Plasma IP-10 Is Increased in Immunological NonResponders and Associated With Activated Regulatory T Cells and Persisting Low CD4 Counts

Birgitte Stiksrud, MD,† Kristina Berg Lorvik, PhD,*‡§|| Dag Kvale, MD, DMSc,*†|| Tom Eirik Mollnes, MD, DMSc,||¶#**†† Per Magne Ueland, MD, DMSc,‡‡§§ Marius Trøseid, MD, PhD,*||||||¶¶ Kjetil Taskén, MD, DMSc,*‡§|| and Anne M. Dyrhol-Riise, MD, DMSc*†||‡‡*

Objective: To explore immune mechanisms and identify biomarkers associated with an inadequate immune recovery in patients with HIV with efficient antiretroviral therapy.

Design: A cross-sectional study of 67 HIV-infected patients on antiretroviral therapy for ≥24 months with HIV RNA ≤20 copies per milliliter; 41 were defined as immunological nonresponders (INR) (CD4 < 400 cells per microliter) and 26 as immunological responders (CD4 > 600 cells per microliter). CD4 counts were also registered 2 years after inclusion.

Methods: Cytokines, soluble markers of microbial translocation, and tryptophan catabolites were measured in plasma by multiplex assay, ELISA, or mass spectrometry. T-cell activation, differentiation, and regulatory T cells (Tregs) were analyzed by flow cytometry in 2 subgroups with comparable nadir CD4 counts.

Results: Plasma interferon-inducible protein-10 (IP-10) levels were higher ($P < 0.05$), the T cells were more activated ($CD38^{+}HLA\text{-}DR^{+}$) ($P < 0.05$), the naive/effector memory T-cell ratio was lower ($P < 0.01$) and the proportion of resting Tregs ($CD4^{+}CD45RA^{+-}FoxP3^{+}$) was reduced ($P < 0.001$) in INR patients compared with immunological responders. INR patients with CD4 counts ≤300 cells per microliter also demonstrated a higher fraction of activated Tregs (aTreg) ($CD4^{+}CD147^{high}CD25^{high}$) ($P < 0.05$). In the INR group, the aTreg percentages correlated with plasma IP-10 levels and inversely with CD4 counts (both $P < 0.01$). IP-10 levels ($P < 0.05$) and kynurenine/tryptophan ratio ($P < 0.01$) were negatively associated with the CD4 count 2 years after inclusion.

Conclusion: Patients with HIV with inadequate CD4 responses had higher levels of IP-10, more activated and differentiated T-cell phenotypes, as well as aTreg, compared with patients with satisfactory CD4 gain. High IP-10 levels were also associated with lower CD4 counts after 2 years.

Key Words: HIV, immunological nonresponders, IP-10, CXCL-10, Tregs, T-cell activation

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From the *Department of Infectious Diseases, Oslo University Hospital, Oslo, Norway; †Department of Infectious Diseases, Institute of Clinical Medicine, University of Oslo, Oslo, Norway; ‡Centre for Molecular Medicine Norway, Nordic EMBL Partnership, Oslo University Hospital, University of Oslo, Oslo, Norway; §Biotechnology Centre, University of Oslo, Oslo, Norway; ||K.G. Jebsen Centre for Inflammation Research, Institute of Clinical Medicine, University of Oslo, Oslo, Norway; ¶Department of Immunology, Oslo University Hospital, Oslo, Norway; #Research Laboratory, Nordland Hospital, Bodø, Norway; **Faculty of Health Sciences, K.G. Jebsen TREC, University of Tromsø, Norway; ††Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim, Norway; ‡‡Department of Clinical Science, University of Bergen, Bergen, Norway; §§Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway; ||||Research Institute of Internal Medicine, Division of Cancer Medicine, Surgery and Transplantation, Oslo University Hospital, Oslo, Norway; and ¶¶Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, Oslo, Norway.

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A.M.D.-R. and B.S. designed the study. B.S. included the patients, performed the laboratory experiments, analyzed the data, and wrote the article. K.B.L. performed the flow cytometry experiments, analyzed these results, and drafted parts of the article. A.M.D.-R., K.T., and D.K. took part in the evaluation of the flow cytometry experiments and A.M.D.-R. outlined central aspects of the article. M.T., P.M.U., and T.E.M. contributed to the LPS, tryptophan, and multiplex analyses, respectively. All authors have critically revised the article.

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Correspondence to: Anne M. Dyrhol-Riise, MD, DMSc, Department of Infectious Diseases, Oslo University Hospital, Ullevål, P. O. Box 4950, Nydalen N-0424 Oslo, Norway (e-mail: a.m.d.riise@medisin.uio.no).

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INTRODUCTION

Antiretroviral therapy (ART) with effective control of viral replication and subsequent immunologic reconstitution has dramatically reduced HIV-related morbidity and mortality.[1] Despite persistent virus suppression about 15%–30% fails to restore their CD4 count to normal levels >500 cells per

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microliter,[2–4] hence termed immunological nonresponders (INR). These patients have an elevated risk of developing AIDS, non-AIDS clinical complications such as cancer and cardiovascular disease, as well as all-cause mortality compared with those who achieve a normal CD4 count.[5–7] Increased residual chronic immune activation and inflammation are plausible causes of these adverse outcomes.[8–10]

Low nadir CD4 count, long duration of HIV infection before ART, coinfections such as hepatitis C and older age are factors associated with an incomplete immune recovery.[3,11,12] Studies have also identified several possible underlying immunological mechanisms; decreased $CD4^+$ T-cell production could be caused by exhaustion of lymphopoiesis,[13] thymic insufficiency,[14,15] and/or disturbed peripheral homeostasis of naive $CD4^+$ T cells.[16,17] Furthermore, persistent immune activation, as well as accelerated T-cell turnover and apoptosis[8,18,19] may be induced by continuous residual viral replication in lymphoid tissue[20–22] or by translocation of microbial products into the circulation across a damaged mucosal barrier.[23]

The role of regulatory T cells (Tregs) in immune reconstitution remains debated, whether they are useful down-regulators of harmful unspecific immune activation or dampen beneficial HIV-specific immune responses. Recent studies report increased fractions of Tregs in INR compared with patients with an adequate CD4 gain.[10,24–26] Activated Tregs (aTreg) are apparently more frequent,[10,25,26] whereas the percentage of resting Tregs (rTreg) seems to be reduced.[25] We have previously identified CD147 as a marker of activated and highly suppressive human Tregs within the $CD4^+FoxP3^+$ subset,[27] but this Treg subpopulation has not been investigated in patients with HIV.

Another relevant pathway in HIV pathogenesis is tryptophan catabolism and indoleamine 2, 3 dioxygenase (IDO1) activity measured by kynurenine/tryptophan ratio (KTR). KTR is upregulated in HIV infection[28] and 2 studies have recently found that higher KTR predicts lower CD4 recovery in patients on ART,[29,30] but its association in INR patients has not yet been determined.

To further investigate these immunological disturbances in INR and to identify possible biomarkers associated with a discordant response to ART, we conducted a cross-sectional study comparing a cohort of INR patients to patients with HIV who had developed normal CD4 T cell counts (IR, immunological responders). Owing to the strong association of nadir CD4 with low CD4 recovery, flow cytometry analyses were performed in 2 INR and IR subgroups with comparable nadir CD4 count. Finally, we aimed to explore if any of the analyzed markers were related to the CD4 gain or CD4 count 2 years after inclusion.

METHODS

Patients and Study Design

Sixty-seven HIV-infected patients were recruited between October 2012 and April 2013 at Oslo University Hospital, Norway. All patients were >18 years old and had received continuous ART for minimum 24 months with HIV RNA ≤20 copies per milliliter for the last 18 months. Those with CD4 count <400 cells per microliter were defined as INR, whereas patients with CD4 count >600 cells per microliter were classified as IR. Exclusion criteria were concomitant or sporadic use of immune modulating therapies, acute or chronic severe illnesses, including opportunistic infections, autoimmune diseases, active chronic hepatitis B and C, cancer, or tuberculosis. Two subgroups of 21 patients from each cohort, with comparable age and nadir CD4 count, were included in the flow cytometry analyses (see Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A835). CD4 counts were also recorded for all patients approximately 2 years after inclusion.

All participants provided written informed consent. The study was approved by the Regional Ethics Committee (1.2007.83).

Blood Sampling and Peripheral Blood Mononuclear Cell Isolation

EDTA plasma was snap frozen, centrifuged within 20 minutes at 2000*g* and 4°C for 20 minutes and stored in pyrogen-free tubes at −80°C until further analyses. Peripheral blood mononuclear cells (PBMC) were isolated and cryopreserved at −150°C until analyses. Clinical biochemistry parameters, CD4 and CD8 T-cell counts, and HIV RNA were performed according to clinical routine and obtained from fresh samples.

Analyses of Soluble Markers

Lipopolysaccharide was analyzed in plasma by Limulus Amebocyte Lysate colorimetric assay (Lonza, Walkersville, MD) as previously described.[31] Interferon-inducible protein-10 (IP-10), interleukin (IL)-6, and soluble (s)CD14 were analyzed by Quantikine ELISA kits (R&D Systems Europe, Abingdon, United Kingdom) in line with the manufacturer's instructions, but with the following modifications; samples were run with dilution 1:300 for sCD14 and 1:2 for IP-10. All analytes were run in batches with all samples and in duplicates. For >95% of the samples, the inter-assay CV% was <10%.

A multiplex cytokine assay (Bio-Plex Human Cytokine 16-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA) was used to measure the concentrations of 16 different interleukins and chemokines in plasma; IL-1β, IL-1 receptor antagonist (IL1-ra), IL-2, IL-6, IL-7, IL-10, IL-15, IL-17a, IL-21, IL-22, IL-23, interferon gamma (IFNγ), macrophage chemoattractant protein 1 (MCP-1)/CCL2, macrophage inflammatory protein 1 alpha (MIP-1α)/CCL3, MIP-1β/CCL4, and tumor necrosis factor (TNF). The samples were analyzed on a multiplex analyzer using Bio-Plex Manager 6.0 (Bio-Rad Laboratories) according to instructions from the manufacturer. Samples were run in single copies and the intra-assay and interassay coefficients of variation were <12% for all analytes. Eight of the soluble markers were excluded from further statistical analyses because of low plasma levels (>65% below the lower detection level (LDL) of the assay). For the remaining 8 markers (IL-1β, IL-6, IL-7, IL-10, IL-22, MCP-1/CCL2, MIP-1β/CCL4, and

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TNF), the occasional values (<25%) below the LDL were replaced by a value below the LDL which allowed for statistical analyses.

Plasma concentrations of tryptophan, the tryptophan metabolite kynurenine, and neopterin were analyzed by liquid chromatography-tandem mass spectrometry by Bevital A/S (www.bevital.no).[32]

Flow Cytometry Analyses

Flow cytometry analyses were performed on PBMCs from the INR and IR subgroups (see Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A835). The following monoclonal antibodies from BD biosciences (San Jose, CA) (unless otherwise specified) were used according to the manufacturer's instructions: anti-CD3 PB (UCHT1), anti-CD4 PerCP (L200), anti-CD8 APC-H7 (SK1), anti-CD25 PE (4E3; Miltenyi, Lund Sweden), anti-CD27 PE-Cy7 (M-T271), anti-CD38 PE (HB7), anti-CD45RA APC-H7 or FITC (clone HI100), anti-CD45RO PerCP-Cy5.5 (UCHL1), anti-CD127 PE-Cy7 (eBioRDR5; eBioscience, San Diego, CA), anti-CD147 FITC (HIM6), anti-CD279 APC (MIH4), anti-FoxP3 Alexa Fluor 647 (259D/C7), and anti-HLA-DR BV605 (G46-6). Samples were acquired with BD LSR Fortessa and analyzed with the FlowJo software (version 10; BD LSR Fortessa, TreeStar Inc., Ashland, OR). A strict lymphocyte gate was set in the forward versus side scatter plot to exclude dead cells and debris. Fluorescence minus one and 2 (for anti-HLA-DR, and anti-CD38, and anti-CD279, respectively) and biologically negative cell populations were used as staining controls. The gating strategy is shown in Figure S1, Supplemental Digital Content, http://links.lww.com/QAI/A835. aTreg was determined using surface markers ($CD147^{high}CD25^{high}$)[27] and compared with $CD45RA^{-}FoxP3^{high}$ gating of aTreg (denoted traditional aTreg), see Figure S2, Supplemental Digital Content, http://links.lww.com/QAI/A835. rTreg was defined as $CD45RA^{+}Foxp3^{+}$ (see Figure S2, Supplemental Digital Content, http://links.lww.com/QAI/A835).

Statistical Analyses

All continuous variables are presented as median and interquartile range. Owing to lack of normal distribution of the data, nonparametric statistics were generally applied; Kruskal–Wallis test and Mann–Whitney *U* test for comparison between the subgroups or cohorts, Fischer Exact Test or Pearson χ^2 test for testing differences in categorical variables between the groups, and Spearman rank correlation for assessing correlation between parameters. Significant differences between the main cohorts were further analyzed by binary logistic regression adjusting for age, nadir CD4 count, and duration of viral suppression and with INR as outcome. The immunological variables were divided in tertiles, with first tertile used as reference. A 2-tailed significance level of 0.05 was used and considering the study's exploratory intention, adjustments for multiple testing were not performed. Statistical analyses and graphical presentations were performed in SPSS statistics 22 (IBM Corp., Armonk, NY) and GraphPad Prism V6.03 software (GraphPad, San Diego, CA).

RESULTS

Study Participants and Baseline Characteristics

Forty-one INR and 26 IR HIV-infected patients were included (Table 1). The median CD4 count in the INR group was 285 cells per microliter compared with 810 cells per microliter in the IR group ($P < 0.001$). The INR patients had significant lower nadir CD4 count ($P < 0.01$), lower CD4/CD8 ratio ($P < 0.001$), a higher frequency of comorbidities ($P = 0.04$), and shorter duration of viral suppression ($P = 0.01$), but there was no significant difference in the duration of continuous ART use (Table 1). Regarding the 2 INR (n = 21) and IR (n = 21) subgroups with similar nadir CD4 counts selected for flow cytometry analyses, the IR patients had higher viral load at initiation of continuous ART ($P = 0.01$), longer duration of viral suppression ($P = 0.01$), as well as slightly increased duration of continuous ART ($P = 0.03$) (see Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A835). Otherwise, the INR and IR groups were similar regarding demographics and clinical characteristics (Table 1 and Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A835).

INR Patients Had Higher Levels of Plasma IP-10 Compared With Responders

Patients in the INR cohort had higher levels of plasma IP-10 than the IR patients [104.5 pg/mL (83.5–148.0) vs 78.7 pg/mL (63.4–109.1), $P = 0.03$] (Fig. 1A). When adjusting for age, nadir CD4, and duration of viral suppression for all patients, the highest IP-10 tertile still predicted INR (odds ratio = 9.6; CI: 1.7 to 52.9, $P = 0.01$). Within the IR and INR subgroups with comparable nadir CD4 count and age, the difference in IP-10 levels between INR and IR was even more pronounced [110.5 pg/mL (83.8–146.8) vs 72.2 pg/mL (57.3–101.1), $P < 0.01$] (Fig. 1B) and IP-10 correlated negatively with the CD4 counts ($r = -0.30$, $P = 0.05$) (Fig. 1C).

We aimed to further characterize systemic inflammation by analyzing a number of cytokines, as well as CRP and β2-microglobulin, but the levels of these markers were not different in the INR than in the IR patients. Thus, IP-10 was the only inflammatory marker included, which showed significant differences between the cohorts.

T Effector Cell Activation Was Increased in INR Patients

In INR patients, the fraction of activated cells ($CD38^{+}HLA\text{-}DR^{+}$) was increased compared with the IR group for both the $CD4^{+}$ ($P < 0.01$) and $CD8^{+}$ ($P = 0.02$) T-cell compartments (Figs. 2A, B). In terms of differentiation status, INR had lower proportions of $CD4^{+}$ and $CD8^{+}$ naive T cells ($CD45RA^{+}CD27^{+}$) compared with the IR (both $P = 0.02$)

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TABLE 1. Characteristics of the Study Cohort at Inclusion

Total Study Population	INR (n = 41)	IR (n = 26)	INR vs IR, *P*
Age, IQR	49.9 (41.4–57.9)	45.0 (39.2–54.5)	NS
Male sex, n (%)	35 (85.4)	18 (69.2)	NS
Ethnicity, n (%)			
White	29 (70.7)	17 (65.4)	NS
Risk group, n (%)			
MSM	22 (53.7)	13 (50)	NS
Other*	19 (46.3)	13 (50)	NS
Smoking, n (%)			
Current smoking	8 (19.5)	10 (38.5)	NS
Comorbid diseases, n (%)			
Cardiovascular	6 (14.4)	0 (0)	NS
Hypertension	7 (17.1)	1 (3.8)	NS
Previous cancer	8 (19.5)	1 (3.8)	NS
Previous TBC	4 (9.8)	0 (0)	NS
Any comorbidity†	21 (51.2)	6 (23.1)	0.04
CMV IgG pos	41 (100)	25 (96.2)	NS
HIV characteristics, IQR or n (%)			
Yrs since HIV diagnosis	8.6 (6.3–15.7)	9.2 (6.9–14.4)	NS
Yrs of continuous ART	5.5 (3.1–7.1)	6.6 (4.4–8.8)	NS
Viral load at ART initiation, copies/mL	67,500 (28,150–130,000)	10,000 (50,000–330,000)	NS
Time to viral suppression, mo	6.4 (3.8–14.2)	6.7 (4.4–10.5)	NS
Duration of viral suppression, yrs	3.8 (2.0–5.8)	6.1 (4.0–7.8)	0.01
Viral blip‡	12 (30.8)	11 (42.3)	NS
Viral rebound§	9 (23.1)	9 (34.6)	NS
Current use of PI	14 (34.4)	16 (61.5)	0.04
Current use of INSTI	8 (19.5)	0 (0)	0.02
Experience of ≥2 ART regimens	32 (78)	22 (84.6)	NS
Previous use of ART	7 (17.1)	5 (19.2)	NS
CD4 count nadir, cells/μL	100 (20–159)	180 (114–225)	<0.01
CD4 count, cells/μL	285 (232–350)	810 (739–887)	<0.001
CD8 count, cells/μL	670 (501–896)	1005 (808–1589)	<0.001
CD4/CD8	0.43 (0.32–0.58)	0.79 (0.59–1.00)	<0.001

Data are presented as number (%) of patients or median (interquartile range (IQR)) values.
*Other: heterosexual or unknown. There were no intravenous drug abusers.
†One or more of the following comorbidities; cardiovascular disease, hypertension, diabetes, renal disease, osteoporosis, chronic obstructive pulmonary disease, neurodegenerative disease, previous cancer or TBC.
‡Isolated measurement of HIV RNA 50–500 copies per milliliter preceded and followed by another value with full viral suppression.
§Either a HIV RNA value >50 copies per milliliter at 2 consecutive visits, or 1 HIV RNA value >500 copies per milliliter. Mann–Whitney *U* test. Pearson χ^2 and Fischer exact test.
NS, not significant; CMV, cytomegalovirus; INSTI, integrase strand transfer inhibitor; IQR, interquartile range; MSM, men who have sex with men; PI, protease inhibitor; TBC, mycobacterium tuberculosis infection.

(Figs. 2C, D). The fractions of both activated $CD4^+$ and $CD8^+$ T cells correlated negatively (r = −0.71, $P < 0.001$ and r = −0.56, $P < 0.01$) and the fractions of naive T cells were positively correlated with the CD4 count (both r = 0.41, $P < 0.01$), when the cohorts were studied together. The INR patients showed higher percentages of effector memory (EM, $CD45RO^+CD27^-$) $CD4^+$ T cells ($P = 0.02$) (Fig. 2E), whereas the fraction of EM $CD8^+$ T cells was similar between the groups (Fig. 2F). Taken together, the naive/EM ratios were lower in the INR than in the IR group for both T-cell subsets (both $P = 0.01$) (Figs. 2G, H). The proportions of central memory (CM, $CD45RO^+CD27^+$) T cells and programed death-1, a marker of T-cell exhaustion, were comparable for the INR and IR groups (data not shown).

In INR the Frequency of Activated Treg Correlated With IP-10 Levels and Negatively With the CD4 Count

The INR group displayed a higher aTreg/rTreg ratio compared with the IR group ($P < 0.01$) (Fig. 3A). This difference was seen both when aTreg was defined traditionally as $CD45RA^-FoxP3^{high}$ or alternatively as $CD147^{high}CD25^{high}$ (shown in Figure S2, Supplemental Digital Content, http://links.lww.com/QAI/A835) because the 2 aTreg phenotypes were highly correlated (r = 0.79, $P < 0.0001$). The change in ratio was predominantly caused by a significantly lower percentage of rTreg in INR than in the IR group ($P < 0.001$) (Fig. 3A) as there were no significant differences in aTreg between the groups (Fig. 3A).

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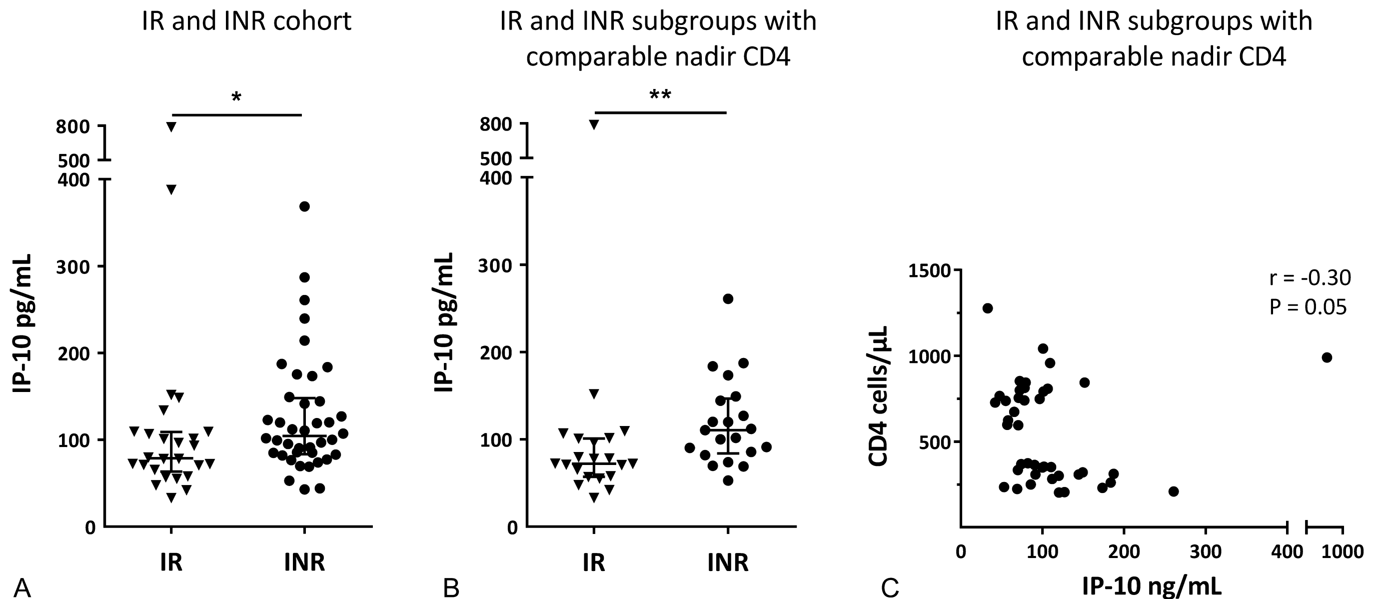

FIGURE 1. Comparison of IP-10 levels in the IR and INR cohorts and its correlation with CD4 count. IP-10 levels in the IR and INR cohorts (A) and in the IR and INR subgroups with comparable nadir CD4 count (B). C, Correlation between IP-10 levels and CD4 counts in the IR and INR subgroups combined. Mann–Whitney *U* test. * $P < 0.05$, ** $P < 0.01$. Lines indicate the median and interquartile range.

Among the INR, CD147^{high} aTreg correlated strongly with the plasma IP-10 levels (r = 0.59, $P < 0.01$) (Fig. 3B), but these associations were not observed in the IR group (Fig. 3B). Moreover, a marked negative association was found between the fraction of CD147^{high} aTreg and the CD4 count (r = −0.61, $P < 0.01$). It should be noted that the total fractions of Tregs (Foxp3^{+}CD25^{high} or CD127^{low}CD25^{high}), were similar in the 2 groups (data not shown).

Increased levels of aTreg in INR with CD4 count <200 cells per microliter has previously been reported.[25] As we had no patients with CD4 <200 cells per microliter in our INR group, we stratified the INR into 2 groups with CD4 count ≤300 and 301–400 cells per microliter, according to levels close to the median CD4 count in the INR group (see Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A835). With this stratification, a higher proportion of CD147^{high} aTreg and a lower proportion of rTreg were detected in the INR group with CD4 count ≤300 compared with both the INR patients with CD4 count 301–400 (P = 0.03) and the IR group (P = 0.02) (Fig. 3C). However, such differences were not seen when aTreg was identified by the traditional gating strategy (CD45RA^{-}FoxP3^{high}) (Fig. 3C).

IP-10 and Activated Treg Correlated With Soluble Inflammation Markers

Microbial translocation and subsequent monocyte activation were measured by circulating levels of lipopolysaccharide, sCD14 and neopterin, respectively. We observed no significant differences in any of these parameters between the INR and IR cohorts. Neither did the levels of tryptophan, kynurenine, or KTR vary between the groups. However, the soluble inflammation markers IL-6, β2-microglobulin, sCD14, neopterin, kynurenine, and KTR correlated moderately with IP-10 levels when the INR and IR cohorts were analyzed together (see Table S2, Supplemental Digital Content, http://links.lww.com/QAI/A835). IL-6 (r = 0.56, P = 0.01) and β2 microglobulin (r = 0.46, P = 0.04) were also associated with the fractions of aTreg in the INR group.

High IP-10 Levels at Baseline Correlated With Low CD4 Counts 2 Years Later

CD4 counts were also measured median 2.4 years after inclusion. The INR cohort experienced a median CD4 gain of 45 cells per microliter (−15 to 105), but only 12 patients (30.7%) had reached CD4 count >400 at that time point. There were no differences in clinical and basic immunological characteristics at inclusion, between these patients and those who were still defined as INR after 2 years. Younger age (r = −0.41, P = 0.01) and high nadir CD4 count (r = 0.45, $P < 0.01$) were the only parameters associated with prospective CD4 count. However, in the INR group with CD4 count ≤300 at inclusion, 13 (59.1%) had still CD4 <300 after 2 years and both age, duration of viral suppression, IP-10 levels, and KTR were inversely related to the CD4 count and the CD4 gain after 2 years (Figs. 4A–H). Furthermore, IP-10 and KTR correlated strongly with aTregs (r = 0.78, P = 0.01 and r = 0.71, P = 0.02, respectively). IP-10 also correlated negatively with the prospective CD4 count in analyses when both cohorts were included (r = −0.29, P = 0.02).

DISCUSSION

We show in this study that INR patients had higher levels of plasma IP-10 and more activated T cells compared

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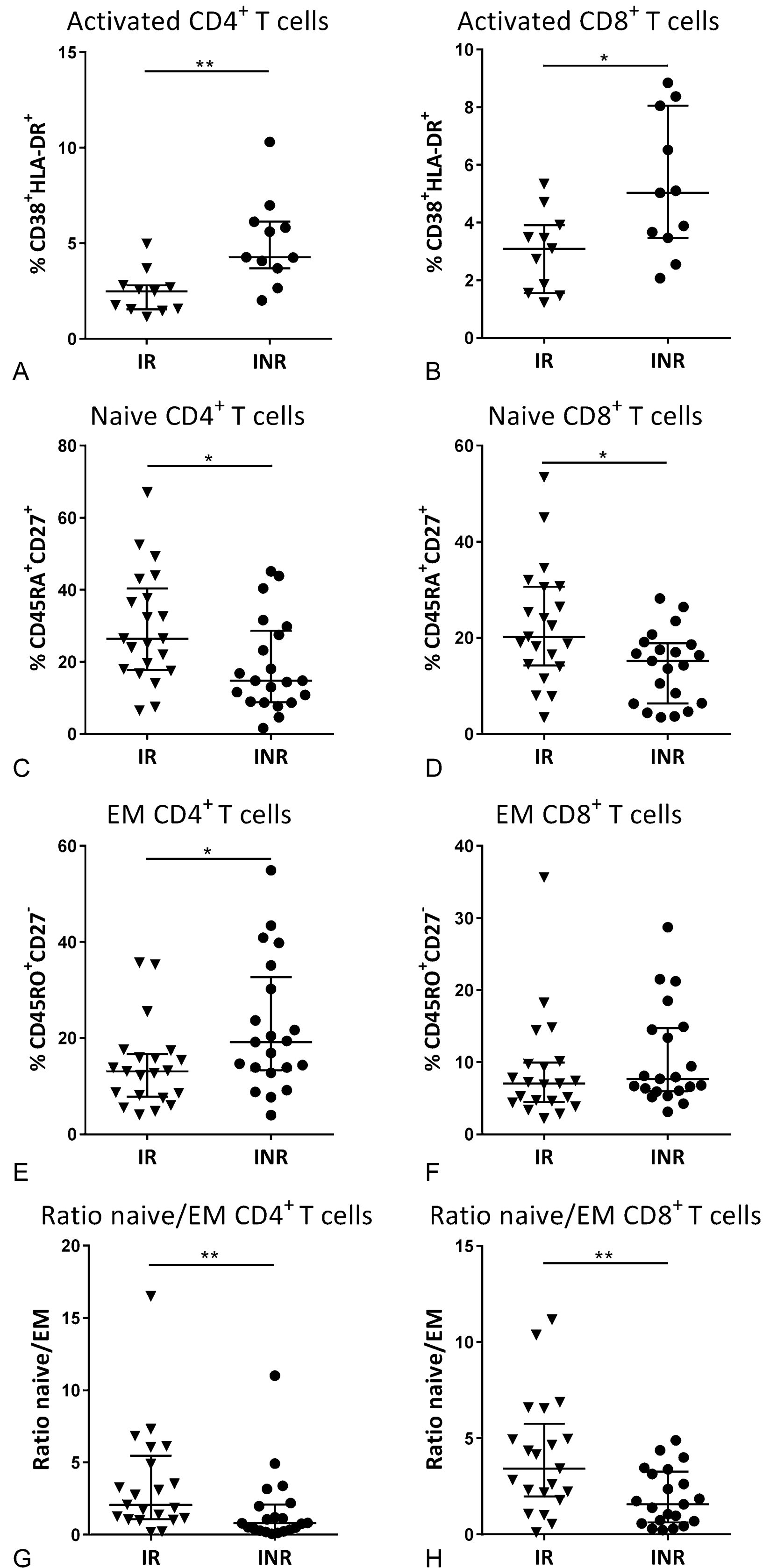

FIGURE 2. Activation and differentiation status of the T cells in IR and INR. The percentages of activated CD4+ (A) and CD8+ (B) T cells determined as CD38+HLA-DR+. The percentages of CD45RA+CD27+ naive CD4+ (C) and CD8+ (D) T cells. The percentages of CD45RO+CD27− EM CD4+ (E) and CD8+ (F) T cells. The ratio of percent naive/EM CD4+ (G) and CD8+ T cells (H). Mann–Whitney *U* test. * $P < 0.05$, ** $P < 0.01$. Lines indicate the median and interquartile range. N = 11–21. EM, effector memory.

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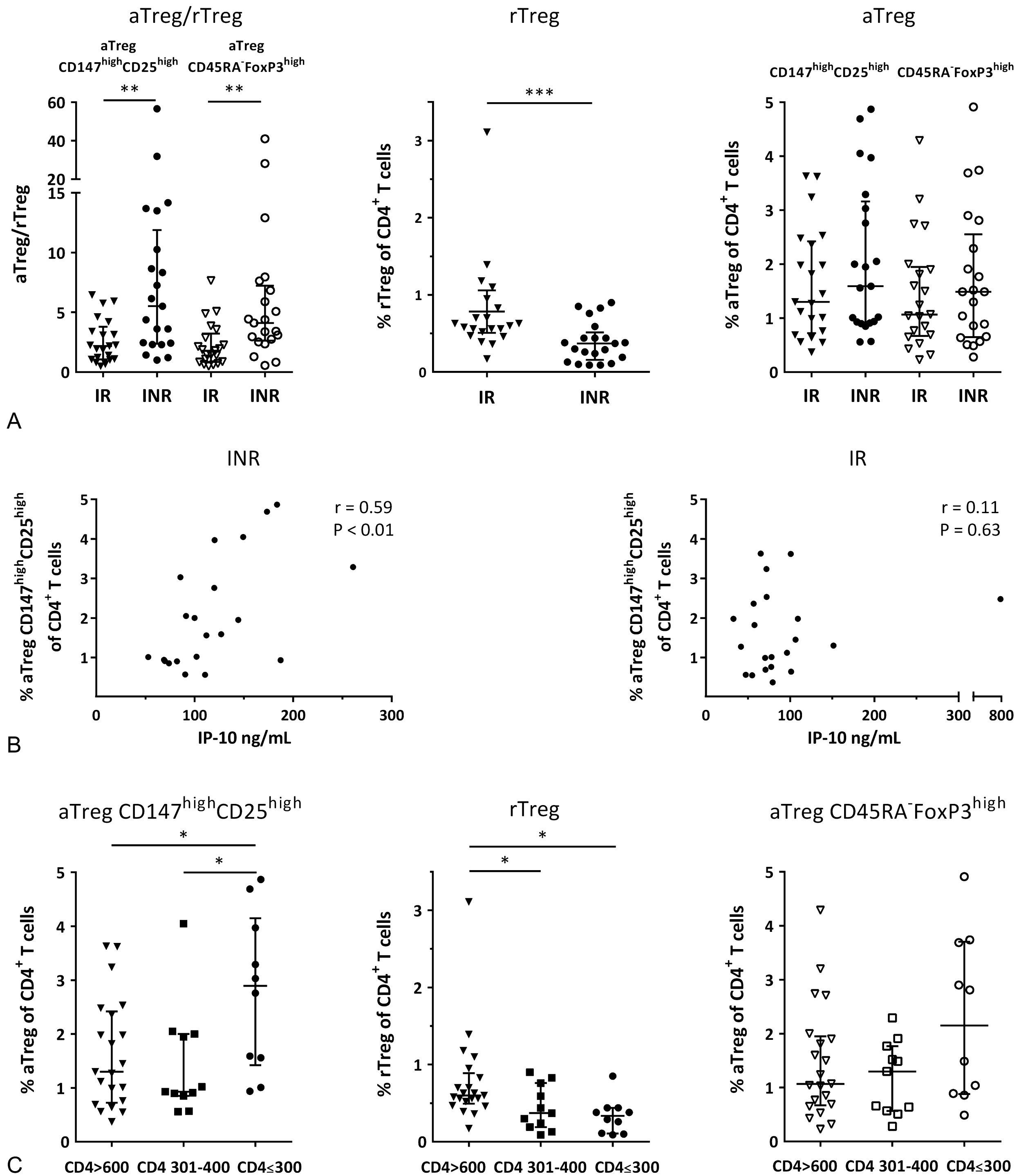

FIGURE 3. Frequencies of Treg subsets and correlation with IP-10 levels. A, Differences between the IR and INR; the ratio of aTreg/rTreg (left panel). Filled and open symbols indicate aTreg identified by alternative ($CD147^{high}CD25^{high}$) (filled symbols) or traditional ($CD45RA^{-}FoxP3^{high}$) (open symbols) gating strategy, respectively. The percentages of resting (middle panel) and activated (right panel) Tregs identified by alternative or traditional gating strategy. B, Correlation of $CD147^{high}$ aTreg with plasma IP-10 in INR (left panel) and IR (right panel). C, The percentages of resting (middle panel) and activated Treg identified by alternative (left panel, filled symbols) or traditional (right panel, open symbols) gating strategy in the 2 INR subgroups with CD4 counts between 301–400 cells per microliter or ≤300 cells per microliter and in the IR group with >600 cells per microliter. Mann–Whitney *U* test and Spearman rank-order correlation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Lines indicate the median and interquartile range. N = 10–21.

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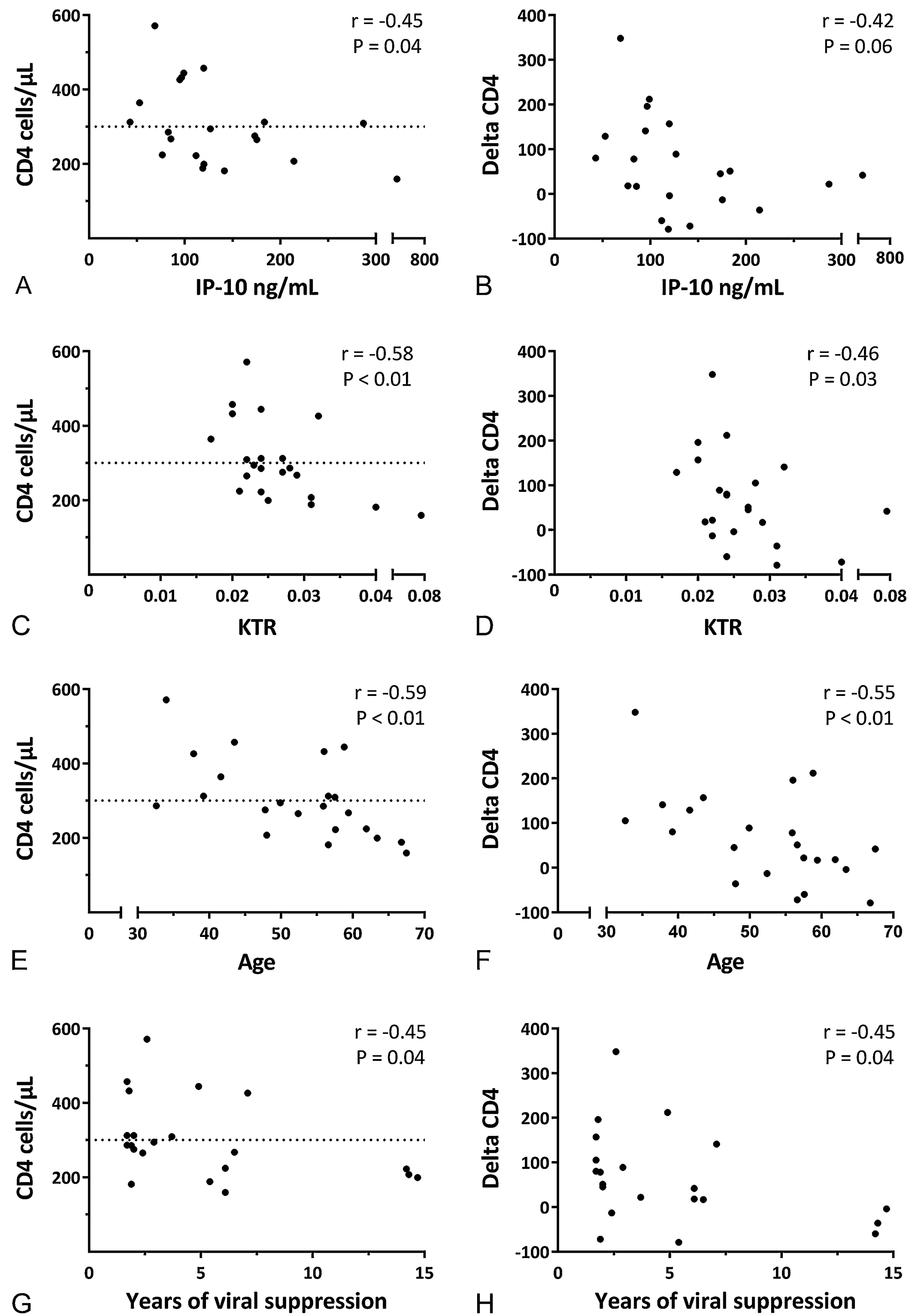

FIGURE 4. Correlation of IP-10 levels, KTR, age, and duration of viral suppression with CD4 count and CD4 gain after 2 years, in INR with CD4 count ≤300 cells per microliter at inclusion. Correlation of IP-10 levels (A and B), KTR (C and D), age (E and F), and years of viral suppression (G and H) with the CD4 count and the CD4 gain after 2 years, respectively. Lines indicate the upper limit of the CD4 count in this subgroup at inclusion (300 cells per microliter) (B). Spearman rank order correlation.

with the IR, and in the INR patients with the lowest CD4 counts, also fractions of CD147^{high} aTregs were elevated. Moreover, high plasma IP-10 was related to a lower CD4 gain 2 years after inclusion. To our knowledge, this is the first article reporting an association between high IP-10 levels and present INR status, as well as future inadequate immune reconstitution. In addition, we present novel data of the new CD147^{high} aTreg subset in HIV-infected patients, using a more simplified method of defining aTreg as permeabilization is not required.[27]

IP-10 levels are increased in HIV infection and in untreated patients, related to viremia and disease progression.[13,33,34] After ART, studies disagree to whether the levels are normalized or not.[33–39] Furthermore, IP-10 is shown to be negatively related to nadir CD4[40] and is also associated with age in HIV infection.[36,38] Of note, in the subgroups with comparable nadir CD4 count and age, INR patients still demonstrated significantly elevated levels of IP-10 compared with IR. This is in line with an earlier study reporting persistently elevated IP-10 levels to be associated with

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immunological treatment failure after 1 year on ART.[37] Also in HIV controllers, IP-10 levels were negatively correlated with CD4 count after 12 and 24 months.[40] Thus, IP-10 should be evaluated as a possible biomarker of incomplete immune recovery in a larger, prospective study including HIV-infected patients at ART initiation.

IP-10, also known as CXCL-10, is a chemokine secreted from various cell types including monocytes and leucocytes, mainly in response to IFNγ. It binds to the CXCR3 receptor on activated T-, B- and antigen-presenting cells and induces primarily chemotaxis of these cells toward inflamed areas.[41] In response to HIV-1 stimulation in vitro, IP-10 was shown to be produced mainly by innate immune cells with the largest contribution from monocytes[42] and 2 other studies have recently linked IP-10 strongly to monocyte activation in HIV-infected patients.[38,43] In this study, IP-10 correlated with the monocyte activation markers sCD14 and neopterin, as well as other soluble markers of inflammation, but we did not find any difference in the levels of these markers between the INR and IR groups. The contribution by different cellular monocyte subsets in INR is also unknown.

In INR patients with CD4 cells ≤300 at baseline, both the levels of IP-10 and the KTR were negatively associated with CD4 count and CD4 gain after 2 years, as well as strongly correlated with the aTreg fraction. IDO1 is upregulated by interferons[44] and increased IDO activity is shown to suppress T-cell responses[45] and favor conversion of naive T cells to Tregs in HIV infection.[46] Two studies have recently demonstrated that high KTR predicts reduced CD4 recovery in patients on ART,[29,30] and our results may indicate that increased interferon and IDO1 activity contribute to hamper immune reconstitution, possibly through increased levels of aTreg, and this was particularly seen in the patients with the lowest CD4 counts.

It has recently been shown that 48 weeks of rosuvastatin in addition to ART significantly reduced the levels of IP-10, other inflammation makers, and T-cell activation and monocyte activation compared with both ART alone and placebo.[47] If such intervention is beneficial for INR has not yet been explored. Furthermore, the probiotic VSL#3 seems to reduce IP-10 secretion and IP-10–mediated T-cell transmigration in vitro.[48] In contrast, we could not detect reduction of plasma IP-10 levels after 8 weeks of administration of a multistrain probiotic to ART-treated HIV-infected patients with CD4 count <500 cells per milliliter.[49] Thus, whether probiotic could affect systemic IP-10 levels are unknown, but studies of INR patients are ongoing.

The INR patients displayed significant alterations in the Treg subsets with a higher proportion of aTreg relative to rTreg compared with patients with an adequate immune recovery and comparable nadir CD4. This change was more pronounced among those with the lowest CD4 levels. In agreement with our results, Gaardbo et al[25] also demonstrated increased proportions of aTreg and decreased fractions of rTreg in INR with CD4 <200 compared with IR having similar nadir CD4 counts. Likewise, Saison et al[26] found higher percentages of aTreg in patients with reduced immune reconstitution, but without adjustments of the factors listed above.

Among the INR patients, the CD147^{high} aTreg fraction correlated with both IP-10 and other soluble markers of inflammation, and inversely with the CD4 count, but not with T-cell activation. This association was not observed in patients with adequate CD4 responses, indicating that the alterations in the Treg subsets seen in INR perhaps are more related to general inflammation than T-cell activation. This is consistent with other studies, which report no association between Tregs and T-cell activation.[24,25] In murine models, IFNγ signaling and increased IP-10 levels are shown to attract CXCR3 (IP-10 receptor) positive, highly suppressive Tbet^{+} Tregs to sites of inflammation and thus reduce the recruitment of pro-inflammatory immune cells.[50,51] Whether this mechanism is essential in HIV infection remains to be elucidated, but it could imply that the general immune activation drives the alterations in the Treg subsets seen in INR.[52]

Furthermore, we found higher T-cell activation and differentiation phenotypes in the INR group, potentially indicating increased cell turnover in patients with low CD4 count, in agreement with previous studies.[8,25,53] Surprisingly, we did not notice any differences in exhaustion in any of the analyzed T-cell subsets nor did we detect signs of increased microbial translocation as a possible explanation of reduced CD4 count. However, we did not analyze residual HIV viral replication or CMV DNA, which both could possible contribute to the immune activation observed in INR patients.[54] Whether the increased occurrence of any comorbidity in the INR cohort is a cause or consequence of dysregulation of immune responses is still uncertain, but large prospective studies have demonstrated that INR remain at higher risk of severe clinical events than responders.[5,7,55]

The INR patients had a shorter duration of continuous viral suppression compared with the IR and the length of viral suppression correlated moderately with the CD4 gain after ART start (data not shown) indicating that some of these patients may still improve in CD4 count. However, as most of the INR patients remained low in CD4 count after 2 years, many of these patients may have reached their potential of immune reconstitution.

Our study has some limitations. Few patients with CD4 cells <200, possibly those with the most profound immune dysregulation, were included. Still, even patients with suboptimal CD4 counts (<500 cells per microliter) apparently have poorer clinical outcome,[5,56] giving the rationale for studying the patients with suboptimal immune reconstitution as well. Furthermore, analyses were performed on thawed PBMC, which may have contributed to the relative low percentages of Tregs detected,[57] and we cannot rule out that selective death of cells may have affected programed death-1 positive cells particularly, and thus impacted these results.

In conclusion, patients with HIV with inadequate CD4 responses had higher levels of IP-10, more activated and differentiated T-cell phenotypes, and higher aTreg/rTreg ratios compared with patients with satisfactory CD4 T-cell gain and comparable CD4 nadir counts. Plasma IP-10 levels were also negatively associated with CD4 counts 2 years later. We suggest that IP-10 should be evaluated as a potential biomarker for incomplete immune recovery in larger prospective studies.

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