

Neutrophils in antiretroviral therapy-controlled HIV demonstrate hyperactivation associated with a specific IL-17/IL-22 environment

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Background: Despite control of HIV infection under antiretroviral therapy (ART), immune T-cell activation persists in patients with controlled HIV infection, who are at higher risk of inflammatory diseases than the general population. PMNs play a key role in host defenses against invading microorganisms but also potentiate inflammatory reactions in cases of excessive or misdirected responses.

Objective: The aim of our study was to analyze PMN functions in 60 ART-treated and controlled HIV-infected patients (viral load, <20 RNA copies/mL; CD4 count, ≥ 350 cells/mm³) with (HIV[I] group) and without (HIV[NI] group) diseases related to an inflammatory process and to compare them with 22 healthy control subjects.

Methods: Flow cytometry was used to evaluate PMN functions in whole-blood conditions. We studied in parallel the activation markers of T lymphocytes and monocytes and the proinflammatory cytokine environment.

Results: Blood samples from HIV-infected patients revealed basal PMN hyperactivation associated with deregulation of the apoptosis/necrosis equilibrium. Interestingly, this hyperactivation was greater in HIV(I) than HIV(NI) patients and contrasted with a lack of monocyte activation in both groups. The percentage of circulating cells producing IL-17 was also significantly higher in HIV-infected patients than in control subjects and was positively correlated with markers of basal PMN activation. In addition, the detection of IL-22 overproduction in HIV(NI) patients suggests that it might contribute to counteracting chronic inflammatory processes during HIV infection.

Conclusions: This study thus demonstrates the presence of highly activated PMNs in HIV-infected patients receiving effective ART and the association of these cells with a specific IL-17/IL-22 environment. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: Neutrophils, HIV, inflammation, IL-18, IL-17, IL-22

HIV-1 establishes persistent infection in human subjects. Although antiretroviral therapy (ART) prevents AIDS-related complications and prolongs life expectancy, HIV-1-infected patients have several physiologic impairments and comorbidities (eg, atherosclerosis, osteoporosis, malignancies, cognitive impairment, and frailty) that are usually observed during the human aging process.¹ These clinical manifestations are thought to result from chronic increased immune activation that occurs despite effective HIV control by ART.² Activated adaptive (eg, T lymphocytes) and innate (mainly monocytes) cells secrete proinflammatory mediators, leading to the chronic low-grade inflammation that is a hallmark of these non-AIDS comorbidities.²

PMNs are a key component of the early innate response to bacterial and fungal pathogens.³ In response to pathogens, PMNs rapidly migrate from the blood to inflamed tissues, where their activation triggers such microbicidal mechanisms as rapid production of reactive oxygen species (ROS) in oxidative bursts.³ ROS can be harmful to their environment, including cells and tissues, especially in cases of excessive release, extracellular release, or both.³ After they kill microbes, PMNs die spontaneously, mainly through apoptosis. Although they have a very short lifespan, their activation by circulating microbial products, as well as by proinflammatory mediators, promotes their survival and is a critical mechanism in their effectiveness against pathogens.⁴ Nevertheless, inappropriate PMN survival might lead to a chronic persistent inflammatory state with ongoing release of inflammatory mediators and damage-associated molecular patterns through PMN necrosis.⁴ Thus although PMNs are primarily protective, their inappropriate, excessive, or prolonged activation presents the risk of tissue injury and organ dysfunction and has been involved in various inflammatory diseases, including cardiovascular and osteoarticular disorders.⁵⁻⁷ The severity of these inflammatory processes is controlled, at least in part, by factors that regulate PMN functions, cell death, and survival, mainly proinflammatory cytokines, such as IL-8, TNF- α , IL-1 β , IL-6, GM-CSF, and IL-18.^{4,8}

The role of PMNs in HIV-1 disease has mainly been examined from the point of view of patients' increased susceptibility to bacterial and fungal infections. HIV-1 does not infect PMNs but leads to impaired PMN responses (phagocytosis, oxidative burst,

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Abbreviations used

7-AAD:	7-Amino-actinomycin D
APC:	Allophycocyanin
ART:	Antiretroviral therapy
FITC:	Fluorescein isothiocyanate
HC:	Healthy control subject
HIV(I):	HIV-infected patients with inflammatory diseases
HIV(NI):	HIV-infected patients without an inflammatory disease
MDC-8:	6-Sulfo LacNAc (Slan) DC
NRTI:	Nucleoside reverse transcriptase inhibitor
PE:	Phycoerythrin
ROS:	Reactive oxygen species
sCD14:	Soluble CD14
TLR:	Toll-like receptor

and bacterial killing) and a higher rate of PMN death.⁹⁻¹¹ ART basically corrects these abnormalities associated with HIV infection, especially CD4 cell depletion.¹⁰ However, despite the persistence of increased levels of proinflammatory cytokines² that can, as stated above, stimulate PMN activities, the possible link between excessive PMN responses and chronic inflammation in ART-treated HIV-infected patients has never been explored. One reason might be the difficulty of studying PMN functional responses, especially given the need to handle these samples immediately after taking them.

The aim of our study was to analyze PMN functions and survival in ART-treated patients with controlled HIV-1 infection, as well as activation markers of T lymphocytes and monocytes and the proinflammatory cytokine environment. We also investigated associations between these indicators and the presence of clinical disorders related to inflammatory processes.

METHODS**Study design and patients**

This cross-sectional study took place in a cohort of 60 HIV-1-infected subjects from the Infectious Diseases Department of the Pitié Salpêtrière Hospital (Paris, France). General inclusion criteria included documented HIV infection, age of 18 years or greater, stable ART treatment, and viral (viral load, ≤ 20 RNA copies/mL) and immunologic (CD4 count, ≥ 350 cells/mm³) control. Exclusion criteria included current opportunistic infections, HIV/hepatitis B virus/hepatitis C virus coinfection, acute inflammatory or infectious conditions, and corticoid or immunosuppressive therapy.

Next, we classified the included patients according to the presence of diseases related to an inflammatory process. Because chronic inflammatory diseases are a heterogeneous group of conditions that can affect any organ or system, we decided to select patients with (1) at least 1 of the active conditions (major criteria) of cardiovascular disease (atherosclerosis, coronaropathy, stroke), chronic obstructive pulmonary disease, vasculitis, or chronic arthritis or (2) at least 2 of the (minor criteria) conditions of hypertension, metabolic syndrome, lipodystrophy, diabetes type 2 with vascular complications, or osteoporosis/osteopenia. Patients meeting either criteria were assigned to the group of HIV-infected patients with inflammatory diseases (HIV[I] group, $n = 18$). The other group of HIV-infected patients (HIV[NI] group, $n = 42$) included those with no inflammatory conditions, according to our inclusion criteria.

All HIV-infected patients were enrolled after a physical examination at which blood pressure, height, weight, and temperature were measured and recorded. An extensive review was conducted of each patient's chart (Table 1). Demographic characteristics were collected, including smoking status and ethnicity. For comparison, peripheral blood from 22 age-matched healthy control subjects (HCs) was collected at the Etablissement

Français du Sang (Pitié Salpêtrière Hospital, Paris, France) after ethical approval for the use of such material by the institutional review committees of INSERM and Etablissement Français du Sang (convention 12/EFS/079). Exclusion criteria in the control group included acute or chronic inflammatory conditions and corticoid or nonsteroidal anti-inflammatory drugs; smoking status and ethnicity were unavailable for these patients. The study was approved by the local institutional ethics committee (Comité de Protection des Personnes of the Pitié Salpêtrière Hospital). After written informed consent had been obtained from the patients and control subjects, whole blood was sampled, kept on ice, and transported immediately to the laboratory.

Immunophenotyping of T cells and monocytes

The expression of cell-surface molecules in the T-cell and monocyte subpopulations was assessed by using 10-color flow cytometry (Gallios Flow Cytometer; Beckman Coulter, Fullerton, Calif). The detailed staining procedure is described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Determination of surface molecule expression on PMNs

Heparin whole-blood samples were either kept on ice or incubated at 37°C with PBS, Pam₃CSK₄ (Toll-like receptor [TLR] 1/2 ligand, 1 μ g/mL; InvivoGen, San Diego, Calif), LPS from *Escherichia coli* serotype R515 (TLR4 ligand, 10 ng/mL; Invivogen), or TNF- α (5 ng/mL; R&D Systems, Abingdon, United Kingdom) for 45 minutes. Samples were stained with phycoerythrin (PE)-anti-human CD11b (DakoPatts, Glostrup, Denmark), allophycocyanin (APC)-anti-human CD62L (BD Biosciences, San Jose, Calif), and fluorescein isothiocyanate (FITC)-anti-human CD16b antibodies (Beckman Coulter).¹² Samples were then analyzed by means of flow cytometry, as described in the [Methods](#) section in this article's Online Repository.

Measurement of oxidative burst of PMNs and monocytes

Superoxide anion ($O_2^{\cdot-}$) production by PMNs and monocytes was measured with a flow cytometric assay derived from the hydroethidine oxidation technique.¹² Heparin whole-blood samples (500 μ L) were loaded for 15 minutes with 1500 ng/mL hydroethidine (Sigma Aldrich, St Louis, Mo) at 37°C and then incubated for 45 minutes with PBS or various stimuli, as described above. Samples were then treated with PBS or 10^{-6} mol/L formyl-methionyl-leucyl-phenylalanine (Sigma Chemical, St Louis, Mo) for 5 minutes. Samples were then analyzed by means of flow cytometry, as described in the [Methods](#) section in this article's Online Repository.

Measurement of PMN apoptosis/necrosis

PMN cell death in whole blood was quantified with Annexin V and the impermeant nuclear dye 7-amino-actinomycin D (7-AAD). Whole-blood samples were incubated for 20 hours at 37°C with PBS or various stimuli, as described above. Samples were incubated with APC-anti-CD15, FITC-Annexin V, and 7-AAD (BD Biosciences).¹² Samples were then analyzed by means of flow cytometry, as described in the [Methods](#) section in this article's Online Repository.

Cytokine and soluble mediator assays

Intracellular analyses of IL-17 and IL-22 production were performed by means of flow cytometry. A detailed procedure is described in the [Methods](#) section in this article's Online Repository. Cytokines and chemokines were detected from serum with the Cytometric Bead Array kit (FACSCanto II, BD Biosciences) or ELISA assays (Platinum ELISA; eBioscience, San Diego, Calif). Soluble CD14 (sCD14) was detected from serum with an ELISA assay (Quantikine ELISA, R&D Systems).

TABLE I. Characteristics of HIV-infected and uninfected subjects participating in the study

	HCs (n = 22)	HIV-1-infected (n = 60)	P value	HIV(NI) patients (n = 42)	HIV(I) patients (n = 18)	P value
Age (y), (median [minimum-maximum])	44 (21-62)	48 (27-76)	.126	46 (27-60)	49 (30-76)	.17
Male sex, no. (%)	10 (45)	49 (82)	<.02	33 (78)	16 (89)	>.4
Ethnicity, no. (white/black)	ND	47/13	—	31 (11)	16 (2)	>.1
CD4 nadir, median (minimum-maximum)	—	251 (33-998)	—	243 (33-998)	288 (60-468)	.9
CD4 count (cells/mm ³), median (minimum-maximum)	716 (510-1037)	664 (353-1499)	NS	679 (343-1499)	601 (423-929)	.09
CD4/CD8 ratio, median (minimum-maximum)	1.9 (1.2-3.1)	0.87 (0.11-3.18)	<.001	0.89 (0.11-1.7)	0.74 (0.39-1.25)	.07
Neutrophil count (cells/mm ³), median (minimum-maximum)	5000 (2000-7000)	3040 (880-8236)	NS	3115 (880-8236)	3020 (1750-7440)	.88
ART with NRTIs, no. (%)	—	46 (77)	—	34 (80)	12 (66)	>.2
ART with protease inhibitors, no. (%)	—	25 (42)	—	16 (38)	9 (50)	>.3
Treatment duration (y), median (minimum-maximum)	—	9.4 (0.17-20.01)	—	7.7 (0.18-20.01)	13.96 (0.17-19.47)	.13
Undetectability duration (y), median (minimum-maximum)	—	5.2 (0.03-13.07)	—	4.8 (0.03-12.02)	5.8 (0.08-13.07)	.7
Pathologies related to inflammatory process:						
Chronic arthritis					2	
Osteoporosis + lipodystrophy					1	
Stroke with persistent neurologic symptoms					2	
HT + diabetes type 2 + coronaropathy					1	
HT + diabetes type 2 + peripheral vascular complications					3	
HT + metabolic syndrome					4	
HT + renal vascular complications					1	
Coronaropathy + metabolic syndrome					1	
Behçet disease with vasculitis					1	
Chronic obstructive pulmonary disease					1	
Metabolic syndrome + lipodystrophy					1	
hsCRP (mg/mL), median (minimum-maximum)	1.01 (0.19-1.97)	1.1 (0.18->10)	NS	1.05 (0.18->10)	1.65 (0.2->10)	.23

Statistical analysis of race/ethnicity, male sex, and type of treatment was performed with the χ^2 test.

hsCRP, High-sensitivity C-reactive protein; HT, hypertension; ND, not determined; NS, not significant.

Statistical analysis

Data are reported as means $\pm$ SEMs. The Mann-Whitney *U* test or 2-way ANOVA was used to determine whether differences were significant between groups, and Spearman correlation was used to examine the associations between PMN activation and cytokine production. All analyses were performed with GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif). The significance level was set at a *P* value of less than .05.

RESULTS

Patients' characteristics

Table I summarizes patients' clinical and HIV-related characteristics and notes the 18 HIV(I) group patients who had at least 1 chronic disease related to an inflammatory process. The 2 groups of patients, HIV(I) and HIV(NI), were similar for age, ethnicity, sex, smoking rate, duration and type of treatment, duration of HIV undetectability, CD4/CD8 ratios, nadir CD4 counts, PMN counts, and levels of C-reactive protein detected by using a highly sensitive test (high-sensitivity C-reactive protein). Moreover, the indicators unrelated to disease and available for both groups did not differ between patients and HCs.

In addition, the ultrasensitive test of viral load found less than 1 copy/mL in 46 patients, between 1 and 10 copies/mL in 8 patients, and between 10 and 20 copies/mL in 4 patients. Furthermore, of the 14 HIV-infected patients who did not receive nucleoside reverse transcriptase inhibitors (NRTIs), 9 received NNRTIs combined with either protease inhibitor (*n* = 2), integrase inhibitor (*n* = 5), protease inhibitor plus integrase inhibitor (*n* = 1), or integrase inhibitor plus CCR5 antagonist (*n* = 1); 5 patients received either a protease inhibitor alone (*n* = 2) or a protease inhibitor plus integrase inhibitor (*n* = 1), a protease inhibitor plus integrase inhibitor plus CCR5 antagonist (*n* = 1), or

an integrase inhibitor plus CCR5 antagonist (*n* = 1). Clinicians prescribed these various treatment combinations, taking account of the duration of infection, ART drug resistance, and cumulative toxicity of the different types of ART.

Highly activated circulating PMNs in HIV-infected patients

PMN activation is associated with the modulation of surface molecules, specifically a decrease in L-selectin (CD62L) or FC γ RIIIB (CD16b) and an increase in β_2 -integrins (CD11b/CD18), through either stimulus-induced shedding^{13,14} or translocation from intracellular granules.¹⁵ As Fig 1 shows, we observed lower expression of CD62L and CD16b associated with higher CD11b expression on resting PMNs from HIV-infected patients than from HCs (Fig 1, A and B). Because PMNs are reported to trigger microbicidal mechanisms on activation,³ in unstimulated whole-blood samples we measured ROS production and found that it was significantly higher in HIV-infected patients than in HCs (Fig 1, C). These results reflect the basal hyperactivation of PMNs from HIV-infected patients.

Control of PMN death by apoptosis is paramount for effective resolution of inflammation. In contrast, PMN death by necrosis induces the release of harmful intracellular contents and thus promotes proinflammatory responses.⁴ Therefore we measured the percentage of PMNs undergoing spontaneous apoptosis and necrosis. Cultured whole-blood PMNs from HCs died rapidly, mainly through apoptosis (43%) or necrosis (1.6%), as expected.¹² The percentage of apoptotic PMNs was significantly lower in HIV-infected patients because of their increased percentage of necrotic PMNs (Fig 1, D). This deregulation of the balance

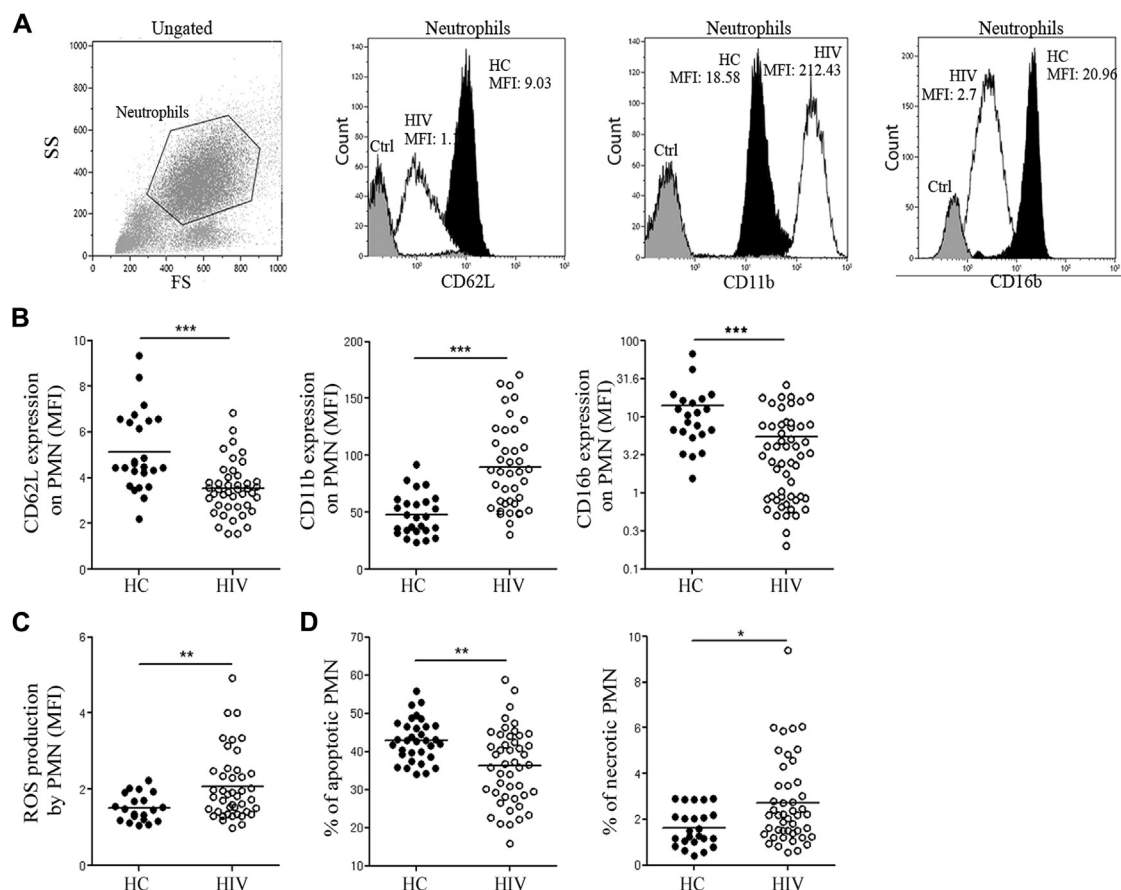


FIG 1. Highly activated circulating PMNs from HIV-infected patients. **A** and **B**, Expression of cell-surface molecules on resting PMNs was studied on whole-blood samples maintained at 4°C. One histogram representative of CD62L, CD11b, and CD16b expression at the surface of PMNs from HCs (black) and HIV-infected patients (white) is shown; the isotype control is shown in gray (Fig 1, A). Average mean fluorescence intensity (MFI) in each group is shown in Fig 1, B. **C**, Production of ROS by unstimulated PMNs. **D**, Spontaneous PMN death was measured by incubating whole-blood samples with PBS for 20 hours at 37°C; results are expressed as percentages of apoptotic (left) and necrotic (right) PMNs. * $P < .05$, ** $P < .01$, and *** $P < .001$.

between apoptosis and necrosis, combined with basal PMN hyperactivation, might play a role in establishing chronic inflammation in these patients.

To evaluate the effect of the duration of viral undetectability on PMN hyperactivation, we performed a sensitivity analysis that excluded patients whose viral control was recent (<1 year) and observed that the PMN abnormalities described above were still detectable (data not shown). These data demonstrate that the subgroup of HIV-infected patients with recent viral control was not the main driver of the findings.

Lack of monocyte activation in HIV-infected patients

Because the mononuclear phagocyte lineage also plays a pivotal role in innate immunity and inflammatory responses, we investigated whether this basal PMN hyperactivation in our cohort was associated with an increased level of activated markers in the monocyte population. Expression of the monocyte activation markers CD44 and CD11b and the percentage of CD14⁺HLA-DR^{high} cells were similar in HIV-infected patients and HCs (Fig 2, A), as was the sCD14 level (Fig 2, B) and distribution of the different monocyte subpopulations: classical, intermediate, and nonclassical (also called proinflammatory)

monocytes (Fig 2, C).¹⁶ Among the proinflammatory monocytes, we also investigated the 6-Sulfo LacNAc (SlaN) DC (MDC-8)⁺ subset, which is a major producer of TNF- α in response to LPS and has been found in inflamed tissues during chronic inflammatory diseases, such as Crohn disease and rheumatoid arthritis.¹⁷ As previously reported,¹⁸ the percentage of MDC-8⁺ cells did not differ in aviremic HIV-infected patients and HCs (Fig 2, D). Finally, in contrast to the findings described above for PMNs, unstimulated monocytes did not produce ROS at higher rates in HIV-infected patients than in HCs (Fig 2, E).

Normal functional responses of PMNs from HIV-infected patients

We next analyzed the functional capacity of PMNs to respond to various stimuli, including TNF- α and TLR agonists. Consistent with previous reports that ART restores PMN functions,^{10,19} TLR1/2 and TLR4 agonists, as well as TNF- α , induced normal CD62L shedding (see Fig E1, A, in this article's Online Repository at www.jacionline.org), normal CD11b translocation (see Fig E1, B), and normal priming of oxidative bursts in response to the bacterial formyl peptide formyl-methionyl-leucyl-phenylalanine (see Fig E1, C) in PMNs from HIV-infected

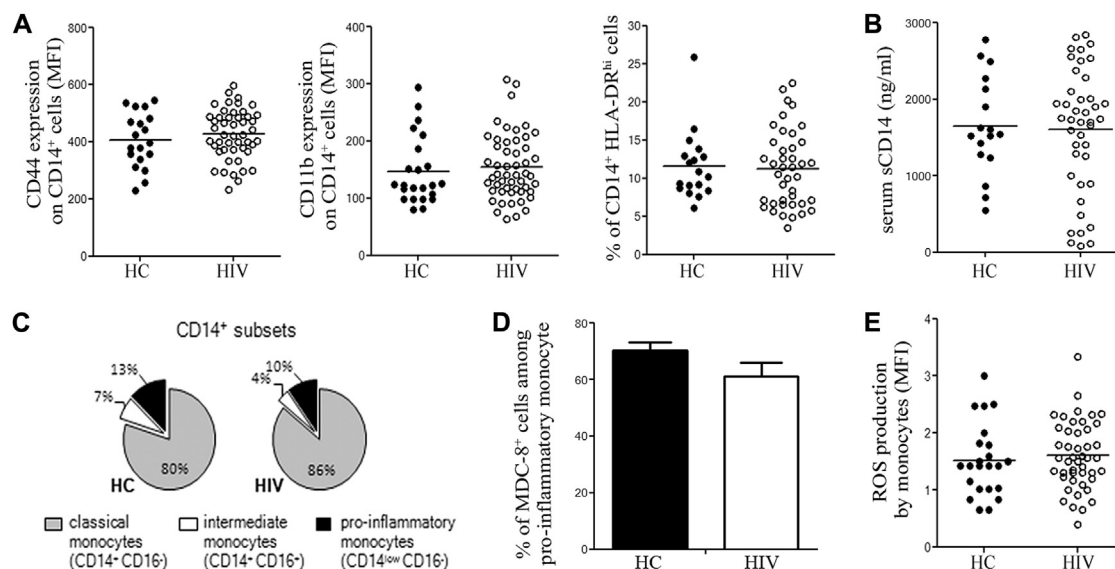


FIG 2. Lack of monocyte activation in HIV-infected patients. **A**, Surface expression of CD44 and CD11b on monocytes was evaluated as mean fluorescence intensity (MFI) on total CD14⁺ cells; HLA-DR expression on monocytes was estimated by the percentage of CD14⁺ cells that highly expressed HLA-DR. **B**, Serum level of sCD14. **C**, Average percentages of classical, intermediate, and proinflammatory monocytes among total CD14⁺ cells in each group. **D**, Percentage of proinflammatory monocytes that expressed the MDC-8 marker. **E**, ROS production by unstimulated monocytes.

patients. Furthermore, the capacity of TLR1/2 and TLR4 agonists to increase PMN survival did not differ between HIV-infected patients and HCs (see Fig E1, D).

Level of T-lymphocyte activation in HIV-infected patients

Persistent T-cell activation in patients with HIV infection has been associated with accelerated immune senescence, leading to the accumulation of highly differentiated CD8⁺ and CD4⁺ cells.²⁰ As expected,^{21,22} ART-treated HIV-infected patients had higher percentages of CD38⁺HLA-DR⁺ (activation markers) and CD57⁺ (immunosenescence marker) CD8 and CD4 memory T cells than did HCs (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). Percentages of CD57⁺ CD8 and CD4 memory T cells correlated with percentages of the corresponding CD38⁺HLA-DR⁺ CD8 and CD4 memory T cells (see Fig E2, C).

Higher level of PMN activation in HIV-infected patients with than in those without inflammatory diseases

Excessive or inappropriate PMN stimulation is reported to potentiate inflammatory responses.²³ Thus we investigated whether the PMN abnormalities described above in HIV-infected patients compared with HCs were greater in patients with chronic diseases related to inflammatory process (HIV[I] group) than in the others (HIV[NI] group). First, we observed that basal hyperactivation of PMNs was detectable even in HIV-infected patients without inflammatory diseases. Furthermore, unstimulated PMNs from the HIV(I) group showed stronger modulations in basal CD62L, CD11b, and CD16b expression at the cell surface and higher basal ROS production than PMNs from the HIV(NI) group (Fig 3, A and B). These results reflect a higher

level of basal PMN activation in patients with inflammatory diseases. In addition, the percentage of necrotic PMNs was significantly higher in the HIV(I) group compared with that seen in the HIV(NI) group (Fig 3, C). Finally, PMN abnormalities did not differ according to the regimen type, in particular according to past use of some NRTIs (zidovudine, didanosine, and stavudine) and protease inhibitors that have been involved in deleterious effects, such as mitochondrial toxicity, lipodystrophy, and cardiovascular complications (data not shown).^{24,25}

In contrast, we observed no significant differences in monocyte and lymphocyte activation between the 2 groups of patients. Monocytes from patients in the HIV(I) group did not express more cell-surface activation markers (Fig 4, A), or produce more sCD14 (Fig 4, B) or ROS (Fig 4, C) or have a higher percentage of the MDC-8⁺ monocyte subpopulation (Fig 4, D) than those from the HIV(NI) group. These findings point to a lack of monocyte activation, even in HIV-infected patients with a history of inflammatory diseases. In addition, the percentages of CD38⁺HLA-DR⁺ and CD57⁺ memory T cells were not significantly higher in the HIV(I) group than in the HIV(NI) group (Fig 4, E and Fig 4, F).

Specific cytokine environment in HIV-infected patients

PMN hyperactivation during chronic inflammatory diseases has been widely associated with excess production of proinflammatory cytokines.²⁶ Therefore we investigated the cytokine environment in HIV-infected patients. Serum IL-18, TGF- β , IL-22 (Fig 5, A), and IL-8 (see Fig E3 in this article's Online Repository at www.jacionline.org) levels were significantly higher in HIV-infected patients than in HCs; in contrast, levels of various other proinflammatory cytokines were either less than the detection limit in both groups or similar in HIV-infected patients and HCs (see Fig E3).

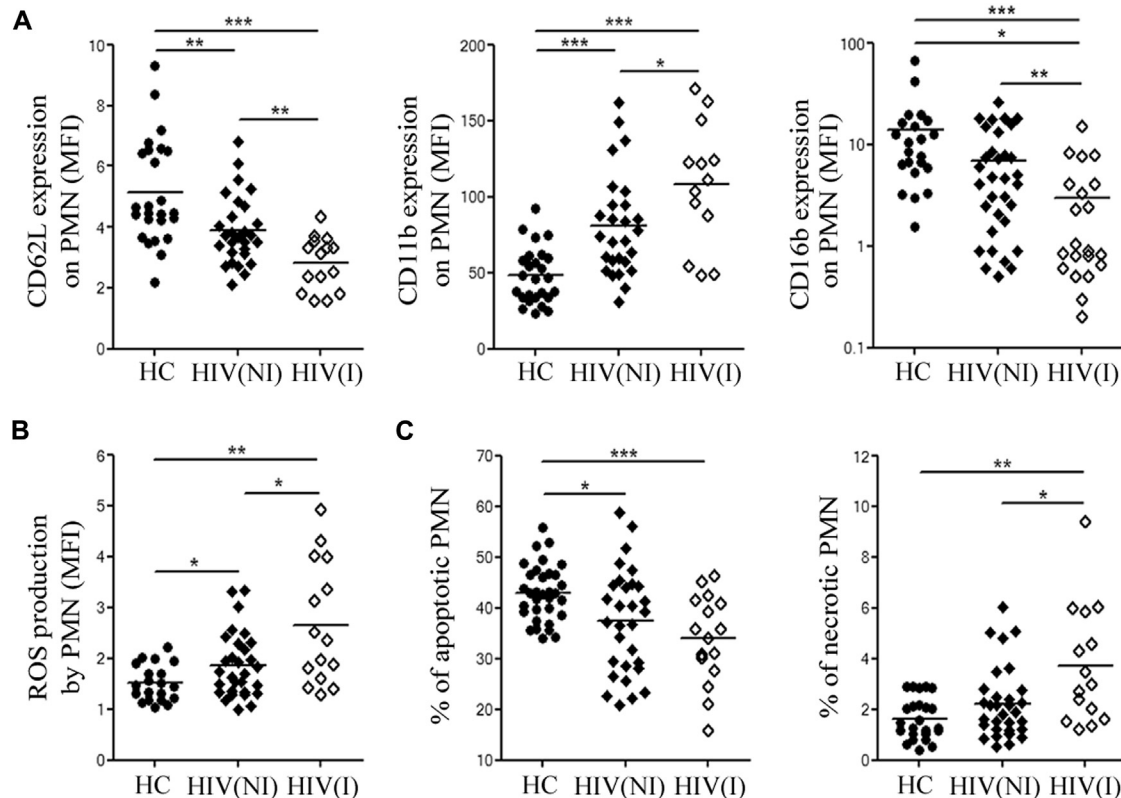


FIG 3. Higher level of PMN activation in HIV-infected patients with a history of inflammatory diseases than in the HIV-infected patients without inflammatory diseases. **A**, Expression of cell-surface molecules (CD62L, CD11b, and CD16b) on resting PMNs. **B**, ROS production by unstimulated PMNs. **C**, Percentages of apoptotic and necrotic PMNs. All measurements were made in HCs and patients from the HIV(I) and HIV(NI) groups. * $P < .05$, ** $P < .01$, and *** $P < .001$.

IL-17 is widely documented to play a key role in establishing inflammatory processes and is a critical mediator in PMN recruitment, migration, and activation.²⁷ Because IL-17 was not detected in the serum of HIV-infected patients (see Fig E3), we completed this analysis by studying intracellular production by PBMCs of this cytokine, as well as of IL-22, which is usually produced at the same time as IL-17.²⁷ After staphylococcal enterotoxin B stimulation, PBMCs from HIV-infected patients showed a normal capacity for intracellular production of IL-17/IL-22 (see Fig E4, B and C, in this article's Online Repository at www.jacionline.org), which was in accordance with previous reports that long-term ART induces normalization of the capacity of T cells to produce IL-17/IL-22.^{28,29} More importantly, in the absence of any stimulation, the percentages of IL-17⁺ or IL-22⁺-producing PBMCs were significantly higher in HIV-infected patients than in HCs (Fig 5, B and C). The phenotypic analysis showed that these circulating IL-17⁺ and IL-22⁺ cells were mainly T helper cells (CD3⁺CD4⁺; see Fig E4, D).

We also analyzed the cells producing IL-17 or IL-22 in the HIV(I) and HIV(NI) groups. Interestingly, these cytokines had opposite profiles in the 2 groups: the percentage of IL-17⁺ cells was significantly lower in the HIV(NI) group than in the HIV(I) group (Fig 5, D), whereas the percentage of IL-22⁺ cells was significantly higher (Fig 5, E). In addition, the percentage of IL-22⁺ cells (Fig 5, E), as well as the serum level of IL-22 (Fig 5, F), in patients in the HIV(I) group did not differ from that observed in HCs.

Finally, to investigate the relation between the percentage of circulating IL-17⁺ cells and basal PMN activation, we set the threshold for positivity at 0.03% of IL-17⁺ cells (corresponding to a tripling over the isotype control). We found that the percentage of IL-17⁺ cells correlated positively with basal PMN expression of CD11b and ROS production and negatively with basal PMN expression of CD62L (Fig 5, G).

DISCUSSION

Our results demonstrate the presence of continuous basal hyperactivation of PMNs in patients with HIV infection controlled by ART and thus extend the data previously reported for uncontrolled treated and untreated patients.^{9,30} PMN hyperactivation was associated with deregulation of the equilibrium between PMN apoptosis/necrosis observed in HIV-infected patients; this imbalance could be related to impaired phagocytosis of apoptotic PMNs by macrophages isolated from HIV-1-infected patients³¹ and might be a supplemental factor promoting the development of chronic inflammation. These abnormalities were associated with a specific cytokine environment characterized by increased production of IL-18, IL-17, and IL-22. Interestingly, we show that PMN activation was exacerbated in the HIV(I) group compared with that seen in the HIV(NI) group; the former also expressed higher levels of IL-17 and low levels of IL-22. In contrast, T-cell activation was similar in both groups, and circulating

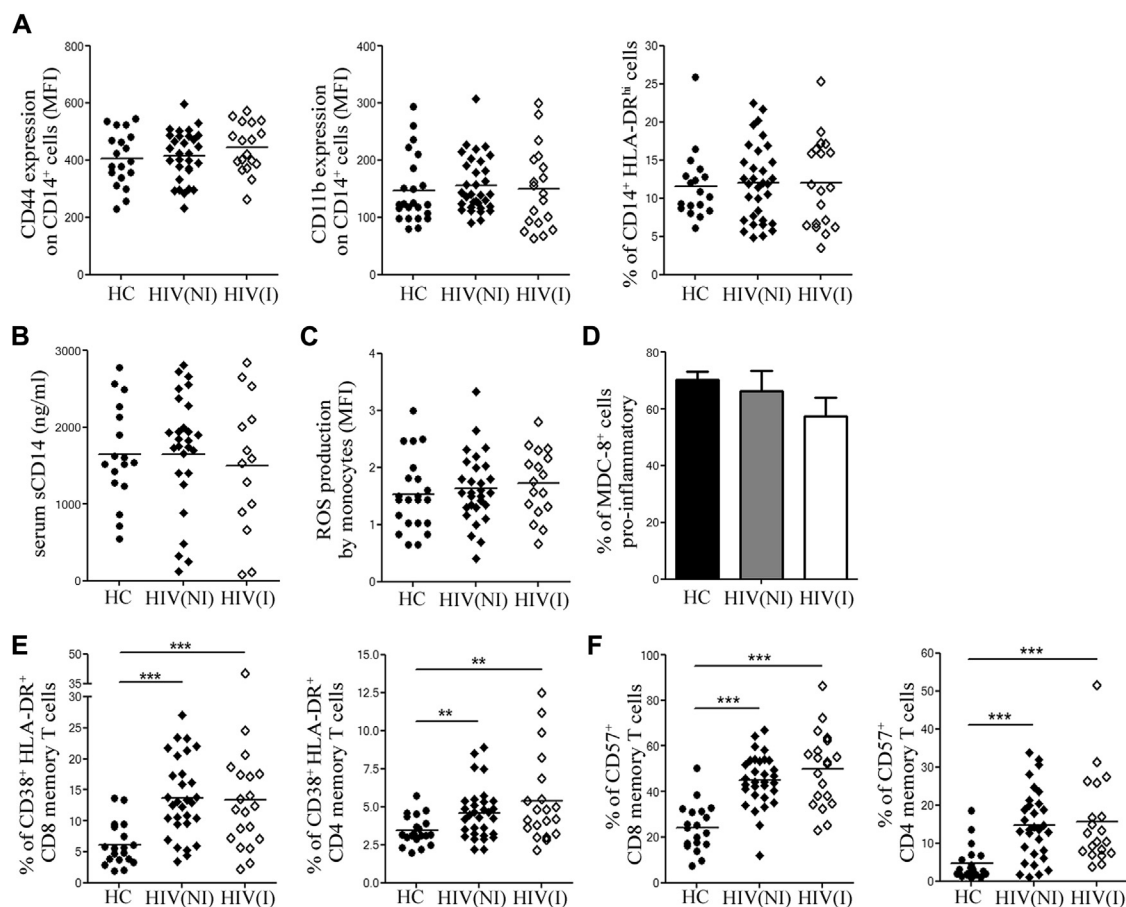


FIG 4. Similar level of monocyte and T-cell activation in HIV-infected patients with a history of inflammatory diseases and HIV-infected patients without inflammatory diseases. **A-D**, CD44, CD11b, and HLA-DR expression on monocytes (Fig 4, A); serum level of sCD14 (Fig 4, B); ROS production by unstimulated monocytes (Fig 4, C); and percentages of proinflammatory monocytes that expressed the MDC-8 marker (Fig 4, D). **E** and **F**, Percentage of memory CD3⁺CD8⁺ cells (left) or CD3⁺CD4⁺ cells (right) that expressed both CD38 and HLA-DR (Fig 4, E) or CD57 (Fig 4, F) markers. All measurements were made in HCs and patients in the HIV(I) and HIV(NI) groups. ** $P < .01$ and *** $P < .001$.

monocytes were not activated in either, not even among the patients in the HIV(I) group.

Chronic immune activation in HIV-infected patients receiving ART has previously been associated with increased levels of circulating classical proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6.² Nevertheless, the level of these cytokines in our study were less than the detection limit or similar to those of HCs. These results are supported by the findings of Cozzi-Lepri et al,³² who observed median plasma concentrations of these cytokines of less than 10 pg/mL in HIV-infected patients receiving long-term ART. Our finding that levels of monocyte chemoattractant protein 1 were normal further extends our demonstration of the lack of monocyte activation. Previous studies have reported higher circulating levels of proinflammatory cytokines and monocyte activation in various cohorts of HIV-infected patients treated for a maximum of 2 years and with inclusion criteria for the plasma viral load that were higher than in our study.³³⁻³⁹ These less strict viral load criteria suggest the possibility of incomplete viral suppression. The data obtained from our HIV cohort (median treatment duration, 9.4 years/viral load ≤ 20 RNA copies/mL) suggest that a longer duration of ART and consequently of viral suppression can restore some HIV-associated immunologic

abnormalities, such as increased proinflammatory cytokine levels and monocyte hyperactivation.

In contrast, serum IL-18 levels were significantly greater in HIV-infected patients than in HCs, also as expected. IL-18 production requires activation of the inflammasome, a group of multimeric protein complexes that sense stress signals, activate caspase 1, and thus cause its proteolytic cleavage.⁴⁰ HIV-1 is reported to induce *in vitro* IL-18 production by macrophages.⁴¹ In addition, Pontillo et al⁴² demonstrated that HIV can induce the specific expression of NALP3 inflammasome components in dendritic cells within moments of virus-cell interaction. Although ART induced undetectable plasma viral load in the HIV-infected patients included in our study, HIV clearly persists in tissues through multiple mechanisms and thus might activate the inflammasome.⁴³ It might also be activated by increased plasma levels of extracellular mitochondrial DNA released from damaged or dead cells, such as necrotic PMNs.⁴⁴ Interestingly, we found a higher percentage of necrotic PMNs in HIV-infected patients than in HCs.

IL-18 enhances PMN functions, including priming PMN oxidative bursts and degranulation.^{45,46} In turn, activated PMNs produce IL-18,⁴⁷ a process that might maintain a vicious cycle.

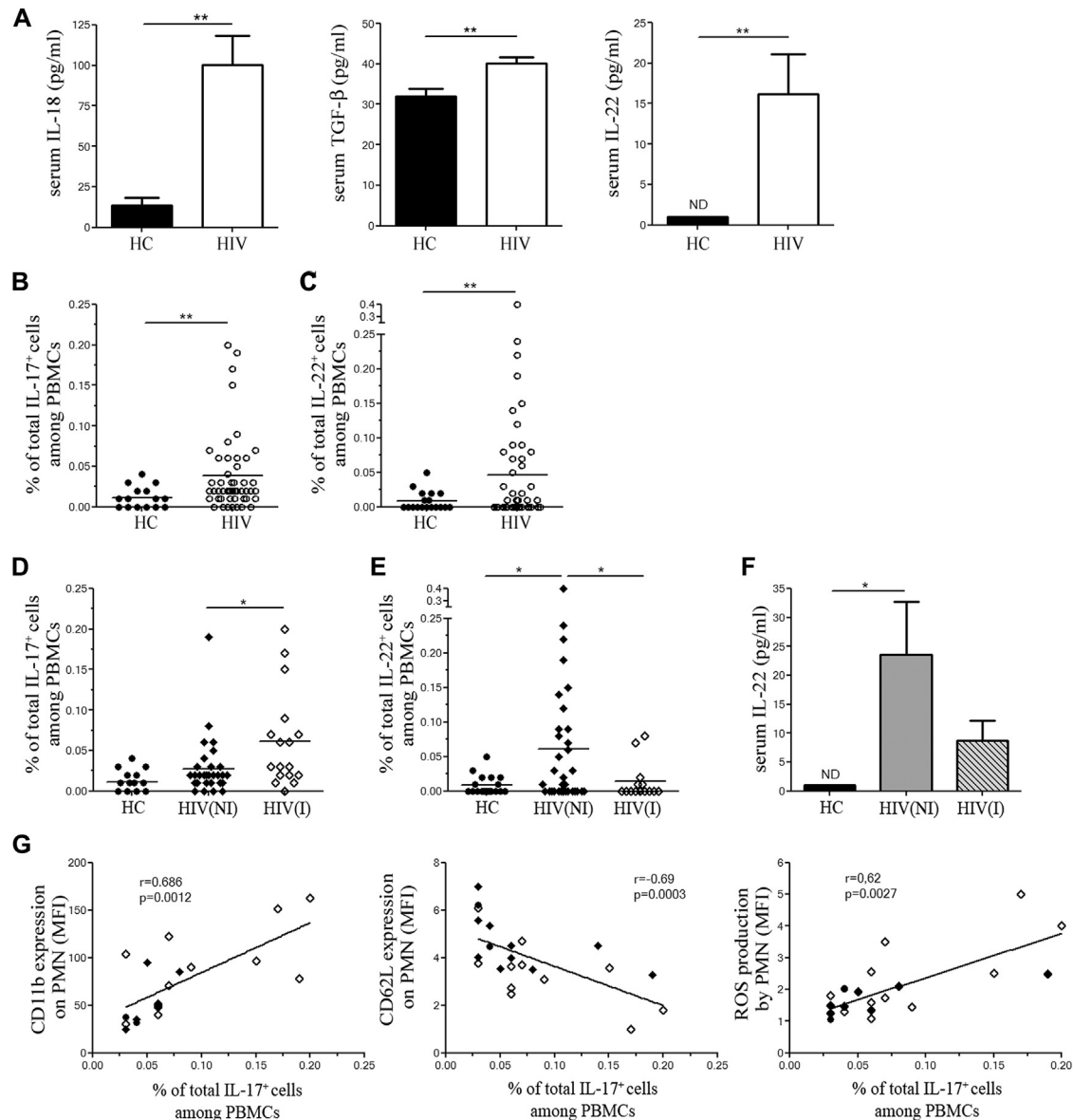


FIG 5. Specific cytokine environment in HIV-infected patients. **A-C**, Serum levels of IL-18 (*left*), TGF- β (*middle*), and IL-22 (*right*; Fig 5, A) and percentages of total IL-17⁺ (Fig 5, B) and IL-22⁺ (Fig 5, C) cells in HCs ($n = 22$) and HIV-infected patients ($n = 60$). **D-F**, Percentage of IL-17⁺ (Fig 5, D) or IL-22⁺ (Fig 5, E) cells among PBMCs and serum IL-22 concentration (Fig 5, F) in HCs and patients in the HIV(I) and HIV(NI) groups. **G**, Correlation between the percentage of total IL-17⁺ cells and CD11b (*left*) or CD62L (*middle*) expression on resting PMNs and ROS production by unstimulated PMNs (*right*). * $P < .05$ and ** $P < .01$.

Increased IL-18 production that matches increased TGF- β levels might be involved, at least in part, in the expansion of cells expressing IL-17 in HIV-infected patients.⁴⁸ Furthermore, IL-17 production was higher in the HIV(I) group than in the HIV(NI) group and was positively correlated with basal PMN hyperactivation. IL-17 plays a central role in both host defense and the induction and progression of multiple chronic inflammatory diseases.^{27,49} Various immunohistochemical studies of inflammatory diseases, such as rheumatoid arthritis and atherosclerosis, have highlighted a high frequency of IL-17-expressing cells entering tissue. The presence of IL-17⁺ cells has been associated with recruitment of PMNs, which is in turn related, at least in part, to IL-17-induced production by endothelial cells of IL-8,⁴⁹⁻⁵¹ a

major chemokine involved in PMN activation and survival.⁵² In agreement with those findings, we observed a significantly higher circulating level of IL-8 in HIV-infected patients than in HCs.

IL-22, a member of the IL-10-related cytokine family, is usually produced together with IL-17.²⁷ Consistent with this general rule, we observed a higher percentage of circulating IL-22⁺ cells in our cohort. IL-22 can play a pathogenic or protective role in patients with chronic inflammatory diseases, depending on the type of tissue and the local cytokine milieu.⁵³⁻⁵⁵ In particular, IL-22 has properties that repair and regenerate epithelium,⁵⁶ and its protective role against HIV-induced damage to the gut epithelium has recently been reported.²⁹ Interestingly, expression of IL-22 in the HIV(I) group was poor. It might be hypothesized that the

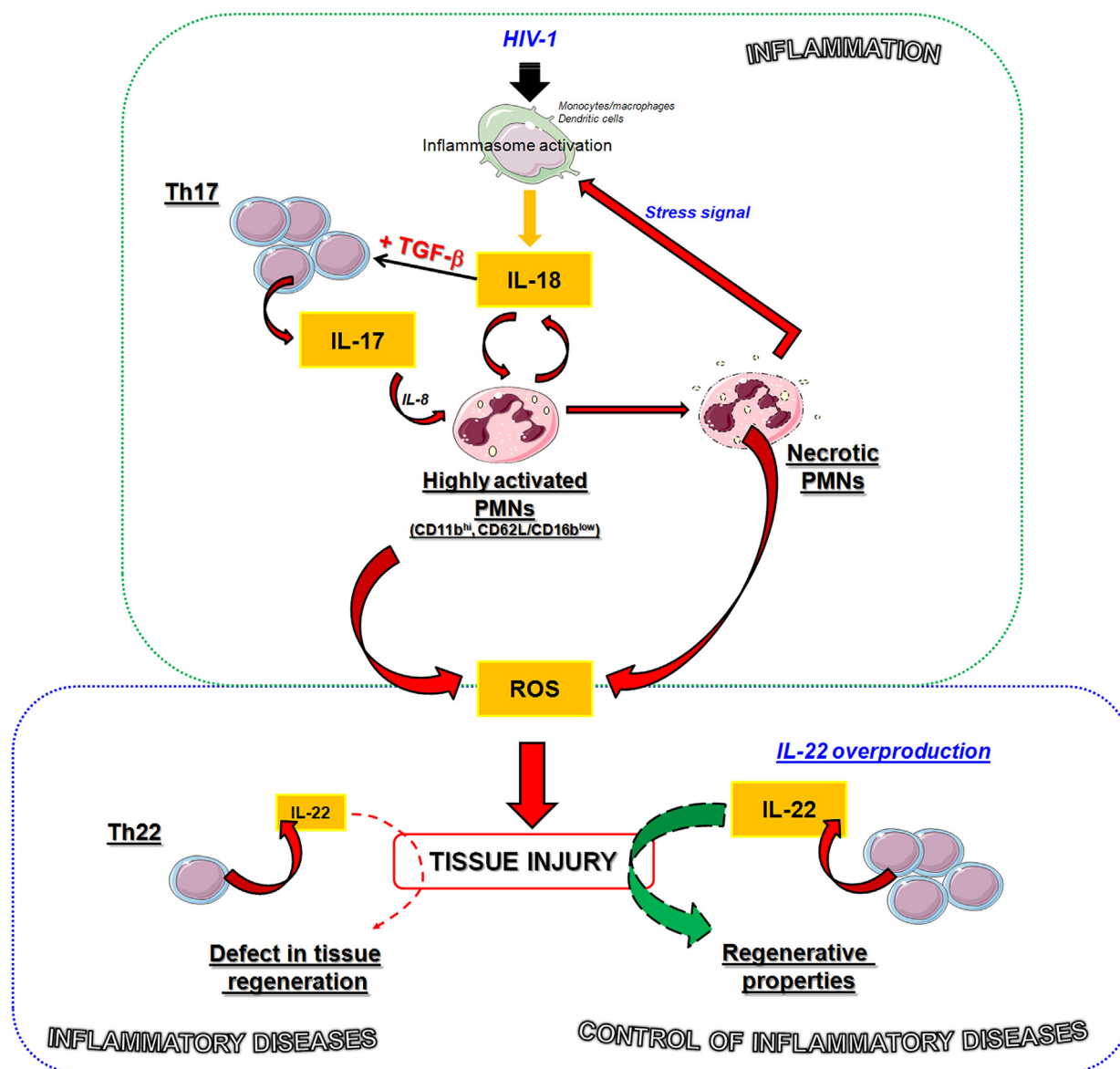


FIG 6. Model for HIV-associated PMN hyperactivation. HIV-1 activates the inflammasome, leading to production of IL-18, which, combined with TGF- β , plays a role in the expansion of IL-17-producing cells. IL-17 production in tissue leads to IL-8 secretion by endothelial cells, which in turn induces massive recruitment and activation of PMNs. IL-18 might also activate PMNs directly; this in turn might produce IL-18. Cytotoxic contents released by activated and necrotic PMNs lead to tissue injury. Vascular and tissue lesions might be counteracted by IL-22, depending on the level of this cytokine.

higher levels of IL-22 observed in the HIV(NI) group exert a beneficial effect by moderating and controlling the tissue damage mediated by PMN hyperactivation. The mechanisms involved in the absence of IL-22 production observed in HIV-infected patients with inflammatory diseases also remain to be elucidated.

To sum up, IL-18 might act through one of 2 major mechanisms, either directly by activating PMN functions or indirectly by inducing the expansion of the IL-17-producing cells involved in PMN recruitment and activation. Activated PMNs in turn produce IL-18, and necrotic PMNs stimulate the inflammasome and thereby amplify IL-18 production. Activated and necrotic PMNs induce vascular and tissue lesions that might be counteracted by IL-22, depending on the level of this cytokine (Fig 6).

The limitations of the present study pertain to its observational design and the use of samples from patients who already presented signs of inflammatory disease. Longitudinal assessment of PMN activation, the IL-18/IL-17/IL-22 environment, and the effect of both on the development and severity of inflammatory diseases will be needed. In addition, we had a relatively limited number of patients, and our observations must be corroborated in larger cohorts. This small headcount could explain the absence of significant differences concerning the markers of T lymphocytes between the HIV(NI) and HIV(I) groups. Nevertheless, our study makes clear that PMN activation is the most sensitive biological marker for discriminating between patients in the HIV(NI) and HIV(I) groups.

In conclusion, our clinical study performed in patients receiving ART with controlled HIV infection brought to light new immunologic features that might play a role in their increased risk of chronic inflammatory pathologies, such as cardiovascular and bone diseases.

We thank V. Cornette for his helpful contribution.

Key messages

- Neutrophil hyperactivation is observed in ART-treated HIV-infected patients and associated with a specific IL-17/IL-22 environment.
- This hyperactivation is more accentuated in HIV-infected patients with chronic diseases related to an inflammatory process.

REFERENCES

- Deeks SG, Phillips AN. HIV infection, antiretroviral treatment, ageing, and non-AIDS related morbidity. *BMJ* 2009;338:a3172.
- D'Ettore G, Paiardini M, Ceccarelli G, Silvestri G, Vullo V. HIV-associated immune activation: from bench to bedside. *AIDS Res Hum Retroviruses* 2011;27:355-64.
- Borregaard N. Neutrophils, from marrow to microbes. *Immunity* 2010;33:657-70.
- Gabelloni ML, Trevani AS, Sabatté J, Geffner J. Mechanisms regulating neutrophil survival and cell death. *Semin Immunopathol* 2013;35:423-37.
- Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, et al. NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal* 2006;8:691-728.
- Garlachs CD, Eskafi S, Cicha I, Schmeisser A, Walzog B, Raaz D, et al. Delay of neutrophil apoptosis in acute coronary syndromes. *J Leukoc Biol* 2004;75:828-35.
- Mirshafiey A, Mohsenzadegan M. The role of reactive oxygen species in immunopathogenesis of rheumatoid arthritis. *Iran J Allergy Asthma Immunol* 2008;7:195-202.
- El-Benna J, Dang PM-C, Gougerot-Pocidallo M-A. Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. *Semin Immunopathol* 2008;30:279-89.
- Elbim C, Prevot M, Bouscarat F, Franzini E, Chollet-Martin S, Hakim J, et al. Polymorphonuclear neutrophils from human immunodeficiency virus-infected patients show enhanced activation, diminished fMLP-induced L-selectin shedding, and an impaired oxidative burst after cytokine priming. *Blood* 1994;84:2759-66.
- Michailidis C, Giannopoulos G, Vigklis V, Armenis K, Tsakris A, Gargalianos P. Impaired phagocytosis among patients infected by the human immunodeficiency virus: implication for a role of highly active anti-retroviral therapy. *Clin Exp Immunol* 2012;167:499-504.
- Heit B, Jones G, Knight D, Antony JM, Gill MJ, Brown C, et al. HIV and other lentiviral infections cause defects in neutrophil chemotaxis, recruitment, and cell structure: immunorestorative effects of granulocyte-macrophage colony-stimulating factor. *J Immunol* 2006;177:6405-14.
- François S, El Benna J, Dang PMC, Pedruzzi E, Gougerot-Pocidallo M-A, Elbim C. Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad. *J Immunol* 2005;174:3633-42.
- Moldovan I, Galon J, Maridonneau-Parini I, Roman Roman S, Mathiot C, Fridman WH, et al. Regulation of production of soluble Fc gamma receptors type III in normal and pathological conditions. *Immunol Lett* 1999;68:125-34.
- Venturi GM, Tu L, Kadono T, Khan AI, Fujimoto Y, Oshel P, et al. Leukocyte migration is regulated by L-selectin endoproteolytic release. *Immunity* 2003;19:713-24.
- Sengeløv H, Kjeldsen L, Diamond MS, Springer TA, Borregaard N. Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. *J Clin Invest* 1993;92:1467-76.
- Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood* 2010;116:e74-80.
- De Baey A, Mende I, Baretton G, Greiner A, Hartl WH, Baeuerle PA, et al. A subset of human dendritic cells in the T cell area of mucosa-associated lymphoid tissue with a high potential to produce TNF-alpha. *J Immunol* 2003;170:5089-94.
- Dutertre C-A, Amraoui S, DeRosa A, Jourdain J-P, Vimeux L, Goguet M, et al. Pivotal role of M-DC8+ monocytes from viremic HIV-infected patients in TNF overproduction in response to microbial products. *Blood* 2012;120:2259-68.
- Levine AM, Karim R, Mack W, Gravink DJ, Anastos K, Young M, et al. Neutropenia in human immunodeficiency virus infection: data from the women's interagency HIV study. *Arch Intern Med* 2006;166:405.
- Papagno L, Spina CA, Marchant A, Salio M, Rufer N, Little S, et al. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. *PLoS Biol* 2004;2:E20.
- Vinikoor MJ, Cope A, Gay CL, Ferrari G, McGee KS, Kuruc JD, et al. Antiretroviral therapy initiated during acute HIV infection fails to prevent persistent T-cell activation. *J Acquir Immune Defic Syndr* 2013;62:505-8.
- Fernandez S, French MA, Price P. Immunosenescent CD57+CD4+ T-cells accumulate and contribute to interferon-gamma responses in HIV patients responding stably to ART. *Dis Markers* 2011;31:337-42.
- Ricevuti G. Host tissue damage by phagocytes. *Ann N Y Acad Sci* 1997;832:426-48.
- Garg H, Joshi A, Mukherjee D. Cardiovascular complications of HIV infection and treatment. *Cardiovasc Hematol Agents Med Chem* 2013;11:58-66.
- Payne BAI, Wilson IJ, Hateley CA, Horvath R, Santibanez-Koref M, Samuels DC, et al. Mitochondrial aging is accelerated by anti-retroviral therapy through the clonal expansion of mtDNA mutations. *Nat Genet* 2011;43:806-10.
- Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. *Trends Immunol* 2011;32:452-60.
- Eyerich S, Eyerich K, Cavani A, Schmidt-Weber C. IL-17 and IL-22: siblings, not twins. *Trends Immunol* 2010;31:354-61.
- He Y, Li J, Zheng Y, Luo Y, Zhou H, Yao Y, et al. A randomized case-control study of dynamic changes in peripheral blood Th17/Treg cell balance and interleukin-17 levels in highly active antiretroviral-treated HIV type 1/AIDS patients. *AIDS Res Hum Retroviruses* 2012;28:339-45.
- Kim CJ, Nazli A, Rojas OL, Chege D, Alidina Z, Huibner S, et al. A role for mucosal IL-22 production and Th22 cells in HIV-associated mucosal immunopathogenesis. *Mucosal Immunol* 2012;5:670-80.
- Boros P, Gardos E, Bekesi GJ, Unkeless JC. Change in expression of Fc gamma RIII (CD16) on neutrophils from human immunodeficiency virus-infected individuals. *Clin Immunol Immunopathol* 1990;54:281-9.
- Torre D, Gennaro L, Baccino F, Speranza F, Biondi G, Pugliese A. Impaired macrophage phagocytosis of apoptotic neutrophils in patients with human immunodeficiency virus type 1 infection. *Clin Vaccine Immunol* 2002;9:983.
- Cozzi-Lepri A, French MA, Baxter J, Okhuysen P, Plana M, Neuhaus J, et al. Resumption of HIV replication is associated with monocyte/macrophage derived cytokine and chemokine changes: results from a large international clinical trial. *AIDS* 2011;25:1207-17.
- Almeida M, Cordero M, Almeida J, Orfao A. Relationship between CD38 expression on peripheral blood T-cells and monocytes, and response to antiretroviral therapy: a one-year longitudinal study of a cohort of chronically infected ART-naïve HIV-1+ patients. *Cytometry B Clin Cytom* 2007;72:22-33.
- Calmy A, Gayet-Ageron A, Montecucco F, Nguyen A, Mach F, Burger F, et al. HIV increases markers of cardiovascular risk: results from a randomized, treatment interruption trial. *AIDS* 2009;23:929-39.
- Baker JV, Neuhaus J, Duprez D, Kuller LH, Tracy R, Belloso WH, et al. Changes in inflammatory and coagulation biomarkers: a randomized comparison of immediate versus deferred antiretroviral therapy in patients with HIV infection. *J Acquir Immune Defic Syndr* 2011;56:36-43.
- Regidor DL, Detels R, Breen EC, Widney DP, Jacobson LP, Palella F, et al. Effect of highly active antiretroviral therapy on biomarkers of B-lymphocyte activation and inflammation. *AIDS* 2011;25:303-14.
- Burdo TH, Lentz MR, Autissier P, Krishnan A, Halpern E, Letendre S, et al. Soluble CD163 made by monocyte/macrophages is a novel marker of HIV activity in early and chronic infection prior to and after anti-retroviral therapy. *J Infect Dis* 2011;204:154-63.
- Funderburg NT, Zidar DA, Shive C, Lioi A, Mudd J, Musselwhite LW, et al. Shared monocyte subset phenotypes in HIV-1 infection and in uninfected subjects with acute coronary syndrome. *Blood* 2012;120:4599-608.
- Longenecker C, Funderburg N, Jiang Y, Debanne S, Storer N, Labbato D, et al. Markers of inflammation and CD8 T-cell activation, but not monocyte activation, are associated with subclinical carotid artery disease in HIV-infected individuals. *HIV Med* 2013;14:385-90.
- Van de Veerdonk FL, Netea MG, Dinarello CA, Joosten LAB. Inflammasome activation and IL-1β and IL-18 processing during infection. *Trends Immunol* 2011;32:110-6.

41. Iannello A, Boulassel M-R, Samarani S, Tremblay C, Toma E, Routy J-P, et al. HIV-1 causes an imbalance in the production of interleukin-18 and its natural antagonist in HIV-infected individuals: implications for enhanced viral replication. *J Infect Dis* 2010;201:608-17.
42. Pontillo A, Silva LT, Oshiro TM, Finazzo C, Crovella S, Duarte AJS. HIV-1 induces NALP3-inflammasome expression and interleukin-1 β secretion in dendritic cells from healthy individuals but not from HIV-positive patients. *AIDS* 2012;26:11-8.
43. Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, et al. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci U S A* 1997;94:13193-7.
44. Cossarizza A, Pinti M, Nasi M, Gibellini L, Manzini S, Roat E, et al. Increased plasma levels of extracellular mitochondrial DNA during HIV infection: a new role for mitochondrial damage-associated molecular patterns during inflammation. *Mitochondrion* 2011;11:750-5.
45. Elbim C, Guichard C, Dang PMC, Fay M, Pedruzzi E, Demur H, et al. Interleukin-18 primes the oxidative burst of neutrophils in response to formyl-peptides: role of cytochrome b558 translocation and N-formyl peptide receptor endocytosis. *Clin Diagn Lab Immunol* 2005;12:436-46.
46. Wyman TH, Dinarello CA, Banerjee A, Gamboni-Robertson F, Hiester AA, Englund KM, et al. Physiological levels of interleukin-18 stimulate multiple neutrophil functions through p38 MAP kinase activation. *J Leukoc Biol* 2002;72:401-9.
47. Fortin CF, Ear T, McDonald PP. Autocrine role of endogenous interleukin-18 on inflammatory cytokine generation by human neutrophils. *FASEB J* 2009;23:194-203.
48. Campillo-Gimenez L, Cumont M-C, Fay M, Kared H, Monceaux V, Diop O, et al. AIDS progression is associated with the emergence of IL-17-producing cells early after simian immunodeficiency virus infection. *J Immunol* 2010;184:984-92.
49. Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, et al. Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. *Blood* 2012;120:3793-802.
50. Wang Z, Lee J, Zhang Y, Wang H, Liu X, Shang F, et al. Increased Th17 cells in coronary artery disease are associated with neutrophilic inflammation. *Scand Cardiovasc J* 2011;45:54-61.
51. Fontao L, Brembilla NC, Masouyé I, Kaya G, Prins C, Dupin N, et al. Interleukin-17 expression in neutrophils and Th17 cells in cutaneous T-cell lymphoma associated with neutrophilic infiltrate of the skin. *Br J Dermatol* 2012;166:687-9.
52. Kobayashi Y. The role of chemokines in neutrophil biology. *Front Biosci* 2008;13:2400-7.
53. Ma H-L, Liang S, Li J, Napierata L, Brown T, Benoit S, et al. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. *J Clin Invest* 2008;118:597-607.
54. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* 2008;118:534-44.
55. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* 2008;29:947-57.
56. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol* 2011;12:383-90.

METHODS

Immunophenotyping of T cells and monocytes

Expression of cell-surface molecules in the T-cell and monocyte subpopulations was analyzed with the Gallios flow cytometer. The following fluorochrome-conjugated anti-human mAbs were used: CD45RA-FITC (clone 2H4), CD27-PE (clone 1A4CD27), CD28-Electron Coupled Dye (ECD) (clone CD28.2), CD38-PC5.5 (clone T16), CCR7-PC7 (clone 3D12), HLA-DR-APC (clone Immu-357), CD8-AF700 (clone B9.11), CD3-AF750 (clone UCHT1), CD57-Pacific Blue (clone NC1), CD4-Krome Orange (clone 13B8.2), MDC-8-FITC (clone DD1), CD44-PE (clone J.173), CD14-ECD (clone RMO52), CD11b-PC7 (clone Bear1), and CD16-PC7 (clone 3G8). All antibodies were purchased from Beckman Coulter, except for CCR7, which was obtained from BD Biosciences, and MDC-8, which was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). EDTA whole-blood samples were labeled for 30 minutes at 4°C with a cocktail of antibodies. The blood was then lysed with BD FACS Lysing Solution, and cells were then resuspended with Cell Fix 1X (BD Biosciences). At least 20,000 events corresponding to lymphocytes or monocytes according to their forward- and side-scatter characteristics were acquired with the Gallios flow cytometer and further analyzed with Kaluza software (Beckman Coulter).

Flow cytometric analysis of PMN and monocyte functions

PMNs and monocytes were analyzed with the Gallios flow cytometer and Kaluza software. All fluorescence measurements were conducted with identical settings. To determine the expression of surface molecules on PMNs and ROS production by PMNs, forward- and side-scatter characteristics were used to identify the PMN population and to gate out other cells and debris, and 10,000 events were counted per sample. Results were expressed as mean fluorescence intensity. An anti-CD14 antibody was used to identify the monocyte population, which appeared on the ECD-CD14 antibody

fluorescence histogram as highly fluorescent CD14⁺ cells (CD14^{high} cells), to measure ROS production by monocytes. CD14 expression was the same in HIV-infected patients and control subjects. A total of 10,000 CD14^{high} cells were counted per sample.

PMNs were identified as CD15^{high} cells to measure cell death in whole blood by means of flow cytometry, and 2×10^5 events were counted per sample. Results were expressed as the percentages of apoptotic (Annexin V⁺/7-AAD⁻) and necrotic (Annexin V⁺/7-AAD⁺) PMNs.

Intracellular assays of IL-17 and IL-22 expression

Five hundred thousand PBMCs were cultured overnight in RPMI 1640 supplemented with 10% FCS, 1% glutamine, 1% pyruvate, 1% antibiotics (Invitrogen GIBCO, Carlsbad, Calif), and 5 µg/mL Brefeldin A (Sigma-Aldrich, St Louis, Mo) to block trans-Golgi trafficking (secretion pathway). For stimulated conditions, staphylococcal enterotoxin B (2 µg/mL, Sigma-Aldrich) was added 30 minutes before Brefeldin A. PBMCs were first stained with eFluor 450 Fixable Viability Dye (eBioscience) and anti-human CD14-ECD, CD8 AF700, CD3-AF750, CD4-Krome Orange (Beckman Coulter), iNKT-PE (clone 6B11, BD Biosciences) and T-cell receptor γδ-FITC (clone B1.1, eBioscience). For intracellular analyses, cells were fixed and permeabilized for 45 minutes at 4°C with the Cytofix/Cytoperm kit (BD Biosciences) and incubated with an anti-human IL-17A-eFluor 660 (clone eBio64DEC17) and anti-human IL-22-peridinin-chlorophyll-protein complex eFluor 710 (clone 22URT1)-conjugated antibodies (eBioscience) for 30 minutes at 4°C. Cells were then washed and fixed with BD Cell Fix 1X.

Two hundred fifty thousand events were acquired with the Gallios flow cytometer and analyzed with Kaluza software to determine the percentages of IL-17⁺ and IL-22⁺ cells. Cells were first gated on the basis of forward- and side-scatter characteristics. Then only singlets and live cells (defined by the Fixable Viability marker) were included in the analysis.

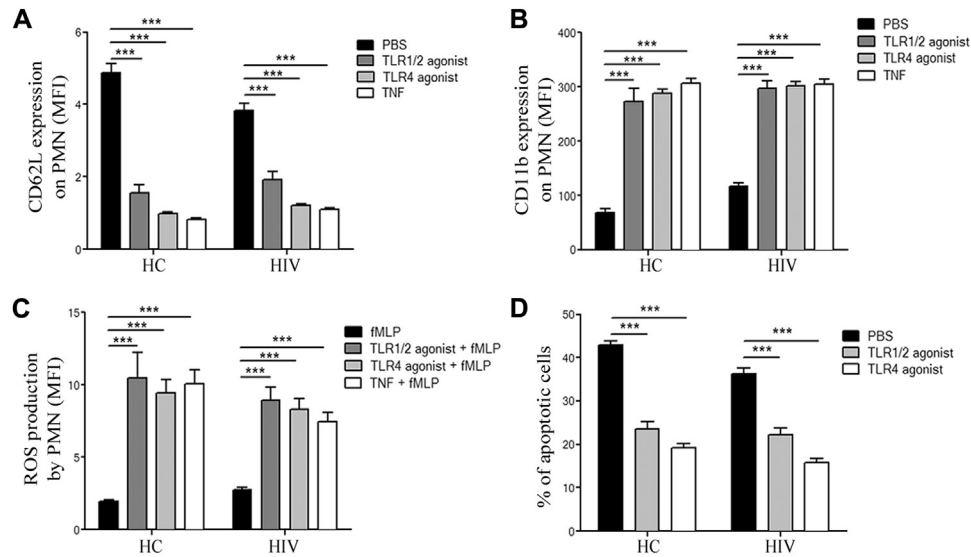


FIG E1. Normal functional capacities of PMNs from HIV-infected patients depending on the agonist. **A** and **B**, CD62L (Fig E1, A) and CD11b (Fig E1, B) expression on the PMN surface was measured after incubation of whole-blood samples for 45 minutes with PBS, TLR1/2 agonist, TLR4 agonist, or TNF- α . **C**, ROS production by PMNs was measured after pretreatment of whole-blood samples for 45 minutes with PBS, TLR1/2 agonist, TLR4 agonist, or TNF and stimulation for 5 minutes with formyl-methionyl-leucyl-phenylalanine (fMLP). **D**, PMN survival was analyzed by incubating whole-blood samples with PBS, TLR1/2 agonist, or TLR4 agonist for 20 hours at 37°C; the percentage of apoptotic PMNs was determined, as described in the legend of Fig 1. All measurements were taken in HCs and HIV-infected patients. Values are means $\pm$ SEMs. *** $P < .001$.

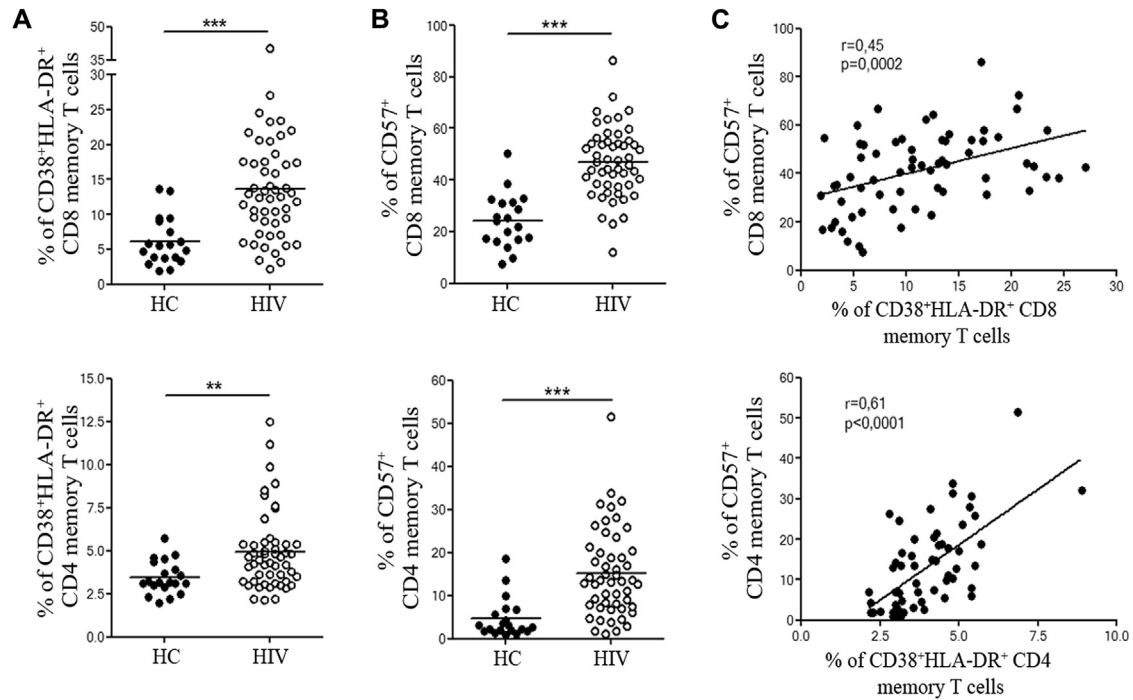


FIG E2. Levels of T-cell activation and immunosenescence. **A**, Percentage of memory CD3⁺CD8⁺ (upper panel) or CD3⁺CD4⁺ (lower panel) cells that express both CD38 and HLA-DR markers. **B**, Percentage of memory CD3⁺CD8⁺ (upper panel) or CD3⁺CD4⁺ (lower panel) cells that express the CD57 marker. Memory cells were defined by means of Boolean gating with Kaluza Software (CD4⁺ or CD8⁺ and not naive cells). **C**, Correlation between markers of T-lymphocyte activation and T-lymphocyte immunosenescence.

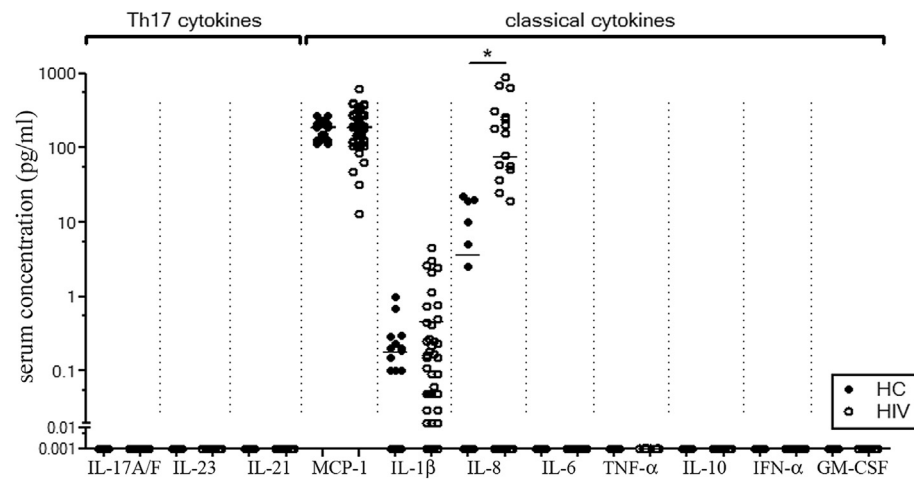


FIG E3. Cytokine quantification in HIV-infected patients. Multiple cytokines and chemokines were quantified in serum from HCs and HIV-infected patients. IL-23, IL-21, and IL-1 β levels were measured by means of ELISA, whereas IL-17A/F, monocyte chemoattractant protein 1 (*MCP-1*), IL-8, IL-6, TNF- α , IL-10, IFN- α , and GM-CSF were detected by using the Cytometric Bead Array. * $P < .05$.

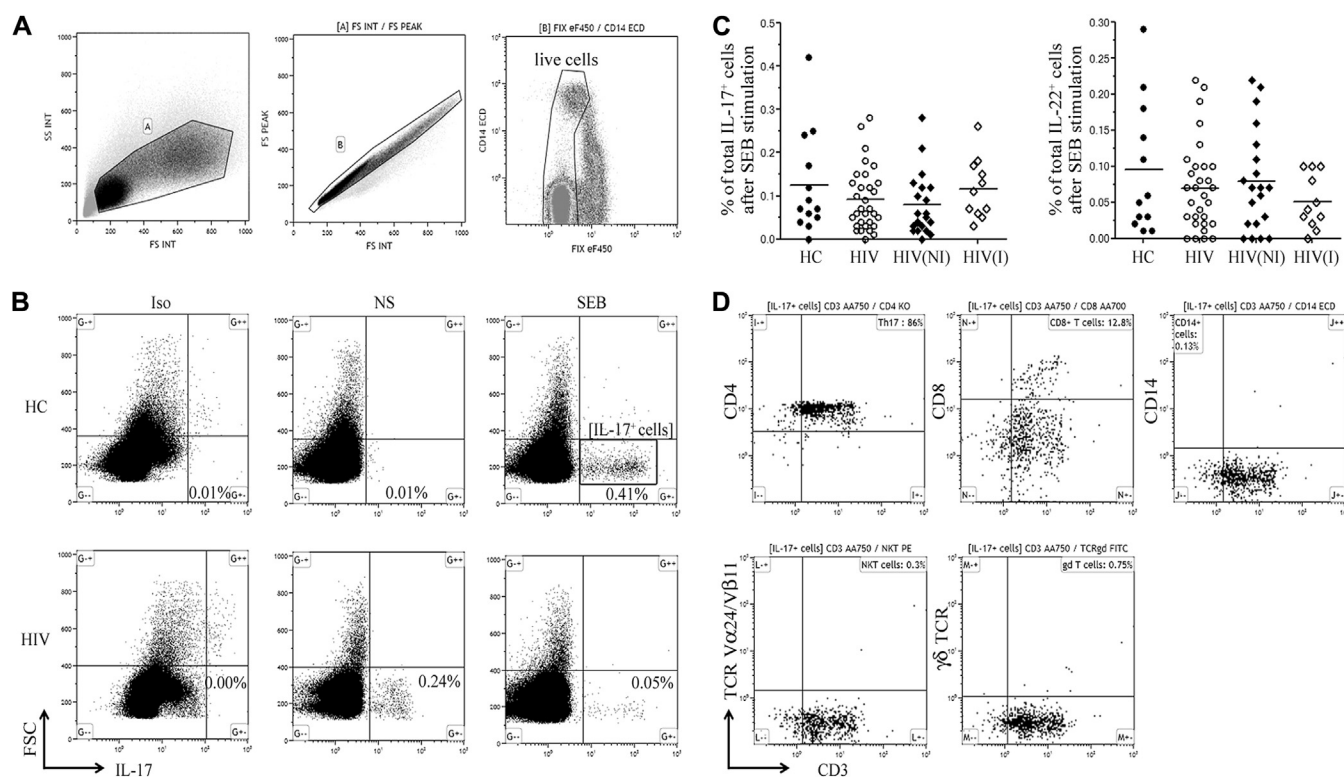


FIG E4. Intracellular cytokine analysis. **A**, Gating process. Cells were first gated on the basis of forward-scatter (FS) and side-scatter (SS) characteristics (gate A). Then only singlets (gate B) and live cells (defined by the Fixable Viability marker eF450) were included in the analysis. **B**, Representative dot plots of IL-17⁺ cells after incubation without (NS) or with staphylococcal enterotoxin B (SEB) in 1 HC and one HIV-infected patient. The negative control used isotype fluorochrome-conjugated antibodies (Iso) corresponding to anti-IL-17 fluorochrome-conjugated antibody. **C**, Percentage of total IL-17⁺ (left) and IL-22⁺ (right) cells among PBMCs after SEB stimulation in HC and HIV-infected patients with (HIV(I)) or without (HIV(NI)) inflammatory diseases. **D**, After gating on total IL-17⁺ or IL-22⁺ cells, phenotype analyses were performed. We used the expression of CD3 and CD4 markers to define T helper cells, the CD14 marker to define monocytes, and the iNKT (TCRVα24-Vβ11) and Tγδ (TCRγδ) markers. Each selected dot plot represents 1 HIV-infected patient.