

Characteristics of Natural Killer Cells in Malaysian HIV Patients Presenting with Immune Restoration Disease After ART

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Abstract

Objectives: Natural killer (NK) cell function was investigated in Malaysian HIV patients beginning antiretroviral therapy (ART) with advanced immunodeficiency. Some patients experienced immune restoration disease (IRD) presenting as exacerbations of pre-existing infections. Whilst most IRD are attributed to interferon-gamma (IFN γ) produced by T-cells, NK cells may also contribute.

Methods: Blood leukocytes were collected prospectively from 100 HIV patients over 1 year on ART, plus 36 healthy controls. Eleven patients who experienced an IRD and 14 matched controls were assayed. Cells producing IFN γ were quantitated by ELISpot after stimulation with an NK target (K562 cells) or antigens from pathogens associated with the IRD. NK cell subsets, CD16 and perforin expression were determined by flow cytometry.

Results: NK cell IFN γ responses were lower in HIV patients at baseline ($p < 0.001$), improved by Week 24 ($p < 0.01$) but remained lower than uninfected controls ($p < 0.05$). Proportions of CD56^{hi} NK cells increased ($p < 0.01$) above controls at Week 24. Perforin expression on these cells was higher than controls at baseline ($p < 0.01$), but declined on ART. Proportions of CD56^{lo} NK cells were similar in patients and controls throughout. IRD patients showed lower CD16 expression on CD56^{lo} NK cells than non-IRD patients before treatment ($p < 0.05$).

Conclusions: NK cells profiles were restored on ART, but NK cell IFN γ production remained impaired. Low CD16 expression on CD56^{lo} NK cells may mark a predisposition for an IRD.

Keywords: Antiretroviral therapy; HIV; Immune restoration disease; Natural killer cells

Introduction

Chronic human immunodeficiency virus (HIV) infection is characterized by defects in the immune system including depletion of CD4⁺ T-cells and impaired T-cell function. Successful antiretroviral therapy (ART) suppresses viral replication. The subsequent recovery of T-cell responses and the decline of opportunistic infections are well documented.

Natural killer (NK) cells are part of the innate immune system and can secrete T-helper 1 (Th1)-associated pro-inflammatory cytokines like interferon-gamma (IFN γ) and tumor necrosis factor-alpha (TNF α). NK cells are important in controlling tumors and cytomegalovirus (CMV), mycobacterial and fungal infections [1-4]. Effects of untreated HIV disease on NK cells include depletion of the large CD56^{lo} NK subset, expansion of a dysfunctional CD56^{neg}CD16⁺ NK subset, functional abnormalities and perturbation of the NK cell receptor repertoire [5-8].

Studies of the restoration of NK cell functions after ART have yielded inconsistent results [9-12]. We reported that NK cell IFN γ responses and proportions of CD56^{lo} NK cells are lower in Caucasian HIV patients on long-term ART (2-5 years) than in healthy controls [13]. However, no previous studies of NK cells in HIV disease have considered Asian populations. This is important because Asian patients are typically younger and affected by more opportunistic infections than Caucasians [14,15]. Moreover genetic profiles pertinent to NK cells vary with ethnicity [16].

In a subset of patients, a favorable virological response to ART is accompanied by an atypical presentation of diseases associated

with pre-existing opportunistic pathogens [17,18]. These are known as immune restoration disease (IRD) or immune reconstitution inflammatory syndrome (IRIS). IRD associated with Mtb and *Cryptococcus neoformans* parallel robust IFN γ responses evident *in vitro* following stimulation of blood leukocytes with relevant antigens [19-22]. These are attributed to CD4⁺ T-cells but monocytes and NK cells may contribute if stimulated via pattern recognition receptors (PRR).

Less is known about the immuno-pathogenesis of IRD associated with herpes viruses [CMV or Varicella Zoster Virus (VZV)]. Caucasian HIV patients with CMV IRD had higher numbers of activating Killer Immunoglobulin-like Receptor (KIR) genes than non-IRD patients or healthy controls [23]. KIR are expressed by all NK cells and some CD8⁺ T-cells [24,25]. High numbers of activated NK cells were found in the cerebral spinal fluid of a patient with VZV IRD presenting as transverse myelitis [26]. Together, these results suggest NK cells may contribute to viral IRD.

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Here we describe the recovery of NK cells in our cohort of Malaysian HIV patients sampled prospectively during their first year of ART. This is the first study of the recovery of NK cell function in Asian HIV patients beginning ART with advanced immunodeficiency and multiple opportunistic infections. Some patients from the cohort presented with IRD associated with Mtb, cryptococcal, CMV or VZV. These patients are followed individually.

Materials and Methods

Study subjects

Eleven male HIV patients with IRD [Mtb (n=6), Mtb/CMV retinitis (n=1), cryptococcal meningitis (n=1), cryptococcal meningitis/Kaposi's sarcoma (KS; n=1), CMV retinitis (n=1), dermatomal VZV (n=1)] were identified from a cohort of 100 patients enrolled into an ongoing study of immune reconstitution in patients at the Infectious Disease Outpatient Clinics at University Malaya Medical Centre (UMMC; Kuala Lumpur, Malaysia). Fourteen patients with no evidence of IRD were selected for comparison. These were matched with the IRD patients by sex (male) and ethnicity (Chinese and Malay). All patients received 2 nucleoside analog reverse transcriptase inhibitors (3TC with d4T or AZT) and 1 non-nucleoside reverse transcriptase inhibitors (EFV or NVP). Healthy Chinese and Malay donors (n=36) residing in Kuala Lumpur were included as controls.

Non-IRD patients and healthy controls had no known history of cryptococcal, CMV and VZV disease, but several patients had a history of Mtb, and vaccination with the mycobacterial antigen Bacillus Calmette-Guérin (BCG) is standard practice in Malaysia. HIV patients were slightly older than the healthy controls (p=0.02), but the IRD and non-IRD groups were similar in age (p=0.35; Table 1). Institutional ethics approval was obtained for the study and informed consent was given by all participants.

Sample collection, plasma HIV RNA level and CD4⁺ T-cell counts

Whole blood was collected into EDTA tubes from patients at baseline and approximately 6, 12, 24 and 48 weeks after commencement of ART, and once from control donors. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll gradient centrifugation and cryopreserved in liquid nitrogen. Plasma HIV RNA and absolute CD4⁺ T-cell counts were assessed before treatment and on 1 or 2 occasions during the first year of ART, by routine clinical laboratories at UMMC. Plasma HIV-1 RNA was measured using the COBAS Amplicor HIV-1 Monitor Test, v1.5 (Roche Diagnostics, Indianapolis, IN, USA). The cutoff defining an undetectable viral load was <50 copies/ml.

IFN γ ELISpot assay

IFN γ enzyme-linked immunospot (ELISpot) assay was performed

as described [13]. PBMC were stimulated for 20hr with K562 cells (effector:target ratio of 10:1), PPD (10 μ g/ml; Statens Serum Institute, Copenhagen, Denmark), *Cryptococcus neoformans* (50 μ g/ml), CMV and VZV antigens. A mannoprotein preparation of *Cryptococcus neoformans*, (acapsular strain Cap67) was provided by Dr Stuart M. Levitz (University of Massachusetts Medical School, Worcester, MA, USA) [27]. CMV (strain AD169) and VZV (VARIVAXTM vaccine, Oka/Merck strain; Merck, Sharp and Dohme; South Granville, NSW, Australia) were cultured in MRC-5 cells, antigens were prepared as described [28] and optimal concentration were determined by IFN γ ELISpot using PBMC from sero-positive healthy controls. Spots were counted using AID ELISpot Reader v2.9 software (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Flow cytometry

Thawed PBMC were washed in 1% BSA/PBS and 5 x 10⁵ cells were surface stained with CD3-APC, CD16-APC-H7 and CD56-PE (BD Pharmingen, San Jose, CA, USA). To detect intracellular perforin, cells were fixed and permeabilized using Cytofix/Cytoperm KitTM (BD Pharmingen) and incubated with Perforin-FITC (BD Pharmingen). Cells were then fixed with 1% paraformaldehyde before being acquired on a FACSCanto II cytometer (BD Pharmingen). 50,000-100,000 events were recorded per tube and analyzed using the FlowJo program v5.7.2 (Tree Star, Ashland, OR, USA). Gating strategies for NK cell subsets have been described [13].

Statistical analyses

Mann-Whitney tests were used to compare groups of individuals. As Week 48 samples were not available for some patients, Wilcoxon matched-pair tests were used to compare data from Week 0 and the closest sample to Week 24 to evaluate changes amongst IRD or non-IRD patients during ART. Spearman's test was used to evaluate non-parametric correlation coefficients. For all comparisons, p-values below 0.05 were considered to be statistically significant.

Results

Clinical and immunological characteristics of the study population, with verification of a response to ART

The cohort comprised IRD patients, non-IRD patients and healthy controls (Table 1). Nadir CD4⁺ T-cell counts were <110 cells/ μ l in IRD and non-IRD patients (p=0.40). ART increased CD4 T-cell counts [p<0.0001 by week 16(11-37) in IRD and non-IRD patients] and percentages [p<0.005 by week 24(13-32) in IRD and non-IRD patients]. All patients had >100,000 copies HIV RNA/ml plasma pre-ART and 24/25 patients suppressed their plasma HIV RNA (<50-437 copies/ml) over 9-39 weeks of ART. The remaining patient had no follow-up HIV RNA data, but achieved 12-fold increases in CD4⁺ T-cell

	IRD patients ^a	Non-IRD patients	Healthy Controls
n	11	14	36
Age (years)	44 (34-56)	41 (33-64)	36 (23-71) ^b
CD4⁺ T-cells/μl blood [Nadir, Week 0]	22 (0-58)	27 (0-104)	NA
CD4⁺ T-cells/μl blood [Week 16 (11-37)]	142 (78-307)	176 (54-338)	NA
%CD4⁺ T-cells of lymphocytes [Week 0]	2.0 (0.5-9.4)	3.9 (0.2-10.8)	35.2 (20.0-46.4) ^c
%CD4⁺ T-cells of lymphocytes [Week 24 (13-32)]	10.2 (4.1-15.7)	8.6 (2.8-20.5)	35.2 (20.0-46.4) ^c

Values are presented as median (range), NA = not available

^aAll parameters assessed were similar to non-IRD patients

^bLower than all HIV patients (p < 0.05)

^cHigher than all HIV patients (p < 0.05)

Table 1: Demographics of study cohort.

count (22 to 262 cells) after 37 weeks. HIV RNA levels were similar in IRD and non-IRD patients.

All cases of IRD presented as atypical opportunistic infections or inflammatory disease after ART [29]. Four patients experienced 'paradoxical' Mtb IRD defined by treatment for active Mtb disease before ART, followed by lymphadenopathy, lymph nodes evolving into abscesses or cold abscess enlargement on ART. Three patients with no history of Mtb infection pre-ART developed 'unmasking' Mtb IRD, including one who also experienced CMV retinitis as an IRD. Two patients experienced 'unmasking' cryptococcal IRD presenting as cryptococcal meningitis, including one with worsening of Kaposi sarcoma coincident with his cryptococcal IRD. Two patients presented with CMV retinitis as an IRD (one also experienced Mtb IRD) and one patient experienced dermatitis associated with VZV infection after ART.

IRD usually parallel IFN γ responses to pathogen antigens

Most IRD patients showed increased IFN γ production against antigens from the provoking pathogens at the time of IRD and for up to 48 weeks. Levels were above the median value of non-IRD patients and healthy controls (Figure 1A, B, D). This was evident with 5/7 Mtb, 2/2 cryptococcal and 1/1 VZV IRD patients. The patient who experienced both CMV and Mtb IRD showed a moderate increase in IFN γ responses to CMV during his IRD, whilst the other CMV IRD patient had persistently low responses (<10 spots per 200,000 PBMC; Figure 1C)

NK IFN γ responses remained impaired after Week 24 in all patients, irrespective of IRD

NK cell IFN γ responses were deficient in IRD and non-IRD patients

at baseline compared to healthy controls ($p < 0.001$; Figure 1G). These increased significantly by Week 24 ($p < 0.01$) but remained lower than healthy controls ($p < 0.05$) and did not resolve after 48 weeks (Figure 1E, 1F). No differences were observed between IRD and non-IRD patients at Week 0 or Week 24 of ART. NK cell IFN γ responses remained low in most IRD patients at the time of IRD. One Mtb IRD patient showed a peak in IFN γ production during his IRD. Two IRD patients with CMV retinitis (+/- Mtb IRD) and one other Mtb IRD patient showed an increased NK cell IFN γ soon after their IRD was diagnosed (Figure 1E). However, several non-IRD patients also displayed elevated NK IFN γ responses (Figure 1F).

Proportions of CD56^{hi} NK cells increased on ART, with a peak in a few IRD patients

CD3⁺ lymphocytes were distinguished by expression of CD56 creating NK subsets that can be quantitated as proportions of lymphocytes. CD56^{lo} NK cells are naturally more cytotoxic and express higher levels of CD16 than CD56^{hi} NK cells. CD56^{hi} NK cells are less cytotoxic, express low levels of CD16 and are more able to make cytokines (e.g. IFN γ and TNF α) [13,30]. NK IFN γ responses correlated with proportions of CD56^{lo} NK cells in Australian HIV patients after >2 years of ART. Intracellular cytokine staining assay confirmed that CD56^{lo} NK cells produced IFN γ in response to K562 cells [13].

Here, proportions of CD56^{lo} NK cells were similar across all groups of patients and were relatively stable during one year of ART, irrespective of an IRD (Figure 2A, 2B, 2C). NK IFN γ responses correlated with proportions of CD56^{lo} NK cells in Malaysian controls ($r = 0.35$, $p = 0.05$), but not in Malaysian HIV patients at Week 0 or Week 24 ($r = 0.13$, $p = 0.56$ and $r = 0.19$, $p = 0.38$ respectively).

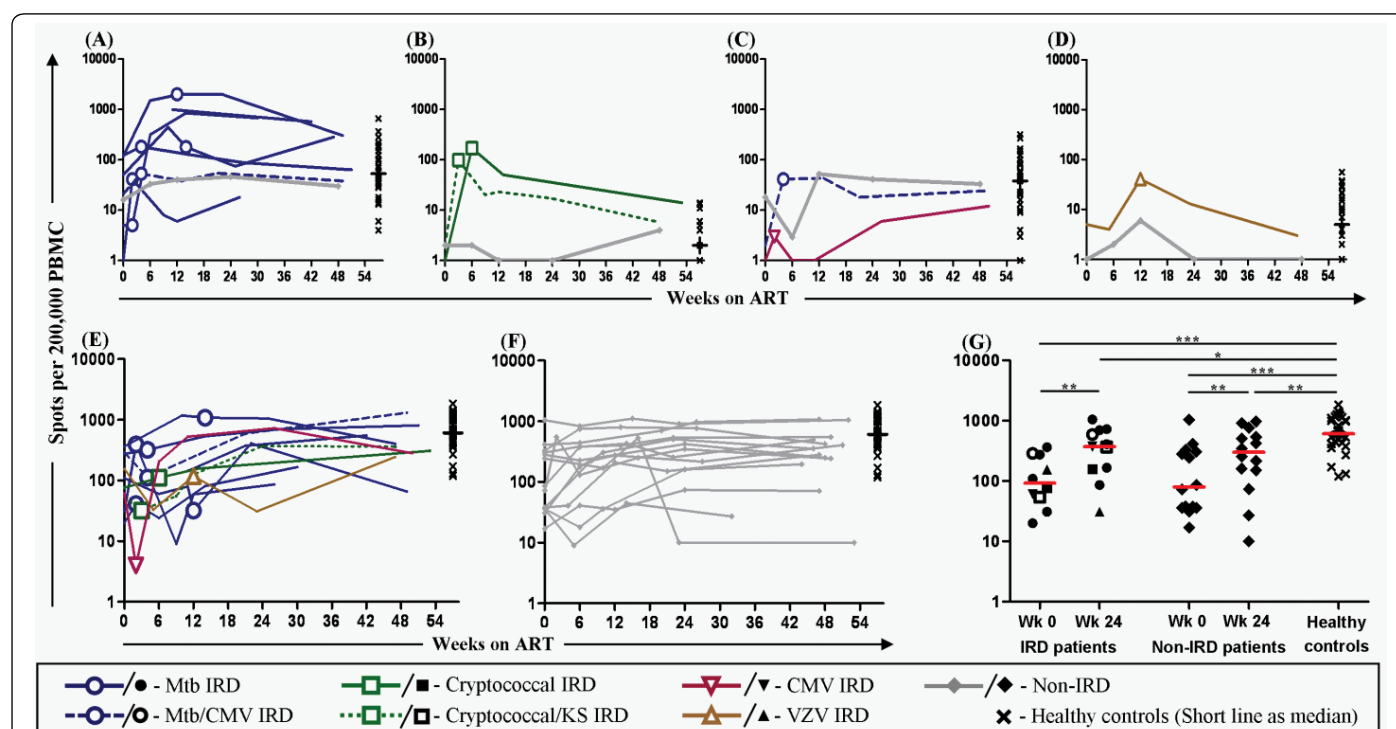


Figure 1: ELISpot analysis of IFN γ -producing PBMC after stimulation with the following antigens: (A) PPD, (B) *Cryptococcus neoformans* mannoprotein, (C) CMV lysate and (D) VZV lysate. Median values for non-IRD patients at week 0, 6, 12, 24 and 48 are represented by filled symbols connected by a grey line in each graph. IFN γ production against a pan-NK cell target (K562 cells) in PBMC from HIV patients who experienced an IRD (E) and non-IRD patients (F). Cross-sectional comparison of IFN γ production against K562 cells between IRD patients, non-IRD patients and healthy controls (G). Horizontal lines represent median values. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

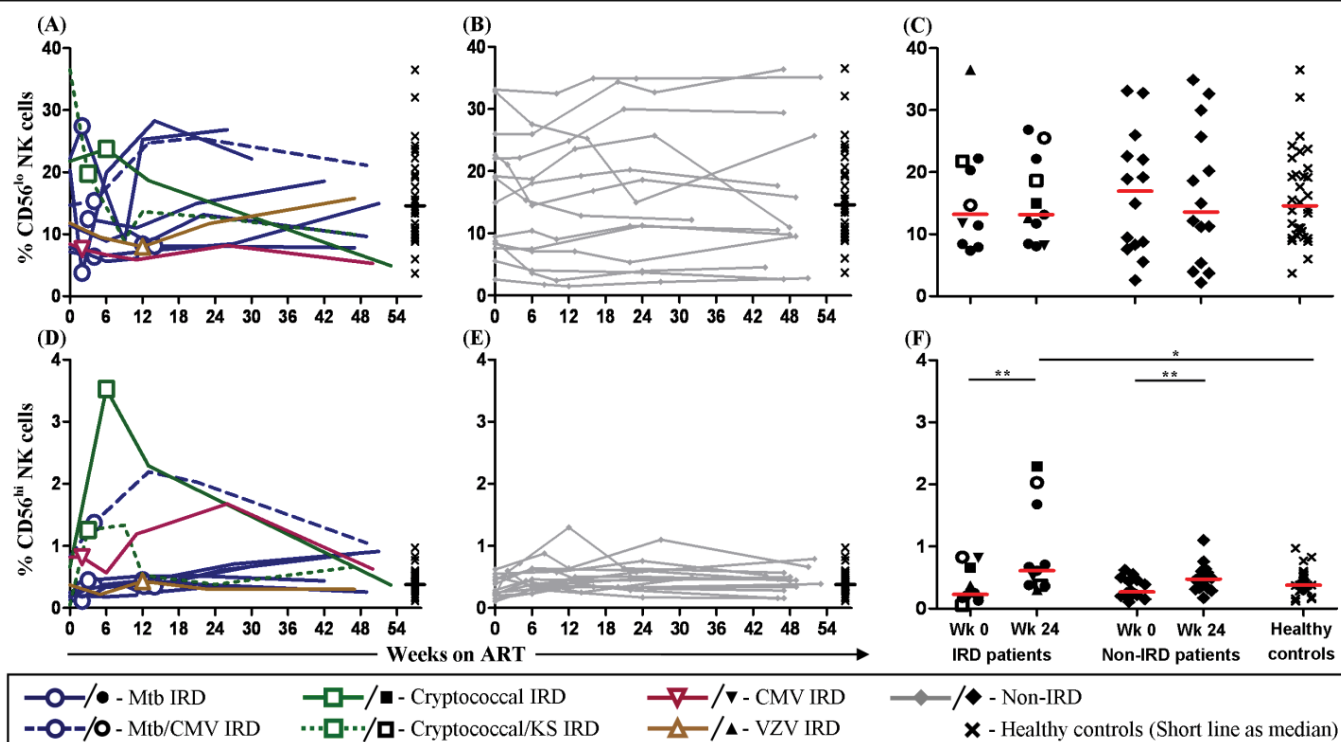


Figure 2: Longitudinal evaluation of proportions of CD56^{lo} NK cells (A,B,C) and CD56^{hi} NK cells (D,E,F) in the lymphocyte population of HIV patients who experienced IRD (A,D) and in non-IRD patients (B,E). Cross-sectional comparison of NK cell subsets between IRD patients, non-IRD patients and healthy controls (C,F). Horizontal lines represent median values. * = p<0.05, ** = p<0.01, *** = p<0.001.

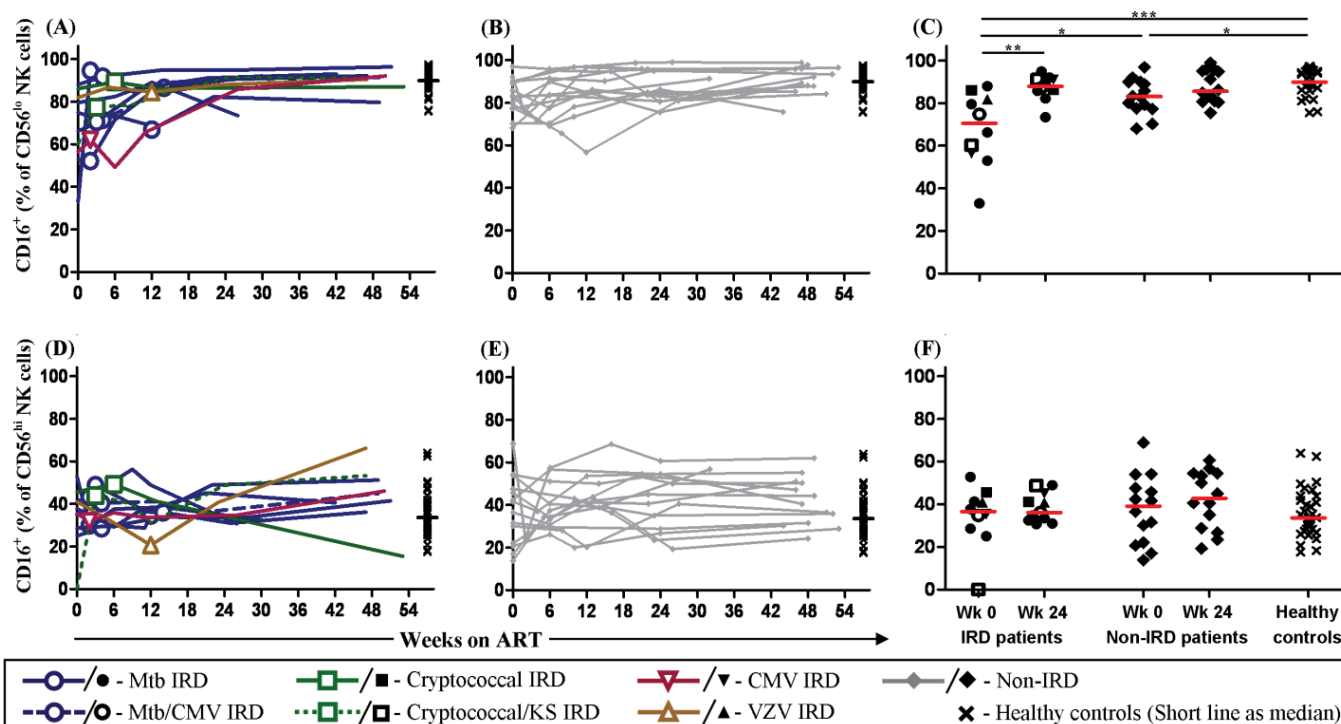
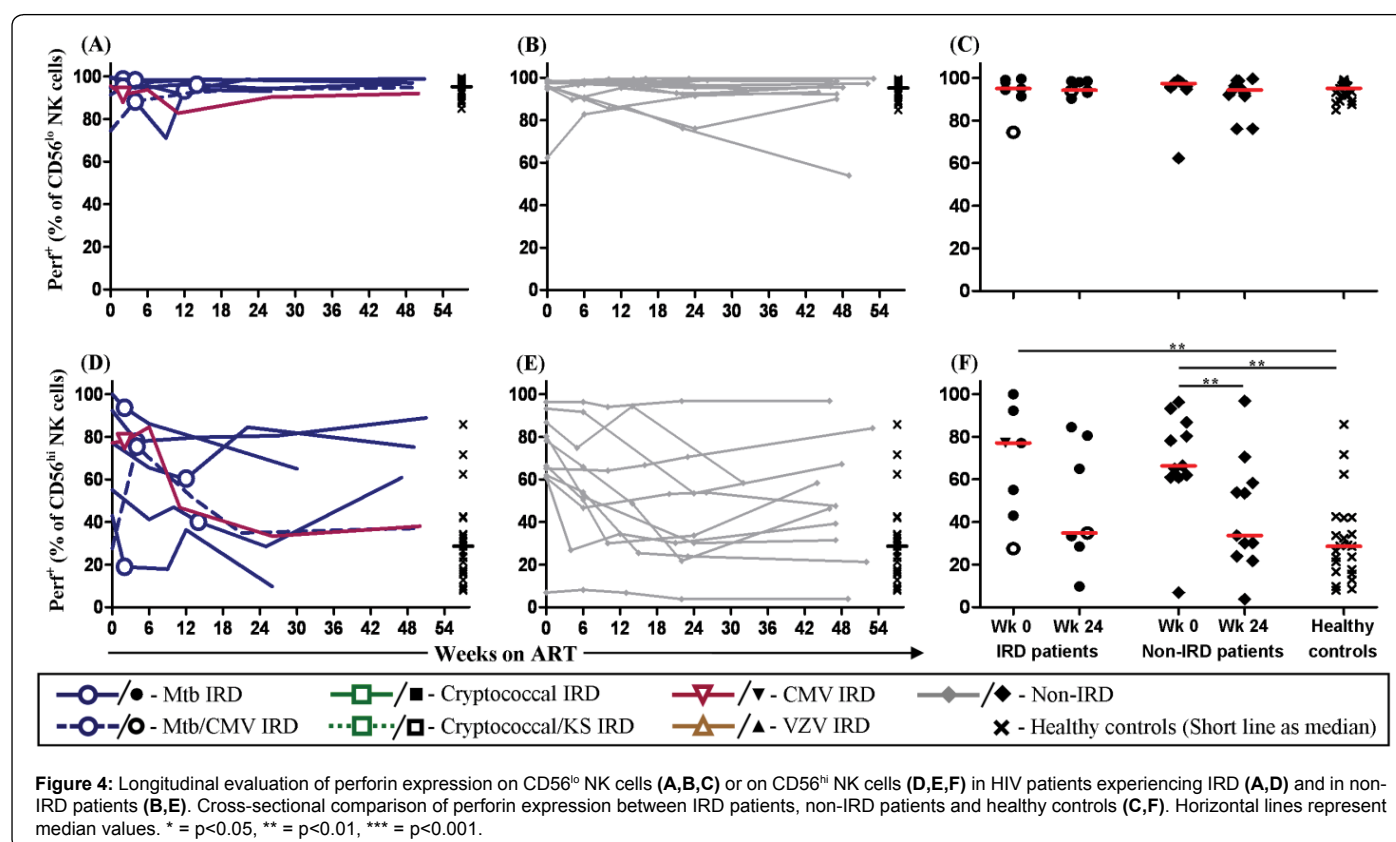


Figure 3: Longitudinal evaluation of CD16 expression on CD56^{lo} NK cells (A,B,C) or on CD56^{hi} NK cells (D,E,F) in HIV patients experiencing IRD (A,D) and in non-IRD patients (B,E). Cross-sectional comparison of CD16 expression between IRD patients, non-IRD patients and healthy controls (C,F). Horizontal lines represent median values. * = p<0.05, ** = p<0.01, *** = p<0.001.



Proportions of CD56^{hi} NK cells increased in IRD and non-IRD patients from Week 0 to Week 24 of ART (Figure 2F, $p<0.01$). IRD patients had slightly more CD56^{hi} NK cells at Week 24 than healthy controls (Figure 2D, 2F). This pattern held in several patients to Week 48. The two cryptococcal IRD patients showed increased proportions of CD56^{hi} NK cells coinciding with peaks in cryptococcal IFN γ responses during their IRD (Figure 1B). The two patients with CMV retinitis (+/- Mtb IRD) also showed increased proportions of CD56^{hi} NK cells on ART above other patients. The CMV/Mtb IRD patient had higher IFN γ responses to CMV (Figure 1C) and proportions of CD56^{hi} NK cells (Figure 2D).

CD16 expression on CD56^{lo} NK cells was low in most IRD patients at baseline.

CD16 expression on CD56^{lo} NK cells was lower at baseline in IRD ($p<0.001$) and non-IRD patients ($p<0.05$) compared to healthy controls (Figure 3A, 3B, 3C) and increased to levels seen in controls by Week 24 (Figure 3C). CD16 expression displayed equivalent trends when assessed as Mean Fluorescence Intensity ($p<0.05$; data not shown). Baseline CD16 expression was lower in IRD patients than non-IRD patients ($p<0.05$; Figure 3C). An examination of the individual plots (Figure 3A) shows that the low baseline values in patients with IRD associated with Mtb ($n=3$), CMV ($n=1$) and cryptococcal meningitis/KS ($n=1$). These IRD occurred whilst CD16 expression was low. In contrast, CD16 expression on CD56^{hi} NK cells was similar in all groups and did not reflect individual IRD events (Figure 3D, 3E, 3F).

Expression of perforin on CD56^{hi} NK cells was high in all patients

Intracellular perforin was assessed in seven IRD patients (5 Mtb, 1 Mtb/CMV and 1 CMV IRD), 10 non-IRD patients and 23 healthy

controls. Perforin expression in CD56^{lo} NK cells was not deficient in HIV patients at baseline and remained high to Week 48 with no difference between IRD and non-IRD patients (Figure 4A, 4B, 4C). Perforin expression on CD56^{hi} NK cells was higher at baseline in IRD and non-IRD patients ($p<0.01$) compared to healthy controls, with a decline by week 24. Expression remained similar in IRD and non-IRD patients (Figure 4D, 4E, 4F).

Discussion

Our study establishes that defects in NK cell IFN γ responses in Asian HIV patients persist for at least 1 year on ART. The K562 cell line only presents ligands for NK cell activatory receptors [31], so the data reflect maximum NK cell responses – responses to other targets may be even lower. NK cell IFN γ responses to K562 cells also remained low in Australian (Caucasian) HIV patients on long-term ART (2-5 years) [13]. Defects in NK cell IFN γ responses increase risk of tumors and infections [1-4]. This may explain the increasingly high incidence of non-AIDS-related malignancies seen in patients with optimal stable responses to ART [32].

NK cell IFN γ responses were limited by proportions of CD56^{lo} NK cells in Australian patients on long-term ART (2-5 years) [13]. Here no correlations were observed with the proportions of CD56^{lo} NK cells or CD56^{hi} NK cells in patients. Expansion of dysfunctional CD56^{neg}CD16⁺ NK cells in HIV viremic patients has been linked to impaired function of the total NK cell population. Compared to CD56⁺ NK cells, CD56^{neg}CD16⁺ NK cells have poorer cytotoxic function and secrete lower levels of IFN γ and TNF α [8]. However we did not quantitate these cells as our antibody panel did not allow us to gate out monocytes.

Perforin expression on CD56^{lo} NK cells was not deficient in the

Malaysian HIV patients suggesting that the cytotoxic potential of their NK cells is not impaired. Functional assessment of cytotoxic NK cells are warranted as other studies have demonstrated impaired NK cell cytotoxicity in HIV viremic and aviremic patients [7,12,33]. Defects affecting the degranulation pathway or receptor recognition may limit NK function, despite normal or elevated levels of perforin. Higher expression of perforin in CD56^{hi} NK cells from HIV patients sampled at baseline (Figure 3D) may reflect immune activation as CD56^{hi} NK cells can increase expression of perforin after stimulation with IL-2 [34].

One can envisage mechanisms whereby NK cells may be protective or pathological in an IRD. Studies of mycobacterial and cryptococcal IRD conclude that antigen load is a risk factor for developing IRD [35-37], so NK cells may minimize the risk of developing IRD through their capacity to control mycobacterial, cryptococcal, CMV and VZV infections[4,38,39]. Alternatively, excessive NK cell responses restored on ART may contribute to the elevated cytokine responses implicated in IRD pathogenesis[19,22]. Clark et al. [2004] described compartmentalization of NK cell activation to the central nervous system in a patient with neurological VZV IRD [26].

Here, NK IFN γ responses only increased before the IRD in one patient (Mtb IRD; Figure 1E), so NK cells are unlikely to contribute cytokines that mediate IRD. No significant changes were observed with the proportion of CD56^{lo} NK cells in circulation during the development of IRD. However, proportions of CD56^{hi} NK cells did increase in the two IRD patients who presented with cryptococcal meningitis coinciding with peaks in cryptococcal IFN γ responses during their IRD. These patients were not distinguishable by other parameters such as nadir CD4⁺ T-cell count, rate of CD4⁺ T-cell recovery or NK IFN γ responses (data not shown), so this warrants further investigation as a mechanism of IRD affecting a secluded site (such as the central nervous system).

CD16 (Fc γ R3a) is an activatory receptor which can mediate antibody-dependent cell cytotoxicity (ADCC) [40]. Here expression of CD16 on CD56^{lo} NK cells normalized by Week 24 of ART, which may reflect normal capability to elicit ADCC. CD16 expression on CD56^{lo} NK cells was particularly low in IRD patients before ART (Figure 3A). Impaired CD16 expression may affect the recognition, activation and ADCC function of NK cells against antibody-coated pathogens [41,42]. This may limit antigen clearance before ART, increasing the risk of IRD. ADCC should be addressed directly in further studies, as the response of NK cells toward K562 cells does not depend on CD16.

In conclusion, we showed partial restoration of NK cell profiles with recovery of CD16 expression on CD56^{lo} NK cells and perforin expression on CD56^{hi} NK cells to levels of healthy controls after approximately 6 months of ART. However, NK cell IFN γ responses remained impaired compared to healthy controls. We found no evidence of a role for NK cells in the pathogenesis of IRD as responses varied between patients with similar IRD, with no consistent peaks preceding diagnosis. However, NK deficiency may promote IRD by impairing antigen clearance before ART as CD16 expression of CD56^{lo} NK cells was low at baseline in most IRD patients. This warrants independent replication. Pathogen-infected target cells (e.g. Mtb-infected monocytes or CMV-infected fibroblasts) should be used to investigate responses of NK cells during specific IRD. NK genotypes (eg: KIR) should also be considered.

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