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Myeloid and lymphoid activation markers in AIDS and non-AIDS presenters

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ABSTRACT

HIV infection is characterized by a state of chronic activation of the immune system, which is not completely reversed by antiretroviral treatment (ART). The aim of this study was to assess myeloid and lymphoid activation markers during HIV infection, before and one year after ART initiation, in AIDS and non-AIDS presenters. Treatment naïve HIV positive patients were enrolled in this study. Myeloid dendritic cell (mDC), plasmacytoid dendritic cell (pDC), slanDC, monocyte and T-lymphocyte cell counts and activation status, were assessed by flow cytometry in peripheral blood samples. Soluble (s)CD14 and sCD163 were assessed in plasma samples using ELISA assays. Statistical analyses were performed using GraphPad Prism and Minitab Express.

Thirty-four ART naïve HIV-1 infected subjects were enrolled in this study (22 non-AIDS and 12 AIDS presenters). Seventeen healthy donors (HD) were included as control group. Although circulating mDC levels resulted unchanged, HLA-DR expression was decreased on mDCs of HIV positive subjects compared to HD ($p < 0,0001$). AIDS presenters showed the lowest level of expression of HLA-DR on mDCs. Circulating levels of pDCs were decreased in HIV patients compared to HD ($p < 0,001$), without any changes in HLA-DR expression. SlanDC cell counts were extremely reduced in AIDS presenters, compared to non-AIDS presenters and HD ($p < 0,01$ and $p < 0,0001$, respectively) and showed higher HLA-DR expression in HIV patients compared to HD ($p < 0,01$). Intermediate monocyte (IM) cell counts were increased in AIDS and non-AIDS presenters compared to HD ($p < 0,001$ and $p < 0,001$ respectively). Furthermore, IM expansion was directly correlated to HIV viral load ($p = 0,036$) and independent from CD4 cell counts and activation levels. Plasma concentrations of sCD14 and sCD163 resulted increased in HIV infected subjects compared to HD ($p < 0,0001$ and $p < 0,001$), with the highest levels observed in AIDS presenters. After 1 year, ART was able to increase pDC and decrease IM absolute cell counts and modify HLA-DR expression on mDCs and slanDCs, approaching the levels observed in HD. ART reduced also CD4 and CD8 activation levels. In conclusion, in untreated HIV infected subjects circulating dendritic cells resulted altered either in numbers or in HLA-DR expression, especially in AIDS presenters. IM absolute counts were equally increased in AIDS and non-AIDS presenters. ART was able to reduce myeloid and lymphoid inflammation in both advanced and non-advanced HIV patients, confirming the role of ART in hampering disease progression and immune activation associated non-AIDS events.

1. Introduction

With the advent of highly active and less toxic antiretroviral therapies, both life expectancy and quality of life of persons living with HIV have improved significantly (Samji et al., 2013). However, the persistence of the virus in the host and its ongoing replication, lead to a state of chronic activation of the immune system, which is not thoroughly reversed by antiretroviral treatment (ART) (Appay and Sauce, 2008; Hearps et al., 2012). This has brought to the emergence of a series of complications, known as non-AIDS-related diseases (e.g. cardiovascular diseases, renal impairment, bone alterations, cancer, mild neurocognitive impairment), which represent the most important contributors to morbidity and mortality in HIV infected people nowadays (Novoa et al., 2008). Lymphoid and myeloid cells both contribute to the genesis of chronic inflammation in HIV infection (Younas et al., 2016). Among the cellular components of the innate immune system, dendritic

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cells (DCs) are specialized antigen presenting cells (APCs) that are able to prime naïve $CD4^+$ and $CD8^+$ T-lymphocytes (Banchereau and Steinman, 1998). During HIV infection, a decrease in circulating plasmacytoid (pDCs) and myeloid (mDCs) dendritic cells has been described, together with an impairment of their function, consisting of an alteration in the expression of surface markers (such as HLA-DR) and the production of cytokines (Donaghy et al., 2001; Pacanowski et al., 2001; Sachdeva et al., 2015; Tufa et al., 2016).

Schäkel and colleagues identified "slanDCs" as an additional population of myeloid cells that shares functional and phenotypic characteristics with mDCs. These cells selectively express the 6-sulfo LacNAc1 (slan) carbohydrate modification of P-selectin glycoprotein ligand-1 (PSGL-1), which is specifically recognized by the monoclonal antibody M-DC8 (Schäkel et al., 1998). A few studies in viremic HIV positive patients, have reported an increase in frequency and numbers of slanDCs, which are major producers of pro-inflammatory cytokines, such as TNF-α and IL1β (Dutertre et al., 2012; Lichtner et al., 2016; Schäkel et al., 2002; Tufa et al., 2016). Besides DCs, innate immune responses rely also on monocytes, which represent a heterogeneous population consisting of $CD14^{++}CD16^-$ classical monocytes (CM), $CD14^{++}CD16^+$ intermediate monocytes (IM) and $CD14^{low}CD16^{++}$ non-classical monocytes (NCM) (Ziegler-Heitbrock, 2014). An expansion of $CD16^+$ inflammatory monocytes has been described during the course of HIV infection, together with an increased production of pro-inflammatory cytokines, such as TNF-α and IL-1α (Hearps et al., 2012; Thieblemont et al., 1995). Moreover, the increase in circulating IM in HIV infection has been related to disease progression and the development of coronary syndromes and central nervous system complications (Fischer-Smith et al., 2001; Funderburg et al., 2012; Han et al., 2009). Soluble markers of monocyte/macrophage activation are also increased during HIV infection. The soluble form of the bacterial lipopolysaccharide (LPS) receptor CD14 (sCD14), shed from the surface of activated monocytes, is a well-known marker of microbial translocation, possibly contributing to disease progression and immune activation (Krastinova et al., 2015; Nockher et al., 1994; Sandler et al., 2011). In addition to this, increased expression of the scavenger receptor CD163 on the surface of monocytes and macrophages, along with elevated levels of its soluble form, sCD163, have been described in HIV positive patients, in association with a concomitant increase in circulating $CD16^+$ monocytes and activated $CD8^+$ T-lymphocytes (Burdo et al., 2011a,b; Tippett et al., 2011). Besides, high plasma concentrations of sCD163 in HIV positive individuals seem to contribute to the development of coronary plaques, neurocognitive impairment, and predict all-cause mortality (Burdo et al., 2013; Burdo et al., 2011a,b; Knudsen et al., 2016; Vita et al., 2017), suggesting a prominent role of monocyte activation in the development of non-AIDS events. Considering the lymphoid compartment, increased expression of activation markers, such as CD38 and HLA-DR, by $CD8^+$ and $CD4^+$ T-lymphocytes has been described during HIV infection as an independent risk factor for disease progression, only partially controlled by ART (Deeks et al., 2004; Giorgi et al., 1993; Kestens et al., 1994). Changes in both myeloid and lymphoid activation due to ART have been pointed out in several studies. Early start of ART leads to a decrease of T-lymphocytes activation, especially for $CD8^+$ T-cells (Deeks et al., 2004). Conversely, monocyte activation is only partially controlled by ART, even when long-term viral suppression is achieved, and changes occur especially during the first year of ART (Hattab et al., 2015; Wada et al., 2015). To our knowledge, the role of myeloid and lymphoid activation markers in predicting immunological recovery and virological suppression has been investigated in a few studies so far.

The aim of our study was to assess myeloid and lymphoid activation markers during HIV infection, before treatment initiation, their reciprocal influence and the impact in predicting immunological recovery, by using an easy-to-perform flow cytometric analysis on peripheral blood. In a subgroup of subjects, antiretroviral treatment-induced modifications on myeloid and lymphoid activation markers were also assessed.

2. Materials and methods

2.1. Study populations and sample collection

Treatment naïve HIV positive patients willing to participate were consecutively enrolled in this study over a period of 12 months. HIV viral load and CD4 cell counts were routinely assessed. Healthy volunteers were age and sex matched with HIV positive patients according to a 1:2 ratio. HIV-1 infected patients as well as healthy donors were enrolled in two HIV clinics of the Department of Public Health and Infectious Diseases of Sapienza University of Rome. The study was approved by the local ethic committee (protocol number 131/13) and all patients signed a written informed consent before enrolment in the study. Blood samples were collected using EDTA tubes, to avoid any interference of heparin on CD16 expression (Tippett et al., 2011).

2.2. Peripheral blood DCs, monocytes and T-lymphocyte immune activation

Peripheral blood DC and monocyte cell counts were assessed using flow cytometry, following a previously published protocol from our group (Lichtner et al., 2016). Briefly, a lyse-no-wash method was utilized, according to which 50 µl of whole blood were stained with the following monoclonal antibodies: CD3-PerCP (clone BW264/56), CD20-PerCP (clone LT20), CD56-PerCP (clone REA196), HLA-DR-APC (clone AC122), CD14-VioGreen (clone TUK4), CD11-PE (clone MJ4-27G12), M-DC8-FITC (clone DD-1) from Miltenyi Biotec (Bergisch Gladbach, Germany); CD235a-PerCP (clone HI264, diluted 1:100 with Phosphate buffer saline, PBS), CD16-PE Vio770 (clone 3G8), CD123-BV (clone 6H6) from BioLegend, Inc (San Diego, CA, USA). After an incubation of 30 min, at room temperature (RT), in the dark, a fixed volume of lysing solution (BD Biosciences, San Jose, CA, USA) was added and, after another 10-minute incubation, samples were acquired on a MACSQuant flow cytometer, equipped with a peristaltic syringe for absolute cell counting. Calibration of the instrument was performed on a daily base using MACSQuantTM Calibration Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Fluorescence compensation was performed with a dedicated software by using compensation beads (BDTM CompBeads Anti-Mouse Ig, k and BDTM CompBeads Negative Control) from BD Biosciences (San Jose, CA, USA).

Activation of $CD4^+$ and $CD8^+$ T-lymphocytes was analysed in a single tube with 50 µl of whole blood and a mix of the following monoclonal antibodies: CD45-VioBlue (clone 5B1), HLA-DR-FITC, (clone AC122) CD38-APC (clone IB6), CD8 PerCP (clone BW135/80) and CD4-PE (clone M-T466), from Miltenyi Biotec (Bergisch Gladbach, Germany). After an incubation period of 20 min, at RT and in the dark, a fixed volume of lysing solution was added and after 10 min of incubation tubes were acquired on a MACSQuant flow cytometer and analysed, as previously indicated. Data were analysed using FlowJo software v.10 (FlowJo LLC, Ashland, OR, USA) and gating strategies are shown in supplementary Fig. 1 and 2.

2.3. sCD14 and sCD163 plasma levels

Plasma samples were stored at -80° until analysed. Soluble immune activation markers sCD14 and sCD163 were determined using ELISA assays (human sCD14 Quantikine ® ELISA, R&D Systems, Inc. Minneapolis, MN, USA and Human sCD163 Ready-SET-Go, Affymetrix eBioscience, San Diego, CA, USA) according to manufacturer's instructions and samples were tested in duplicate.

2.4. Statistical analyses

Statistical analyses were performed using GraphPad Prism ver. 6.0 and Minitab Express ver. 1.5.0 software. Mann-Whitney and Kruskal-Wallis tests were used to compare independent data between two and

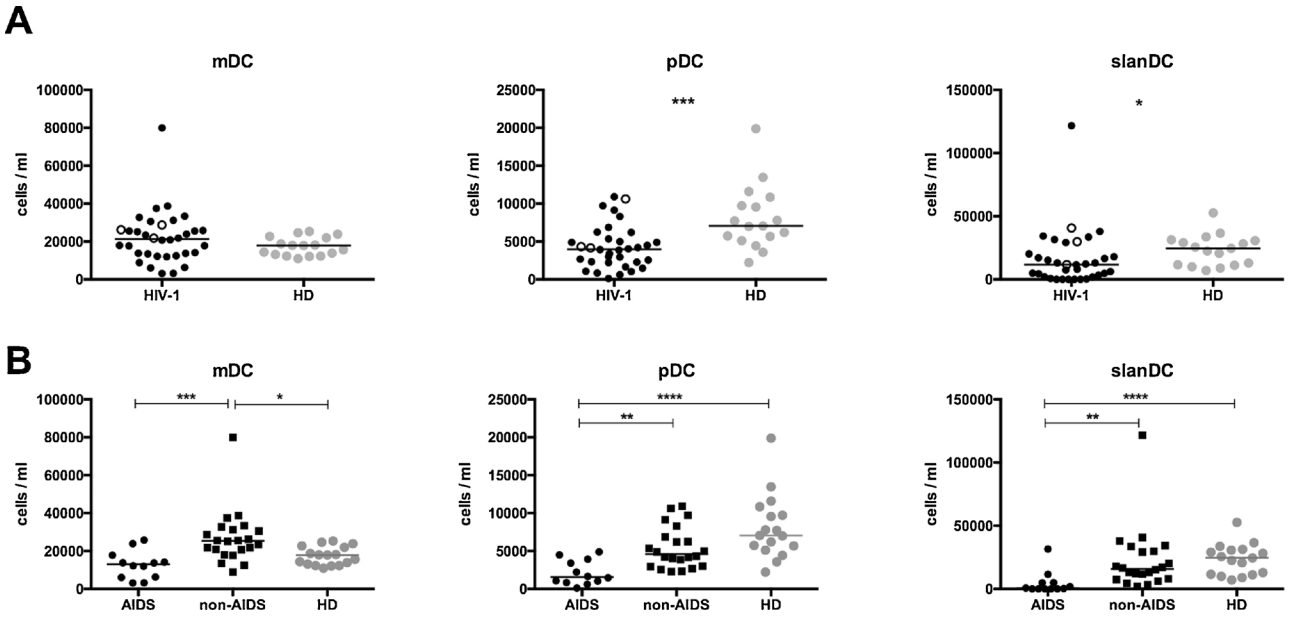

Fig. 1. Dendritic cell numbers in HIV positive subjects and healthy donors.
Dendritic cell numbers were evaluated in HIV infected subjects and HD: mDC cell counts (medians): 21.328 vs 17.835 cells/ml p = 0,23; pDC cell counts (medians): 3.976 vs 7.043 cells/ml, p < 0,001; slanDC cell counts (medians): 11.644 vs 24.538 cells/ml p = 0,02. Statistical analysis was performed by using the Mann-Whitney test (A).
After stratification according to clinical stage, pDC, slanDC and mDC cell counts were compared among AIDS presenters, non-AIDS presenters and HD. For mDC (medians): 12.950 vs 25.333 vs 17.835 cells/ml p < 0,001. For pDC (medians): 1.562 vs 4.601 vs 7.043 cells/ml, p < 0.0001. For slanDC (medians): 511 vs 15.847 vs 24.538 cells/ml, p < 0,001. Kruskal-Wallis test was employed for statistical analysis. Dunnett's post-test was used for comparing the different groups and asterisks indicate the level of significance (B).
Empty circles represent the values observed in the elite controller and the long-term non-progressors. Lines represent median values.
mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells. slanDCs: slan dendritic cells. HD: healthy donors.
*: 0.01 < p < 0,05; **: 0,001 < p < 0,01; ***: 0.0001 < p < 0,001; **** p < 0,0001.

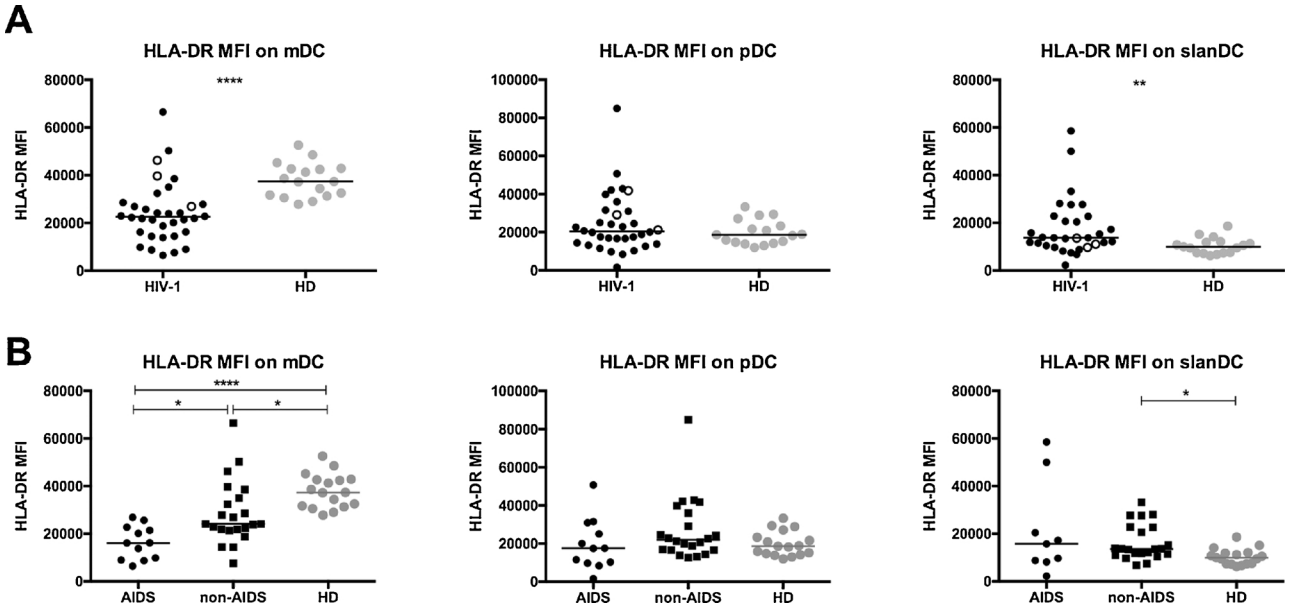

Fig. 2. Dendritic cell HLA-DR expression on DC of HIV positive subjects and healthy donors.
HLA-DR expression on dendritic cells was evaluated in HIV infected subjects and HD. HLA-DR on mDCs (medians): 22.556 vs 37.358 p < 0,0001. HLA-DR on slanDCs (medians): 13.680 vs 9979 p = 0,005, respectively. HLA-DR on pDCs (medians): 20.388 vs 18.602 p = 0,49. Statistical analysis was performed by using the Mann-Whitney test (A).
After stratification according to clinical stage HLA-DR expression on pDCs, slanDCs and mDCs were compared among AIDS presenters, non-AIDS presenters and HD. HLA-DR on mDCs (medians): 16.198 vs 24.166 vs 37.358 p < 0,0001. The analysis also showed a linear trend from AIDS through non-AIDS to HD (ANOVA for linear trend p < 0,0001). HLA-DR on pDCs (medians): 17.525 vs 21.938 vs 18.602 p = 0,19. HLA-DR on slanDCs (medians): 15.748 vs 13.623 vs 9979 p = 0,02. Kruskal-Wallis test was employed for statistical analysis. Dunnett's post-test was used for comparing the different groups and asterisks indicate the level of significance (B).
Empty circles represent the values observed in the elite controller and the long-term non-progressors. Lines represent median values.
mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells. slanDCs: slan dendritic cells. HD: healthy donors.
*: 0.01 < p < 0,05; **: 0,001 < p < 0,01; ***: 0.0001 < p < 0.001; **** p < 0,0001.

Table 1
Demographic and clinical characteristics of HIV-1 positive subjects and healthy donors.

	HIV-1	HD
Number	34	17
Age: median [IQR]	37 [28–44]	37 [30–49]
Sex: M/F	26/8	13/4
VL: median [IQR]	4,9 log/ml [4,1–5,5]	NA
# CD4: median [IQR]	434 cells/μl [101–656]	NA

IQR: interquartile range; M: male; F: female; NA: not applicable; HD: healthy donors.

three groups, respectively. Wilcoxon test was employed for paired data analysis. Correlations analyses were performed using the Spearman test. Results were considered statistically significant if the p value was < 0.05.

3. Results

3.1. Patients

Thirty-one subjects recently diagnosed with HIV-1 infection, one HIV-1 positive elite controller and two long-term non-progressors were enrolled in the study. Median age was 37 (interquartile range [IQR] 28–44) with a prevalence of male sex (males: 26, females: 8). Median HIV-1 viral load was 4,9 log/ml (IQR: 4,1-5,5) and median CD4 cell count was 434 cells/μl (IQR: 101-656) (Table 1). Considering clinical features and according to CDC classification, 20 patients met the criteria for the clinical category "A" (13 were A1, 5 were A2 and 2 were A3), 5 for the category "B" (1 was B1, 3 were B2 and 1 was B3) and 9 for the clinical category "C" (all C3). According to CDC classification, 22 were non-AIDS and 12 were AIDS presenters. Antiretroviral treatment (ART) was administered to 31 patients, while the elite controller and the two long-term non-progressor patients did not receive any treatment, according to national guidelines at the time of enrolment in this study. Considering ART regimen, 15 patients were started on a protease inhibitor (PI)-based first line regimen, 10 patients on a non-nucleoside reverse-transcriptase inhibitor (NNRTI) and 6 patients on an integrase strand transfer inhibitor (INSTI) (Table 2). No differences in HIV-1 viral load or CD4 cell counts were observed among the three therapeutic groups at baseline. Considering comorbidities three patients had had a previous hepatitis B virus (HBV) infection while no patients were co-infected with Hepatitis C virus (HCV).

To minimize the well-known effect of age on monocytes (Seidler et al., 2010) age and sex matching (patient/control ratio 2/1) was performed, including a group of 17 healthy donors (Table 1).

Table 2
Demographic and clinical characteristics of HIV-1 positive subjects according to CDC classification.

	Non-AIDS presenters	AIDS presenters
Number	22	12
Age: median [IQR]	34 [27–44]	42,5 [31–45]
Sex: M/F	17/5	9/3
VL: [IQR]	4,3 log/ml [3,9–5,1]	5,5 log/ml [5,3–6.0]
# CD4: median [IQR]	569 cells/μl [456–796]	50 cells/μl [21–101]
CDC classification	A1: 13 A2: 5 B1: 1 B2: 3	A3: 2 B3: 1 C3: 9
ART (PI/NNRTI/INSTI)[a]	7/7/5	8/3/1
Patients without treatment[b]	3 (1 EC and 2 LTNP)	0

IQR: interquartile range; M: male; F: female; NA: not applicable; CDC: center for disease control; ART: antiretroviral treatment. PI: protease inhibitor; NNRTI: non-nucleoside reverse-transcriptase inhibitor; INSTI: integrase strand transfer inhibitor; EC: elite controller; LTNP: long term non-progressors.

[a] ART started after the enrolment in this study.

[b] Patients who did not need to start ART according to Italian guidelines at the time of enrolment.

3.2. Dendritic cells at baseline

Before starting ART DC, monocyte, T-cell counts and activation status were assessed through flow cytometric analysis of peripheral blood, with a lyse-no-wash protocol. Specifically, DCs were identified in the lineage^{neg}HLA-DR^{+} gate as CD14$^{low/-}$CD123^{+} events for pDCs and CD14$^{low/-}$CD123-CD11c^{+} events for mDCs. Furthermore, the slanDCs subset (also known as M-DC8^{+} cells) was identified in the CD14^{low}CD16^{++} gate as CD11c^{+}M-DC8^{+} events. At baseline, pDCs and slanDCs were reduced in HIV-1 infected patients compared to HD ($p < 0.001$ and $p = 0.02$, respectively), while no differences were observed in mDC count when comparing the HIV-1 infected subjects with HD (Fig. 1A). After stratifying patients according to the CDC clinical classification in AIDS and non-AIDS presenters, pDCs, slanDCs and mDCs were significantly reduced in AIDS presenters than in non-AIDS presenters and HD ($p < 0.0001$; $p < 0.001$; $p < 0.001$, for pDCs, slanDCs and mDCs, respectively) (Fig. 1B). Interestingly, mDCs seemed to be increased in non-AIDS rather than in AIDS presenters and controls (Fig. 1B).

Considering HLA-DR as an activation marker, we found that its expression was decreased on mDCs and increased on slanDCs of HIV-1 infected subjects compared to HD ($p < 0.0001$ and $p = 0.005$, respectively). No differences in levels of expression of HLA-DR on pDCs were observed (Fig. 2A). After stratifying HIV positive patients according to the CDC clinical stage, AIDS presenters showed the lowest values for HLA-DR MFI on mDCs, when compared to non-AIDS presenters and HD, with an increasing linear trend going from AIDS through non-AIDS presenters, to HD (ANOVA with post-test for linear trend $p < 0.0001$). No significant differences were observed in the expression of HLA-DR on pDCs and slanDCs between AIDS and non-AIDS presenters (Fig. 2B). mDC, pDC and slanDC absolute counts and the corresponding HLA-DR expression obtained from the elite controller and the two long-term non-progressors were apparently similar to those measured in the control group, even if a statistical analysis was not performed, owing to the limited number of subjects (Figs. 1 and 2A).

3.3. Monocytes at baseline

Taking into consideration CD14 and CD16 expression on monocytes, three subpopulations were identified: classical monocytes (CM: CD14^{++}CD16^{-}), intermediate monocytes (IM: CD14^{++}CD16^{+}) and non-classical monocytes (NCM: CD14^{low}CD16^{++}). No differences in NCM absolute cell counts were observed when comparing HIV-1 infected patients and HD at baseline. Conversely, CM and IM absolute cell counts were increased in HIV-1 infected patients compared to HD ($p = 0.035$ and $p < 0.001$ respectively). Considering monocyte subsets relative counts as percentages of the total monocytes, only IM percentages were significantly increased in HIV-1 positive subjects compared to HD ($p < 0.0001$) (Fig. 3A). Concerning HLA-DR expression, IM showed the highest median fluorescence intensity (MFI) among the three different monocyte subsets, in both HIV-1 infected subjects and HD ($p < 0.0001$) (Fig. 3B). Considering CDC clinical stage, CM were increased in non-AIDS presenters compared to AIDS-presenters and controls ($p = 0.011$) and after applying the post-test analysis the difference remained statistically significant only when comparing non-AIDS with HD. NCM absolute counts were reduced in AIDS presenters compared to non-AIDS presenters and HD ($p = 0.02$) and the difference was statistically significant only between AIDS and non-AIDS presenters, after applying the Dunnett's post-test. Although IM absolute counts were higher in AIDS and non-AIDS patients than in HD ($p < 0.0001$), no differences were observed between the two groups of HIV-infected subjects (Fig. 3C). HLA-DR MFI was increased in CM of non-AIDS presenters compared to HD, while no other statistically

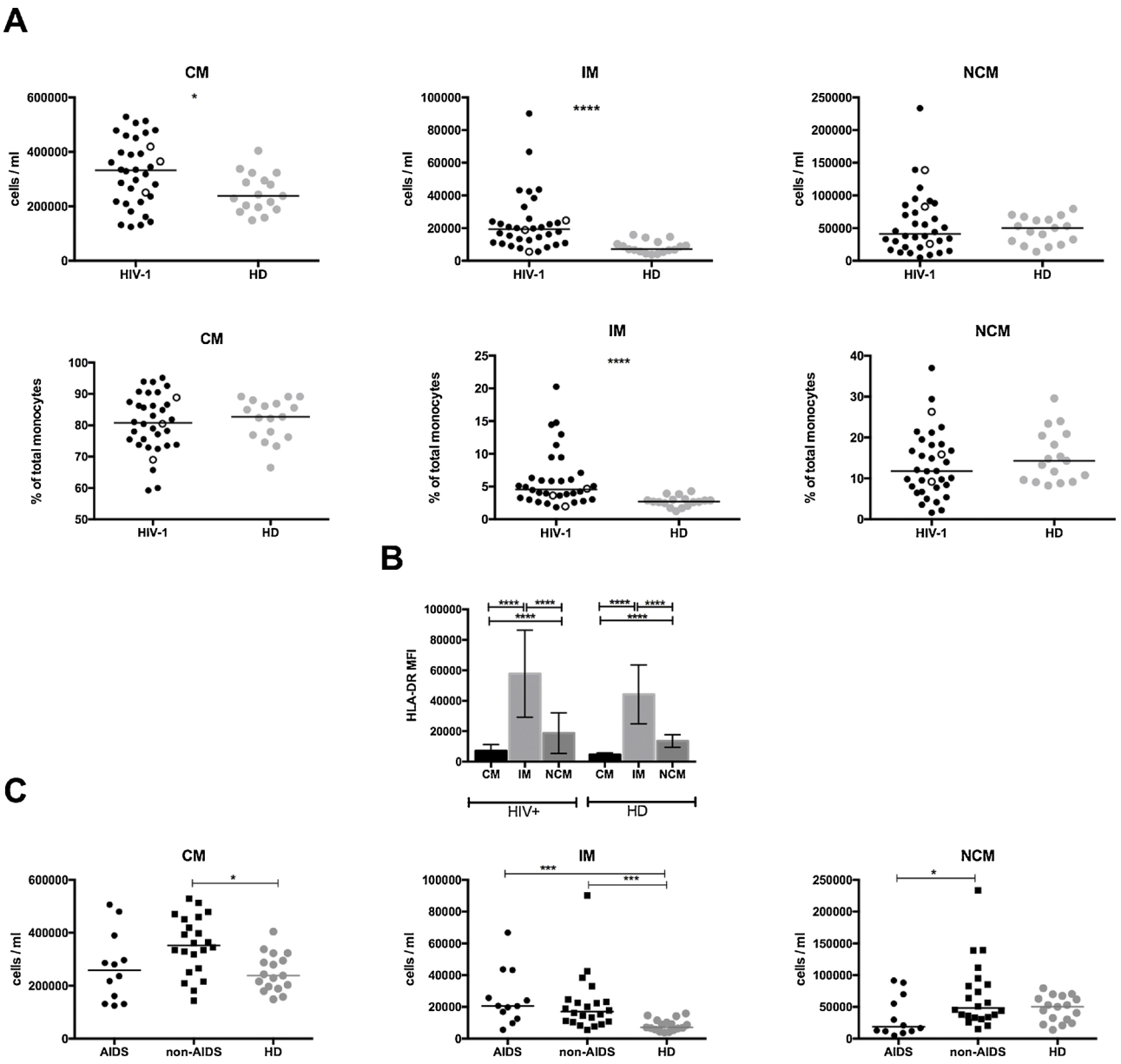

Fig. 3. Monocyte subsets in HIV-1 positive subjects and healthy donors.

Monocyte absolute and relative counts were evaluated in HIV infected subjects and HD. CM absolute counts (medians): 332.110 vs 238.049 cells/ml p = 0,035. IM absolute counts (medians): 19.369 vs 7.157 cells/ml p < 0,001. NCM absolute counts (medians): 41.010 vs 50.268 cells/ml p = 0,99. Monocyte relative counts (percentages of total monocytes) were evaluated. CM relative counts (medians): 80,78 vs 82,70 % p = 0,76. IM relative counts (medians): 4,58 vs 2,7 %, p < 0,0001. NCM relative counts (medians): 11,7 vs 14,31 % p = 0,27. Statistical analysis was performed by using the Mann-Whitney test (A).

HLA-DR expression on monocyte subsets. For HIV-1 infected subjects, CM HLA-DR (means): 7.134, IM HLA-DR (means): 57.790 and NCM HLA-DR (means): 18.683, repeated-measures one-way ANOVA p < 0.0001. For HD, CM HLA-DR (means): 4.659, IM HLA-DR (means): 44.183 and NCM HLA-DR (means): 13.545 repeated-measures one-way ANOVA p < 0.0001). Post-test analysis, with Geisser-Greenhouse correction for sphericity, was used for comparing the different groups and asterisks indicate the level of significance. The height of the bar represents the mean value. Whiskers represent standard deviations (B).

Considering CDC clinical stage, CM, IM and NCM were compared among AIDS, non-AIDS presenters and controls. CM absolute counts (medians) 258.947 vs 352.870 vs 238.049 cells/ml, p = 0,011. IM absolute counts (medians): 20.590 vs 17.012 vs 7.157 cells/ml. NCM absolute counts (medians): 772 vs 48.166 vs 50.268 cells/ml. Kruskal-Wallis test was used for statistical analysis. Dunnett's post-test analysis was used for comparing the different groups and asterisks indicate the level of significance (C).

CM: classical monocytes ($CD14^{++}CD16^{-}$). IM: intermediate monocytes ($CD14^{++}CD16^{+}$). NCM: Non-classical Monocytes ($CD14^{low}CD16^{++}$). Empty circles represent the elite controller and the long-term non-progressors. Lines represent median values. HD: healthy donors.

*: 0.01 < p < 0,05; **: 0,001 < p < 0,01; ***: 0.0001 < p < 0.001; **** p < 0,0001.

significant differences were observed for HLA-DR MFI on IM and NCM subsets (supplementary Fig. 3)

As a merely descriptive observation, due to the limited number of subjects, IM cell counts in the elite controller and one of the two long-term non-progressors were higher than the median value observed in the control group, while HLA-DR expression on CM was increased in the elite controller and the long-term non-progressors (Fig. 3).

3.4. Activated T-Lymphocytes at baseline

T-lymphocyte immune activation was assessed by determining the percentages of $CD4^{+}$ and $CD8^{+}$ T-cells expressing both HLA-DR and

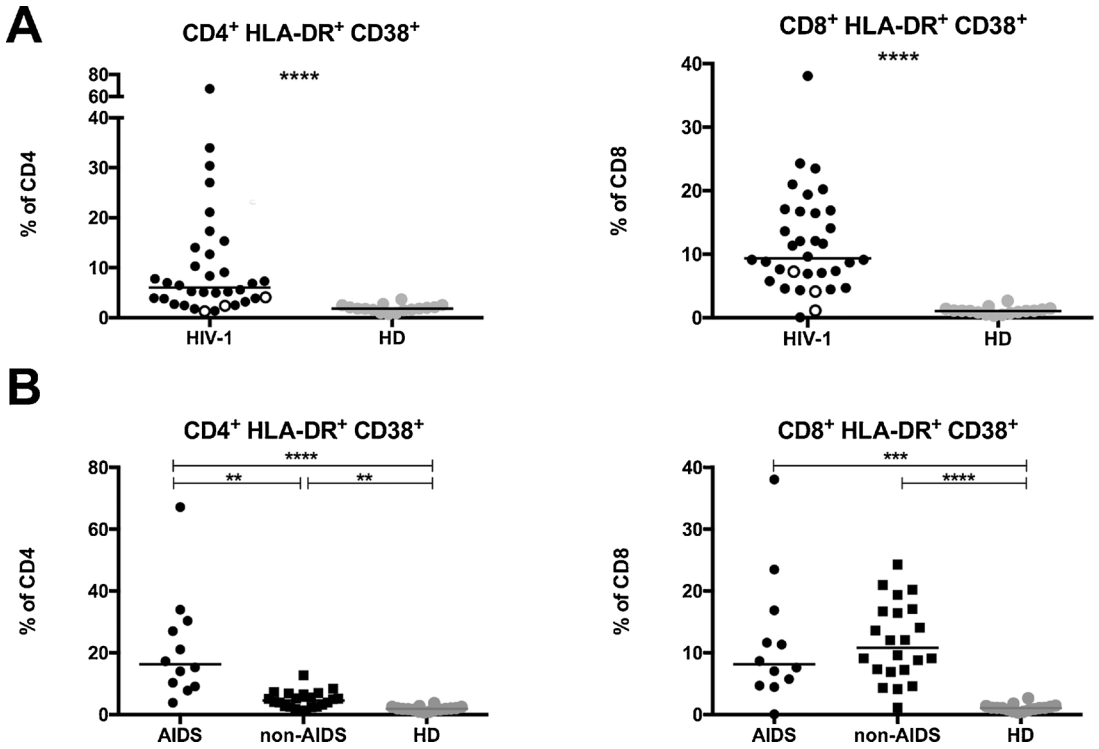

Fig. 4. T-Lymphocyte immune activation in HIV-1 positive subjects and healthy donors.
CD4 and CD8 immune-activation levels were measured as the percentages of cells expressing both HLA-DR and CD38 and HIV-1 infected patients were compared to HD. $CD4^+HLA\text{-}DR^+CD38^+$ percentages (medians): 6,0 vs 1,8% $p < 0{,}001$: $CD8^+HLA\text{-}DR^+CD38^+$ percentages (medians): 9,4% vs 1,1% $p < 0{,}001$. Statistical analysis was performed by using the Mann-Whitney test (A). Considering CDC clinical stage, CD4 and CD8 immune activation levels were compared among AIDS, non-AIDS and HD. $CD4^+HLA\text{-}DR^+CD38^+$ percentages (medians): 16,3 vs 4,5 vs 1,8%. $CD8^+HLA\text{-}DR^+CD38^+$ percentages (medians): 8,2 vs 10,8 vs 1,1 %. Kruskal-Wallis test was used for statistical analysis. Dunnett's post-test analysis was used for comparing the different groups and asterisks indicate the level of significance (B). Empty circles represent the elite controller and the long-term non-progressors. Lines represent median values. HD: healthy donors.
*: $0.01 < p < 0{,}05$; **: $0{,}001 < p < 0{,}01$; ***: $0.0001 < p < 0.001$; **** $p < 0{,}0001$.

CD38. HIV-1 infected subjects showed increased levels of CD4 and CD8 immune-activation compared to HD ($p < 0.001$ for both CD4 and CD8 immune activation) (Fig. 4A). Furthermore, AIDS presenters showed the highest level of CD4 immune activation, when compared with non-AIDS presenters and HD ($p < 0.0001$). Conversely, CD8 immune activation did not change according to CDC clinical stages but was equally increased in AIDS and non-AIDS patients compared to HD (Fig. 4B). In the elite controller and the long-term non-progressors the levels of both CD4 and CD8 immune activation were closer to those observed in the control group (Fig. 4A).

3.5. Soluble markers of myeloid activation

The quantification of myeloid activation soluble markers in plasma showed increased levels of sCD14 and sCD163 in the HIV-1 group compared to HD ($p < 0.001$ and $p = 0.001$ respectively) (Fig. 5A). After stratifying HIV patients according to the CDC clinical stage, AIDS presenters showed the highest levels of sCD14 and sCD163 compared to non-AIDS presenters and HD ($p < 0.0001$ and $p = 0.003$, respectively) (Fig. 5B). Considering the elite controller and the long-term non-progressors, sCD14 and sCD163 plasma levels seemed to be similar to those observed in controls (Fig. 5A).

3.6. Correlation of Myeloid and lymphoid immune activation parameters

Correlation between lymphoid and myeloid parameters was evaluated by applying the Spearman test. A correlation matrix was obtained, showing a close interconnection between myeloid and lymphoid activation markers. CD4 absolute cell count was directly correlated with mDC, pDC, slanDC, CM, NCM absolute counts and HLA-DR expression on mDCs. Similarly, CD4/CD8 ratio was directly correlated with mDC, pDC, slanDC, CM absolute counts and HLA-DR expression on mDCs. Conversely, CD4 immune activation was negatively correlated to CD4 cell counts and CD4/CD8 ratio and mDC, pDC, slanDC, CM absolute counts and HLA-DR expression on mDCs (supplementary table). Summarizing these results, lower CD4 cell counts, lower CD4/CD8 ratios and higher levels of CD4 immune activation were associated with a more severe depletion of dendritic cells and CM and alteration of mDC phenotype, with reduction in HLA-DR expression.

Interestingly, although increased in HIV-1 infected subjects, IM values did not correlate with CD4 cell counts, CD4/CD8 ratios or lymphoid activation markers. As previously shown in this paper, after stratifying the patients according to the CDC clinical stage, IM cell counts did not differ between AIDS and non-AIDS presenters, but were increased in both groups when compared to HD. Considering age, CD4 cell count, CD4/CD8 ratio and viremia of HIV-1 infected patients at enrolment, only viremia was directly correlated with IM cell counts (Spearman r: 0.36 and $p = 0.036$).

Baseline viral load was also positively correlated with CD4 immune activation levels (Spearman r: 0.50 and $p = 0.0024$) and negatively correlated with CD4 (Spearman r: -0.46 and $p = 0.0065$), pDC (Spearman r: -0.34 and $p = 0.047$), slanDC absolute counts (Spearman r: -0.52 and $p = 0.0016$), CD4/CD8 ratio (Spearman r: -0.65 and $p < 0.0001$) and HLA-DR expression on mDCs (Spearman r: -0.45 and $p = 0.0075$).

3.7. Longitudinal evaluation of immunological parameters

In a subgroup of 8 HIV-1 infected patients (3 were A1, 1 was A2, 1 was A3, 1 was B2 and 2 were C3) immunological parameters were

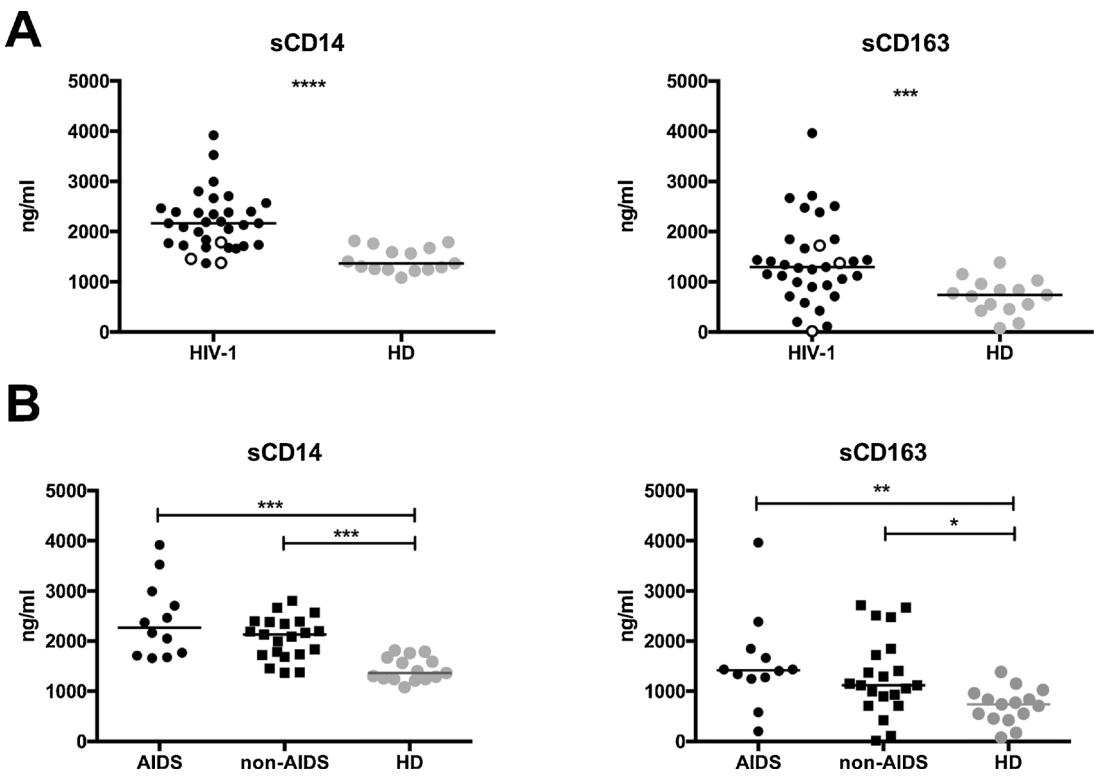

Fig. 5. Soluble markers dosage in HIV-1 positive subjects and healthy donors.
Plasma levels of sCD14 and sCD163 were measured in HIV infected subjects and HD, by ELISA assays. sCD14 plasma concentrations (medians): 2163 vs 1363 ng/ml $p < 0{,}001$, respectively. sCD163 plasma concentrations (medians) 1292 vs 741 ng/ml $p = 0.001$. Statistical analysis was performed by using the Mann-Whitney test (A).
sCD14 and sCD163 plasma levels were compared among AIDS, non-AIDS presenters and HD. sCD14 plasma concentrations (medians): 2.269 vs 2.133 vs 1363, $p < 0{,}0001$. sCD163 plasma concentrations (medians): 1420 vs 1120 vs 741, $p = 0{,}003$. Kruskal-Wallis test was used for statistical analysis. Dunnett's post-test analysis was used for comparing the different groups and asterisks indicate the level of significance (B). Empty circles represent the elite controller and the long-term non-progressors. Lines represent median values.
*: $0.01 < p < 0{,}05$; **: $0{,}001 < p < 0{,}01$; ***: $0.0001 < p < 0.001$; **** $p < 0{,}0001$. HD: healthy donors.

evaluated after one year of effective ART. Considering this subgroup, 50% of the patients were treated with a PI and 50% with a NNRTI, while there were no patients under INSTI. After one year of treatment all the patients had a viral load under 100 copies/ml. On resampling, HIV-1 viral load was undetectable (below 37 copies/ml) in 5 patients, while it was detectable in 3 patients (49, 61 and 79 copies/ml, respectively) who were on a PI based regimen. The three patients with detectable viremia were also AIDS-presenters (1 A3 and 2 C3 at T0). Comparing the immunological parameters at baseline and after the first year of ART, we observed an increment in pDC absolute cell counts ($p = 0.02$), while mDC and slanDC changes in absolute counts were not statistically significant (Fig. 6A). Concerning monocyte subsets, IM absolute counts were drastically reduced after one year of treatment ($p = 0.02$), while CM and NCM cell count changes did not reach the statistical significance (Fig. 6B). An increment in HLA-DR expression levels on mDCs was observed ($p = 0.02$), without reaching the levels observed in mDCs from HD (Fig. 6C). HLA-DR expression on slanDCs and NCM tended to reduce and approach the levels observed in HD ($p = 0.02$ and $p = 0.02$, respectively) (Fig. 6C).

Finally, as expected, both CD4 and CD8 immune activation were reduced after the first year of ART ($p = 0.008$ and $p = 0.008$, respectively). Notably, CD8 immune activation was still significantly elevated when compared to healthy donor levels after 1 year of ART ($p < 0.0001$) (Fig. 7).

4. Discussion

Myeloid and lymphoid immunological parameters are altered in patients with HIV infection before starting ART. In our study we used a comprehensive, rapid and simple method to assess dendritic cell, monocyte, $CD4^+$ and $CD8^+$ T-lymphocyte cell counts and phenotypic characteristics from whole blood samples, with the aim to identify the interconnection between myeloid and lymphoid parameters and their relationship with the clinical presentation at HIV diagnosis, stratifying our patients in AIDS and non-AIDS presenters.

Our results confirmed previous reports showing a reduction of pDC absolute counts in viremic HIV infected patients (Donaghy et al., 2001; Hosmalin et al., 2008). Specifically, we showed that, although pDCs are strongly reduced in AIDS presenters, HLA-DR expression remained at normal levels, as high as in HD. In a previous study, our group suggested a role of low baseline levels of pDCs in predicting virological failure during ART (Lichtner et al., 2008). Conversely, mDC absolute counts were unchanged in HIV infected subjects, although their HLA-DR expression was reduced, compared to HD. Interestingly, after stratification according to clinical stage, non-AIDS presenters showed increased mDC absolute counts compared to both AIDS presenters and HD. Yang et al. reported similar findings in an animal model, showing increased numbers of $CD1c^+$mDC (which represents the main subtype of circulating mDCs) in simian immunodeficiency virus (SIV) and simian/human immunodeficiency virus (SHIV) infected rhesus macaques, at 70 and 84 days post-infection. The increased proportion of $CD1c^+$mDC was interpreted as a quantitative compensation for their functional impairment (Yang et al., 2011). Our results showed similar findings in non-AIDS HIV infected subjects. Furthermore, a decreasing linear trend for HLA-DR expression on mDCs was found, suggesting a progressive functional impairment of this cell subtype, which increases

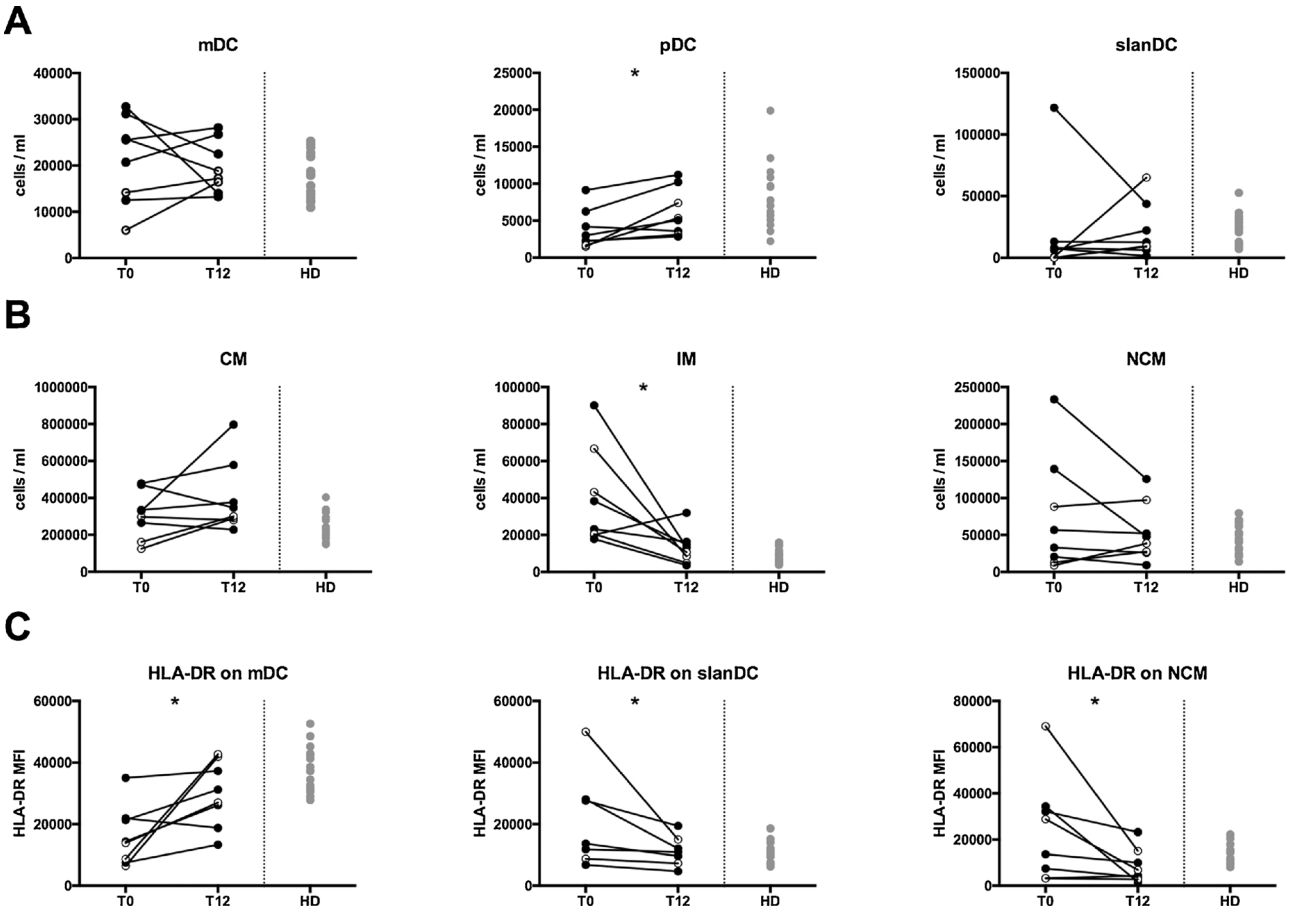

Fig. 6. Myeloid immunological parameters after 1 year of ART.

DC subsets were compared before (T0) and 1 year after (T12) ART initiation. mDC cell counts (medians): 23.146 vs 18,006 cells/ml p = 0,9. pDC cell counts (medians): 2.641 vs 5.197 cells/ml p = 0,02. slanDC cell counts (medians): 6.788 vs 11.104 cells/ml p = 0,6. Statistical analysis was performed by using the Wilcoxon test for repeated measures (A).

Monocyte subsets were compared before (T0) and 1 year after (T12) ART initiation. CM counts (medians): 313.195 vs 323.845 cells/ml p = 0,19. IM counts (medians): 30.786 vs 11.985 cells/ml p = 0,02. NCM counts (medians): 44.900 vs 43.253 cells/ml p = 0,6. Statistical analysis was performed by using the Wilcoxon test for repeated measures (B).

HLA-DR expression levels were measured on DC and monocyte subsets as MFI and were compared before (T0) and 1 year after (T12) ART initiation. HLA-DR on mDCs (medians): 14.116 vs 29.130 p = 0,02. HLA-DR on slanDCs (medians): 13.685 vs 10.986 p = 0,02. HLA-DR on NCM (medians): 21.230 vs 5.579 p = 0,02. Statistical analysis was performed by using the Wilcoxon test for repeated measures (C).

Empty circles represent AIDS presenters (who had also detectable viremia after 1 year of ART). Values measured in HD are shown in grey.

mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells. slanDCs: slan dendritic cells. HD: healthy donors. CM: classical monocytes (CD14 + + CD16-). IM: intermediate monocytes (CD14 + + CD16 +). NCM: Non-classical Monocytes (CD14 + CD16 + +). HD: healthy donors; T0: baseline; T12: after 1 year of ART.

*: 0.01 < p < 0,05; **: 0,001 < p < 0,01; ***: 0.0001 < p < 0,001; **** p < 0,0001.

with disease severity. A previous report showed the association of altered mDC function and modifications in mDC phenotype (such as lower expression of CD80, CD86, CD40 and HLA-DR) in HIV positive patients, particularly in those with CD4 cell counts lower than 250 cell/μl (Sachdeva et al., 2015). Considering our results, ART was able to partially restore pDC absolute counts and HLA-DR expression on mDCs, after the first year of treatment, without reaching the levels observed in HD.

A few studies have addressed the issue of slanDC function and number alterations in HIV infection. In the present study, slanDC absolute counts were found to be decreased in HIV positive patients, essentially in AIDS presenters, with extremely low slanDC absolute counts. Previous studies reported increased slanDC absolute counts in treatment naïve HIV infected patients (Dutertre et al., 2012; Tufa et al., 2016), but these results cannot be compared with our findings because of the different demographic and viro-immunological characteristics of the patients enrolled in the studies. Furthermore, differently from previous reports, we included AIDS presenters with opportunistic infections and all our patients were treatment naïve at baseline. In our cohort, AIDS presenters were almost all (9/12) CDC-C (with active opportunistic infections at baseline) and had high T-lymphocyte immune activation levels. The recruitment of slanDCs in inflamed tissues and lymph nodes could explain their decreased absolute numbers in peripheral blood of AIDS patients, as observed in other inflammatory conditions (Bsat et al., 2015; Hänsel et al., 2013, 2011; Vermi et al., 2014). Moreover, the function of slanDCs seems to be altered during HIV infection, as suggested by phenotypic changes in surface markers and increasing pro-inflammatory cytokine secretion. Previous reports showed increased production of IL-1β and TNF-α by slanDCs in response to LPS stimulation in untreated viremic HIV-infected patients (Dutertre et al., 2012; Tufa et al., 2016). Furthermore, isolated slanDCs are able to produce high levels of IL-23 after LPS stimulation (Kunze et al., 2016). Tufa et al., reported also phenotypic modifications in slanDCs from untreated viremic HIV infected patients, such as increased expression of CD40, CD80, CD86, HLA-DR and membrane-TNF-α (Tufa et al., 2016). In our study, HLA-DR was increased in slanDCs of ART naïve HIV-infected patients and was reduced after one year of effective treatment. Although we did not evaluate cytokine production by

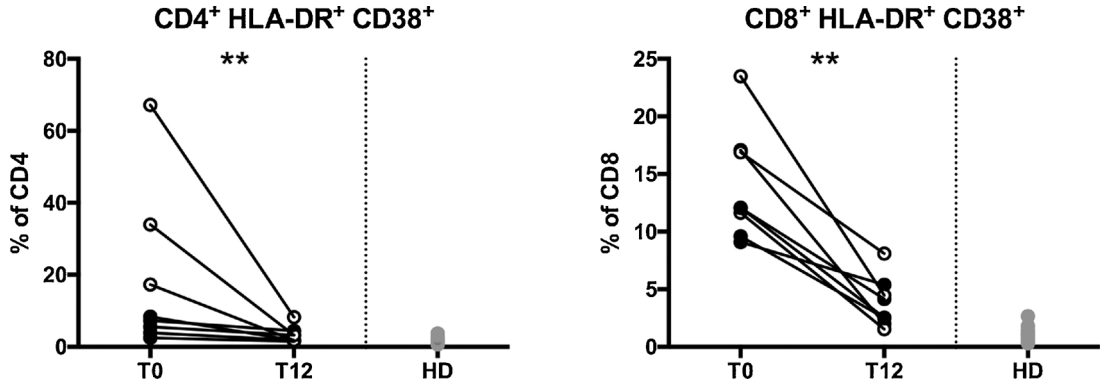

Fig. 7. T-lymphocyte immune activation after 1 year of ART.
T-lymphocyte immune activation levels were compared before (T0) and 1 year after (T12) ART initiation. $CD4^+HLA\text{-}DR^+CD38^+$ percentages (medians): 7,8 vs 1,7% p = 0,008. $CD8^+HLA\text{-}DR^+CD38^+$ percentages (medians): 12,1 vs 3,4 % p = 0,008. Statistical analysis was performed by using the Wilcoxon test for repeated measures.
T0: baseline; T12: after 1 year of ART. HD: healthy donors. Empty circles represent AIDS presenters (who had also detectable viremia after 1 year of ART). Values measured in HD are shown in gray.
*: $0.01 < p < 0,05$; **: $0,001 < p < 0,01$; ***: $0.0001 < p < 0.001$; **** $p < 0,0001$.

slanDCs after LPS stimulation, increased HLA-DR expression can be considered as a marker of activation, associated with increased cytokine secretion. Considering the increased levels of sCD14 in HIV infected patients, we can speculate that in treatment naïve HIV positive patients slanDCs are activated by circulating bacterial products and produce large amounts of pro-inflammatory cytokines, contributing to systemic inflammation. In AIDS-presenters slanDCs could be recruited in tissues, especially in mucosal associated lymphoid tissue (such as Peyer's patches) where they can sustain inflammation, CD4 activation and consequently HIV replication. ART has been shown to be effective in restoring slanDC normal phenotype and function after only one year of therapy.

In another set of experiments, we assessed the role of $CD16^+$ inflammatory monocytes in AIDS and non-AIDS presenters. The expansion of $CD16^+$ inflammatory monocytes has been well characterized during HIV infection (Hearps et al., 2012; Schäkel et al., 2002). Circulating $CD16^+$ monocytes can have a dual role in HIV pathogenesis, being infected by HIV (Zhu, 2002) and recruited in tissues, where they differentiate in mDCs or macrophages (Ancuta et al., 2006). Considering all the available data, $CD16^+$ monocyte can contribute to the expansion of the viral reservoir. In our study, an increase in absolute and relative counts of peripheral blood $CD14^{++}CD16^+$ IM was observed in naïve HIV-1 infected patients, compared to HD. We confirmed previous observations showing that IM cell counts are positively correlated to HIV viral load and tend to decrease after ART initiation (Han et al., 2009). Similarly to previous studies, we showed that IM had the highest HLA-DR MFI among the three monocyte subsets, implying that this subset may possess superior antigen presenting abilities and T-cell stimulatory functions (Han et al., 2009; Wong et al., 2011). Moreover, the increase in IM cell counts seemed to be independent of clinical stage and CD4 absolute counts at baseline, being IM absolute counts increased in both AIDS and non-AIDS presenters and ART was able to reduce IM cell counts, after one year. Interestingly, it has been shown that IM were increased also in the elite controllers (Krishnan et al., 2014), even in the absence of detectable HIV replication in peripheral blood.

Although some reports showed an increase in $CD14^{low}CD16^{++}$ NCM counts (Han et al., 2009; Thieblemont et al., 1995), in our study no changes were observed when comparing HIV infected patients and HD. Furthermore, a decrease in NCM absolute counts was observed in AIDS presenters compared to non-AIDS presenters. This finding could be related to the concomitant presence of opportunistic infections in the majority of the AIDS presenters, determining a decrease of NCM, as already discussed for slanDCs. Further studies are needed to provide a better definition of monocyte subset absolute counts and functions in AIDS presenters.

Soluble markers of monocyte/macrophage activation sCD14 and sCD163 have been found to be increased during HIV infection and associated to disease progression and non-AIDS related events, respectively (Burdo et al., 2013; Burdo et al., 2011a,b; Burdo et al., 2011a,b; Krastinova et al., 2015; Nockher et al., 1994; Romero-Sánchez et al., 2012; Sandler et al., 2011; Tippett et al., 2011; Vita et al., 2017). In the present study both sCD14 and sCD163 were increased and reached the highest levels in AIDS presenters, confirming their role in predicting clinical progression towards AIDS.

Lymphoid immune-activation during HIV infection has been well characterized in several studies in treatment naïve (Orendi et al., 1998) and HAART experienced patients (Zhang et al., 2013), underlining the inability of antiretroviral treatments to completely normalize CD4 and CD8 immune activation levels. In our study CD4 and CD8 immune activation was increased at baseline in HIV infected patients, with the highest CD4 activation values observed in the AIDS presenter group. ART contributed to the decrease in both CD4 and CD8 immune activation levels, although alterations persisted after one year of treatment. CD8 immune activation was elevated in untreated HIV viremic patients, without any difference between AIDS and non-AIDS patients, after stratification according to clinical presentation.

In the present study we looked at both myeloid and lymphoid activation and we could observe a close relationship between the alterations observed in the two peripheral blood compartments. Increased levels of CD4 immune activation, lower CD4 absolute counts and reduced CD4/CD8 ratios paralleled with pDC and slanDC absolute count decreases, HLA-DR reduced expression on mDCs and HLA-DR increased expression on slanDCs. Taken together, these results showed the presence of complex alterations involving innate and adaptive immune-activation during HIV infection.

Interestingly, IM cell count elevation seemed to be independent from the other activation markers. Furthermore, we showed that effective ART was able to reduce HIV viral load contributing to lower lymphoid and myeloid immune activation markers.

Our results showed also that after the first year of treatment, immunological parameters were highly influenced by therapy, with a reduction in inflammatory monocytes and lymphoid immune activation levels and an increase in pDC cell counts. Furthermore, we observed a trend towards the normalization of HLA-DR expression on mDCs, slanDCs and NCM. It should be pointed out that some parameters, although improved, remained still altered when comparing treated HIV patients and HD, such as HLA-DR expression on mDCs and CD8 immune activation levels.

Of note, animal and human studies showed that mDCs may play a key role in chronic inflammation and atherogenesis (Miles et al., 2014; Puddu et al., 2010). Furthermore, Dopheide et al. found increased levels of mDCs with reduced HLA-DR expression, along with increased $CD14^{++}CD16^{+}$ cell counts in the peripheral blood of patients with peripheral arterial disease (Dopheide et al., 2012). Based on these data we can speculate that alterations in mDCs numbers and functions can contribute to sustain a state of chronic inflammation leading to non-AIDS related events, such as atherosclerosis and ageing of the immune system. The observed expansion in circulating levels of mDCs in non-AIDS presenters prior to ART initiation, might be both a response to their functional impairment, or an evidence of their contribution to immune activation. On the other hand, the lack of increase of mDCs in AIDS-presenters might be a consequence of the state of profound immune deficiency, with a reduction in the control of infections and the development of opportunistic complications. Therefore, mDCs may play a dual role in HIV infection, depending on the clinical stage of the disease.

In conclusion in this study, using an easy-to-perform flow cytometric method, we were able to analyse the three classes of circulating dendritic cells, monocyte and lymphocyte subsets on peripheral blood samples, evaluating simultaneously myeloid and lymphoid activation markers in HIV infected subjects with advanced and non-advanced disease stages, before ART initiation.

Some limitations of our study were the small number of patients resampled at 12 months after ART initiation for flow cytometry analyses, and the low percentage of patients under INSTI treatment.

5. Conclusion

The main findings of this work were that in HIV infected subjects the three subtypes of circulating dendritic cells were altered either in the number (pDCs), HLA-DR expression (mDCs) or both parameters (slanDCs), with greater alterations observed in AIDS compared to non-AIDS presenters. Regarding monocyte subsets, IM cell counts were equally increased in AIDS and non-AIDS presenters compared to HD. Furthermore, IM expansion seemed to be independent from clinical stage, CD4 absolute cell counts and CD4/CD8 ratio and directly related to HIV viral load. After ART initiation pDC absolute counts tended to increase and HLA-DR expression on mDCs, slanDCs and NCM tended to the levels observed in HD, along with CD4 and CD8 immune activation reduction. Given their role in inflammation-related organ disorders, the considerable decrease in IM cell counts observed after 1 year of ART in both advanced and non-advanced HIV patients, enforces the need for early therapy initiation in people living with HIV, in order to prevent AIDS progression and limit the development of severe non-AIDS related events.

Competing interests

None to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2018.11.011.

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