

**Increased proportions of dendritic cells and recovery of IFN γ responses in HIV/HCV
co-infected patients receiving ART**

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ABSTRACT

Dendritic cell (DC) numbers and functions can be affected by HIV and HCV disease, but the effects of antiretroviral therapy (ART) on DC and the implications of these changes are unclear. We examined circulating DC in samples from Indonesian patients beginning ART with advanced HIV disease and documented mild/moderate HCV hepatitis. Frequencies of myeloid and plasmacytoid DC increased after 6 months on ART, but frequencies of DC producing IL-12 or IFN α following stimulation with TLR agonists (CL075, CpG) did not change. IFN γ responses to CL075, HCV and other antigens rose over this period. Hence increased IFN γ responses during ART may be associated with increased DC frequencies rather than changes in their functional capacity.

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1. Introduction

HIV infection is characterised by generalised immune dysfunction that affects CD4⁺ T-cells, NK cells, B cells and dendritic cells (DC). DC are essential for the activation of naïve and memory T-cells and induction of immune responses against invading pathogens. Circulating DC constitute about 1% of peripheral blood mononuclear cells (PBMC) and can be identified by their lack of lineage markers (Lin⁻) and high expression of HLA-DR. They comprise 2 subsets: CD11c⁺CD123^{low} myeloid DC (mDC) and CD123^{high}CD11c⁻ plasmacytoid DC (pDC) [1]. Upon recognition of pathogen-associated molecular patterns by toll-like receptors (TLRs), pDC produce large amounts of the antiviral cytokine interferon-alpha (IFN α), whereas mDC secrete primarily IL-12 and prime Th1 responses.

During untreated HIV infection, the numbers of both DC subsets are diminished [2-6]. Loss of circulating DC has been attributed to direct infection by HIV [2,7], apoptosis and migration to lymph nodes [8-10]. In addition to loss of DC, the ability of pDC to produce IFN α after stimulation with viral products [11,12] and the ability of mDC and pDC to stimulate allogeneic T lymphocyte proliferation [2] were diminished in HIV-infected patients. Suppression of HIV viremia by antiretroviral therapy (ART) was associated with recovery of pDC but not mDC numbers in chronically infected patients [5,13,14] and individuals presenting with primary HIV infection [15]. However other investigators report increased numbers of pDC and mDC [4] or no changes in DC numbers [6] after the initiation of ART. Therapy was associated with partial improvement in DC functions [11]. Discrepancies in the literature may reflect HCV status.

HCV patients who are not co-infected with HIV also have lower absolute numbers and percentages of pDC compared to healthy controls [16]. Furthermore, DC functions may be impaired [17-19]. For example, production of IFN α by pDC in response to TLR9 agonist

and the ability of mDC to induce a mixed lymphocyte reaction was diminished in chronically HCV-infected patients [20].

HIV/HCV co-infection is common as both viruses are transmitted through use of intravenous drugs and has a poor clinical outcome. In a cross-sectional study of HIV patients stable on ART, percentages of pDC were lower in those co-infected with HCV [21]. Dual infection also impaired T-cell responses to HCV proteins [22,23] and reduced DC function [24] in untreated HIV patients. HCV co-infection also reduced the ability of monocyte-derived DC to produce IL-12p70 after stimulation with CD40L [25].

Longitudinal studies linking DC numbers and functions with HCV-specific immune responses in HIV/HCV co-infected patients starting ART are lacking and few studies of HCV/HIV co-infection have assessed patients in the developing world where this scenario is common and challenging. Here we determine if DC percentages and functions recover in HIV/HCV co-infected patients after starting ART. We examined frequencies of mDC and pDC at baseline and at 1, 3 and 6 months of ART. IL-12 and IFN α responses by mDC and pDC (respectively) were assessed after stimulation with the TLR7/8 agonist, CL075. The data were correlated with IFN γ production by PBMC following incubation with several stimuli, including CL075 and recombinant HCV antigens (core and NS3).

2. Patients and Methods

2.1 Patients

A cohort of HIV/HCV co-infected patients commencing ART at the HIV/AIDS Clinic of Cipto Mangunkusumo Hospital (Jakarta, Indonesia) have been investigated in a series of studies based on plasma samples [26] and liver biopsies [27]. Inclusion criteria were age 17-50 years, ART and HCV-therapy naïve and a baseline CD4⁺ T-cell count <200 cells/ μ L.

Patients who were pregnant or had HBsAg, liver cirrhosis, kidney or heart failure were excluded. All patients had a history of intravenous drug use but were not actively using at the time of recruitment. HCV diagnosis was based on positivity for anti-HCV antibody using the Elecsys Anti-HCV Assay (Roche Diagnostics, Rotkreuz, Switzerland). CD4⁺ T-cell counts were quantified by flow cytometry. HIV and HCV viral loads were measured using a Cobas Amplicor Monitor (Roche Molecular Diagnostics, United States). The study included PBMC from 23 male patients and 17 healthy controls. Controls were matched with the patients by gender, age and ethnicity. They were all resident in Jakarta, most were health care workers and none declared risk of infection with HIV or HCV. PBMC were isolated by density centrifugation, cryopreserved in 10% DMSO/FCS and stored in liquid nitrogen [28]. Ethics committees of the Faculty of Medicine (University of Indonesia) and Royal Perth Hospital (Western Australia) approved the study protocol, and written informed consent was obtained from all participants.

2.2 Cytokine-producing DC detected by flow cytometry

PBMC resuspended in 10% FCS/RPMI-1640 were stimulated for 6h at 37°C with TLR ligands: CL075 (10ug/mL; Invivogen, San Diego, CA, USA) or CpG 2336 (5uM; Invivogen). Brefeldin-A (BFA; BD Biosciences) was added for the last 5h incubation in cultures stimulated with CL075, while stimulation with CpG was optimal without BFA. PBMC were also cultured without stimulation to assess spontaneous cytokine production. DC subsets were detected with CD123-PerCP-Cy5.5 (clone 7G3), HLA-DR-PE (clone L243), Lin1-FITC (BD Biosciences, Franklin Lakes, NJ) and CD11c-PECy7 (clone 3.9) (BioLegend, San Diego, CA, USA). Intracytoplasmic staining was performed using the BD Cytofix/CytopermTM Kit and with IL-12-APC (clone C11.5) or IFN α -AF547 (clone 7N4.1) (BD Biosciences). Stained

cells were washed twice and 4-colour data were acquired on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA). Analyses were performed using FlowJo (Treestar, San Carlos, CA, USA). DC were identified as Lin⁻HLA-DR^{high}, and mDC and pDC were distinguished based on expression of CD11c^{high} and CD123^{high}, respectively. Representative flow cytometry plots defining dendritic cells and cells producing cytokines are in Supplementary Figure 1. Percentages of cytokine-producing cells were determined by subtracting percentages in unstimulated cultures from those in stimulated cultures.

2.3 IFN γ -producing cells quantified by ELISpot assay

Nitrocellulose plates (Millipore, Danvers, MA, USA) were coated with anti-human IFN γ antibody (15ug/mL; Mabtech, Stockholm, Sweden). PBMC in 10% FCS/RPMI-1640 were plated in duplicate at 2×10^5 viable cells/well and stimulated for 24h at 37°C with recombinant HCV proteins: core (genotype 1b) or NS3 (genotype 1b), (2.5ug/mL; MyBioSource, San Diego, CA, USA) in the presence of the co-stimulatory molecules, anti-CD28 and anti-CD49d (1ug/mL, BD Biosciences). Co-stimulatory molecules were added to unstimulated PBMC as a negative control. PBMC were also stimulated with anti-CD3 (Mabtech) as a positive control, cytomegalovirus (CMV) lysate [28] and purified protein derivative (PPD) (Statens Serum Institute, Copenhagen, Denmark). Spots were detected with biotinylated anti-human IFN γ antibody (Mabtech), streptavidin horseradish peroxidase conjugate (BD Pharmingen, San Diego, CA, USA) and tetramethylbenzidine substrate (Mabtech) and counted using AID ELISpot v2.9 software (Autoimmun Diagnostika GmbH, Strassberg, Germany). Frequencies of reactive cells were determined by subtracting average numbers of spot-forming cells in negative control wells from number in stimulated wells and are expressed per 2×10^5 PBMC.

2.4 Statistics

All data are presented as median (range). Statistical analyses were performed using Graphpad Prism Version 5.01 (Graphpad Software, San Diego, CA, USA). Differences between patients and controls and changes over time on ART were evaluated using Mann-Whitney tests, as missing data points (1-5 per timepoint) precluded paired analyses of the complete dataset. Correlation coefficients were determined by the Spearman's Rank Correlation Test. p -values <0.05 are considered to be statistically significant.

3. Results

3.1 Patient characteristics

Samples from 23 chronically HIV-infected male patients were evaluated at baseline and after 1, 3 and 6 months on ART. Median (range) CD4⁺ T-cell counts increased from 22 (3–182) cells/uL at baseline to 84 (7-238) cells/uL at 1 month, 108 (31-225) cells/uL at 3 months and 123 (25-290) cells/uL at 6 months of ART (Fig. 1A). Despite this increase, 21 out of 23 patients still had <200 CD4⁺ T-cells/uL at 6 months. Plasma HIV RNA loads decreased so 22 (96%) patients had undetectable viremia (<400 copies/mL) at 6 months ($p<0.0001$) (Fig. 1B).

3.2 Frequencies of DC were low in HIV/HCV co-infected patients and rose on ART

At baseline, HIV/HCV patients exhibited lower proportions of mDC (Fig. 1C) and pDC than healthy controls (Fig. 1D). Frequencies of mDC and pDC remained unchanged during the first 3 months of ART but at 6 months were similar to the healthy controls. This increase was significant for mDC ($p=0.027$) but not pDC ($p=0.149$).

Frequencies of mDC and pDC did not correlate with CD4⁺ T-cell counts at any time (data not shown). HIV RNA correlated inversely with percentages of pDC ($r = -0.56$, $p=0.076$) and mDC ($r = -0.48$, $p=0.036$) at baseline.

3.3 Frequencies of cytokine-producing DC producing cytokines after TLR ligand stimulation were not depressed in HIV/HCV co-infected patients

After stimulation with CL075, proportions of IL-12-producing mDC (Fig. 1E) and IFN α -producing pDC (Fig. 1F) were unchanged during ART and remained similar to controls. Similarly, proportions of IFN α -producing pDC after stimulation with CpG, were unchanged on ART and similar to controls (Fig. 1G).

Frequencies of IL-12-producing mDC and IFN α -producing pDC did not correlate with HIV viral loads at baseline (mDC: $r = -0.27$, $p=0.28$ and pDC: $r = -0.12$, $p=0.71$) nor with CD4⁺ T-cell counts pre- and post-ART (mDC: $r = -0.16$ to -0.44 , $p=0.06-0.51$ and pDC: $r = -0.13$ to 0.30 , $p=0.37-0.68$).

3.4 IFN γ responses of PBMC to TLR agonists, HCV and other antigens rose on ART

At baseline, IFN γ responses to CL075 were low compared to controls (Fig. 2A). At 3 and 6 months of ART, numbers of IFN γ -producing cells were higher than at baseline and were similar to controls. Numbers of CMV-specific IFN γ -producing T-cells increased marginally at 3 and 6 months of ART (Fig. 2B). IFN γ responses to PPD were marginally higher at 6 months of ART (Fig. 2C) compared to baseline and controls. Following stimulation with HCV core antigen, IFN γ responses increased at 3 and 6 months of ART (Fig. 2D). Smaller rises were seen following stimulation with NS3 antigen (Fig. 2E). IFN γ responses to HCV

NS3 antigen was detected in 5 healthy controls whilst IFN γ responses to core antigen was observed in 9 healthy controls. This may reflect cross reactivity with influenza or exposure of healthy controls (health care workers) to HCV [29-31].

Frequencies of IFN α -producing pDC and IL-12-producing mDC following stimulation with TLR ligands did not correlate with TLR ligand-specific or antigen-specific IFN γ responses (data not shown).

4. Discussion

HIV and HCV dual infection is common in Indonesia, particularly among injecting drug users [32]. Co-infection with HCV may impair recovery of CD4⁺ T-cells in HIV-infected patients starting ART [33]. Here, we describe changes in mDC and pDC frequencies and their cytokine responses in co-infected patients who started therapy with <200 CD4⁺ T-cells/ μ L. Our results confirm previous studies demonstrating a loss of both DC subsets in HIV-infected patients compared to healthy controls [3,5,8]. Reduced DC frequencies in patients co-infected with HCV may be attributed to HIV infection as percentages of DC were previously shown to be similar in HCV mono-infected patients compared to healthy controls [3,24]. However this finding is controversial as we and others have reported significantly lower percentages of both circulating DC subsets in HCV mono-infected patients [16,34,35]. Decreased frequencies of DC during HIV infection can be caused by migration of these cells to the lymphoid tissue or the apoptosis of circulating DC [8-10]. In HCV infection, there is evidence for increased homing of DC to the liver [36] or increased apoptosis of DC [37-39], which may explain the loss of these cells from the peripheral blood.

To our knowledge, this is the first study to demonstrate increased percentages of mDC after 6 months of ART in HIV/HCV co-infected patients. Increased numbers of mDC but not

pDC during ART have been reported in Asian HIV mono-infected patients [8,40]. The restoration of circulating mDC numbers on ART may reflect reduced expression of the proapoptotic marker caspase-3 following viral suppression [10]. Recovery of pDC may depend on the baseline HIV viral loads and CD8⁺ T-cell activation [13]. A limitation of this study is that HIV-infected patients were not included to delineate the role of HCV, but we established that the loss of DC in co-infected patients with severe HIV disease is reversible.

No associations between DC percentages and CD4⁺ T-cell counts were found. This contradicts previous studies that reported a positive correlation between DC numbers with CD4⁺ T-cell counts in untreated and treated chronically HIV-infected individuals [5,41,42], patients with primary HIV infection [15], a cohort comprising HIV and HIV/HCV co-infected patients [24] and in a longitudinal study that followed HIV-infected patients during 60 weeks of ART [40]. The absence of a correlation probably reflects selection bias as all participants in our study started ART with CD4⁺ T-cell counts <200 cells/ μ L, whereas prior cross-sectional studies included patients with a wider range of baseline CD4⁺ T-cell counts. Furthermore a broader range of CD4⁺ T-cell count recovery was observed (25-535 cells/uL) during ART in the longitudinal study [40] compared to our study (3-290 cells/uL).

Our data showing an inverse association between DC frequencies and HIV viral loads is consistent with previous studies [15,41,42] and is consistent with a role for DC in the control of HIV replication. This may involve the antiviral cytokine, IFN α , present at higher levels in HIV controllers than viremic patients [43].

Here, frequencies of cytokine-producing DC were similar in HIV-infected patients and controls after TLR7/8 stimulation and were not affected by ART. The results contradict reports of reduced cytokine production by DC in patients with dual infection [24] and recovery of DC functions in HIV-infected patients receiving ART [40,41]. The lack of

improvements in DC cytokine responses may reflect impaired CD4⁺ T-cell recovery during ART [44]. Furthermore, increases in levels of TLR7 and TLR8 expression may compensate for reduced percentages of mDC and pDC. Lester *et al.* reported higher expression of TLR7 and TLR8 in PBMC from untreated chronic HIV-infected patients than healthy controls, with a trend for reduced TLR7 expression following ART [45]. We did not investigate TLR7 or TLR8 expression by DC, but this warrants further studies as it also may be increased by immune activation. HIV mono-infected patients from Kuala Lumpur had increased immune activation assessed by HLA-DR expression and this persisted on ART [46].

Cytokines produced by DC can affect virus-specific adaptive immune responses. For example, production of IFN α by pDC promotes differentiation of virus-specific B-cells into mature plasma cells. This is clinically relevant as pre-treatment HCV-specific antibody titres can predict a sustained virological response to pegylated IFN α and ribavirin in chronic HCV infection [47]. Moreover expression of IL-12 by peripheral blood DC was directly related to HCV-induced CD4 T-cell proliferation [48] and hence to sustained virological responses [49]. Here, CD4⁺ T-cell recovery on ART paralleled restoration of IFN γ responses to bacterial and viral antigens. This is consistent with prior studies following HCV-specific and [22,50] CMV-specific [28,51,52] CD4⁺ T-cell responses during ART. In addition, we demonstrated increased IFN γ responses to CL075 on ART. However, no correlations were evident between IFN γ production and frequencies of cytokine-secreting DCs. In Australian HCV mono-infected patients, we described positive correlations between cytokine responses by DC and IFN γ responses in PBMC cultures stimulated with CL075 or CpG at baseline and during IFN α and ribavirin combination therapy [34]. The lack of an association in this study suggests that CL075 and antigen-induced IFN γ responses are not limited by the frequencies of cytokine-producing DC. However, we cannot rule out the possibility that the amount of

cytokine produced per DC may be increased in the present cohort. It is also possible the additional IFN γ may be derived from NK cells activated directly by the TLR agonists. In response to another TLR7/8 agonist, R848, NK cells produced significant amounts of IFN γ when cultured with monocytes [53].

In conclusion, our data showed increased DC frequencies and recovery of IFN γ responses in HIV/HCV co-infected patients receiving ART, but DC function was unchanged. Future studies are warranted to validate these results in a larger cohort.

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Figure 1. CD4⁺ T-cell counts (A), plasma HIV RNA (B), percentages of mDC (C) and pDC (D), and cytokine responses by DC (E-G) in HIV/HCV co-infected patients beginning ART. Proportions of mDC and pDC were calculated as a percentage of PBMC. Frequencies of IL-12-producing (E) and IFN α -producing cells (F) after stimulation with CL075 and proportion of IFN α -producing cells after stimulation with CpG (G) were calculated as a percentage of mDC or pDC. Data are presented as box and whisker plots where the central line represents the median value. p-values were determined using Mann Whitney Tests.

Figure 2. Numbers of IFN γ -producing cells after stimulation of PBMC with the TLR7/8 agonist CL075 (A), CMV (B), PPD (C), HCV core protein (D) or HCV NS3 protein (E). Results are presented as spot forming units (SFU) per 2×10^5 PBMC. Data are presented as box and whisker plots where the central line represents the median value. p-values were determined by Mann-Whitney tests.

Supplementary Figure 1. Gating strategy used to identify DC expressing IFN α and IL-12. Total PBMC were gated out from cell debris (A) and total DC were distinguished by their high levels of HLA-DR and lack of Lin1 expression (B). CD11c and CD123 expression identified mDC (C) and pDC (D), respectively. mDC were further analysed for their expression of IL-12 and pDC for their expression of IFN α in the absence of any stimulation (E and F) and after stimulation with TLR ligands (G and H).

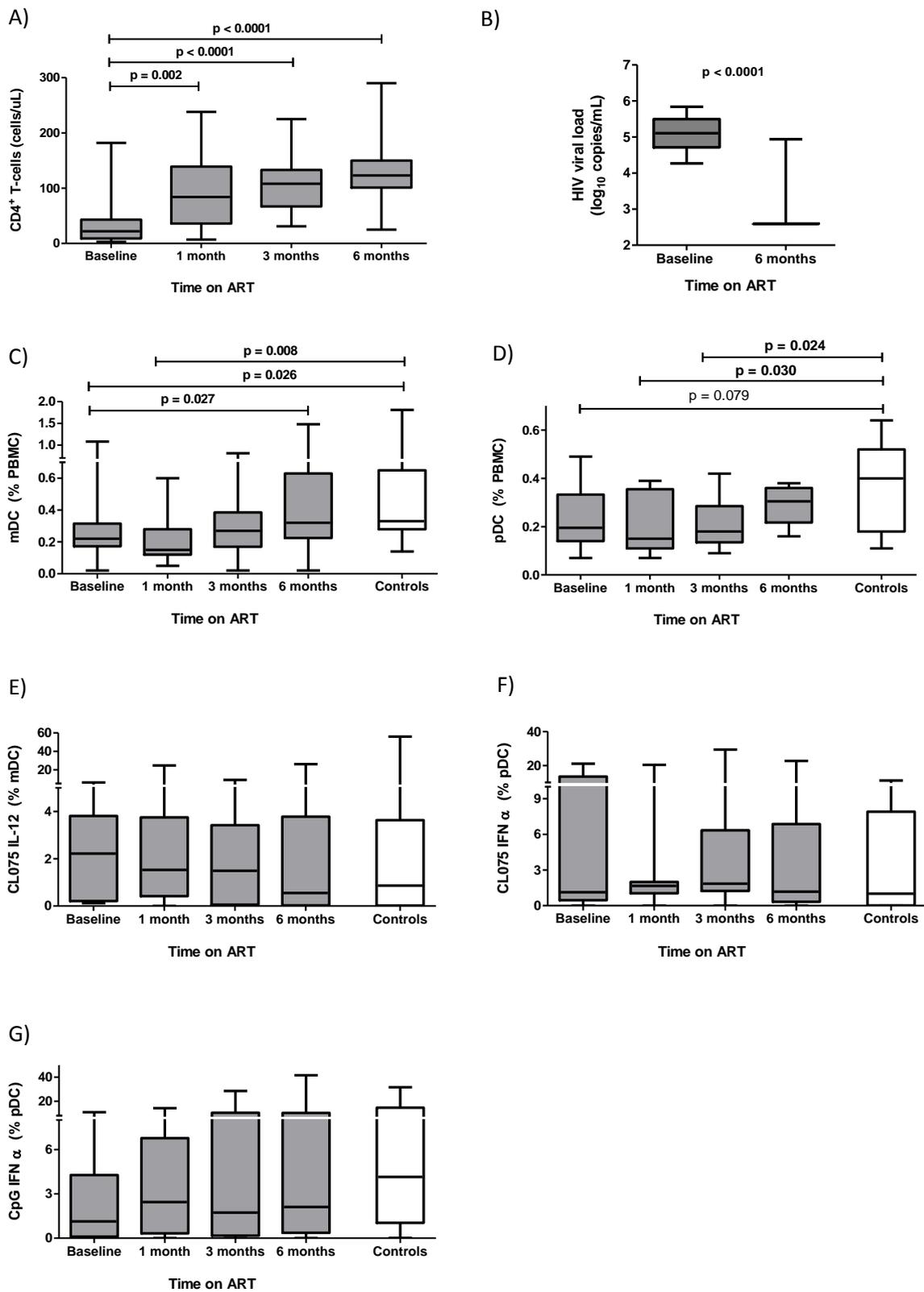


Fig 1

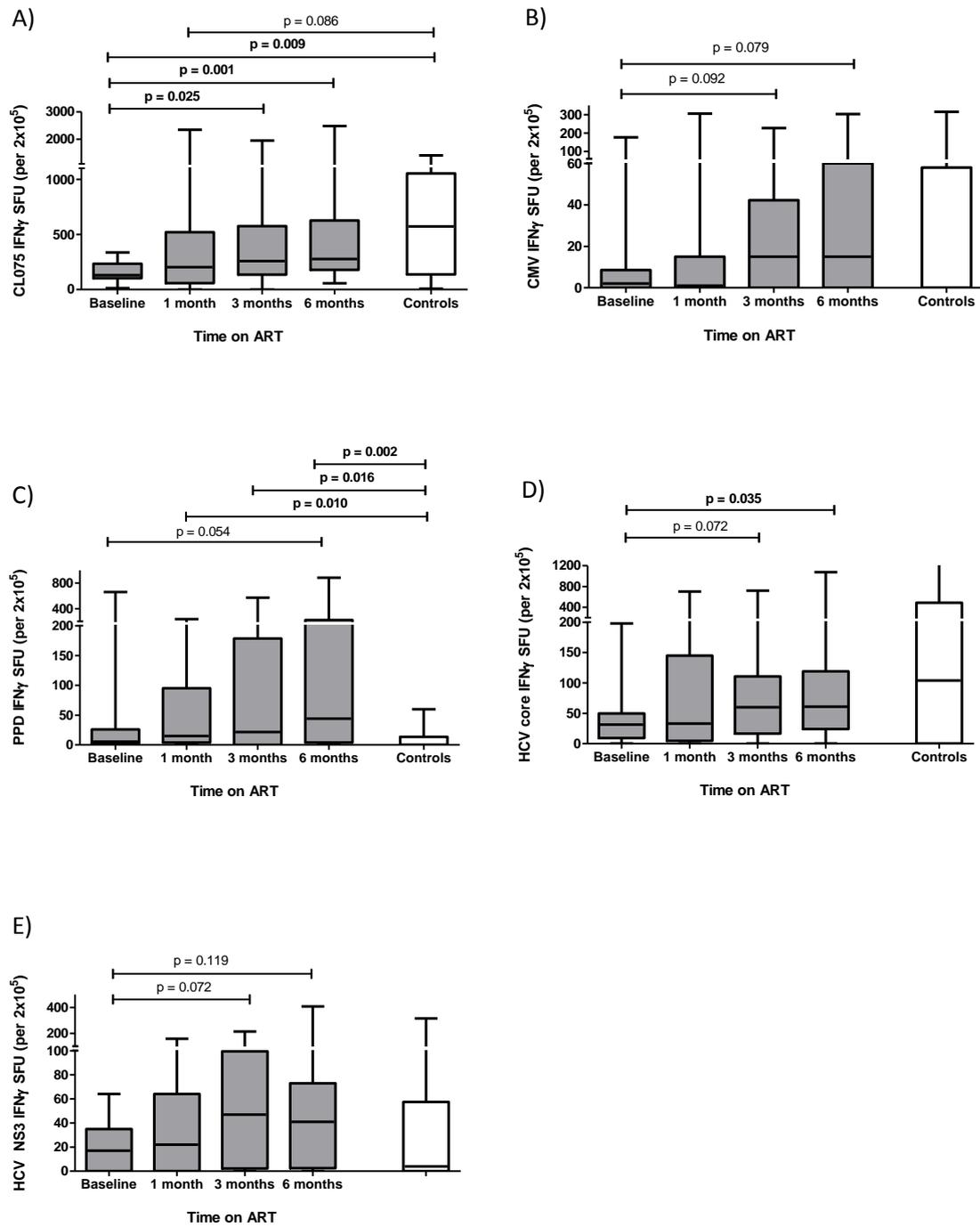
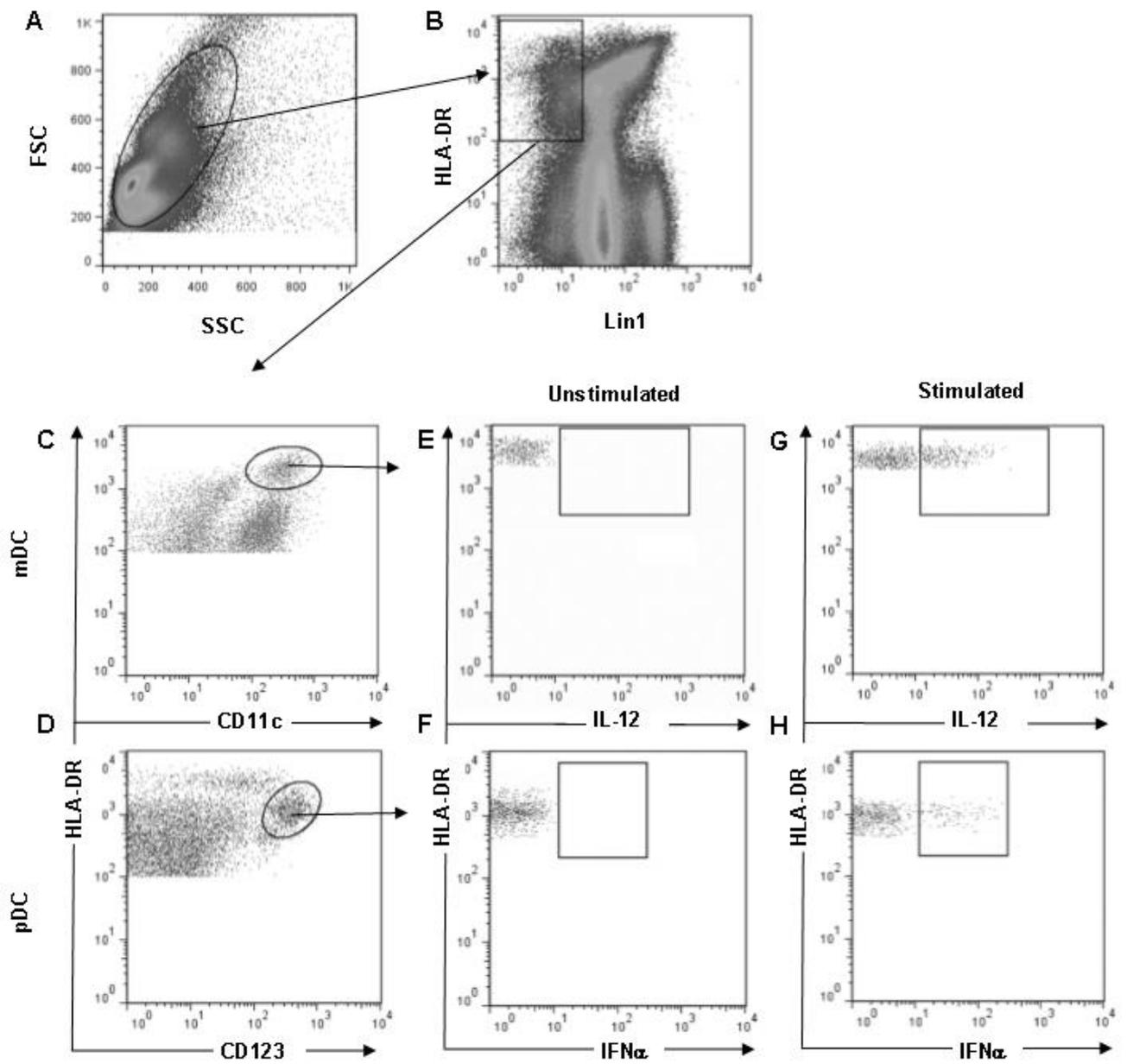


Fig 2



Supplementary Figure 1