distributed into four groups: 22 healthy controls and 23 HIV-1-seropositive patients, most of whom had $CD4^+$ T-cell counts of < 400 cells/μl, 20 patients with leprosy, classified according to the bacillary load,[17,18] and 17 co-infected patients with both leprosy and HIV-1 infection, all recruited at the Leprosy Outpatient Clinics at both Para and Sao Paulo sites. The median age of all participants was 37 years (IQR 35–48 years) and most were men (81%). No difference in gender distribution was observed between the groups for the leprosy and co-infected groups. Most patients had paucibacillary presentation at the time of diagnosis for both leprosy groups. Our results demonstrated that healthy controls had higher $CD4^+$ T-cell counts (median 917 cells/mm^3, IQR 687–1170) when compared with HIV-1-infected patients (median 391 cells/mm^3, IQR 272–536) and co-infected patients (median 285 cells/mm^3, IQR 235–480), $P < 0.001$. Leprosy patients had higher numbers of $CD4^+$ T cells (median 733 cells mm^3, IQR 699–870) when compared with co-infected patients ($P < 0.001$). For $CD8^+$ T-cell counts, healthy controls (median 556 cells/mm^3, IQR 376–735) had lower numbers when compared with co-infected patients (median 806 cells/mm^3, IQR 578–1548), $P < 0.05$ (Table 1).

Reduced NKT cell frequencies in peripheral blood of *M. leprae*-infected subjects

The NKT cells represent a subset of lymphocytes, defined operationally as bearing both the T-cell receptor and the NK cell marker CD161 (NK1.1 in mice).[19] We defined NKT cells as those with the $CD3^+$ $V\alpha24^+$ $V\beta11^+$ phenotype

Table 1. Demographic and laboratory data

	Controls ($n = 22$)	HIV patients ($n = 23$)	Leprosy patients ($n = 20$)	HIV/Leprosy patients ($n = 17$)
Demographics				
Age (years), median (IQR)	24 (23–36) ($n = 17$)	34 (33–39.75) ($n = 22$)	41 (37–49) ($n = 17$)	34 (30–40) ($n = 17$)
Gender, % women	64.7% ($n = 17$)	4.54% ($n = 22$)	15% ($n = 20$)	23.52% ($n = 17$)
Gender, % men	35.3% ($n = 17$)	95.45% ($n = 22$)	85% ($n = 20$)	76.48% ($n = 17$)
Laboratory findings				
HIV-RNA copies/ml, median	–	<400 ($n = 22$)	–	<400 ($n = 13$)
$CD3^+/CD4^+$ cells/μl, median (IQR)	916 (687–1170) ($n = 17$)	391 (272–536) ($n = 22$)	733 (699–870) ($n = 13$)	285 (235–480) ($n = 16$)
$CD3^+/CD8^+$ T cells/μl, median (IQR)	556 (376–735) ($n = 17$)	904 (458.5–1098.7) ($n = 22$)	526 (468–681) ($n = 13$)	806 (578–1548) ($n = 15$)
Bacillary load, *n* (%)				
Paucibacillary	–	–	9 (45%)	13 (77%)
Multibacillary			11 (55%)	4 (23%)

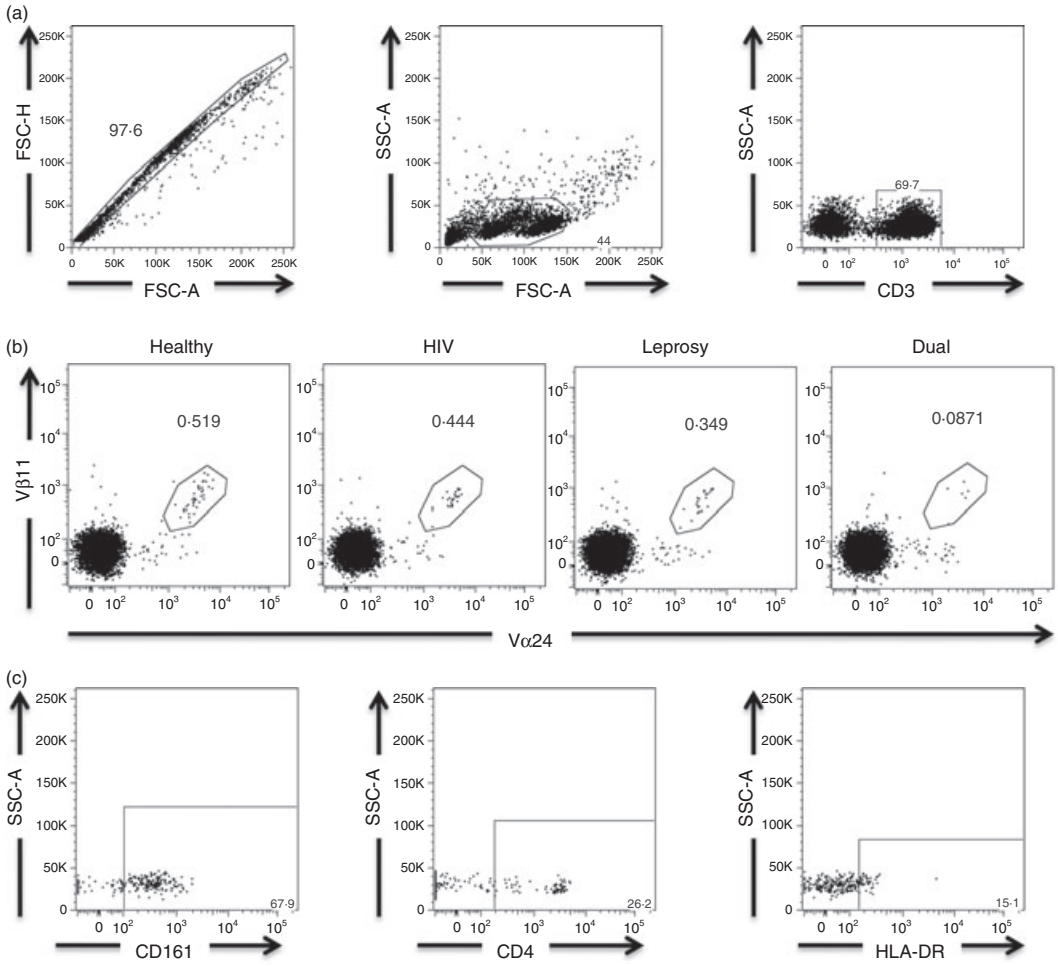

Figure 1. Representative flow cytometry plots. (a) From left to the right: single cell gating, lymphocytes, $CD3^+$ T cells; (b) from left to the right: an example of natural killer T (NKT) cells in each group, double-positive, $V\alpha24^+$ and $V\beta11^+$, defining NKT cells; (c) NKT cells $CD161^+$, $CD4^+$ and $HLA\text{-}DR^+$ subsets.

(Fig. 1a), and further subdivided NKT cell subsets using CD4, CD161 and HLA-DR. The gating strategy enabled delineation of $CD4^+$ NKT subsets (Fig. 1b). Because of the variability of NKT cell frequencies and limitations of available PBMC, data were included in this study if > 30 events were collected within the NKT gate. Berzins *et al.*[20] reported an NKT cell frequency in adult blood ranging from 0.006 to 0.78%. Our results demonstrated that the healthy controls had more NKT cells in the peripheral blood (median 0.077%, IQR 0.032–0.405) than co-infected patients (median 0.022%, IQR 0.007–0.051), $P < 0.01$. Co-infected patients also had fewer NKT cells when compared with HIV-1-infected patients (median 0.072%, IQR 0.030–0.160), $P < 0.05$ (Fig. 2a).

Expression of $CD4^+$ $CD161^+$ $HLA\text{-}DR^-$ NKT cell subset in *M. leprae* infection

The CD4 molecule distinguishes two phenotypic and functionally distinct subsets of NKT cells. $CD4^+$ NKT cells were found to produce both T helper type 1 and type 2 cytokines, whereas $CD4^-$ NKT cells mainly produce T helper type 1 cytokines.[21,22] In peripheral blood from healthy adult volunteers, close to 50% of NKT cells are $CD4^-$ with no, or low, expression of CD8.[23] We observed that leprosy patients have more $CD4^+$ $CD161^+$ $HLA\text{-}DR^-$ NKT cells (median 21.40%, IQR 3.65–59.95) compared with HIV-1-infected patients (median 0.375, IQR 0.00–19.30), $P < 0.05$ (Fig. 2b), but this was not statistically different from healthy controls or co-infected patients.

Activation and maturation NKT cells markers are not significantly affected in *M. leprae*/HIV-1 co-infection

We used CD161 and HLA-DR as activation markers to determine the activation profile of NKT cells. Although 5–10% of human peripheral blood T cells express CD161,[5,24] there were more $CD161^+$ NKT cells in healthy individuals than in leprosy patients.[25] There were no

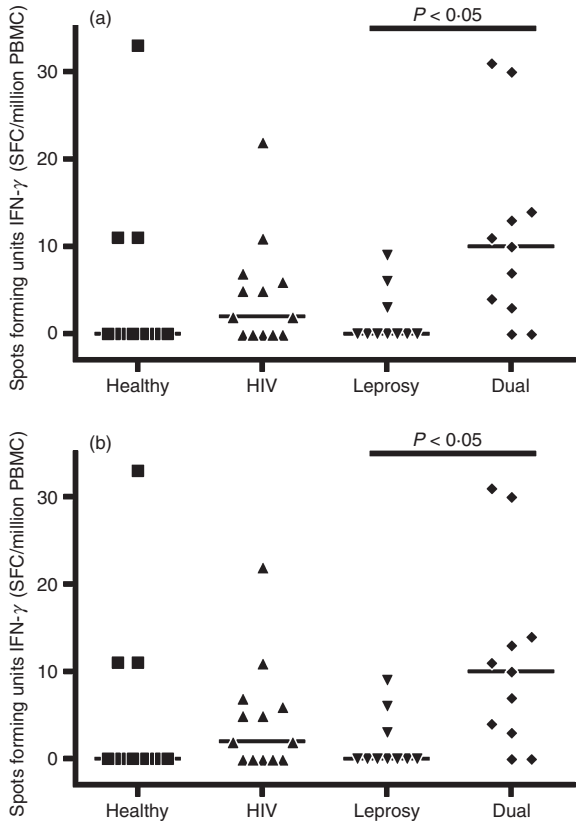

Figure 2. Expression of total and subsets of natural killer T (NKT) cells. (a) Representative of NKT cells (log) showing a decreased expression in dual infection (HIV-1 and leprosy) when compared with HIV-1 patients ($P < 0.05$) and healthy donors ($P < 0.01$). (b) The percentage of dual expression of $CD161^+$ $CD4^+$ HLA-DR^- was increased in leprosy patients when compared with HIV-1-infected patients ($P < 0.05$). Comparisons in all groups were carried out using the analysis of variance non-parametric test.

significant differences in CD161 expression on NKT cells between all four groups. The NKT cells can became activated during a variety of infections and inflammatory responses,[26] but HLA-DR expression was not significantly different between study groups.

PBMCs from co-infected patients produce IFN-γ in response to α-GalCer

The NKT cells are activated in response to the glycolipid antigen α-GalCer and antigen presentation occurs through CD1d.[7] The ELISPOT assay is a sensitive method for detecting and quantifying antigen-reactive cells in a population of lymphocytes with multiple specificities.[27] To determine the frequency of α-GalCer-reactive cells, we analysed PBMCs in a single-colour ELISPOT assay using the DX-α-GalCer stimulation method.[28] Cells secreting IFN-γ and IL-4 were detected from all four groups. Results were expressed in spot-forming units (SFU) per million cells. We demonstrated that, when stimulated with specific antigen α-GalCer, PBMC from co-infected patients showed greater secretion of IFN-γ (median 10 SFU, IQR 3–14) compared with leprosy mono-infected patients (median 0 SFU, IQR 0.0–5.5), $P < 0.05$ (Fig. 3a). No difference in IL-4 secretion by NKT cells was detected between the groups (Fig. 3b). However, IFN-γ frequencies in co-infected patients were positively correlated with the percentage of $CD161^+$ NKT cells ($r = 0.81$, $P = 0.02$) (data not shown).

Discussion

In this study, we demonstrated that patients co-infected with *M. leprae* and HIV-1 had lower frequencies of NKT cells in peripheral blood than healthy subjects and HIV-1-mono-infected patients.

Although many studies have attributed beneficial anti-pathogen responses to NKT cells, they have also been

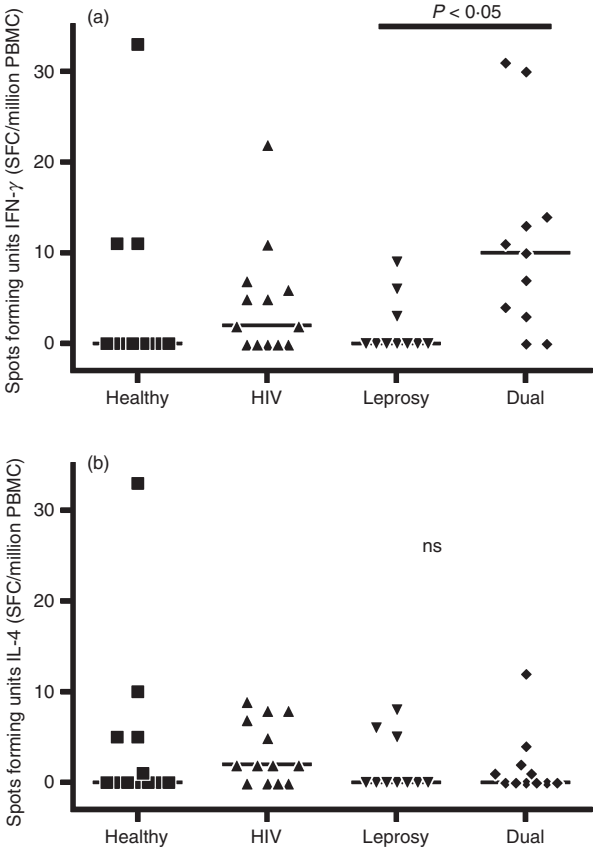

Figure 3. ELISPOT secreting cytokines in total peripheral blood mononuclear cells (PBMC) with α-galactosylceramide (α-GalCer). (a) Significant increase in interferon-γ (IFN-γ) production in dual infection (HIV plus leprosy) when compared with leprosy patients. (b) No significant difference in the production of interleukin-4 (IL-4) between the groups. Comparisons in all groups were carried out using the analysis of variance non-parametric test. SFU, spot-forming units

implicated in detrimental immune responses that lead to immunopathology and disease.[8] In HIV-1-infected individuals, the frequency of NKT cells is markedly reduced in peripheral blood compared with uninfected controls,[2,29,30] and this loss of NKT cells could lead to autoimmunity or to autoimmune-like conditions. Diminished NKT cell-mediated anti-tumour responses could also contribute to increased incidence of infection-related tumours such as Kaposi sarcoma and non-Hodgkin's lymphoma in AIDS patients.[24] In another human retrovirus infection, lower numbers of circulating Vα24$^+$ Vβ11$^+$ NKT cells in individuals infected with human T lymphotropic virus type 1 (HTLV-1) have been demonstrated.[31]

Natural killer T cells also participate in host defence against mycobacterial infection. Some groups have described lower numbers of NKT cells in peripheral blood of patients with mycobacterial infections.[32,33] There are significantly lower percentages of circulating NKT cells in patients with active pulmonary tuberculosis than in subjects uninfected with *Mycobacterium tuberculosis*[33] and these cells become activated upon infection.[32] Activation of NKT cells in *M. tuberculosis*-infected patients could also mediate anti-mycobacterial responses, which may translate into a novel treatment modality for humans.[7] We hypothesized that in the setting of HIV-1 and *M. leprae* co-infection, NKT cells would be reduced in frequency compared with mono-infection alone, and based upon the previous studies of *M. tuberculosis* patients finding activated NKT cells.[33] Our results confirm this hypothesis, indicating that *M. leprae* infection leads to significant changes in the NKT cell population, including the frequency and expression of activation and maturation markers in the peripheral blood. We have previously demonstrated that co-infected patients had higher activation markers on T cells.[34]

CD161 is the homologue of the mouse NK1.1, and is often used to define the maturation state of NKT cell populations, with higher expression reflecting a more mature phenotype.[20] NKT cells in HIV-1-infected patients are compromised and CD161$^+$ CD4$^+$ HLADR NKT cell subsets decline in these patients compared with mono-infected leprosy patients. In this study, we observed that co-infected patients produced greater amounts of IFN-γ when stimulated with α-GalCer. This suggests that NKT cells in co-infected patients may compensate for the lower frequency by increasing the production of IFN-γ. We did not detect the same effect in IL-4 production, but this could be because of differences in the kinetics of cytokine production in the ELISPOT assay. However, these cytokines are not always produced concomitantly at high levels.[35] The importance of NKT cells might depend upon their activation ability early after pathogen infection, with rapid cytokine production (such as IFN-γ) initiating the immune activation cascade.[8] Although CD161 acts as both an activating and an inhibitory receptor, depending on cell type,[36] we observed that in co-infected patients the percentage of NKT cells expressing CD161 correlated positively with the production of IFN-γ. However, one study observed that in HIV-1 infection, impairments of T helper type 1 functions were positively associated with increased frequencies of CD161$^+$ NKT cells.[28] In fact, one important effector mechanism by which NKT cells may contribute to the defence against infection is such production of cytokines.[7]

In summary, our results show that both HIV-1 and *M. leprae* infections can independently have reduced percentages of circulating NKT cells in the peripheral blood, and that co-infection exacerbates the loss, with a further decrease in NKT cell numbers. Interestingly, in dual infection, there appears to be an increase in cytokine produced from NKT cells suggesting a compensatory mechanism whereby a reduced number of cells produce more cytokine. Innate immunity in human subjects is strongly influenced by their spectrum of chronic infections, and in HIV-1-infected subjects, a concurrent mycobacterial infection leads to a further reduction in NKT cell numbers, and skewed innate immunity.

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