Activated dendritic cells and monocytes in HIV immunological nonresponders: HIV-induced interferon-inducible protein-10 correlates with low future CD4+ recovery

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Objective: To explore monocyte and dendritic cell immune responses, and their association with future CD4+ gain in treated HIV patients with suboptimal CD4+ recovery.

Design: A cross-sectional study of HIV-infected, virally suppressed individuals on antiretroviral therapy for at least 24 months; 41 immunological nonresponders (INRs) (CD4+ cell count <400 cells/μl) and 26 immunological responders (CD4+ cell count >600 cells/μl). Ten HIV-infected antiretroviral therapy-naive and 10 HIV-negative healthy persons served as controls. CD4+ cell counts were registered after median 2.4 and 4.7 years.

Methods: Monocyte, dendritic-cell and T-cell activation and regulatory T cells (Tregs) were analyzed by flow cytometry. In INR and immunological responder subgroups matched on age and nadir CD4+ cell count, upregulation of interferon-inducible protein-10 (IP-10) and indoleamine 2,3-dioxygenase in monocytes and dendritic cells and cytokines in cell supernatants were measured *in vitro* in peripheral blood mononuclear cells stimulated with aldrithiol-2-inactivated HIV-1.

Results: The INR group displayed higher spontaneous activation of both monocytes (HLA-DR+) and myeloid and plasmacytoid dendritic cells (HLA-DR+, CD83+ and CD86+) compared with immunological responders, and this was associated with increased T-cell activation (CD38+HLA-DR+), an effector memory T-cell phenotype and activated Tregs. The IP-10 response in monocytes after in-vitro HIV stimulation was negatively associated with prospective CD4+ gain. IP-10, indoleamine 2,3-dioxygenase and cytokines levels were comparable between the groups, but inversely correlated with activated Tregs in INRs.

Conclusion: HIV-infected individuals with suboptimal immune recovery demonstrated more activated monocytes and in particular dendritic cells, compared with patients with acceptable CD4+ gain. A high level of HIV-specific IP-10 expression in monocytes may be predictive of future CD4+ recovery.

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Introduction

Despite modern antiretroviral therapy (ART) and persistent viral suppression, 15–30% of people living with HIV (PLWH) do not normalize their $CD4^+$ cell count, denoted immunological nonresponders (INRs) [1–4]. Low nadir $CD4^+$ cell count, long duration of HIV-infection before ART, coinfections such as hepatitis C and older age are well known factors associated with an incomplete immune recovery [2,4–6]. INRs have increased chronic immune activation and inflammation, which probably contribute to the higher morbidity and mortality seen in this group [7–11]. Given the substantial proportion of PLWH who still initiates ART in late disease, INRs will continue to be of clinical relevance [4,12].

The underlying immunological causes of incomplete immune recovery are multifactorial. Several studies have shown a more activated and differentiated T-cell phenotype [9,13–16] as well as higher percentages of activated regulatory T cells (aTregs) [10,15,16] in INRs. We have recently published increased levels of plasma interferon-inducible protein-10 (IP-10) in an INR cohort, in which IP-10 and kyurenine/tryptophan ratio, a measure of indoleamine 2,3-dioxygenase (IDO) activity, were negatively associated with the $CD4^+$ cell count after 2 years [16]. IP-10 is mainly produced by monocytes and myeloid dendritic cells (mDCs) in HIV-negative persons after in-vitro HIV-1 stimulation [17] and is also associated with monocyte activation in HIV-infection [18]. Increased activation of monocytes is reported even in ART-treated PLWH and is related to cardiovascular disease and markers of inflammation and coagulation [19–23]. IDO-activity predicts mortality in treated HIV-infection [24] and is expressed in mDCs and plasmacytoid dendritic cells (pDCs) both in simian immunodeficiency virus (SIV)-infected macaques [25] and in HIV-negative humans after *in vitro* stimulation with inactivated HIV [26,27], or lipopolysaccharide (LPS) in combination with interferon-gamma (IFN-γ) [28].

Dendritic cells serve as a bridge between innate and adaptive immunity, being major drivers of Th1 responses and persistent IFNα secretion which have antiviral functions, but also contribute to chronic immune activation. Furthermore, dendritic cells upregulate IDO and induce Tregs that could both reduce harmful, general inflammation and dampen beneficial HIV-specific immune responses [29–31]. Even virally suppressed PLWH have signs of dendritic cell dysregulation as some demonstrate subnormal dendritic cell counts in blood [32–36], weakened pDC IFNα secretion after exogenous stimuli [36,37] and impaired mDC induction of Th1 responses [38].

To our knowledge, few studies have investigated monocytes and dendritic cells in INRs. Increased proportion of intermediate monocytes [39,40] and lower absolute pDC count with reduced IFNα production have been reported in INRs compared with PLWH with normalized $CD4^+$ cell count [41].

We set out to study activation of monocytes and dendritic cells in INRs compared with immunological responders, ART-naive PLWH and healthy controls, and in-vitro HIV-specific monocyte and dendritic cell responses in INR and immunological responder subgroups matched on age and nadir $CD4^+$ cell count. We hypothesized that INRs had more activated monocytes and dendritic cell subsets and higher in-vitro production of IP-10, IDO and cytokines than the immunological responder group that might contribute to an inadequate future immune reconstitution in INRs.

Methods

Study participants

Forty-one virally suppressed HIV-infected INRs with $CD4^+$ cell count less than 400 cells/μl and 26 immunological responders with $CD4^+$ cell count more than 600 cells/μl were recruited between October 2012 and April 2013 as previously reported [16]. Both groups had received continuous ART for at least 24 months with HIV-RNA 20 copies/ml or less for the last 18 months. $CD4^+$ cell counts were obtained from fresh samples and recorded at baseline and median 2.4 and 4.7 years after inclusion. The last routine $CD4^+$ cell counts that were available prior to data analyses of the previous [16] and the present reports were used. For comparison, 10 ART-naive individuals with duration of HIV-infection at least one year and 10 HIV-negative healthy controls, all age and sex matched, were included. Peripheral blood mononuclear cells (PBMCs) and EDTA plasma were sampled from all participants at inclusion, frozen and stored for later analyses. All participants provided written informed consent. The study was approved by the Regional Ethics Committee (1.2007.83 and 2015/629).

Flow cytometry analyses of ex-vivo monocyte and dendritic cell activation

Flow cytometry analyses were performed on thawed PBMCs. After 2 h rest, one million (viability >85%) PBMCs were incubated with Fc block (BD Biosciences, San Jose, California, USA) prior to staining with surface markers for 15 min in room temperature. The cells were fixated in 1% BD CellFIX (BD Biosciences) before acquisition on BD FACSCanto II (BD Biosciences). The fluorochrome-conjugated antibodies for the monocyte and dendritic cell panels are listed in Table S1, http://links.lww.com/QAD/B453. Results were analyzed with the FlowJo software version 10.4.1 (Tree Star Inc, Ashland, Oregon, USA). As staining controls, all antibodies in the other channels were combined with concentration matched isotypes for the activation markers, and fluorescence minus one (FMO) was used for anti-CD83. The gating strategy is shown in Fig. S1,

http://links.lww.com/QAD/B453. Monocyte subsets were defined as CD45$^+$HLA-DR$^+$Lineage$^-$Fixable Viability$^-$ and either CD14^{++}CD16$^-$ (classical), CD14^{++}CD16$^+$ (intermediate) or CD14$^+$CD16^{++} (nonclassical). Dendritic cells were characterized as CD45$^+$HLA-DR$^+$Lineage$^-$FixableViability$^-$ and further subdivided into CD1c$^+$ mDCs, CD141^{++} mDCs or CD303$^+$ pDCs.

Intracellular interferon-inducible protein-10 and indoleamine 2,3-dioxygenase detection after in-vitro stimulation with inactivated HIV-1

Owing to the strong association of nadir CD4$^+$ with low CD4$^+$ recovery, 20 INRs and 20 immunological responders with comparable age and nadir CD4$^+$ cell count, and eight age and sex-matched healthy controls were selected for in-vitro HIV-stimulation analyses (Table S2, http://links.lww.com/QAD/B453). One million thawed PBMCs (viability >90%), were cultured for 18 h at 37 °C, 5% CO$_2$ in 200 μl RPMI 1640 (Lonza, Verviers, Belgium) containing 10% heat-inactivated fetal calf serum and penicillin/streptomycine/L-glutamine, with either aldrithiol-2-inactivated (AT-2) HIV-1 (dual-tropic; X4 and R5, lot P4311) at a final concentration of 500 ng/ml p24 [42], or microvesicles with equivalent protein concentration (mock) as negative control [43]. Brefeldin A 1 μl/ml (BD Biosciences) was added after 6 h. Following stimulation supernatants were harvested and the cells stained for either monocyte or dendritic cell surface markers as previously described (Table S1, http://links.lww.com/QAD/B453), subsequently fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) and stained intracellularly for IP-10 and IDO (Table S1, http://links.lww.com/QAD/B453). All IP-10 and IDO responses were calculated with subtraction of the corresponding mock stimulated control. For gating strategy see Fig. S2, http://links.lww.com/QAD/B453.

ELISA and multiplex analyses

Soluble (s)CD163 was analyzed in INRs and immunological responders from snap frozen EDTA plasma in duplicate by immunoassay (DY1607; R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions. The intraassay and interassay coefficients of variation (CV) were less than 10%.

Concentrations of IL-1β, IL-1 receptor antagonist (IL-1ra), IL-6, IL-10, IL-18, IFNα2, IFNγ, tumor necrosis factor, IP-10 and macrophage inflammatory protein 1beta (MIP-1β)/CCL4 were measured in thawed supernatants (10 000 g/10 min/4 °C) by nine-plex and single-plex (Bio-Rad, Oslo, Norway) assays, respectively. The samples were diluted 1 : 7.5 and 1 : 75 as appropriate, analyzed with a Luminex IS 100 instrument (Bio-Rad, Hercules, California, USA) according to instructions from the manufacturer and run in duplicates. Both intraassay and interassay CV were less than 10%. All AT-2

HIV virus responses were found by subtracting concentrations from the corresponding mock stimulated sample.

Multiplex and ELISA analyses of other soluble markers in plasma and flow cytometry phenotyping of T-cell subsets including Tregs have been previously presented [16], and are only used in correlation analyses in the current article. The methods are described in brief in the Supplementary methods, http://links.lww.com/QAD/B453.

Statistical analyses

Statistical analyses and graphical presentations were performed by SPSS statistics 25 (IBM Corp., Armonk, New York, USA) and GraphPad Prism V7.04 software (GraphPad, San Diego, California, USA) using nonparametric statistics. To reduce the percentage of CV (%CV) in the flow cytometry experiments caused by small cell populations, populations consisting of less than 100 cells were excluded for further analyses of percentages of activation markers and when less than 50 cells also for determination of median fluorescence intensity (MFI) [44]. For comparison between more than two groups Kruskal−Wallis test followed by Dunn's post-hoc test with correction for multiple comparisons were applied. Mann−Whitney U test was used when analyzing two groups, Wilcoxon test for paired samples, Fisher's exact test or Pearson chi-squared test for categorical variables and Spearman's rank correlation for correlation between parameters. Factors associated with prospective CD4$^+$ gain were further analyzed with binary logistic regression adjusting for age, nadir CD4$^+$ cell count and duration of ART. The outcome variable was dichotomized on median within the actual patient group. A two-tailed significance level of 0.05 was set.

Results

Characteristics of the study participants

The baseline characteristics for the INR and immunological responder cohorts have previously been described in detail [16]. The ART-naive had median CD4$^+$ cell count 553 (interquartile range 346−667) cells/μl, CD4$^+$/CD8$^+$ ratio comparable with INRs, shorter duration of HIV infection ($P=0.004$) and higher nadir CD4$^+$ cell count ($P<0.001$) than both INRs and immunological responders (Table 1). In the subgroups matched on age and nadir CD4$^+$, selected for AT-2 HIV stimulation assay, the INR group ($n=20$) had lower viral load at initiation of continuous ART ($P=0.02$) and shorter duration of both viral suppression ($P=0.01$) and ART use ($P=0.01$) compared with the immunological responder group ($n=20$) (Table S2, http://links.lww.com/QAD/B453).

Table 1. Characteristics of the study cohort at inclusion.

Total study population	INR, $n = 41$	IR, $n = 26$	ART-, $n = 10$	HC, $n = 10$	P value*
Age (IQR)	49.9 (41.6–57.9)	45 (39.3–53.8)	50.6 (39.6–59.4)	47.2 (43.5–58.0)	NS
Male sex, n (%)	35 (85.4)	18 (69.2)	7 (70)	7 (70)	NS
Ethnicity, n (%)					
White	29 (70.7)	17 (65.4)	5 (50)	10 (100)	NS
Risk group, n (%)					
MSM	22 (53.7)	13 (50)	1 (10)**		**0.04**
Other[a]	19 (46.3)	13 (50)	9 (90)**		**0.04**
Comorbid diseases, n (%)					
Cardiovascular	6 (14.6)	0 (0)	2 (20)		NS
Any comorbidity[b]	21 (51.2)	6 (23.1)	5 (50)		NS
CMV IgG pos	41 (100)	25 (96.2)	9 (90)		NS
HIV characteristics (IQR)					
Years since HIV diagnosis	8.6 (6.4–15.2)	9.2 (7.0–14.1)	3.6 (2.2–6.8)**,***		**0.004**
Years of continuous ART	5.5 (3.1–6.7)	6.6 (4.4–8.7)			NS
Viral load at ART initiation (copies/ml)	67 500 (29 000–110 000)	100 000 (50 000–330 000)			NS
Duration of viral suppression (years)	3.8 (2.0–5.8)	6.1 (4.0–7.6)			0.01
Viral load at inclusion (copies/ml)	≤20	≤20	36 000 (19 750–128 000)**,***		**<0.001**
CD4+ cell count nadir (cells/μl)	100 (20–157)	180 (120–220)	369 (346–512)**,***,****		**<0.001**
CD4+ cell count at inclusion (cells/μl)	285 (232–348)	810 (740–864)	553 (346–677)**,****		**<0.001**
CD8+ cell count at inclusion (cells/μl)	670 (521–890)	1005 (820–1587)	1257 (973–2491)**,****		**<0.001**
CD4+/CD8+ at inclusion	0.43 (0.32–0.57)	0.79 (0.63–0.99)	0.35 (0.25–0.74)***,****		**<0.001**

Data are presented as no. (%) of study participants or median IQR values. ART, antiretroviral therapy; ART-, antiretroviral therapy naive HIV-infected; CMV, cytomegalovirus; HC, healthy control; INR, immunological nonresponder; IQR, interquartile range; IR, immunological responder.
[a]Other. Heterosexual or unknown. There were no intravenous drug abusers.
[b]One or more of the following comorbidities; cardiovascular disease, hypertension, diabetes, renal disease, osteoporosis, chronic obstructive pulmonary disease, neurodegenerative disease, previous cancer or *Mycobacterium tuberculosis* infection.
*P values for Kruskal–Wallis test or Pearson Chi-squared test for comparison between multiple groups, Mann–Whitney U test for comparison INR vs. IR. Significant values are shown in bold.
**P less than 0.05 for comparison INR vs. ART-. Fisher's exact test or Dunn's post-hoc test.
***P less than 0.05 for comparison IR vs. ART-. Dunn's post-hoc test.
****P less than 0.05 for comparison INR vs. IR. Dunn's post-hoc test.

A median of 4.7 years after inclusion the median CD4+ gain was 55 (−9 to 115) cells/μl among INRs and 37% had reached a CD4+ cell count above 400 cells/μl. However, only three persons achieved CD4+ cell count above 500 cells/μl. The median increase in CD4+ from 2.4 until 4.7 years after inclusion was 18 (−29 to 75) cells/μl and 58% of the INRs increased their CD4+ cell count in this period. Nevertheless, the CD4+ cell counts stabilized the last 12 months of follow-up (Fig. S3, http://links.lww.com/QAD/B453). Duration of continuous ART and viral suppression, nadir CD4+ and age were all related to prospective CD4+ cell count and/or CD4+/CD8+ ratio (Table S3, http://links.lww.com/QAD/B453).

Activated monocytes in immunological nonresponders correlate with T-cell activation and low CD4+ cell counts

We first analyzed the phenotypes of the various monocyte subsets. In the INR group, the frequency of monocytes was negatively associated with the CD4+ cell count at inclusion ($r = -0.39$, $P = 0.0012$), and the monocytes constituted a higher fraction of the CD45+ cells within the INR than in the immunological responder group (Fig. S3, http://links.lww.com/QAD/B453). The INRs demonstrated increased MFI of HLA-DR in monocytes compared with immunological responders ($P = 0.043$), particularly in the CD14++CD16+ subset ($P = 0.013$) (Fig. 1a). The HLA-DR expression correlated with T-cell activation, an effector memory (EM) T-cell phenotype and negatively with baseline CD4+ cell count and CD4+/CD8+ ratio ($r = -0.35$, $P = 0.004$) (Table S4, http://links.lww.com/QAD/B453). Overall, the ART-naive had the highest CD16 expression in monocytes ($P = 0.002$) (Fig. S4, http://links.lww.com/QAD/B453). However, we found no differences between the HIV groups neither in the distribution of the various monocyte subsets, nor in the MFI values or frequencies of cells expressing the activation markers CD142 [tissue factor (TF)], CD163 or CD11b on these subsets (Fig. S4, http://links.lww.com/QAD/B453 and Fig. S5, http://links.lww.com/QAD/B453). Nevertheless, all HIV-positive groups showed higher MFI of CD142 and lower MFI and fractions of CD163+ monocytes than healthy controls (Fig. S5, http://links.lww.com/QAD/B453). The expression of CD163 or percentages of CD163+
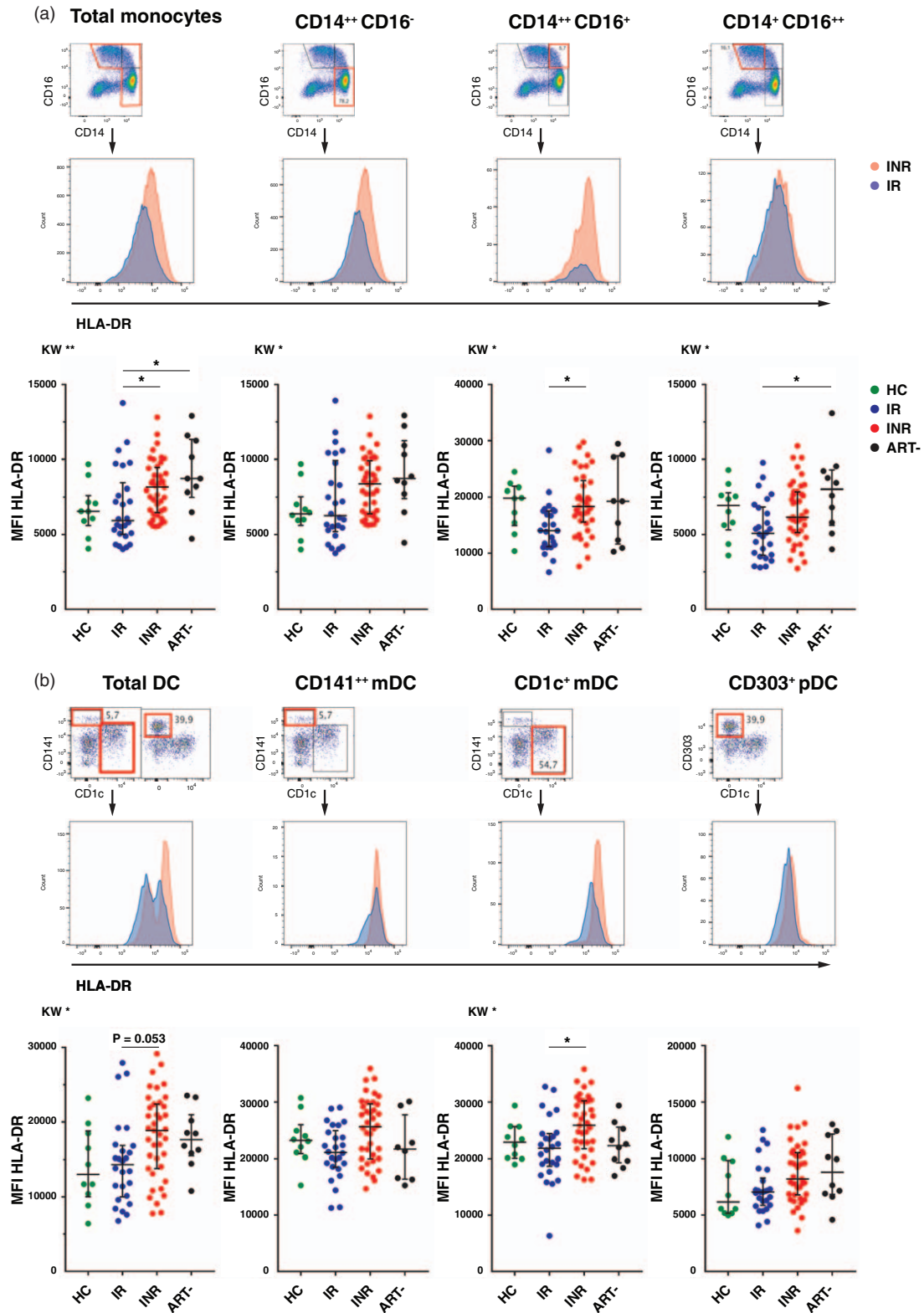
Fig. 1. HLA-DR expression in monocyte and dendritic cell subsets in the different cohorts. Parts (a) and (b) show overlay plots of median fluorescence intensity of HLA-DR in one immunological nonresponder and one immunological responder for various monocyte and dendritic cell subsets, respectively. The graphs display the differences among healthy control, immunological responder, immunological nonresponder and antiretroviral therapy-naive groups in the HLA-DR expression in the specific monocyte (a) and dendritic cell (b) subsets. Kruskal–Wallis test followed by Dunn's post-hoc test for correction for multiple comparisons. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Lines indicate median and interquartile range. ART-, antiretroviral therapy naive HIV-infected; DC, dendritic cells; HC, healthy control; INR, immunological nonresponder; IR, immunological responder; MFI, median fluorescence intensity.

monocytes were not associated with any inflammation markers, T-cell activation or Tregs. Furthermore, analysis of plasma sCD163 revealed no difference between INRs and immunological responders and no correlation with cellular CD163 expressed as MFI or frequencies of CD163$^+$ monocytes.

Dendritic cell activation is increased in immunological nonresponders and correlates with T-cell activation and low CD4$^+$ cell counts

We then investigated the distribution of pDCs and mDCs in the various cohorts. The ART-naive had lower fractions of total dendritic cells compared with both INRs and healthy controls ($P = 0.008$ and $P < 0.001$), but there were no differences between INRs and immunological responders (Fig. S4, http://links.lww.com/QAD/B453). Overall, all HIV groups had more activated dendritic cells than the controls. Still, there was a higher level of dendritic cell activation in the INR compared with the immuno-logical responder group (Figs. 1b and 2b−d) with higher MFI of HLA-DR in the total dendritic cell population, mainly driven by increased expression in CD1$^+$ mDCs ($P = 0.021$) (Fig. 1b). Furthermore, the expression of the maturation marker CD83 in pDCs, the fraction of CD83$^+$ pDCs and CD83$^+$CD141$^+$ mDCs (Fig. 2b), the expression of the T-cell costimulatory molecule CD86 in both mDC subsets (Fig. 2c) and the fraction of CD83$^+$CD86$^+$CD141$^+$ mDCs (Fig. 2d) were all higher in INRs than immunological responders. Finally, activation of pDCs and mDCs correlated strongly with activated T cells, to a lesser degree with the proportion of aTregs/resting (r)Tregs, and all the activated dendritic cell subsets were negatively associated with the baseline CD4$^+$ cell counts (Table S4, http://links.lww.com/QAD/B453). There were no relation between dendritic cell activation and soluble inflammation markers except for a weak correlation between soluble (s)CD14 and HLA-DR expression in CD1c$^+$mDC and CD83 in pDCs ($r = 0.30$, $P = 0.016$, and $r = 0.32$, $P = 0.011$).

HIV-induced upregulation of interferon-inducible protein-10 and indoleamine 2,3-dioxygenase in monocytes and myeloid dendritic cells *in vitro* are comparable in immunological nonresponders, immunological responders and controls

Next, we analyzed the effect of exposure to AT-2 HIV *in vitro* upon the various mDC and monocyte subsets in the INRs, immunological responders and healthy controls. There was a significant upregulation of both MFI of IDO and IP-10 and frequencies of IP-10 positive monocyte and mDC subsets compared with mock-stimulated samples in all cohorts (Fig. 3), with the exception of MFI of IDO in CD141$^+$ mDCs, which decreased. The fraction of IP-10 positive cells increased most in monocytes, whereas IDO expression was highest in mDCs. We found no significant differences between the INR, immunological responder and healthy control

groups neither in frequencies of IP-10$^+$ cells, nor in the MFI values of IP-10 or IDO in monocytes and mDCs after AT-2 HIV stimulation (Fig. 3).

High fractions of activated regulatory T cells are associated with reduced in-vitro HIV-specific responses in immunological nonresponders

We observed clear cytokine responses after AT-2 HIV stimulation, although we did not detect any significant differences between the INRs, immunological responders and healthy controls (Fig. S6, http://links.lww.com/QAD/B453). Changes in soluble IP-10 levels correlated strongly with IP-10 measured by flow cytometry in both monocytes (ΔMFI $r = 0.55$, $P < 0.001$, $\Delta\%$ $r = 0.63$, $P = 0.001$) and mDCs (ΔMFI $r = 0.47$, $P = 0.002$, $\Delta\%$ $r = 0.32$, $P = 0.044$), suggesting that the IP-10 in the supernatants could originate from both monocytes and mDCs. In the INR subgroup the IP-10 expression in monocytes and/or dendritic cells analyzed by flow cytometry corresponded to changes in soluble IFNα2, IL-1ra, IL-18, IFNγ and MIP-1β (Table S5, http://links.lww.com/QAD/B453).

Furthermore, in INRs, the upregulation of both IP-10 and IDO in monocytes and mDCs, as well as the IFNα2, IL-18 and IFNγ responses after exposure to AT-2 HIV, were strongly inversely correlated with the proportion of aTregs (Fig. 4a−c). In immunological responders, there were no such associations.

HIV-specific monocyte interferon-inducible protein-10 responses are negatively associated with future CD4$^+$ gain

Finally, we aimed to explore markers associated with future CD4$^+$ gain in the INR group. The increase of IP-10 expression in monocytes observed after exposure to AT-2 HIV *in vitro*, was negatively correlated with the CD4$^+$ gain seen 2.4 and 4.7 years later (Fig. 4d). In INR with CD4$^+$ cell count 300 cells/μl or less at baseline this association was even stronger ($r = -0.86$, $P = 0.007$) and there was also a negative correlation between the MFI IP-10 response and the prospective CD4$^+$/CD8$^+$ ratio ($r = -0.71$, $P = 0.047$). When adjusting for age and nadir CD4$^+$, an increase in MFI of IP-10 in monocytes above median, could still predict a lower CD4$^+$ gain in INRs after both 2.4 and 4.7 years [2.4 years; odds ratio (OR) = 19.2 (95% confidence interval (CI): 1.1−350.2), $P = 0.046$ and 4.7 years; OR = 21.2 (95% CI: 1.1−399.6), $P = 0.041$]. Nevertheless, when including duration of continuous ART in the model, none of the parameters remained significant.

Discussion

We have explored innate immunity in an INR cohort compared with ART-treated PLWH with satisfactory CD4$^+$ recovery, by characterizing monocyte and
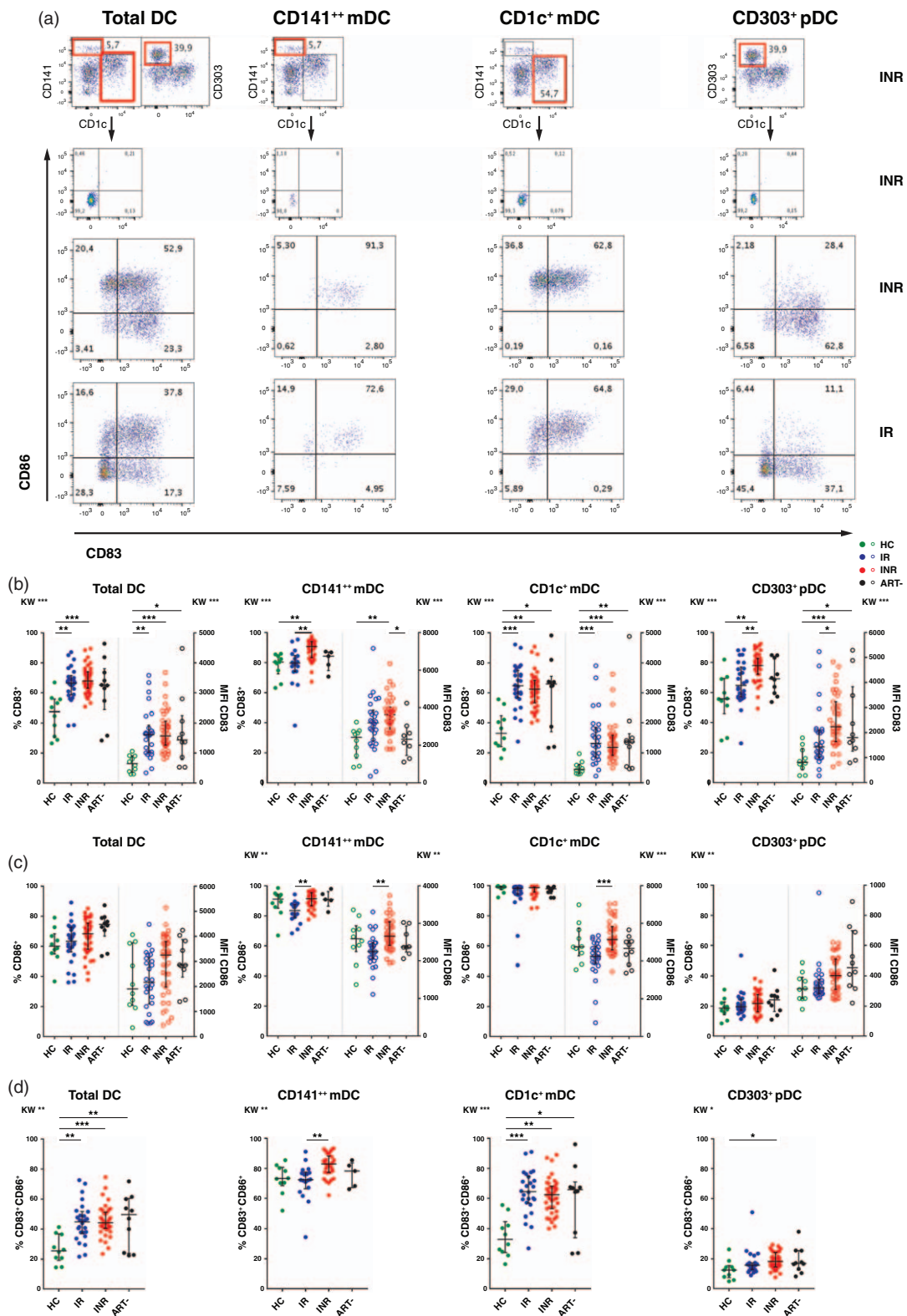
Fig. 2. Dendritic cell activation markers in the various groups. Part (a) illustrates gating strategy of dendritic cell activation markers based on isotype or fluorescence minus one in total dendritic cells and the different dendritic cell subsets. The plots show representative examples from one immunological nonresponder and one immunological responder. In parts (b), (c) and (d), the graphs display both percentage (solid dots) and median fluorescence intensity (open dots) values of the activation markers CD83, CD86 and the coexpression of CD83 and CD86 in the various dendritic cell subsets for each group. Kruskal–Wallis test followed by Dunn's post-hoc test for correction for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001. Lines indicate median and interquartile range. ART-, antiretroviral therapy naive HIV-infected; DC, dendritic cells; HC, healthy control; INR, immunological non-responder; IR, immunological responder; MFI, median fluorescence intensity.
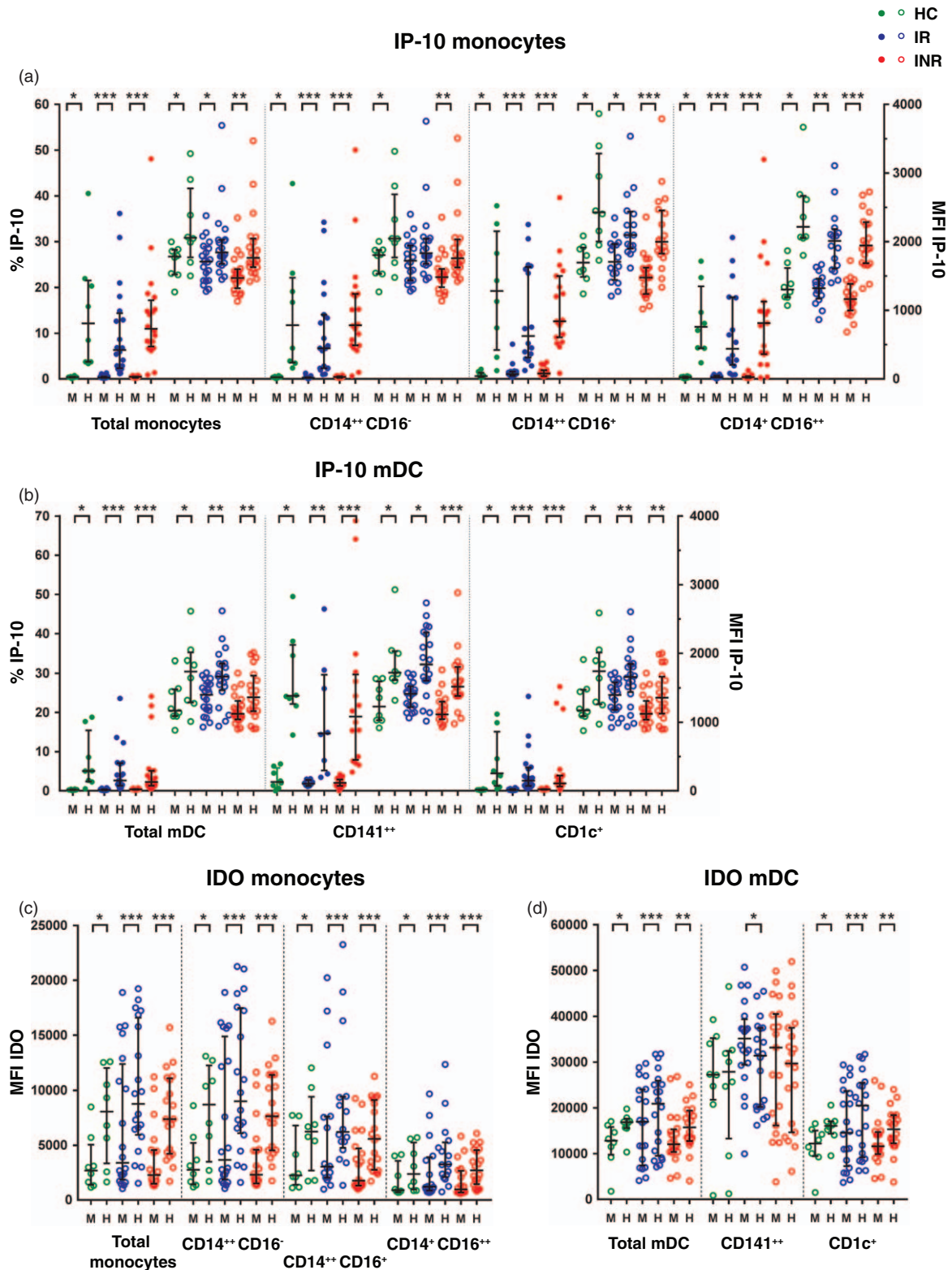
Fig. 3. Interferon-inducible protein-10 and indoleamine 2,3-dioxygenase responses in monocytes and myeloid dendritic cells after exposure to aldrithiol-2 inactivated HIV-1 *in vitro*. Percentages (solid dots) and median fluorescence intensity (open dots) of interferon-inducible protein-10 in monocytes (a) and myeloid dendritic cells (b) after in-vitro stimulation of peripheral blood mononuclear cells with either mock (M) or aldrithiol-2 inactivated HIV-1 (H) in healthy controls, immunological responders and immunological nonresponders. Parts (c) and (d) display indoleamine 2,3-dioxygenase responses in monocytes and myeloid dendritic cells, respectively. Wilcoxon test for pairwise comparisons between mock and aldrithiol-2 inactivated HIV-1 stimulation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Lines indicate median and interquartile range. HC, healthy control; INR, immunological nonresponder; IR, immunological responder; mDC, myeloid dendritic cell; MFI, median fluorescence intensity.
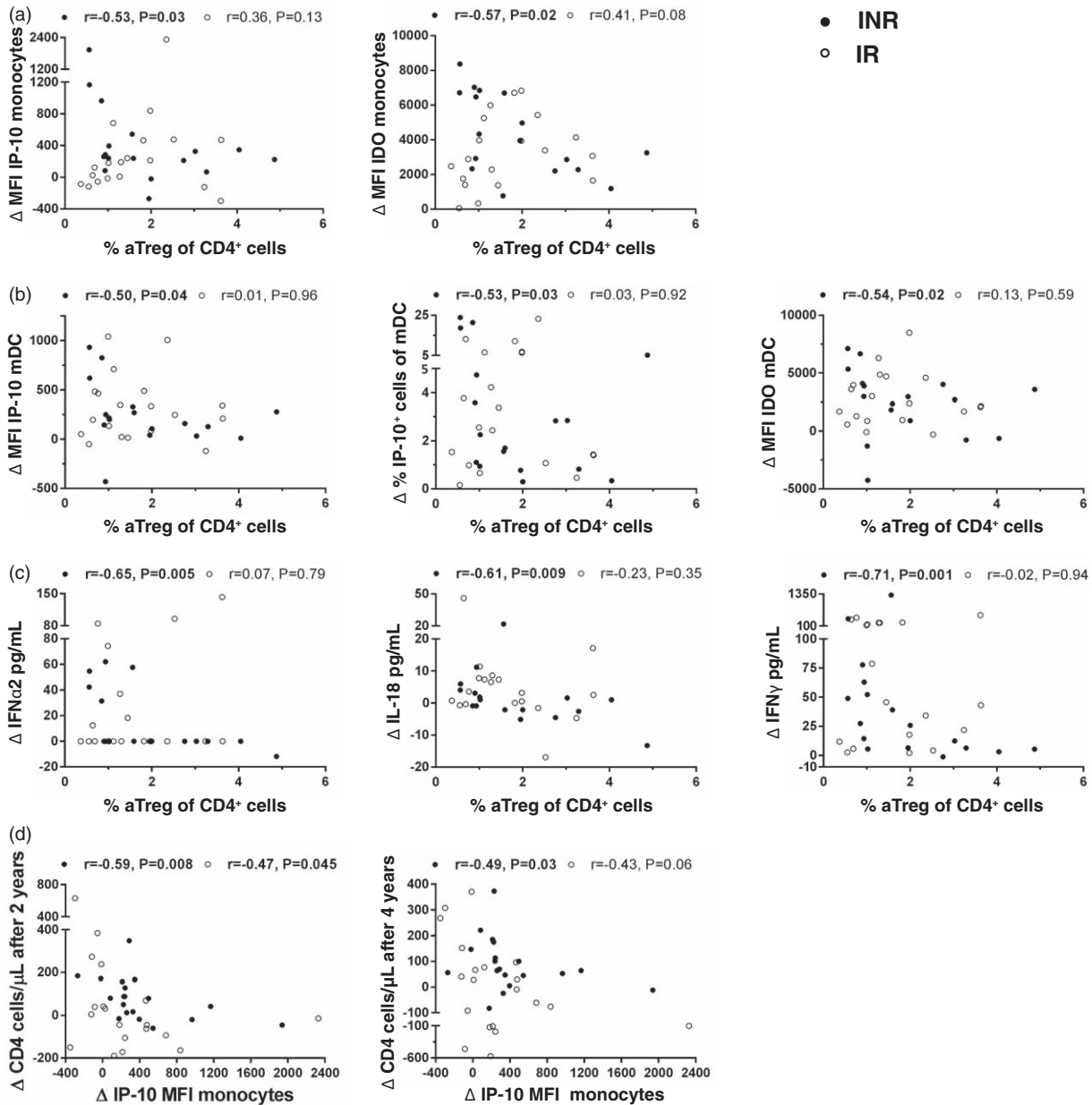
Fig. 4. Associations between fraction of activated regulatory T cells and interferon-inducible protein-10, indoleamine 2,3-dioxygenase and cytokine upregulation after exposure to aldrithiol-2 inactivated HIV-1 *in vitro* and correlation between interferon-inducible protein-10 responses in monocytes and CD4$^+$ recovery after 2 and 4 years. Correlation between percentages of activated regulatory T cells and indoleamine 2,3-dioxygenase and interferon-inducible protein-10 responses in monocytes (a) and myeloid dendritic cells (b) and cytokine responses in supernatants (c) after in-vitro stimulation with aldrithiol-2 inactivated HIV-1. (d) Associations with interferon-inducible protein-10 increase in monocytes after exposure to HIV *in vitro* and prospective CD4$^+$ gain after 2 and 4 years. Spearman rank order correlation. The aldrithiol-2 inactivated HIV-1 responses are calculated with subtraction of the mock stimulated control. •INR, immunological nonresponder; ∘IR, immunological responder; aTregs, activated regulatory T cells (%CD147^{++}CD25^{++} of CD4$^+$); mDC, myeloid dendritic cell; MFI, median fluorescence intensity.

dendritic cell subsets and responsiveness to in-vitro HIV stimulation and their associations with T-cell phenotypes. To our knowledge, this is the first study that presents such thorough characterization of phenotypes and functionality in innate and adaptive immunity in a group of INRs. Our main findings can be summarized as follows: first,

INRs demonstrated increased activation of dendritic cells and monocytes compared with immunological responders. Second, these changes were associated with T-cell activation, a shift towards an EM T-cell phenotype and higher frequency of aTregs. Third, in INRs, the presence of aTregs was correlated with reduced in-vitro

HIV-specific IDO and cytokine responses, and fourth, a high HIV-specific increase in IP-10 expression in monocytes was associated with lower CD4$^+$ recovery after 2 and 4 years.

mDCs are potent antigen-presenting cells and recognize diverse pathogens due to their broad expression of Toll-like receptors (TLRs) [30]. Following TLRs engagement, mDCs upregulate major histocompatibility complex class II, the costimulatory molecules CD80 and CD86, and produce IL-12 which induces Th1 cell responses [30,45]. In our cohorts, INRs showed increased CD86 and HLA-DR expression in mDCs compared with the immuno-logical responder group. Activated pDCs are the most potent producers of IFNα [46] which stimulate a wide range of immune cells. The maturation marker CD83 was higher expressed in pDCs in the INR cohort than in immunological responders and HIV-negative controls. Furthermore, we found that the activated dendritic cells were associated with T-cell activation, an EM T-cell phenotype, as well as the fraction of aTregs/rTregs, and inversely with the CD4$^+$ cell count. Our results thereby suggest that activation of dendritic cells is connected to well known disturbances in the T-cell compartment in both immune-stimulatory and immune-suppressive directions.

HIV and other viruses can activate pDCs directly [34,45,47], whereas mDCs seem to be dependent of exposure to pDC-derived cytokines to mature after exposure to HIV [34,45]. In HIV-infected individuals also LPS, other pathogen-derived factors and unmethy-lated DNA contribute to activation of mDCs and pDCs, respectively [30,48,49]. We found no correlations between dendritic cell activation and soluble inflamma-tion markers in plasma except for a weak correlation with sCD14. Despite lack of association with LPS, dendritic cell activation could still be driven by other microbial products [47,49,50]. Of note, none of the patients had acute infections, hepatitis C or B viremia, but with one exception, all were cytomegalovirus (CMV)-positive and differences in CMV replication and low-level HIV viremia might be partly responsible for the activation of dendritic cells [51].

Contrary to a recent report [39], we discovered higher HLA-DR expression in monocytes in INRs compared with immunological responders. Bandera et al. studied frequencies of HLA-DR$^+$ CD14$^+$ cells and as most monocytes express HLA-DR, such differences could be more difficult to detect. Variations in definition and gating strategies of monocytes and different patient populations can also be a relevant explanation of our diverging results. As seen for dendritic cells, activated monocytes correlated inversely with the CD4$^+$ cell count and were associated with T-cell activation, but not with soluble inflammation markers linked to non-AIDS morbidity in ART-treated PLWH. Nevertheless, in line with two recently published studies, all our HIV groups showed higher expression of TF than controls, which are related to coagulation and cardiovascular disease [22,23].

Despite the increased spontaneous ex-vivo activation of monocytes and dendritic cells seen in INRs, there were no differences between INRs, immunological responders and controls in the in-vitro cell upregulation of IP-10 and IDO or cytokine levels in supernatants. This implies that innate immunity in INRs preserve their capacity to respond to the HIV virus. Our data support other studies reporting similar cytokine production in supernatants in INRs and immunological responders after exposure to single-stranded RNA [52], TLR7 or TLR9 [41], yet IFNα synthesis seemed to be reduced in INRs [41]. Few had a measurable raise in IFNα2 in our study and the numbers are thus too low to draw any conclusions. In line with Simmons et al. [17], we found that monocytes had the highest fractions of IP-10 positive cells after AT-2 HIV stimulation. However, in contrast to Boasso et al. [26], we saw an increase in IDO expression in both monocytes and mDCs indicating that also monocytes could be an important source for IDO activity in HIV infection.

Although the increases in cytokines in supernatants and cellular IP-10 and IDO were similar, the inflammatory responses seemed to be more closely correlated in INRs compared with immunological responders. Only in INRs, the upregulation of IP-10 in monocytes and mDCs were related to the increase in several supernatant cytokines. This could imply that IP-10 responses are accompanied by a more general and possible harmful immune activation in patients with incomplete immune recovery. Moreover, we identified a negative association between the frequency of aTregs and the upregulation of several cytokines and cellular IDO and IP-10 in INRs, suggesting that presence of aTregs may suppress HIV-specific responses in patients with incomplete CD4$^+$ gain.

We recently reported that the level of IP-10 in plasma was negatively associated with the CD4$^+$ cell count after 2.4 years in the individuals with profoundly impaired immune reconstitution at inclusion [16]. In the current study, we found more precisely that the IP-10 response in monocytes after exposure to HIV *in vitro* was negatively correlated with the CD4$^+$ gain after median 2.4 and 4.7 years both for the INR and immunological responder groups, with the strongest association in individuals with baseline CD4$^+$ cell count 300 cells/μl less. Type I interferons seem to be important for IP-10 expression in monocytes after HIV exposure [17], suggesting that pDCs could play a role in the IP-10 increase. IP-10 stimulates HIV-replication *in vitro* [53]. Moreover, in primary HIV-infection, IP-10 levels are strongly correlated with HIV RNA and cell-associated DNA and reported to be a better predictor of disease progression than the level of viremia [54,55]. In addition, IP-10 attracts CXCR3$^+$CD4$^+$ T cells which are major target cells for HIV and also contain the highest amount of integrated DNA in treated HIV infection [56]. Hence,

monocytes which respond to HIV with a high IP-10 production could be important in HIV pathogenesis and potentially unfavorable in the long term also in treated HIV infection.

Our study has some limitations. We have evaluated HIV-specific monocyte and dendritic cell responses, but inclusion of other stimuli such as LPS, flagellin, CpG-containing DNA and CMV, would have given a more comprehensive knowledge of factors contributing to monocyte and dendritic cell activation in INRs. Furthermore, dendritic cell subsets are rare cell populations and are sometimes too small for phenotyping and evaluation of IP-10 and IDO responses. However, studying PBMCs has an advantage over isolated cell populations as cytokine responses and in particular IP-10 is dependent of cross-talk between different immune cells [57]. Finally, about one third of the INRs experienced an increase in their CD4$^+$ cell count to more than 400 cells/ μl after 4 years, in line with other studies [58]. Some of these patients were found in the groups selected for in-vitro AT-2 HIV assays. Although only INRs and immunological responders with similar age and nadir CD4$^+$ cell count were selected for this in-vitro assay, diverse potential for prospective CD4$^+$ recovery in the INR group could have contributed to the observed lack of differences in in-vitro responses between INRs and immunological responders. Thus, for future studies inclusion criteria with even longer duration of ART could be preferable.

In conclusion, ART-treated PLWH with an inadequate immune recovery had a more activated phenotype of monocytes and in particular dendritic cells, changes that were related to activation and regulation in the T-cell compartment. Of note, a high in-vitro HIV-induced IP-10 expression in monocytes was related to a lower future CD4$^+$ recovery. As type I interferons are important for IP-10 expression in monocytes after HIV exposure, an interplay between viral stimulation of pDCs, activation of monocytes and subsequent IP-10 expression could be a relevant pathway involved in inflammation and poor immune recovery during HIV infection.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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