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Natural Killer Cells Phenotype in Antiretroviral Naïve HIV-1 Infected People Living in Cameroon

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Abstract

The impact of antiretroviral naïve HIV-1 infection on the modulation of Natural Killer (NK) cells phenotype has not been fully assessed.

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This study aimed to define the phenotype of NK cell in the context of antiretroviral naïve HIV-1 infection. A total of 85 ARV naïve HIV-1 infected and 55 healthy individuals were included in the study. Purified NK cells alongside bulk Peripheral Blood Mononuclear Cells (PBMC) were surface stained with fluorochrome conjugated antibodies and samples were acquired using a BD FACS canto II flow cytometer. A down-regulation of CD56 $^{+}$/CD16 $^{-}$ and CD56 $^{+}$/CD16 $^{+}$ NK cells (p= 0.003), and a significant expansion (p= 0.03) of CD56 $^{-}$/CD16 $^{+}$ NK cells subset was observed in ARV naïve HIV-1 infection. The high expression of both CD38 (p= 0.02) and HLA-DR (p=0.001) in the CD56^{-}/CD16^{+} NK cells subset, shows the activation status of NK cells from HIV-1 infected people. A reduced expression of activating markers NKp44 and NKp30 and the down regulation of NKG2A was observed in CD56^{+}/CD16^{-} and CD56^{+}/CD16^{+} NK cells from HIV-1 infected people (p= 0.006, p= 0.009, respectively). Antiretroviral naive HIV-1 infected people living in Cameroon show a differential modulation of NK cell phenotype relative to HIV negative controls.

Keywords: Natural killer cells; subsets profile; ARV naïve HIV-1 infection.

1. Introduction

Natural Killer (NK) cells are key players of innate immunity which selectively eliminate virus infected and tumor cells. NK cells comprise 5-20% of human peripheral blood mononuclear cells [1, 2]. They are vital effector cells in innate immune response to many diseases including HIV infection. These cells can spontaneously sense and lyse virus-infected cells without prior sensitization to antigen [3]. In addition to their ability to kill infected cells, activated NK cells secrete various immune modulatory cytokines and chemokines that mediate the suppression of viral replication, contribute to the prevention of viral entry into host cells, and amplify immune response by recruiting and priming cells of the innate and adaptive immunity [1, 4]. NK cells can also perform antibody-dependent cellular cytotoxicity (ADCC) through CD16 (FcγIII receptor) or directly exert their cytotoxic ability by the release of cytotoxic proteases termed perforin and granzyme B [5]. Previously, human NK cells were divided into two major subsets according to their phenotype and functions [6, 7]. The CD56^{bright}/CD16^{-} NK cells represent the minor subset (10% of total NK) in the peripheral blood, with predominantly immune-regulatory properties, and a potent cytokine (mainly IFN-γ) producing capacity. In contrast, the major subset CD56^{dim}/CD16^{+} (90% of total NK cells) is characterized by low levels of cytokine secretion and high cytolytic function [1]. Currently, three subtypes of NK cells are recognized namely, CD56^{dim} /CD16^{+}, CD56^{bright} /CD16$^{+/-}$ and CD56^{-}/CD16^{+} [8]. Chronic NK cells activation could play a potential role in the impairment of their functions. Several markers are associated with NK cells activation and cytolytic function. That include different surface molecules, such as CD69, CD38, and HLADR which are general activation markers. CD38 is a glycoprotein that regulates levels of cytoplasmic calcium and acts as an adhesion molecule expressed in activated NK cells. HLA-DR is an activation marker on NK cells with a potential antigen-presenting function where CD69 is an acute NK cell activation maker [9, 10]. The co-expression of CD38 and HLA-DR translates an activated status of NK cells [11]. In effecting their functions, NK cells deploy different varieties of surface activating and inhibitory receptors. These receptors can be divided into three groups, (i) killer immunoglobulin-like receptors (KIRs), (ii) natural cytotoxicity receptors (NCR) and (iii) NKG2/CD94 heterodimer family (C-type Lectin-like receptors). Natural cytotoxicity receptor (NCR) that includes three type I transmembrane receptors, termed NKp46, NKp44, and NKp30. NCR are activating receptors which are

encoded by the genes, *NCR1*, *NCR2*, and *NCR3*, respectively [12-14], recognize viral derived products [15]. Unlike NKp30 and NKp46, NKp44 is only expressed on activated NK cells. NKp46 has been shown to play a role in NK cell-mediated lysis of several tumor cells and pathogen-infected cell lines[16]. The natural killer group 2, member D (NKG2D) that belong to NKG2 calcium-dependent lectin-like (NKG2 C-lectin) group need another receptor to act [17]. Activating signals delivered through the NKG2D receptor is known to induce the killing of pathogen-infected cells as well as cancer cells [18]. The killer cell immunoglobulin-like receptors (KIRs) include activating and inhibitory receptors that recognize major histocompatibility complex (MHC) class I associated with peptides [12, 15]. KIRs are transmembrane glycoproteins that have been shown to recognize allotypic determinants of human leukocyte antigen (HLA) class I molecules among which, HLA-A, HLA-B and HLA-C [19]. NK cell surveys the expression of MHC class I molecules through inhibitory receptors like NKG2A and KIRs to protect normal cells from autologous NK -cells mediated killing. KIRs remain vital in the initiation and the modulation of NK cell mediated immune responses [20-22]. Previous studies suggest NK cells impairment in number and function during HIV-1 infection [23, 24]. However, it is not known how ARV naive HIV-1 infection would affects the modulation of NK cell phenotype. The ultimate goal of this study is to describe the phenotype of NK cells in the context of ARV naïve HIV-1 infection by assessing NK cell subpopulations profile, activating and inhibitory receptors expression with respect to virological and immunological status of participants. Our hypothesis is that, during antiretroviral (ARV) naïve HIV-1 infection, perturbations in innate immunity can result to detectable alterations in NK cells profile.

2. Materials and methods

2.1. Study population

Eighty-five (85) ARV naïve HIV infected participants and fifty-five (55) HIV negative controls aged between 21 to 65 years were included in the study. Participants with co-infections (including Malaria, Dengue, Hepatitis B and C infections) as well as pregnant women were excluded from the study. Two major groups of participants were enrolled; HIV-1 positive and HIV negative individuals (as HIV negative controls). HIV positive participants were members of the CIRCB AFRODEC (African HIV-1 dendritic cell targeted vaccine) cohort. The AFRODEC cohort is a group of antiretroviral naïve HIV infected people from different Cameroonian regions who have being monitored in Centre International de Reference Chantal BIYA (CIRCB) since 2012 [25].

2.2. Ethical considerations

The present study was approved by the National ethics committee for human Health Research in Cameroon with administrative authorization number 2015/08/631/CE/CNERSH/SP. Participant's enrolment was voluntary and each participant signed an informed consent. We assigned a code to each participant for confidentiality purpose. All clinical data (helper $CD4^+$ T-cell counts, plasmatic viral loads and rapid test results) obtained during this study were provided free of charge to each participant.

2.3. Blood samples collection

Twenty (20) mL of venous blood was collected in Ethylene-Diamine-Tetra-Acetic Acid (EDTA) tubes from each participant by trained clinicians and used for experiment within 2 hours for maximum cells viability. A volume of 2mL from the EDTA blood sample tubes was transferred into a Falcon tube, centrifuged at 1500 rpm (at 21°C for 5 min). Plasma fraction was removed and transferred in sterile 1, 8 mL cryo-tubes in a biosafety cabinet and stored at -20°C until use.

2.4. Helper CD4$^+$ T cells immune-phenotyping

Absolute numbers of helper $CD4^+$ T-cells were determined in whole blood using BD multitest ™ CD3/CD8/CD45/CD4 kit according to the manufacturer's instructions (BD Biosciences, USA).

2.5. Plasmatic HIV-1 viral load determination

Plasmatic HIV-1 viral load was determined with the m2000rt machine using the Abbott Real-Time HIV-1 Assay protocol. The Assay detection limit was 40 viral RNA copies/mL of plasma.

2.6. Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation using Ficoll-hypaque (GE Healthcare Biosciences, Sweden) and washed twice in cold 1x Phosphate Buffered Saline (PBS) without Ca^{2+} and Mg^{2+} (Mediatech, corning). The centrifugation was run at 300g, 4°C for 10 minutes. PBMCs were counted using the trypan blue exclusion method and used for NK cells purification and surface staining.

2.7. NK cells purification

Human NK cells were magnetically sorted from PBMCs through negative selection according to manufacturer's instruction (Miltenyi Biotec, Germany).

2.8. Cells surface staining for flow cytometry

A volume of 50 µL of either PBMCs or purified NK cells suspension ($1x10^6$ cells) was aliquoted into a 96 V-bottom plate. Compensation wells were also designed, one well for each fluorochrome used. Cells were washed twice (centrifuged at 2000 rpm for 2 Minutes at 4°C). The supernatant was discarded by flipping the plate. Fifty (50) µL of the Fragment crystallizable receptor (FcR) blocking solution (diluted 1/100 FcR block in Fluorescence Activated Cell Sorting (FACS) buffer) were then added into each well to avoid the binding of antibodies to the FcR of NK cells. The plate was incubated for 20 minutes at 4°C in the dark. After Fcγ receptors blocking, the cells were washed twice with FACS buffer (1X PBS with Ca^{2+} and Mg^{2+}, 2% FBS, 1% EDTA), surface stained with fluorochrome-conjugated antibodies to CD56 (CD56-APC- R-700), CD16 (CD16-PECF-594), CD38 (CD38- PE-Cy7), HLADR (FITC- HLADR), NKp44 (NKp44-PE), NKp46 (NKp46-PE-Cy5), NKp30 (NKp30-APC), NKG2D (NKG2D-APC), NKG2A (NKG2A-PE), KIR2DL1(KIR2DL1-APC), KIR2DL5 (KIR2DL5-PE), CD3 (CD3- APC-H7), CD14 (CD14- APC-H7), CD19 (CD19- APC-H7), CD20

(CD20- APC-H7) and LIVE/DEAD stain for 20 min at 4°C (or 15 minutes at 37°C) in the dark. Several panels of antibodies were used. All antibodies were from eBioscience. Cells were then washed twice and re-suspended in 400 µL of FACS buffer. Stained samples were then acquired on BD Fortessa X-20 cytometer using BDFACS Diva Software

2.9. Statistical analysis

Flow cytometric data were analyzed using FLOWJO software (Tree Star, Ashland, OR, USA) version 9.8.5. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 17.0 and Prism (GraphPad 9.0, San Diego, CA, USA) software. Participant's clinical characteristics were described as medians (range). To determine if the values come from a Gaussian distribution, Kolmogorov-Smirnov test was performed. For a non-Gaussian distribution, continuous variables were compared using nonparametric tests; Mann- Whitney *U*-test for the comparison between two groups and the Kruskal–Wallis test was used to compare more than two groups. NK cells subpopulations as well as activating and inhibitory markers were compared using the Mann- Whitney *U*-test or Kruskal–Wallis Dunn's test (95% CI). The associations between variables were assessed using Spearman Rank order correlation coefficients. In all statistical tests Two-tailed p-values were reported and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical characteristics of the study population

Table 1: Participant's clinical information

Variable	HIV non-infected participants (HIV-) (n=55)		HIV infected participants (HIV+) (n=85)		*P*-values
	Males	Females	Males	Females	
Participants (%)	26(47)	29(53)	35(41)	50(59)	
Median age (range), years	39 (32-59)		30 (18-46)		*p*=0.3
Median Helper CD4 T cells count (range), cells/mm^3	955 (507- 1400) *		490 (55-959) *		**p*= 0.01
Median Viral Load (range)	**RNA copies/mL**		3058 (40-10,004,094)		
	N/A				N/A
	Log_{10} RNA copies/mL		3.16 (1.60- 6.00)		
Range (minimum–maximum).	n: numbers		N/A (Not Applicable)		*P<0.05

A total of 121 ARV naïve HIV-1 infected people and 85 HIV negative individuals were enrolled. After elimination of all co-infected participants, samples from 85 ARV naïve HIV-1 infected and 55 healthy donors were kept for experiments and analyses. Thus, 36 HIV positive and 10 HIV negative participants were excluded because of malaria, HBV, HCV and dengue infections. The median age of the HIV-1 positive individuals was 39 years (range: 32 to 59) and 30 years (range: 18 to 46) for HIV-1 negative controls respectively (Table I). The median of helper $CD4^+$ T cells count of HIV-1 positive participants was 490 cells/mm3 and 955 cells/mm^3 for HIV negative controls. As expected, the median of helper $CD4^+$ T-cell counts of HIV-1 positive participants was significantly low (p=0.01) when compared to HIV-1 negative participants (table I). Plasmatic viral loads of HIV positive individuals were from 1.6 up to 6 Log RNA copies/mL with a mean of 3.16 Log.

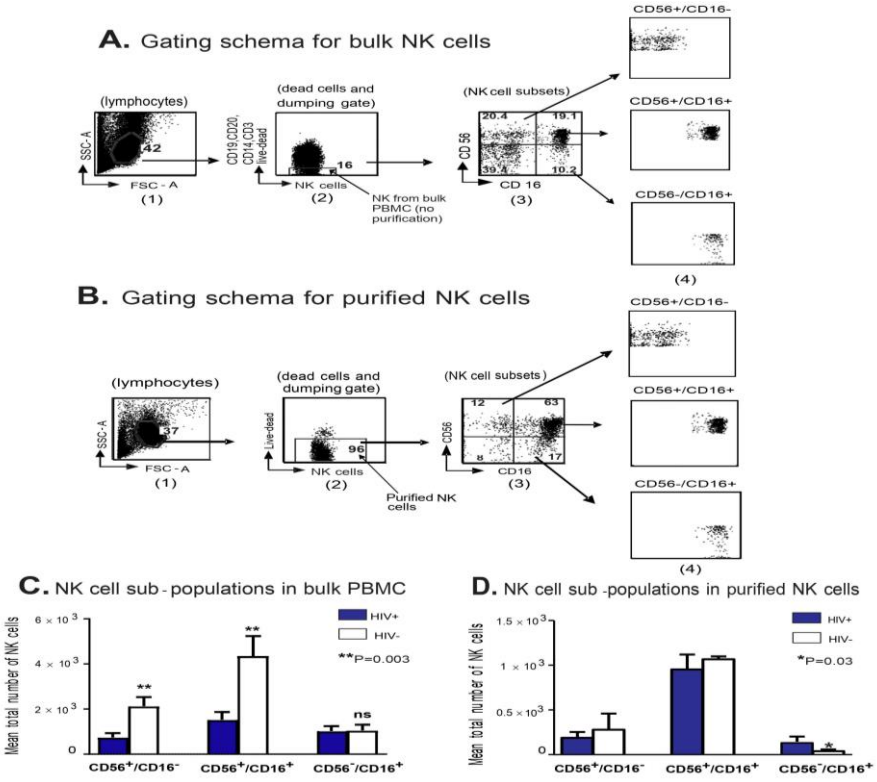


Figure 1: NK cell sub-populations profile

A. Lymphocytes were defined in bulk PBMC from side scatter (SSC) versus forward scatter (FSC) gate (1). NK negative cells and dead cells were excluded from lymphocytes population by gating dumping channel (CD19, CD20, CD14 and CD3) plus live-dead (LD) versus FSC-A (2). NK cells subpopulations were defined by CD56 versus CD 16 gate (3). NK cells subsets ($CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$) are shown in (4).
B. Lymphocytes (bulk NK cells) were defined from side scatter (SSC) versus forward scatter (FSC) gate (1). Dead cells and impurities were excluded from bulk NK cells by gating dumping channel (CD19, CD20, CD14 and CD3) plus live-dead (LD) versus FSC-A (2). NK cells subpopulations were defined based on CD56 and CD16 expression level from purified NK cells (3 and 4)

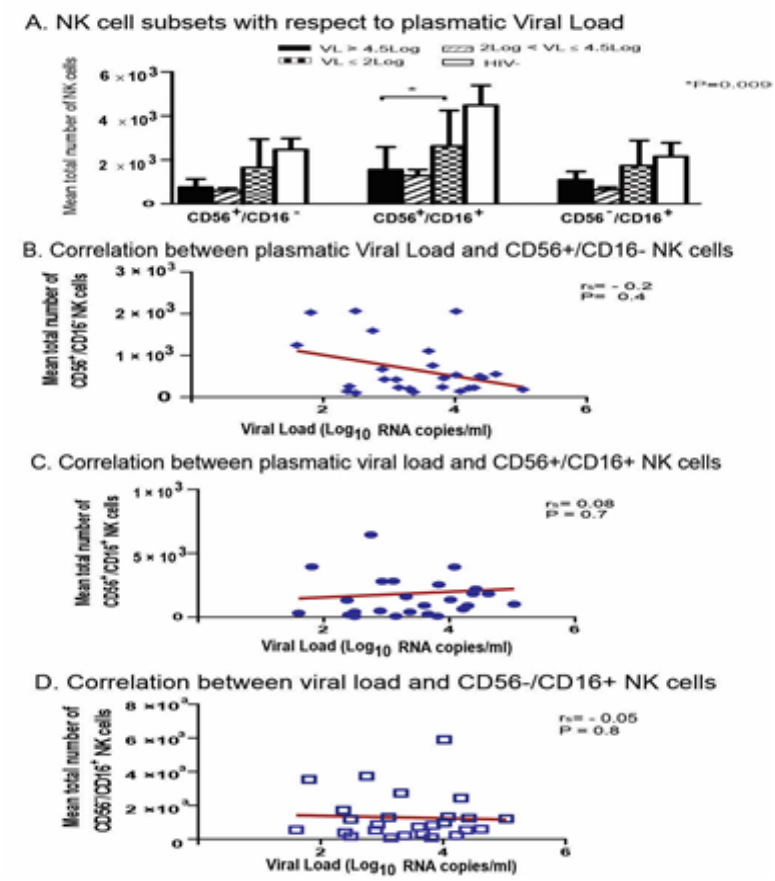
C. A comparison of NK cell subpopulations ($CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$) profile between HIV positive (HIV+) and HIV negative (HIV-) participants is shown. Data are represented as means total number of NK cells ± SD (standard deviation

D. A comparison of NK cell subpopulations ($CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$) profile between HIV positive (HIV+) and HIV negative (HIV-) participants in purified NK cells is shown. Data are represented as means total number of NK cells ± SD (standard deviation). P-values <0.05 were considered as significant, ns (no significant).

3.2. NK cell subpopulations profile

In Figure 1A, lymphocytes were defined from side scatter (SSC) versus forward scatter (FSC) gate (1). NK negative cells and dead cells were excluded from lymphocytes population by gating dumping channel (CD19, CD20, CD14 and CD3) and live-dead respectively (2). NK cells from bulk PBMC and their subsets ($CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$) were defined based on CD56 and CD16 expression level (3) and (4). Purified NK cells were similarly gated and the NK cell subpopulations identified as $CD56^+/CD16^-$, $CD56^+/CD16^+$, and $CD56^-/CD16^+$ in Figure 1B. The purity of NK cells ranged from 90 to 96% with a mean of 93 ± 3%. In bulk PBMCs, results show an elevated (p= 0.003) expression level of $CD56^+/CD16^-$ NK cell subpopulations in HIV-negative controls Vis a Vis HIV-positive group (shown in Fig. 1C). A similar trend was also observed in $CD56^+/CD16^+$ NK cells (p= 0.003). However, $CD56^-/CD16^+$ NK cells present no significant difference (p=0.9) between HIV negative controls and HIV positive participants. However, when we compared the NK cell subpopulations using purified NK cells, there were no significant differences between HIV-positive and HIV-negative participants for the $CD56^+/CD16^-$ and $CD56^+/CD16^+$ subpopulations (p= 0.8 and p= 0.7; respectively). However, the $CD56^-/CD16^+$ NK cell subpopulation significantly increased (p = 0.03) in HIV-positive participants as shown in Figure 1 D, raising the importance of the purification step.

3.3. Impact of plasmatic viral load on NK cells profile



ARV naïve HIV-1 infected people were grouped in three categories according to their viral loads (VL) level: i) VL<2log, ii) 2log< VL ≤ 4.5 log and iii) VL> 4.5 log. Means total number ± SD of $CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$ NK cells were compared within the HIV positive (HIV^+) groups, and with HIV negative (HIV -) participants.
Mean total number of $CD56^+/CD16^-$ NK cells versus VL are represented and a very weak (no) correlation between plasmatic viral load and $CD56^+/CD16^-$ NK cells is shown.
Mean total number of $CD56^-/CD16^+$ NK cells versus VL are represented and no correlation is shown.
Mean total number of $CD56^-/CD16^-$ NK cells versus VL are represented and no correlation is shown.

Figure 2: Impact of plasmatic viral load on NK cells profile.

The relation between NK cells profile and plasmatic viral loads (VL) was next assessed. ARV naïve HIV-1 infected people were grouped into three categories: i) VL<2log, ii) 2log< VL ≤ 4.5 log and iii) VL> 4.5 log. As shown on Figure 2A, NK cell subsets decreased with increased viral loads (VL>4.5log and 2log< VL ≤ 4.5 log). Likewise, HIV-positive participants with low viral loads (VL< 2log) maintain acceptable NK cell subsets levels Vis a Vis HIV-negative participants although not significant (p= 0.06). The number of $CD56^+/CD16^+$ NK cells decreased significantly (p= 0.009) in participants with viral loads more than 2log (VL > 4.5 log and 2log< VL ≤ 4.5 log) when compared to those with viral load less than 2log. A similar trend was observed in $CD56^+/CD16^-$ and $CD56^-/CD16^+$ NK cell subsets but not significant (p= 0.05 and p= 0.07; respectively). A relationship between plasmatic viral load and the three NK cell subpopulations was also assessed. As shown in Figure 2B, the decrease of $CD56^+/CD16^-$ NK cells did not correlate with viral loads (r_s= -0.2, p= 0.4). A similar trend was observed in Figure 2C and 2D between viral loads and $CD56^-/CD16^+$ NK cell expression and between $CD56^+/CD16^+$ NK cells expression and viral loads (r_s= 0.08, p= 0.7, r_s= -0.05, p= 0.8 respectively).

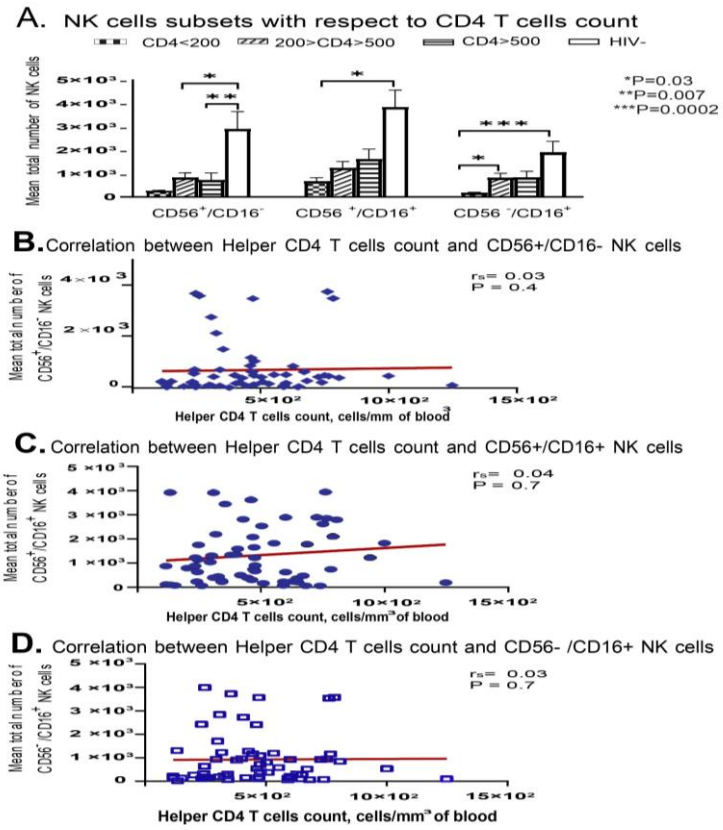


Figure 3: Impact of Helper $CD4^+$ T-cells on NK cells profile

A. ARV naïve HIV-1 infected people were grouped according to helper CD4 T cells count: CD4 < 200, 200 < CD4 ≤ 500 and CD4 > 500. Means total number ± SD of $CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$ NK cells were compared within the HIV positive (HIV+) groups, and with HIV negative (HIV -) participants.

B. Correlation between Helper CD4 T cells count and $CD56^+/CD16^-$ NK cells: Means total number of $CD56^+/CD16^-$ NK cells are represented versus Helpers CD4 T cells count and no correlation is shown.

C. Correlation between Helper CD4 T cells count and $CD56^+/CD16^+$ NK cells: Means total number of $CD56^+/CD16^+$ NK cells are represented versus Helpers CD4 T cells count and no correlation is shown.

D. Correlation between Helper CD4 T cells count and $CD56^-/CD16^+$ NK cells: Means total number of $CD56^-/CD16^+$ NK cells versus Helpers CD4Tcells count are represented and no correlation is shown.

P-value <0.05 was considered as significant. r_s (Spearman coefficient).

3.4. Relationship between the helper $CD4^+$ T-cell count and NK cell subpopulations profile

To evaluate the relationship between helper $CD4^+$ T-cells and NK cell subpopulations profile, ARV naïve HIV-1 infected people were grouped according to helper $CD4^+$ T-cells counts (cells/mm^3 of blood) following the CDC categorization. Taking three groupings consisting of i) HIV positive participants with $CD4^+$ T-cell counts less than 200 cells/mm^3, ii) CD4 between 200 and 500 cells/mm^3, and iii) CD4 more than 500 cells/mm^3. As

shown in Figure 3A, expression of $CD56^-/CD16^+$ NK cells decreased significantly (p= 0.03) when $CD4^+$ T-cells was less than 200 cells/mm^3 compared to 200 < CD4 ≤ 500, and CD4 >500 groups. A similar trend was observed in $CD56^+/CD16^-$ and $CD56^+/CD16^+$ NK cell subsets although not significant. However, a significant reduction in $CD56^+/CD16^-$ and $CD56^+/CD16^+$ NK cell expression was observed (p= 0.03) between HIV positive with CD4 between 200 and 500 cells/mm^3 and HIV negative participants. Moreover, the $CD56^-/CD16^+$ NK cells expression also significantly (p= 0.0002) decreased between HIV positive with CD4 less than 200 cells/mm^3 and HIV negative participants. We then determined the relationship between NK cells profile and helper $CD4^+$ T-cell values. In Figure 3B, 3C and 3D, no correlation between helper $CD4^+$ T-cells and $CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$ NK cell subsets is shown (r_s = 0.03, p = 0.4; r_s = 0.04, p = 0.7 and r_s = 0.03, p = 0.7; respectively).

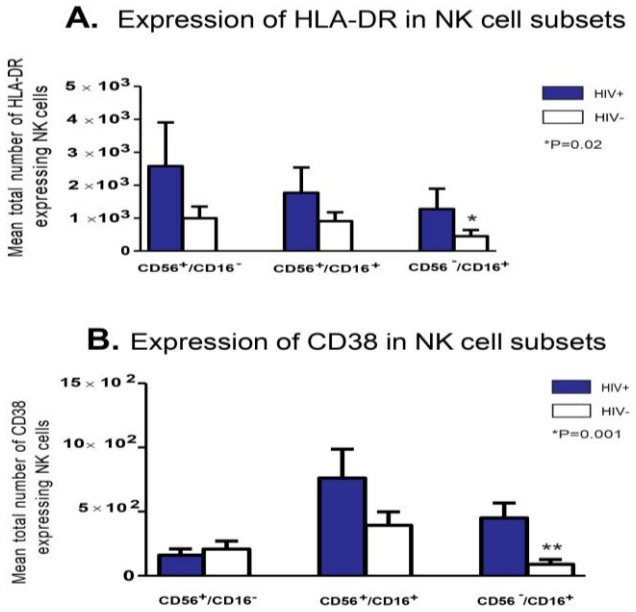


Figure 4: Expression of general activation markers

A. HLA-DR expression in $CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$ NK cells subsets was represented as means total number ± SD of HLA-DR expressing NK cells. A comparison between HIV positive (HIV+) and HIV negative (HIV-) participants is shown.

B. The expression of CD38 in $CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$ NK cells subsets was represented as means total number ± SD of CD38 expressing NK cells. A comparison between HIV positive (HIV+) and HIV negative (HIV-) participants is shown.

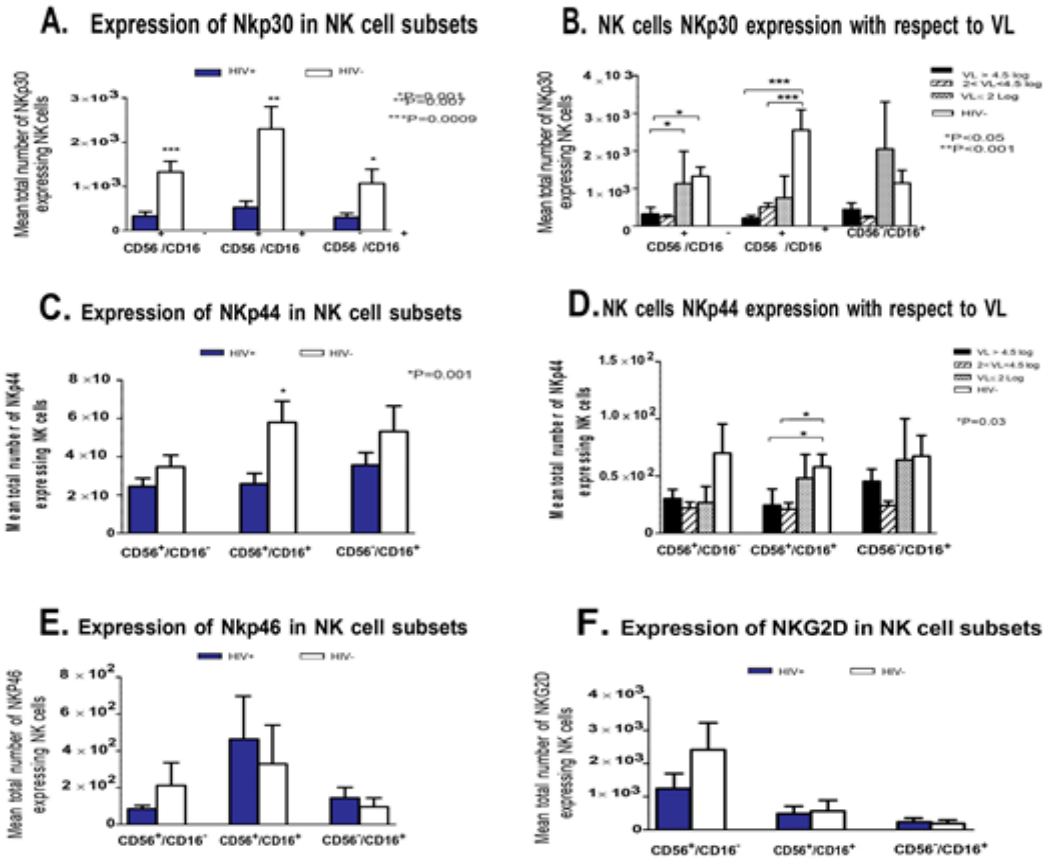
P-values <0.05 were considered as significant.

3.5. Expression of general activation markers

As HIV-1 maintains the immune system in a sustained state of activation, NK cells activation status was further evaluated. To do this, we assessed the expression of CD38 and HLA-DR on NK cell surface for each sub-population. As shown in Figure 4A, HLA-DR was more expressed on HIV positive participants when compared

to HIV negative controls in all NK cells subpopulations ($CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$). However, a significant (p=0.02) increase was observed only in $CD56^-/CD16^+$ NK cells. In Figure 4B, CD38 expression increased significantly (p=0.001) in the $CD56^-/CD16^+$ NK cells subset from HIV positive vis a vis HIV negative control. The same trend was observed with $CD56^+/CD16^+$ NK cells subset, although not significant (p=0.07) while there was no difference between groups in $CD56^+/CD16^-$ NK cells (p=0.6). This increased expression of CD38 on NK cells translates the activated state of NK cells in ARV naïve HIV-1 infection.

3.6. Expression of NK cells cytotoxicity activating markers



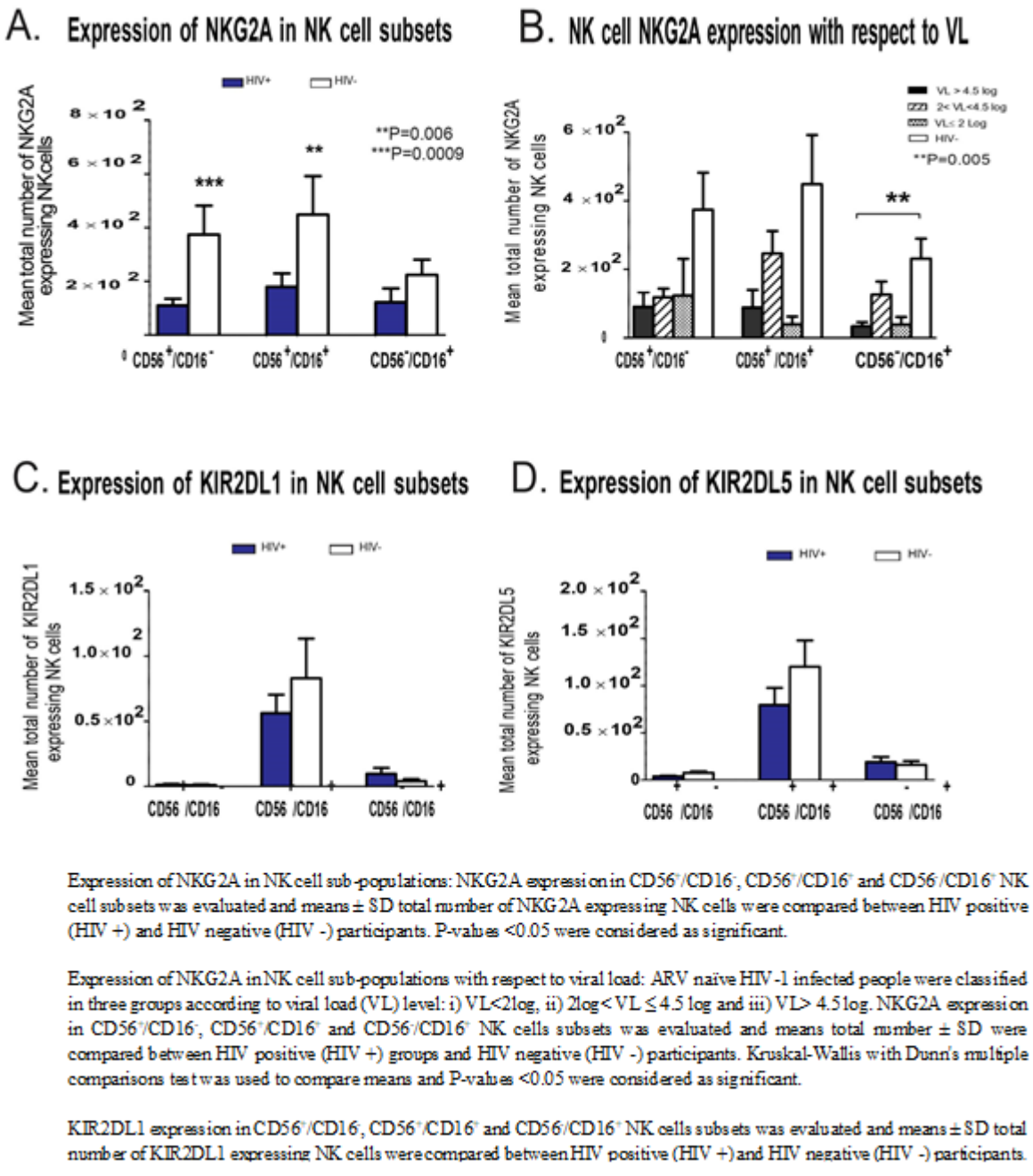
NKp30 expression was evaluated in $CD56^-/CD16^-$, $CD56^+/CD16^-$ and $CD56^-/CD16^+$ NK cell subsets and means ± SD total number of NKp30 expressing NK cells were compared between HIV positive (HIV +) and HIV negative (HIV -) participants.
ARV naïve HIV-1 infected people were classified in three groups according to viral load (VL) level: i) VL<2log, ii) 2log< VL ≤ 4.5 log and iii) VL> 4.5 log. NKp30 expression in $CD56^-/CD16^-$, $CD56^+/CD16^-$ and $CD56^-/CD16^+$ NK cells subsets were evaluated and means total number ± SD were compared within HIV positive (HIV+) groups, and with HIV negative (HIV-) participants.
NKp44 expression was evaluated in $CD56^-/CD16^-$, $CD56^+/CD16^-$ and $CD56^-/CD16^+$ NK cells subsets and means ± SD total number of NKp44 expressing NK cells were compared between HIV positive (HIV +) and HIV negative (HIV -) participants.
ARV naïve HIV-1 infected people were classified in three groups according to viral load (VL) level: i) VL<2log, ii) 2log< VL ≤ 4.5 log and iii) VL> 4.5 log. NKp44 expression in $CD56^-/CD16^-$, $CD56^+/CD16^-$ and $CD56^-/CD16^+$ NK cells subsets were evaluated and means total number ± SD were compared among HIV positive (HIV+) groups, and with HIV negative (HIV -) participants.
NKp46 expression was evaluated in $CD56^-/CD16^-$, $CD56^+/CD16^-$ and $CD56^-/CD16^+$ NK cells subsets and means ± SD

Figure 5: Expression of NK cell cytotoxicity activating markers

The expression of four NK cells activating markers was evaluated; NKp30, NKp44, NKp46 and NKG2D. The impact of viral load on NKp30 and NKp44, was also evaluated. As shown in Figure 5A, NKp30 (activating marker) involved in the natural cytotoxic activity of NK cells was expressed more in HIV negative controls than in HIV positive participants for NK cells subpopulations $CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$ with significant differences (p= 0.0009, p= 0.007 and p= 0.03, respectively). The impact of viral load (VL) on the reduction of NKp30 expression in ARV naïve HIV-1 infected people was subsequently evaluated. As shown in Figure 5B, $CD56^+/CD16^-$ NK cells subset displays a significant reduction of NKp30 expression in HIV-1 positive participants with VL between 2log and 4.5 log compared to those with VL< 2log and HIV negative groups (p= 0.03, p= 0.02, respectively). In the $CD56^+/CD16^+$ NK cells subset a gradual decrease in NKp30 expression between HIV negative individuals and HIV-1 positive people was observed (shown in Fig. 4B). The $CD56^+/CD16^+$ NK cells subset shows a significant reduction in NKp30 expression among HIV-1 positive participants with viral load between 2 to 4.5 log compared to HIV negative (p= 0.0009). The same trend was also observed in HIV-1 positive with VL> 4.5 log (P= 0.0008). In $CD56^-/CD16^+$ NK cells subset, reduced but no-significant expression level of NKp30 was observed in both HIV-1 positive with VL> 4.5 log and 2log< VL ≤ 4.5 log compared to VL> 2 log (p=0.05). Thus, with respect to $CD56^+/CD16^+$ NK cells subset which is able to perform high cytotoxic activity as well as cytokines production, the reduction in NKp30 expression appears to be inversely related to viral load (r_s=- 0.6, p=0.01). As far as NKp44 is concerned, this activating marker was more expressed in $CD56^+/CD16^+$ NK cells subset from HIV negative than ARV naïve HIV-1 positive participants (p= 0.01, shown in Fig. 5C). In contrast, there was no significant (p=0.2) reduction in HIV-1 positive group within both $CD56^+/CD16^-$ and $CD56^-/CD16^+$ NK cells subsets. NKp44 is exclusively expressed on activated NK cells. The expression of this marker shows the activation of NK cells cytotoxicity in both HIV negative and HIV positive people. However, a decreased level of NKp44 might suggest a diminished cytotoxicity of $CD56^+/CD16^+$ NK cells subset. Then, the impact of viral load (VL) on the reduction of NKp44 expression in ARV naïve HIV-1 infected people was assessed. As shown in Figure 5D, $CD56^+/CD16^-$ and $CD56^-/CD16^+$ NK cell subsets showed a diminished but non-significant NKp44 expression in HIV-1 positive participants with VL> 4.5 Log and VL between 2 and 4.5 Log compared to HIV-1 positive participants with VL> 2Log (p= 0.06 and p=0.08, respectively). In the $CD56^+/CD16^+$ NK cells subset there was no difference among HIV-1 positive groups. However, a significant decrease was confirmed in HIV-1 positive participants with VL between 2Log and 4.5 Log compared to HIV negative participants (p= 0.03). The same trend was observed between HIV positive with VL less than 2Log and HIV negative participants (p=0.03). Thus, NKp44 expression in NK cells decreases more with augmented HIV plasmatic viral load but the correlation was not significant (r_s=-0.3, p= 0.4). A third activating marker NKp46 was next assessed. As shown on Figure 5E, there was no difference in the NKp46 expression between HIV positive and HIV negative participants. Yet, in both $CD56^+/CD16^+$ and $CD56^-/CD16^+$ NK cells, a slight but no-significant increased NKp46 expression in HIV positive participants compared to HIV negative individuals was observed (p=0.1 and p= 0.3, respectively). Meanwhile, a decreased expression of this activating marker in the $CD56^+/CD16^-$ NK cell subpopulations from HIV positive participants compared to HIV negative individuals was observed although not-significant (p=0.07). The expression of a fourth but not the less important activating marker was next evaluated. The NKG2D expressing $CD56^+/CD16^-$, $CD56^+/CD16^+$ and $CD56^-/CD16^+$ cells were compared between HIV-1 positive (HIV^+) and HIV negative (HIV^-) participants (shown in Fig. 5F). There was no significant decrease in

the expression of NKG2D in HIV-1 positive participants compared to HIV negative individuals in the $CD56^{+}/CD16^{-}$ NK cells subset (p= 0.06). In addition, no significant differences were also observed between the two groups in both $CD56^{+}/CD16^{+}$ and $CD56^{-}/CD16^{+}$ NK cells sub-populations (p=0.09 and p=0.1, respectively).

3.7. Expression of NK cells cytotoxicity inhibitory receptors



Expression of NKG2A in NK cell sub-populations: NKG2A expression in $CD56^{+}/CD16^{-}$, $CD56^{+}/CD16^{+}$ and $CD56^{-}/CD16^{+}$ NK cell subsets was evaluated and means ± SD total number of NKG2A expressing NK cells were compared between HIV positive (HIV +) and HIV negative (HIV -) participants. P-values <0.05 were considered as significant.

Expression of NKG2A in NK cell sub-populations with respect to viral load: ARV naïve HIV-1 infected people were classified in three groups according to viral load (VL) level: i) VL<2log, ii) 2log< VL ≤ 4.5 log and iii) VL> 4.5log. NKG2A expression in $CD56^{+}/CD16^{-}$, $CD56^{+}/CD16^{+}$ and $CD56^{-}/CD16^{+}$ NK cells subsets was evaluated and means total number ± SD were compared between HIV positive (HIV +) groups and HIV negative (HIV -) participants. Kruskal-Wallis with Dunn's multiple comparisons test was used to compare means and P-values <0.05 were considered as significant.

KIR2DL1 expression in $CD56^{+}/CD16^{-}$, $CD56^{+}/CD16^{+}$ and $CD56^{-}/CD16^{+}$ NK cells subsets was evaluated and means ± SD total number of KIR2DL1 expressing NK cells were compared between HIV positive (HIV +) and HIV negative (HIV -) participants.

Figure 6: Expression of NK cell cytotoxicity inhibitory receptors

As expected, in Figure 6A, $CD56^{+}/CD16^{-}$ and $CD56^{+}/CD16^{+}$ NK cell subpopulations from HIV negative controls expressed increased levels of NKG2A than HIV-1 positive participants (p=0.0009 and p=0.006, respectively). Afterwards, the impact of viral load on the reduced expression of NKG2A in ARV naïve HIV- 1

infected people was assessed. As shown in Figure 6B, there were no significant differences (p=0.06) among HIV-1 positive groups with respect to VL. This result shows that VL was not implicated in the reduction of NKG2A expression in HIV-1 positive participants. Importantly, a significant reduction (p=0.005) of NKG2A expression in $CD56^{-}/CD16^{+}$ NK cells subset for HIV-1 positive (VL> 4.5 log) was confirmed. Thus, the effect of ARV naïve HIV-1 infection was a substantial reduction of NKG2A expression independently of plasmatic viral load. The expression level of inhibitory Killer immunoglobulin-like receptors (KIR) was then evaluated and the results are shown in Figures 6C and 6D. As shown in Figure 5C, KIR2DL1 expression is almost absent in $CD56^{+}/CD16^{-}$ NK cells in both HIV-1 positive and HIV negative participants. However, low levels were observed in $CD56^{-}/CD16^{+}$ NK cells with an elevated but non-significant level in HIV-1 positive participants (p=0.09). Results also show a reduction of KIR2DL1 expression in $CD56^{+}/CD16^{+}$ NK subset in HIV-1 positive participants although non- significant (p=0.08). As shown in Figure 6D, KIR2DL5 expression follows almost a similar trend displayed by KIR2DL1 expression. Low levels were observed in $CD56^{+}/CD16^{-}$ NK cells with a decreased but no-significant (p=0.1) level in HIV-1 positive participants. Moreover, a reduction in KIR2DL5 expression was also observed in $CD56^{+}/CD16^{+}$ NK cells subset from HIV-1 positive participants although no-significant (p=0.07). In $CD56^{+}/CD16^{-}$ NK cells no significant difference was observed between the two groups (p=0.09).

4. Discussion

The present study was designed to characterize NK cells in ARV naïve HIV-1 infected people. In bulk PBMCs, expression levels of $CD56^{+}/CD16^{-}$ and $CD56^{+}/CD16^{+}$ NK cells subpopulations were significantly low in the HIV-1 positive group compared to HIV negative controls, while $CD56^{-}/CD16^{+}$ NK cells subset presented no significant difference between HIV negative controls and HIV positive participants. However, in purified NK cells, there were no significant differences between HIV positive and negative participants for $CD56^{+}/CD16^{-}$ and $CD56^{+}/CD16^{+}$ sub-populations, whereas the $CD56^{-}/CD16^{+}$ NK cells subpopulation significantly increased (p= 0.03) in HIV positive participants. Thus, the $CD56^{-}/CD16^{+}$ NK cells subset was greatly expanded in ARV naïve HIV-1 infected people as previously reported in HIV-infected viremic individuals [24, 26]. Moreover, the expansion of the $CD56^{-}/CD16^{+}$ NK subset in HIV-infected individuals appears to be associated with HIV viremia as also showed in previous studies [26, 27]. However, there was no correlation between the expression of this subset and plasmatic viral loads (rs= -0.05, p=0.8). NK cells activation was evaluated through the expression of CD38 and HLA-DR. The results showed higher co-expression of CD38 and HLA-DR, suggesting the activated state of NK cells in ARV naïve HIV-1 infection as previously suggested by kuri-Cervantes and his colleagues [11]. This can explain the aberrant activation and dysfunction previously suggested in the context of antiretroviral therapy [28]. In addition, NK cells are more activated in ARV naïve HIV-1 participants than in HIV negative controls. This activation might increase cytotoxic activity and high expression of NK cells cytotoxic activating markers. CD336 (NKp44), CD337 (NKp30) and CD335 (NKp46) are members of the natural cytotoxicity receptors (NCRs) which are responsible for NK cell activation in the process of natural cytotoxicity. They are exclusively found on the surface of activated natural killer (NK) cells [14, 29]. The reduction of activating NCR (NKp44 and NKp30) observed on the $CD56^{-}/CD16^{+}$ NK cells subset is in accordance with results obtained by Marvilio and collaborators [24] and could contribute to a reduced NK cells ability to eliminate HIV virus infected cells. In regard to $CD56^{+}/CD16^{+}$ NK subset which is able to perform high

cytotoxic activity as well as cytokines production, the reduction in NKp30 and NKp44 expression was related to high plasmatic viral load. Their expression in NK cells significantly decreases more with augmented HIV plasmatic viral load. This might predict NK cells reduced activity in ARV naïve HIV-1 infection. Nevertheless, the reduction in those NCR may be due to the potentially elevated levels of some cytokines in the serum of AIDS individuals [30, 31] that have been shown to modulate the expression of NCR such as TGF, which down-regulates the levels of NKp30 [32] . In contrast to others [26, 33], NKp46 expression was higher in HIV positive participants NK cells ($CD56^+/CD16^+$ and $CD56^-/CD16^+$) than in HIV negative individuals. Thobakgale and collaborators [34] showed that NK cells that produced IFN-γ in response to HIV peptides had significantly lower expression of NKp46. Therefore, our results suggest a predicted low IFN-γ production by $CD56^+/CD16^+$ and $CD56^-/CD16^+$ subsets. Similarly, reduced NKp46 expression of activated NK cells has also been described previously, but different studies have suggested different conclusions [24, 35, 36]. Moreover, there was no significant difference in the activating natural killer group 2, member D (NKG2D) expression (p= 0.06) in HIV positive participants compared to HIV negative individuals in the $CD56^+/CD16^+$ NK cells subset. This result is different from the one previously suggested with ART-treated HIV positive individuals by Luo and collaborators [37] where NKG2D-expressing NK cells increased in the $CD56^{dim}/CD16^+$ ($CD56^+/CD16^+$) subset among immunologic non-responders ($CD4^+$ T cells below 350 cells/mm3) compared to immunologic responders ($CD4^+$ T cells above 500 cells/mm3) and HIV negative people. NKG2D has been shown to regulate NK cell cytotoxicity and cytokine production [38]. Therefore, from this study, results show that without ART treatment, NK cells especially $CD56^+/CD16^+$ subset is activated and maintains the ability to regulate cytotoxic activity and cytokine production through the expression of NKG2D as previously shown [18, 39]. NK cells cytolytic activity is induced through NCR-target cell interaction and can be inhibited by interaction of target cell HLA molecules with certain killer immunoglobulin-like receptors (KIRs). In our study, we evaluated the expression of three inhibitory receptors namely NKG2A, KIR2DL1 and KIR2DL5. NK cells cytolytic activity is induced through NCR-target cell interaction and can be inhibited by interaction of target cell HLA molecules with certain killer immunoglobulin-like receptors (KIRs). In this study, there was a significant reduction in NKG2A expression in HIV positive when compared to HIV negative participants in both $CD56^+/CD16^-$ and $CD56^+/CD16^+$ NK cells subpopulations (p=0.0009, p= 0.006, respectively). However plasmatic VL was not implicated in the reduction of NKG2A expression in HIV positive participants. The down-regulation of NKG2A suggests an augmented NK cells killing activity as the reduction of this marker is supposed to allows the high expression of activating receptor and increase the cytotoxicity at the same time. The reduction, although non-significant of KIR2DL1 and KIR2DL5 expression in $CD56^+/CD16^+$ NK subset in HIV positive participants observed can be explained by a predicted low level of IL-10, which is known to increase the expression of several inhibitory NK receptors (iNKRs) [40]. However, there was a slight increase of KIR2DL1 (not-significant) in $CD56^-/CD16^+$ subset in HIV positive. The present study shows some alterations in NK cell phenotype and function and outlines the potential mechanisms of these abnormalities by demonstrating that the expression of NCRs is low or negative whereas that of certain iNKRs is elevated on the NK cells subpopulations. The $CD56^+/CD16^-$ and $CD56^+/CD16^+$ down-regulation, the high expression of $CD56^-/CD16^+$ and altered expression of NCRs and iNKRs may reflect persistent cellular activation as previously seen in HIV viremic context [36, 41] and NK cells function in spite of HIV infection.

5. Conclusion

In summary, NK cells from antiretroviral naive HIV-1 infected people living in Cameroon show phenotypic alterations including the down-regulation of $CD56^{+}/CD16^{-}$ and $CD56^{+}/CD16^{+}$ NK cells, the expansion of $CD56^{-}/CD16^{+}$ NK cells subset, the high expression of both CD38 and HLA-DR, the reduced expression of activating receptors NKp44 and NKp30 and the reduction in the expression of some inhibitory receptors including NKG2A, KIR2DL1, and KIR2DL5. As there was no association between NK cell subpopulations profile and either helpers $CD4^{+}$ T cell counts or plasmatic viral loads, additional studies would be needed to elucidate the mechanism driving the dysregulation of NK cell receptors within NK cell subsets in the context of HIV infection. This study provides new prospects for NK-cell-directed immunotherapeutic strategies for the long-term management of HIV-1 infection.

6. Limitations and Recommendations

Due to the WHO recommendation since 2015 (« Test and treat ») it was difficult to realize large cohort studies, as the number of ARV naïve HIV-1 infected people decreased. The ultimate goal been to design novel immunotherapeutic strategies exploiting NK cells for the long-term management of HIV-1 infection, we recommend to carry out additional studies to elucidate the mechanisms driving the dysregulation of NK cells receptors in the context of HIV-1 infection and evaluate the restauration under ARV treatment. NK cells exert immune pressure on HIV and should be specifically targeted in therapeutic and preventive interventions.

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7. Conflicts of Interest

The authors declare no conflict of interest.

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9. Author Contributions

GN and CSS conceived and designed the study; CSS designed and performed the experiments; JCT, GA, LN, AL, TFT, and LD provided the technical assistance at different steps. CSS analyzed all the data, drafted and wrote the manuscript. GN, GA and F-XE revisited the article for important intellectual content.

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Appendix 1: Bulleted list

- Flow cytometry to define NK cell profile in ARV naïve HIV-1 infection
- High activation status of the $CD56^{-}/CD16^{+}$ NK cells subset from ARV naïve HIV-1 infected people

- Down-regulation of $CD56^{+}/CD16^{-}$ and $CD56^{+}/CD16^{+}$ NK cells in ARV naïve HIV-1 infection
- Expansion of $CD56^{-}/CD16^{+}$ NK cells subset from ARV naïve HIV-1 infected people

Appendix 2: Abbreviations List

ADCC: Antibody-Dependent Cellular Cytotoxicity

A700: Alexa fluor 700

APC: Allophycocyanin

ARV: Antiretroviral

CD: Cluster of differentiation

CDC: Centre of Disease Control

EDCTP: European and Developing Countries Clinical Trial Partnership

EDTA: Ethylene diamine-tetra acetic acid

FACS: Fluorescence Activated Cell Sorting

Fc: Fragment crystallizable

FcR: Fragment crystallizable Receptor

FITC: Fluorescein isothiocyanate

FSC: forward scatter

HIV: Human Immunodeficiency Virus

HLA: Human Leucocyte Antigen

HLA-DR: Human Leucocyte Antigen- DR Isotype

INF: Interferon

INKRs: Inhibitory natural killer cells receptors

KIRs: Killer Immunoglobulin like Receptors

MHC: Major histocompatibility complex

NCR: Natural cytotoxicity receptor

NK: Natural killer

NKG2A: Natural-killer group 2, member A

NKG2D: Natural-killer group 2, member D

NKp30: Natural killer cell p30-related protein

NKp44: Natural killer cell p44-related protein

NKp46: Natural killer cell p46-related protein

PBS: Phosphate Buffered Saline

PBMC: Peripheral blood mononuclear cells

PE: Phycoerythrin

RNA: Ribonucleic Acid

SPSS: Statistical Package for Social Sciences

SSC: Side scatter

VL: viral load

WHO: World Health Organization