Immune activation in prolonged cART-suppressed HIV patients is comparable to that of healthy controls

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ABSTRACT

Sustained immune activation during chronic HIV infection is considered to augment co-morbidity and mortality. Effective combination antiretroviral therapy (cART) has shown to dampen immune activation especially during the first year cART, but the effects of long-term cART in patients without major comorbidities remains under-investigated.

We performed a comprehensive analysis including cellular, intracellular and plasma biomarkers to study the effect of cART on immune parameters in 5 groups of 10 HIV patients. All patients were without major co-morbidities and grouped based on cART duration (0, 1, 3, 5, and 10 years). We included 10 matched healthy controls for comparison. Our data show that after the first year of cART, no additional effect on the level of inflammatory markers is observed in HIV infected patients without major co morbidities. Residual immune activation status in well-treated HIV-infection is similar to levels observed in healthy controls.

1. Introduction

With the introduction of combination anti-retroviral therapy (cART), HIV-related mortality and morbidity has decreased dramatically. As a result, studies investigating AIDS-related complications are substituted with studies focusing on long-term health consequences of HIV infection in patients with cART induced viral suppression (Paiardini and Müller-Trutwin, 2013; Funderburg, 2014). These studies draw the picture that successful therapy does not completely reverse/normalize the inflammatory status induced by HIV infection since sustained activation of the immune system is observed which in turn leads to non-AIDS-related morbidity such as cardiovascular disease and non-AIDS related cancers (Lederman et al., 2011; French et al., 2009; Abraham et al., 2015; Funderburg et al., 2012; Deeken et al., 2012).

Studies comparing immune parameters in HIV patients and healthy individuals have shown enhanced levels of the plasma cytokines IL-6 (Armah et al., 2012; Neuhaus et al., 2010; Somsouk et al., 2015; Borges et al., 2015) and sCD14 (Abraham et al., 2015; Somsouk et al., 2015; Hattab et al., 2015; Wada et al., 2015), increased inflammatory profiles

of monocytes (Funderburg et al., 2012; McCausland et al., 2015; Hearps et al., 2012a) and enhanced frequencies of activated CD8^{+} T cells as determined by CD38 and HLA-DR expression (Lederman et al., 2011; Bisset et al., 1998; Cobos Jiménez et al., 2016) which is most pronounced during viremia and in case of poor immunologic recovery. However, not all studies were able to reproduce these findings and conflicting data or only subtle effects of HIV infection on monocytes and plasma cytokines were observed (Hattab et al., 2015; Wada et al., 2015; Hearps et al., 2012b; Shah et al., 2015; Castley et al., 2014; Kooij et al., 2016). Several theories have been suggested to cause the sustained immune activation in HIV infection, which include microbial translocation, continued viral replication and pyroptosis (Brenchley et al., 2006; Doitsh et al., 2014). cART-induced reduction of viral load is likely to diminish the degree of immune activation. Indeed, it has been shown that early after cART is initiated, markers of immune activation drop dramatically (Wada et al., 2015; Deeks et al., 2004). However, the long-term effects of cART treatment have been less well studied. One study showed that markers of immune activation fluctuate substantially over the course of chronic treatment (French et al., 2009), while another study demonstrated that T cell activation markers can

reach levels comparable to uninfected controls as early as 6 months after treatment initiation (Chevalier et al., 2013).

The effect of long-term cART on immune activation after initial viral suppression remains under-investigated. The question whether immune activation persists in well-suppressed patients with good immunological recovery is of paramount importance, especially considering the suspected risks on increased mortality and morbidity. In the current study, we investigated the effect of long-term cART treatment on a comprehensive panel of biomarkers of immune activation in HIV patients. We investigated the temporal effect of cART in cross-sectional matched groups of well-treated HIV infected individuals without clinically apparent co-morbidities. We found that after the first year of therapy-induced HIV suppression no additional effect on inflammation markers is observed. Importantly, the residual immune activation status in this population with well-treated HIV-infection is comparable to levels observed in healthy individuals.

2. Methods

2.1. Subjects

Fifty individuals infected with HIV were recruited between 2012 and 2013 from the outpatient clinic of the Erasmus MC and participated in the cross-sectional TREVI cohort, a Dutch study focusing on neurocognitive disorders in patients living with HIV (Langerak et al., 2015). A selection of 10 individuals per group was performed based on the duration of cART treatment. The groups consisted of patients not receiving treatment, patients during the early phase of treatment (1 year on cART), patients during an intermediate phase of treatment (3 and 5 years on cART) and patients on long-term treatment (10 years of cART). A group of 10 uninfected otherwise healthy individuals was used as control (HC). Groups were matched on age, sex and (with the exception of the control group) smoking. Patient history was assessed for major co-morbidities, such as active hepatitis B or C and malignancies. Although all HIV patients are at risk of major co-morbidities, at the time of sampling no clinical signs were apparent yet. Age-related non-communicable diseases were reduced to a minimum in al groups. All participants provided written informed consent, and the study was approved by the ethical committee of the Erasmus MC.

2.2. Assessment of the frequency of leukocyte subpopulations

Blood was collected in heparin BD Vacutainer® CPT™ tubes, and PBMC were isolated within 24 h and cryopreserved in RPMI-1640 medium and DMSO (20%) for later use. Plasma was collected in 3.2% citrated Vacutainer® tubes, spun twice and cryopreserved for later use. Viable PBMC were stained with the following antibodies: CD3-FITC/PE-Cy-7, CD45RO-PE, HLA-DR-PerCP-Cy5.5, CD8-APC-H7/FITC, CD4-PE-Cy7/APC-H7, CD38-eFluor450, CD14-PE/eFluor450, CD56-APC, CD19-APC-eFluor780, and CD16-eFluor450 (Biolegend/eBioscience/BD Biosciences). Flowcytometry was performed using the MACSQuant® (Miltenyi Biotec) and analyzed using Flowjo software (Treestar). The frequencies of lymphocytes and monocytes were determined on the basis of their forward/sideward scatter (FSC-SSC) profile.

2.3. Intracellular cytokine staining

Cytokine production by monocytes was determined as described before (Liu et al., 2011). Briefly, PBMC were seeded at $0.25 \times 10^6/250\,\mu l$ with serum free X-vivo media (Lonza) alone, or in combination with 2 ng/ml LPS Minnesota (TLR4 agonist, Sigma), or 1 µg/ml R848 (TLR7/8 agonist, Enzo Lifesciences). Cells were incubated for 2 h and treated with 10 µg/ml brefeldin A (Sigma) to block protein secretion. After 7 h of incubation, cells were fixed with 2% formaldehyde and stained for CD14. Cells were then permeabilized with 0.5%

saponin and stained with antibodies against MIP-1β-PE, TNF-PE-Cy7; MCP-1-APC; Tissue Factor (CD142)-PE, IL-6-FITC, IL-8-FITC. For the intracellular staining of NK and T cells, 0.5×10^6 PBMC were incubated in a 96 wells plate for 1 h in 200 µl RPMI-1640 medium with 5% FCS and subsequently fixed with 2% formaldehyde. Cells were then washed once with PBS, permeabilized with 0.5% saponin and stained with antibodies against TRAIL-FITC, granzyme B-PE, perforin-PerCp-Cy5.5, CD56-APC and CD3-eFluor450. 0.5×10^6 PBMC were incubated in a 24 wells plate for 21 h in 250 µl RPMI-1640 medium with 5% FCS in the presence or absence of IL-12 (0.25 ng/ml, Miltenyi) and IL-18 (50 ng/ml, R & D systems). Cells were fixed, stained for CD3 and CD56, permeabilized and stained with antibodies against IFNγ-FITC (Spaan et al., 2016). Flowcytometry and analysis was performed as described earlier. All antibodies were purchased from Biolegend, eBioscience or BD Biosciences.

2.4. Assessment of plasma biomarkers using multiplex immunoassays and ELISA

The ProcartaPlex human multiplex assay was used to detect cytokines in plasma (Affymetrix eBioscience). Targets included sIL-2R, interferon-gamma-inducible protein (IP)10, sTNFRII, IL-10, D-dimer, and CRP. The assays were analysed on the Ap54 microsphere based multiplex LUMINEX 100 FIDIS v3 using ProcartaPlex Analyst 1.0 Software. Plasma levels of sCD14 were measured sandwich ELISA using rat anti-human sCD14 (clone 55-3; BD Biosciences), and biotinylated anti-human sCD14 (clone 3-C39; BD Biosciences). Visualization of the assay was using streptavidin-horseradish peroxidase and TMB, and the optical density (OD) at 450 nm was measured on a Bio-Rad imager.

2.5. Statistical analysis

Statistical analysis was performed using SPSS software (IBM, v21.0). Cell populations of each sample were measured as percentage within the parent population and compared between subgroups. Continuous variables were assessed for normality and significance between groups was detected using a one-way ANOVA. A post-hoc correction for multiple comparisons was applied using Tukey HSD test when equal variances were assumed. For analysis of data with clear heterogeneity of variances, a Games-Howel test was applied.

Table 1
Basic characteristics of HIV-infected cases and uninfected controls.

	HC	0	1	3	5	10
Years on cART	0 (0)	0 (0)	1.1 (0.5)	3.0 (0.3)	5.2 (0.6)	10.8 (2.3)
Age (yr)	44 (4)	45 (4)	42 (8)	44 (2)	45 (8)	45 (5)
Males (%)	100	100	100	100	100	100
Caucasian	90	100	100	100	100	100
CD4 nadir	n.a.	511 (209)	296 (151)	257 (131)	200 (77)	200 (99)
CD4	n.a.	629 (222)	601 (271)	630 (172)	624 (276)	667 (160)
VL < 200 copes/ml	n.a.	0	80	100	100	100
Years since diagnosis	0 (0)	4.4 (2.8)	2.3 (1.2)	4.5 (1.8)	6.9 (2.4)	12.3 (2.4)
Smoking (%)	0	40	50	40	50	50

Each group consists of 10 individuals. n.a = not applicable. HC = healthy controls. 0, 1, 3, 5, 10 = HIV groups with corresponding cART treatment duration (in years). The standard deviation is presented in brackets.

3. Results

Table 1 displays the characteristics of the study population. All HIV patients and HC were male, and almost exclusively Caucasian. The mean age of patients combined was 45 years and did not significantly differ between the HIV groups and HC (p=0.848). The CD4 nadir count and viral load were highest at baseline (both p < 0.0001). The CD4 counts were comparable across HIV groups (p=0.978). To minimize the effect of smoking as a potential confounder, we choose to balance this risk factor across the HIV groups. All patients were free from malignancies and active hepatitis co-infection. Only three patients had been previously diagnosed with comorbidities with a possible effect on inflammation. Two patients had well-controlled diabetes and one patient had minor myocardial ischemia 5 years before sampling for which he received adequate treatment. One diabetes patient was on cART for 3 years; the other diabetes patient and the myocardial ischemia patient were on cART for 10 years. None of the patients demonstrated significantly stronger inflammatory responses compared to other subjects.

3.1. Proportions of lymphocyte subsets not affected by prolonged HIV suppression by cART

Phenotypic characterization was performed to assess the impact of prolonged HIV suppression by cART on the percentage of blood lymphocyte populations. Fig. 1A shows the proportion of $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, $CD56^+CD3^-$ NK cells, and $CD56^+CD3^+$ NKT cells within the lymphocyte population. All HIV positive patients showed significantly lower $CD4^+$ and higher $CD8^+$ T cell percentages in comparison to healthy control subjects and these percentages did not normalize in patients with prolonged cART. Our patient population consists of chronically HIV-infected patients in which treatment was initiated when CD4 counts dropped below 350×10^6 $CD4^+$ T cells/ml following previous guidelines (European AIDS Clinical Society Guidelines, 2014). The proportions of $CD19^+$ B cells, NK cells, and NKT cells did not differ between untreated patients and those treated with cART for 1, 3, 5 or 10 years, and were comparable to those observed for healthy individuals.

3.2. Activation of $CD4^+$ and $CD8^+$ T cells reduced during cART induced HIV suppression

Next, we investigated the impact of duration of cART on the activation status of lymphocyte subpopulations. While no differences in CD45RO expression was observed in $CD4^+$ and $CD8^+$ T cells before and during cART (data not shown), the fraction of $CD4^+$ and $CD8^+$ T cells being $HLA-DR^+CD38^+$ were reduced in patients on cART and seemed to normalize to the low levels observed in healthy controls after 3–5 years (Fig. 1B). Also in patients treated with cART for 1 year only, this effect was observed, albeit not significant (p=0.069).

3.3. Monocytes seem unaffected by HIV infection and subsequent cART initiation

Circulating monocytes can be subdivided on the basis of expression of CD14 and CD16 into inflammatory ($CD14^{++}CD16^+$), patrolling ($CD14^+CD16^+$) and traditional ($CD14^+CD16^-$) monocytes. The proportions of inflammatory, patrolling and traditional monocytes seem unchanged by HIV infection, and cART initiation has no significant impact on their relative distribution (Fig. 2A). Also, the proportions of monocytes producing the pro-inflammatory cytokines TNF, IL-6, MIP-1β and MCP-1 (Fig. 2B) as well as the anti-inflammatory cytokine IL-10 (data not shown) were similar between healthy subjects and HIV patients, irrespective of treatment duration. Only when all HIV patients were pooled, significantly higher proportions of monocytes producing MCP-1 (p=0.003), IL-8 (p=0.008; data not shown) and Tissue Factor (p=0.011; data not shown) were observed as compared to healthy control subjects. However, it is important to note that the levels of induction of pro-inflammatory cytokines by monocytes of HIV-infected patients are variable.

3.4. NK cells seem unaffected by HIV infection and subsequent cART initiation

Under pro-inflammatory conditions, NK cells are able to mount a potent antiviral response, primarily mediated by IL-12 and IL-18. This results in the production of IFNγ, a cytokine crucial in controlling viral infections (Murphey et al., 2012). NK cells are able to induce killing of virus-infected host cells by self-recognition and subsequent production
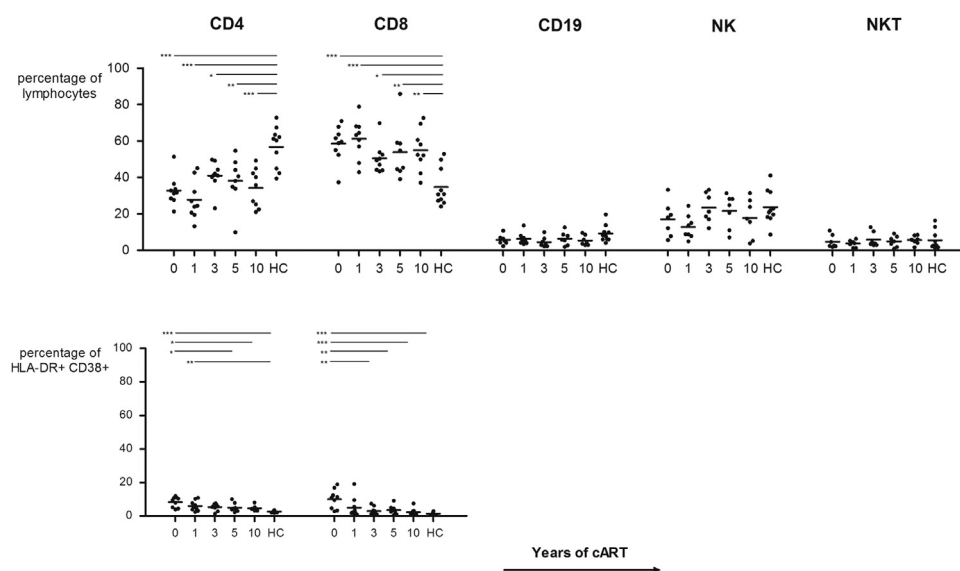

Fig. 1. Distribution of CD4, CD8, CD19, NK and NKT cells within lymphocyte populations. HC had more CD4 cells and less CD8 cells compared to all HIV+ groups. Below: the proportion of HLA DR positivity of corresponding CD4 and CD8 population. A significantly higher percentage of $HLA-DR^+CD38^+$ cells in the CD4 population of untreated HIV+ patients was found compared to patients more than 5 years on treatment and HC. The percentage of $HLA-DR^+$ cells in CD8 population was higher in untreated HIV+ patients compared to patients longer than 3 years on treatment. 0, 1, 3, 5, 10 = HIV infected patients in ascending order of antiretroviral treatment duration (0, 1, 3, 5 or 10 years of therapy). HC = healthy controls. * p≤0.05; ** p≤0.01; *** p≤0.001.
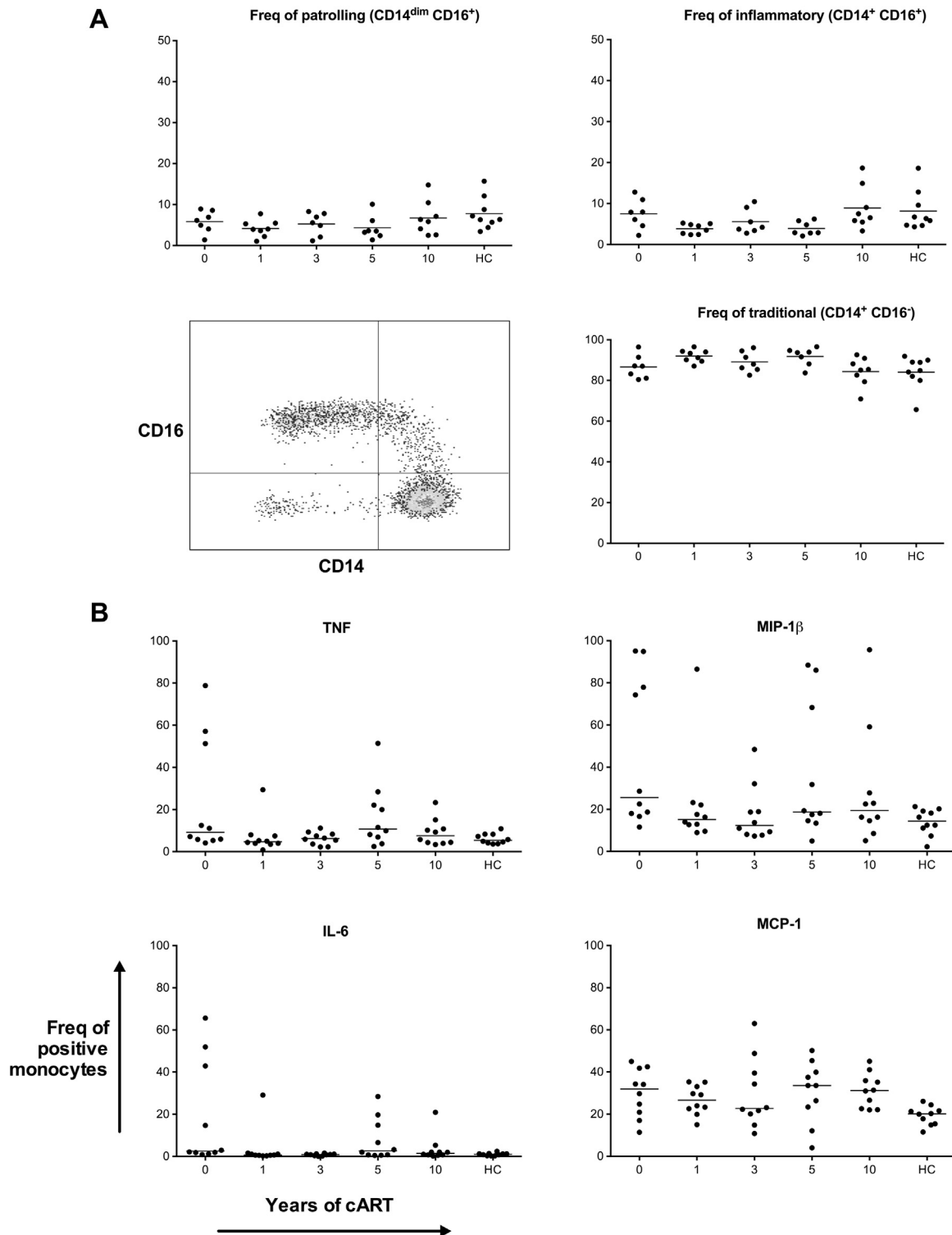
Monocytes

A

Fig. 2. (A) Proportion of traditional (CD14+CD16-), inflammatory (CD14+CD16+) and patrolling (CD14dimCD16+) monocytes in otherwise healthy uninfected controls (HC) and patients with HIV infection in ascending order of cART treatment duration (0, 1, 3, 5 or 10 years of therapy). The variation of CD16 expression within groups was considerable but no statistically significant differences were observed between groups. (B) The frequency of monocytes expressing inflammatory markers of otherwise healthy uninfected controls (HC) and patients with HIV infection in ascending order of cART treatment duration (0, 1, 3, 5 or 10 years of therapy). No significant difference was found across groups in baseline intracellular levels of TNF, IL-6, MIP-1β and MCP1.

of enzymes, like perforin, granzyme B and membrane expression of TRAIL. Fig. 3A illustrates the frequency of NK cells expressing TRAIL, granzyme B and perforin, and demonstrates no significant differences

between expression of these markers in NK cells of patients compared to HC, albeit that the expression of granzyme B appeared higher in patients than controls. Also, no clear association with treatment
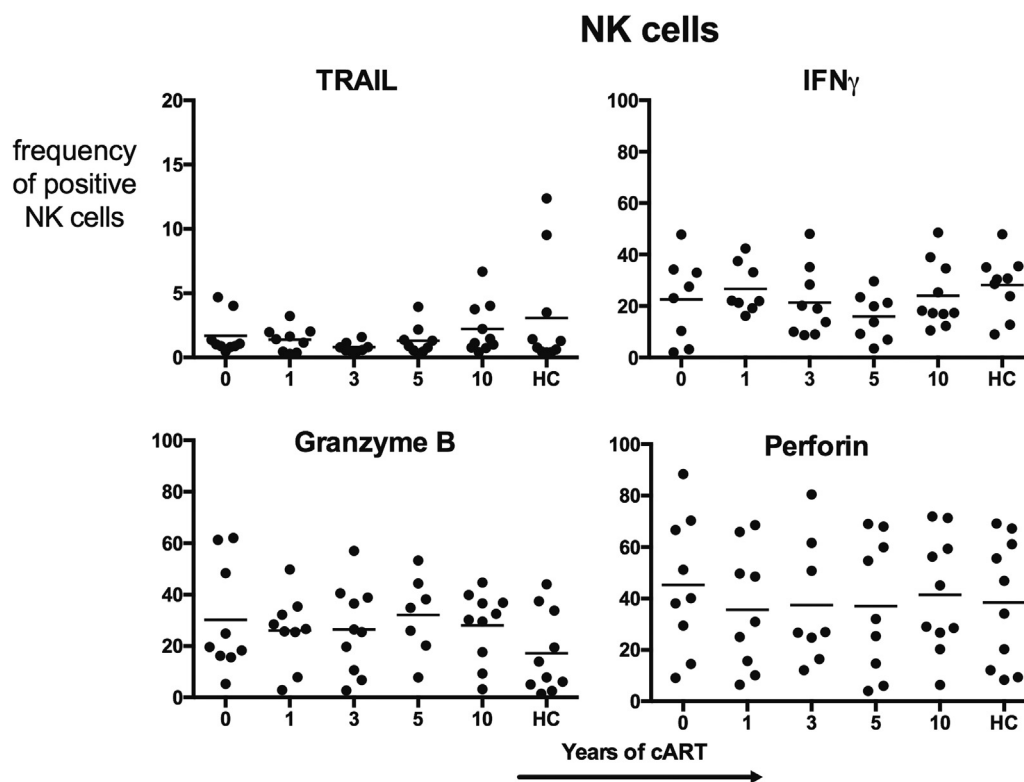
Fig. 3. Intracellular markers associated with immune activation in NK cells. Intracellular expression of TRAIL, granzyme B, IFNγ and perforin by NK cells. For the IFNγ assay, prior stimulation with IL-12 and IL-18 was performed. No unidirectional trend of immune activation level was observed with treatment duration. TRAIL: TNF-related apoptosis-inducing ligand. IFN: interferon.* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

duration was observed. The range of perforin expressing cells was high, probably reflecting a large intra-individual spread of this marker. To evaluate the function of NK cells, IFNγ production was induced with IL-12 and IL-18. The frequencies of IFNγ-producing NK cells were comparable in all HIV groups, and did not statistically differ from HC.

3.5. Except for IP-10, the levels of inflammatory markers in plasma did not decline during cART suppressed HIV-infection

Finally, plasma levels of various pro-inflammatory biomarkers were determined. As shown in Fig. 4, plasma IP-10 levels were higher in untreated HIV patients as compared HC. As a consequence of early, intermediate and long-term cART the plasma IP-10 levels declined to the levels observed in HC. sTNF-RII and sCD14 levels were also significantly higher in untreated HIV patients compared to HC, but did not decline on cART. The plasma levels of sIL-2R and CRP showed a trend towards higher levels in the untreated group as compared to HC, but again the levels did not significantly decline during the course of cART. Plasma D-dimer levels were not increased and displayed no change during the course of cART.

4. Discussion

A number of studies have demonstrated that effective cART treatment of chronic HIV patients result in reduced activation of components of the patient's immune system. Most studies evaluated the immune effects during the first year after start of therapy. We now show the immune effects of HIV suppression up to 10 years after start of cART in a well-defined cross-sectional study cohort. Importantly, albeit that the selected patients are at risk of major co-morbidities, including hepatitis and malignancies, at the time of sampling no clinical signs of co-morbidities were apparent yet. Our findings show that after the first year of therapy-induced HIV suppression no additional effects on inflammation markers were observed in HIV-

infected patients without major co-morbidities. Our observation of no or mild immune activation in suppressed HIV-infection questions the impact of ongoing inflammation on co-morbidity and mortality observed in HIV-infected patients without any previous major co-morbidities.

In line with previous studies, we observed that untreated HIV infection is associated with an increased expression of the activation markers HLA-DR and CD38 on CD4^{+} and CD8^{+} T cells, indicating occurrence of the persistent immune activation in untreated HIV-infected individuals (Paiardini and Müller-Trutwin, 2013; Hunt, 2012; Wittkop et al., 2013). Importantly, the T cell activation is not permanent, since the frequencies of HLA-DR^{+}CD38^{+} T cells drop during the early stage (1 year) after treatment initiation and plateau during the intermediate (3 and 5 years) and long-term (10 years) phase of treatment. We did not detect a significant difference in this marker when comparing intermediate and long-term treatment to healthy controls. This is in line with a number of studies, although conflicting data exists. Some studies investigating the level of activated T cells in well treated HIV infected individuals report levels comparable to uninfected controls (Lederman et al., 2011; Bisset et al., 1998; Chevalier et al., 2013), others find a modest but significant increase (Cobos Jiménez et al., 2016; Vinikoor et al., 2013). These contradictory findings point towards additional confounding factors that influence the activation status of T cells in the absence of viremia. Behavioral and socio-demographic risk factors might play a role, as well as the timing of treatment initiation (Jain et al., 2013).

Different from the effect of chronic HIV infection on T cells, we were unable to find modulation of monocyte and NK cell frequencies, phenotype or function as a consequence of the ongoing infection, and as a result the additional effect of cART could not be assessed. Little information is available in literature on the effect of long-term cART on NK cells, but a number of studies in monocytes have been performed. Our results are in line with studies that show that monocytes do not exhibit an inflammatory profile (Castley et al., 2014; Fischer-Smith
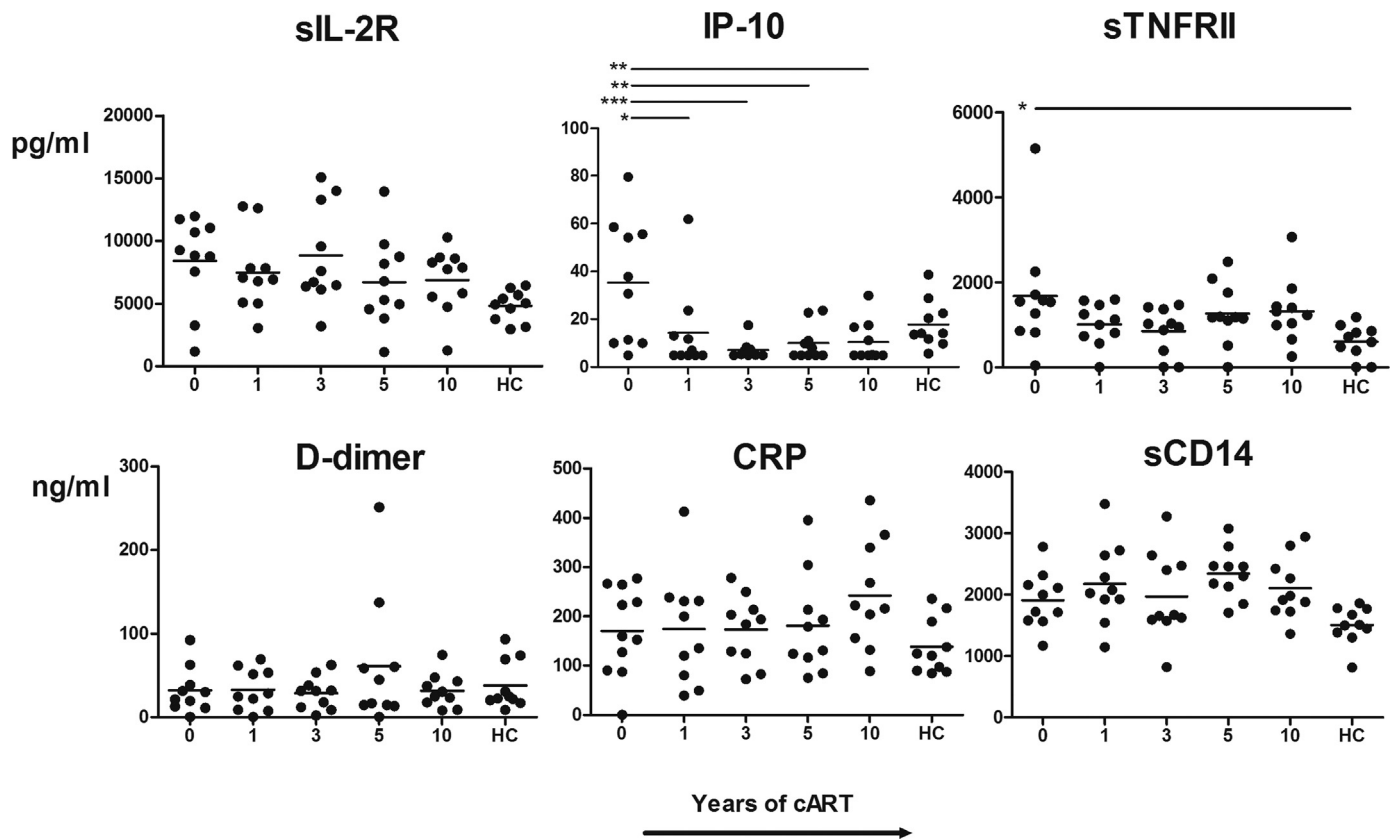
Fig. 4. Plasma biomarkers measured in plasma of HIV infected patients in ascending order of treatment duration and healthy controls. Plasma levels of sIL-2R, IP-10, sTNF-RII, D-dimer, CRP and sCD14 are presented. Results are in pg/ml. sIL-2R= soluble IL-2 receptor. IP-10= Interferon gamma-induced protein 10. TNFRII= TNF receptor II. CRP= C-reactive protein. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

et al., 2008; Amirayan-Chevillard et al., 2000; Jaworowski et al., 2006). In studies where increased CD16^{+} monocyte subsets are identified, these observations are often in the context of viremia (Funderburg et al., 2012; McCausland et al., 2015; Jaworowski et al., 2006).

We observed a large variation on the production of intracellular cytokines in monocytes between individuals. These outliers could represent patients that are at increased risk for illnesses associated with an activated myeloid phenotype. The presence of patrolling and inflammatory monocytes is strongly associated with cardiovascular disease in the setting of HIV infection (McKibben et al., 2015). However, our patients show similar percentages of patrolling and inflammatory monocytes and only a minority of HIV-infected patients shows elevated production by monocytes of pro-inflammatory cytokines. Considering the absence of inflammatory profile in other subjects, we expect this phenomenon not to be HIV-related. It is tempting to speculate that HIV infection does not increase the risk of cardiovascular disease in patients in the absence of prior risk factors. Longitudinal studies are needed to formally address this issue.

In line with the effects of cART on T cell activation, we also observed that plasma IP-10 levels significantly declined during the first year after start of treatment, and normalized during continued treatment as compared to the levels observed in healthy individuals. Our results corroborate with a previous study where no additional effect of treatment was observed after one year of treatment (Wada et al., 2015). However, others showed that the same plasma biomarkers have shown to correlate with morbidity and mortality (Kuller et al., 2008; Kalayjian et al., 2010; Sandler et al., 2011). In this context, IL-6 has been studied most extensively for its predictive value (Kuller et al., 2008; Bétété et al., 2014; Nordell et al., 2014). However, Borges et al. elegantly demonstrated using the data from these 3 major clinical trials representing close to 10,000 patients that plasma IL-6 can be influenced by a variety of other, non-HIV related factors like age,

smoking and co-morbidities (Borges et al., 2015). The aim of our study was to investigate the specific effect of cART on the level of immune activation. We therefore matched all patients on age and smoking status and excluded individuals with potentially confounding co-morbidities. Importantly, the other biomarkers tested, sIL-2R, sTNF-RII, D-dimer, CRP, and sCD14, did not demonstrated a significant reduction, albeit that for some elevated levels were observed in HIV patients, and a trend towards decline was observed on cART.

In conclusion, our findings suggest that in our cross-sectional cohort consisting of HIV patients of the same age and devoid of clinically apparent co-morbidities, the modulation of immune parameters as a consequence of persistent HIV infection is relatively weak. In this, the majority of monocyte and NK cell markers as well as the serum biomarkers tested, resemble those in healthy individuals. cART reduced normalization of the T cell activation status and plasma IP-10 levels were observed early following treatment initiation, and no additional immune effects were observed upon continued treatment for up to 10 years. The data therefore questions the concept of chronic inflammation in HIV as the driving mechanism in the development of long-term co-morbidities in HIV as suggested by others (Cobos Jiménez et al., 2016; Castley et al., 2014; Tenorio et al., 2014), and leave room for alternative explanations, such as the effects of lifestyle factors (smoking, obesity, drug abuse), sexual behavior, aging, and changes in cellular metabolism due to HIV.

5. Sources of support

writing of the report; and in the decision to submit the article for publication.

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