

# CD4<sup>+</sup> T-Cell Deficiency in HIV Patients Responding to Antiretroviral Therapy Is Associated With Increased Expression of Interferon-Stimulated Genes in CD4<sup>+</sup> T Cells

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**Most patients with human immunodeficiency virus (HIV) who remain CD4<sup>+</sup> T-cell deficient on antiretroviral therapy (ART) exhibit marked immune activation. As CD4<sup>+</sup> T-cell activation may be mediated by microbial translocation or interferon-alpha (IFN- $\alpha$ ), we examined these factors in HIV patients with good or poor CD4<sup>+</sup> T-cell recovery on long-term ART. Messenger RNA levels for 3 interferon-stimulated genes were increased in CD4<sup>+</sup> T cells of patients with poor CD4<sup>+</sup> T-cell recovery, whereas levels in patients with good recovery did not differ from those in healthy controls. Poor CD4<sup>+</sup> T-cell recovery was also associated with CD4<sup>+</sup> T-cell expression of markers of activation, senescence, and apoptosis, and with increased serum levels of the lipopolysaccharide receptor and soluble CD14, but these were not significantly correlated with expression of the interferon-stimulated genes. Therefore, CD4<sup>+</sup> T-cell recovery may be adversely affected by the effects of IFN- $\alpha$ , which may be amenable to therapeutic intervention.**

Up to 30% of patients with human immunodeficiency virus (HIV) infection receiving antiretroviral therapy (ART) fail to achieve a normal CD4<sup>+</sup> T-cell count [1], particularly patients who have a nadir CD4<sup>+</sup> T-cell count <100/ $\mu$ L [2]. Even after 10 years of ART, approximately 40% of patients who commenced therapy with a CD4<sup>+</sup> T-cell count <200/ $\mu$ L have a count <500/ $\mu$ L [3]. Although persistent CD4<sup>+</sup> T-cell deficiency was not associated with an increased risk of opportunistic

infections after the first 6 months of ART [4], a study of >23 000 HIV patients (the D:A:D study) demonstrated an increased risk of death [5, 6]. CD4<sup>+</sup> T-cell deficiency in HIV patients receiving ART is associated with an increased risk of cancer, particularly skin cancer [5, 7], cardiovascular disease [8], chronic kidney disease [9], and acute renal failure [10], although it is unclear to what extent these conditions reflect immunodeficiency as opposed to immune activation and inflammation. The development of new therapies to optimize CD4<sup>+</sup> T-cell recovery in HIV patients on ART requires a better understanding of the causes of persistent CD4<sup>+</sup> T-cell deficiency.

Comparisons between patients with good and poor CD4<sup>+</sup> T-cell recovery on ART have identified 2 important factors [1]. First, CD4<sup>+</sup> T-cell deficiency parallels lower naive CD4<sup>+</sup> T-cell counts [11]. This may reflect thymic insufficiency [12, 13], exhaustion of lymphopoiesis [14], and/or impaired peripheral homeostasis of naive CD4<sup>+</sup> T cells [15]. Second, persistently increased immune

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**Table 1. Patient Characteristics and Clinical Data**

	Low CD4 <sup>+</sup> T cells (n = 21)	High CD4 <sup>+</sup> T cells (n = 21)	Healthy controls (n = 10)	<i>P</i> value <sup>b</sup>	<i>P</i> value <sup>c</sup>
Age (years)	55 (44–62) <sup>a</sup>	47 (44–53)	56 (43–60)	.2 <sup>d</sup>	.6
Male:female	20:1	20:1	10:0	1.0 <sup>e</sup>	1.0 <sup>e</sup>
Nadir CD4 <sup>+</sup> T cells/μL	18 (10–48)	18 (5–37)	...	.3	...
Hepatitis C seropositive	3/21	1/21	0/10	.6 <sup>e</sup>	.6 <sup>e</sup>
Months on ART	62 (34–102)	112 (76–126)	...	.015	...
Months with HIV RNA <50 copies/mL	44 (20–74)	72 (52–118)	...	.025	...
Current CD4 <sup>+</sup> T cells/μL blood	224 (143–384)	720 (576–885)	917 (717–1183)	<.0001	.0005
Current CD8 <sup>+</sup> T cells/μL blood	736 (551–1241)	950 (811–1206)	424 (316–689)	.2	.001
Naive CD4 <sup>+</sup> T cells/μL blood	42 (23–74)	145 (113–266)	...	<.0001	...
Naive CD8 <sup>+</sup> T cells/μL blood	133 (61–190)	276 (125–401)	...	.007	...

Abbreviations: ART, antiretroviral therapy; HIV, human immunodeficiency virus.

<sup>a</sup> Median (interquartile range).

<sup>b</sup> Comparing patients with low and high CD4<sup>+</sup> T cells.

<sup>c</sup> Comparing patients with healthy controls.

<sup>d</sup> *P* values obtained by Mann–Whitney *U* test unless otherwise indicated.

<sup>e</sup> *P* values obtained by Fisher exact test.

activation [16] may induce CD4<sup>+</sup> T-cell senescence and increase rates of apoptosis [17, 18]. The effects of immune activation are most evident in the naive CD4<sup>+</sup> T-cell population [18], so these mechanisms may act in parallel.

The effect of HIV infection on CD4<sup>+</sup> T-cell activation and senescence in patients with a plasma HIV RNA level of <50 copies/mL may be related to nonreplicating virions [19] or reactivation of infection from cellular reservoirs [20]. In addition, rapid and sustained depletion of CD4<sup>+</sup> T cells from mucosal tissues during acute HIV infection may promote the translocation of microbial products from the intestinal lumen into the circulation and subsequent activation of immune cells [21–23]. Elevated plasma levels of bacterial lipopolysaccharide (LPS) and 16S ribosomal DNA (16SrDNA) are reported in untreated and ART-treated patients and have been associated with immune activation and blunted CD4<sup>+</sup> T-cell gains in patients receiving ART [24, 25]. Studies of a murine model of thymic ablation suggest that translocation of gut microbial products might be particularly detrimental to naive CD4<sup>+</sup> T-cell homeostasis [26].

CD4<sup>+</sup> T-cell homeostasis may also be influenced by type I interferons, particularly interferon-alpha (IFN-α). Increased production of IFN-α by plasmacytoid dendritic cells (pDC) is associated with disease progression in HIV or simian immunodeficiency virus (SIV) infection [27–29]. IFN-α may promote apoptosis of uninfected bystander CD4<sup>+</sup> T cells by upregulating expression of membrane-bound tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and death receptor 5 (a TRAIL receptor) [30–32]. Studies of SIV infection in non-human primates identify IFN-α as a determinant of immune activation [29]. Furthermore, type I interferon-stimulated gene transcripts are markedly upregulated in activated CD4<sup>+</sup> T cells from untreated HIV patients [33]. IFN-α also influences T-cell

development, as murine fetal thymic organ cultures treated with TLR3 or TLR7 ligands express IFN-α and display decreased proliferation and increased apoptosis of thymocytes [34]. IFN-α can also inhibit the development of human T-cell progenitors and affect their proliferation [35]. These effects may be mediated by IFN-α inhibition of IL-7R signaling [34, 35].

The relationship between CD4<sup>+</sup> T-cell recovery and interferon-stimulated gene expression in CD4<sup>+</sup> T cells of HIV patients receiving ART has not been reported. We present a cross-sectional study of patients receiving effective ART and correlate interferon-stimulated gene messenger RNA (mRNA) levels, plasma/serum markers of microbial translocation, and markers of CD4<sup>+</sup> T-cell apoptosis, activation and senescence with current total and naive CD4<sup>+</sup> T-cell counts and estimated CD4<sup>+</sup> T-cell counts after 10 years of ART. We provide novel evidence that low CD4<sup>+</sup> T-cell counts after long-term ART may be associated with increased interferon activity.

## PATIENTS AND METHODS

### Patients

HIV patients (n = 42) were recruited if they had experienced a nadir CD4<sup>+</sup> T-cell count <100 cells/μL, received ART for >12 months, and maintained a plasma HIV RNA level <50 copies/mL for at least 6 months. Patients were categorized as having high (n = 21) or low (n = 21) CD4<sup>+</sup> T-cell counts based on values above or below the median value (490 cells/μL). A cohort of age- and sex-matched healthy controls (n = 10) was recruited as a comparison group for the assessment of interferon-stimulated genes. Demographic and clinical data are presented in the Table 1. Informed consent was obtained from

all subjects, and the study was approved by an institutional ethics committee.

### T-Cell Phenotyping

Total, naive, and memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells were enumerated in EDTA-treated whole blood using the following fluorescently conjugated monoclonal antibodies (mAbs): CD45 (APC-H7), CD3 (PerCP), CD4 (APC), CD8 (PE-Cy7), CD45RA (FITC), and CD62L (PE) (BD Biosciences). Total CD4<sup>+</sup> and CD8<sup>+</sup> T cells were identified as CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup>. Naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells were identified as CD3<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>+</sup>. For assessment of immune activation and proapoptotic molecules, EDTA-treated whole blood was incubated with the following fluorescently conjugated mAbs from BD Biosciences: CD4 (PerCP-Cy5.5), CD8 (APC-Cy7), CD57 (FITC), HLA-DR (APC), and Fas (PE) for 20 minutes, followed by 1 mL FACSlyse (BD Biosciences) for a further 15 minutes at room temperature. All analyses were performed using a FACSCanto II cytometer (BD Biosciences). Stopping and storage gates were set at 100 000 lymphocyte events defined by forward scatter and side scatter. Files were exported in FCS 3.0 format and visualized using FlowJo software, version 7.2.5 (Tree Star).

### Assessment of Spontaneous T-Cell Apoptosis

Lithium heparin-treated whole blood was fractionated over Ficoll gradients, and  $1 \times 10^6$  freshly isolated peripheral blood mononuclear cells (PBMCs) were cultured in RPMI containing 10% fetal calf serum at 37°C in 5% CO<sub>2</sub>. After 72 hours, cells were washed in cold phosphate-buffered saline and incubated with the fluorescently conjugated mAb CD4 or CD8 (PE), Annexin V (FITC), and 7-AAD (BD Biosciences) for 15 minutes at room temperature. Preapoptotic cells expressed Annexin V but not 7-AAD, and apoptotic cells expressed Annexin V and 7-AAD.

### Measurement of Plasma Levels of LPS, 16SrDNA, and sCD14 and Serum Levels of LPS-Specific Antibodies

Plasma levels of LPS were assayed using a commercial chromogenic limulus amoebocyte lysate assay (Lonza) according to the manufacturer's instructions [36]. Plasma 16SrDNA levels were assessed in DNA extracted from 200 µl EDTA plasma (DNeasy Kit, Qiagen) using a novel and sensitive real-time quantitative TaqMan PCR assay that included pretreatment of the PCR reaction mix with 0.2U shrimp DNase to remove errant double stranded DNA [37]. Each patient sample was assayed in triplicate. The assay had a detection range of 10–10<sup>6</sup> copies per 5 µl plasmid 16SrDNA. Samples lacking added DNA template showed no amplification at a cycle threshold of 40. Plasma levels of soluble (s) CD14 were assayed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) per the manufacturer's instructions modified for half-well plates. Serum

levels of immunoglobulin (Ig) A, IgG, and IgM antibodies to LPS were assayed by an in-house ELISA as described previously [38].

### PCR for Detection of Interferon-Stimulated Genes

CD4<sup>+</sup> and CD8<sup>+</sup> T cells were isolated by sequential positive isolations from each cryopreserved PBMC sample using CD4 and CD8 MicroBeads on an OctoMACS Separation Unit (Miltenyi Biotec) according to the manufacturer's instructions. Purified cells were washed, resuspended in 350 µl RNA lysis buffer (Qiagen), and stored at –80°C for RNA extraction. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed into complementary DNA (cDNA) using Sensiscript RT Kit (Qiagen); cDNA samples were then stored at –80°C. Real-time PCR was performed as described previously [39]. Primers used for IFI6-16 cDNA detection were 5'-cctgctgctctcactgca-3' and 5'-ccgacggccatgaaggt-3'; ISG56 detection 5'-ctgactggcaatagcaagct-3' and 5'-gagggtcaatggcgttctga-3'; and IFI27 detection 5'-ccaggattgctacagttgtgattg-3' and 5'-tgaagccatggcactga-3'.

### Estimation of 10-Year CD4<sup>+</sup> T-Cell Counts

To model CD4<sup>+</sup> T-cell counts obtained from the start of ART until the current time for each patient, we fitted both a linear curve  $y = a + bx$ , and a saturating curve  $y = a + bx^2/(1 + cx^2)$  using linear and nonlinear regression as previously described [40]. The Bayesian Information Criterion (BIC) was used to determine the best model for each patient. This best-fit curve for each patient was then used to estimate their 10-year CD4<sup>+</sup> T-cell count. Curve fitting was performed with Matlab, version R2009a (MathWorks).

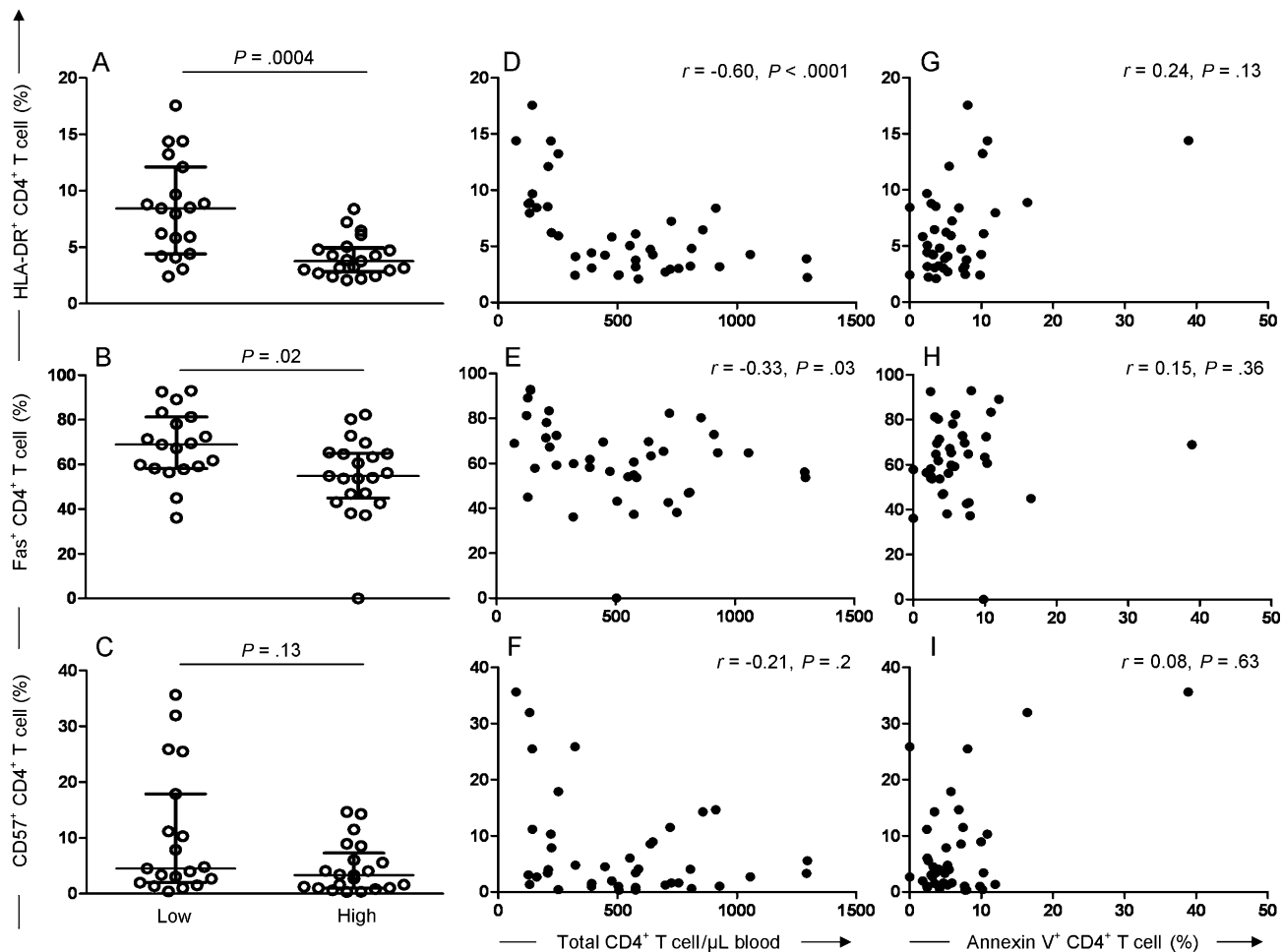
### Statistical Analyses

Analyses were performed with GraphPad Prism software, version 5.01. Statistical significance was assessed by the Mann-Whitney *U* test for continuous variables. Correlation coefficients were determined by the Spearman rank correlation test. For all tests,  $P < .05$  was considered to represent a significant difference.

## RESULTS

### Current CD4<sup>+</sup> T-Cell Counts Correspond With CD4<sup>+</sup> T-Cell Recovery Capacity After 10 Years

To examine the relationship between immune activation and long-term CD4<sup>+</sup> T-cell recovery, an estimated CD4<sup>+</sup> T-cell count 10 years after commencing ART was determined for each patient. Based on their estimated 10-year value, patients were separated into low or high CD4<sup>+</sup> T-cell groups (less or greater than the median count of 555 cells/µL). Only 3 individuals changed classification compared with their current CD4<sup>+</sup> T-cell counts. One of these individuals had been on ART for only 3 years, and the other 2 had CD4<sup>+</sup> T-cell counts close to the median values. Similarly, when patients were separated into 2 groups on the basis of their current naive CD4<sup>+</sup> T-cell count, only 4 individuals changed groups compared



**Figure 1.** Proportions of CD4<sup>+</sup> T cells expressing HLA-DR (median [interquartile range], 8.4% [4.4%–12%] vs 3.8% [2.8%–4.9%]) or Fas (69% [58%–81%] vs 55% [45%–65%]) were higher in patients with low CD4<sup>+</sup> T-cell counts (A, B) and correlated inversely with CD4<sup>+</sup> T-cell counts when the cohort was combined (D, E). Proportions of CD4<sup>+</sup> T cells expressing CD57 (4.5% [2.0%–18%] vs 3.3% [1.0%–7.3%]) did not differ between patients with low or high CD4<sup>+</sup> T-cell counts (C) or correlate with CD4<sup>+</sup> T-cell counts when the cohort was combined (F). Proportions of CD4<sup>+</sup> T cells expressing HLA-DR, CD57, or Fas did not correlate with proportions of apoptotic CD4<sup>+</sup> T cells following 72-hour culture (G–I).

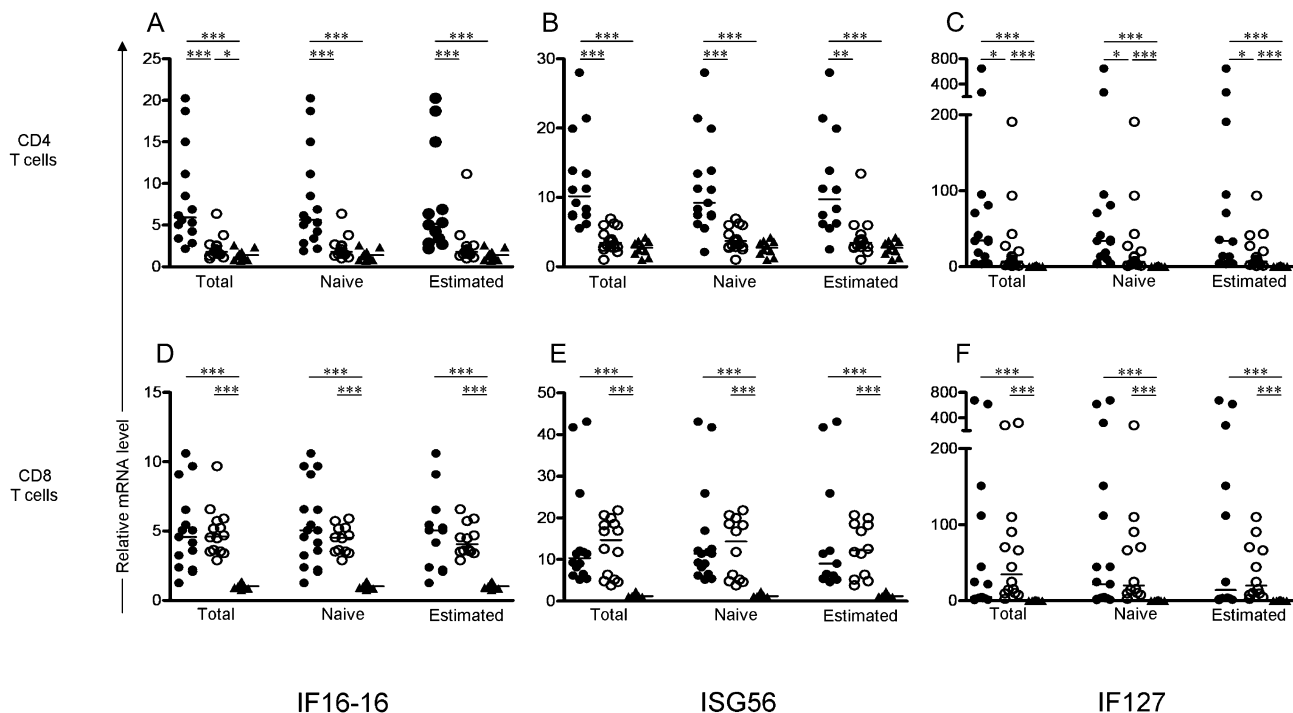
with their current CD4<sup>+</sup> T-cell counts. Hence, overall the current CD4<sup>+</sup> T-cell count corresponded well with the estimated CD4<sup>+</sup> T-cell count after 10 years as well as current naive CD4<sup>+</sup> T-cell counts.

#### Expression of T-Cell Activation Markers Was Higher in Patients With Low CD4<sup>+</sup> T-Cell Counts but Did Not Predict T-Cell Apoptosis in Culture

Expression of markers of activation (HLA-DR), apoptotic potential (Fas), or senescence (CD57) was assessed immediately ex vivo in whole blood. The proportions of CD4<sup>+</sup> T cells expressing HLA-DR or Fas were higher in patients with low CD4<sup>+</sup> T-cell counts than in those with high counts ( $P = .0004$  and  $P = .02$ , respectively; Figure 1A and B) and correlated inversely with CD4<sup>+</sup> T-cell counts when the cohort was assessed as a whole (Figure 1D and E). The proportion of CD4<sup>+</sup> T cells expressing CD57 did not differ between patients with low or high CD4<sup>+</sup>

T-cell counts ( $P = .1$ ; Figure 1C) or correlate with CD4<sup>+</sup> T-cell counts when the cohort was assessed as a whole (Figure 1F). The proportion of CD8<sup>+</sup> T cells expressing CD57 was higher in patients with low CD4<sup>+</sup> T-cell counts ( $P = .04$ ), but this was not observed for HLA-DR or Fas expression ( $P = .7$  and  $P = .3$ , respectively; data not shown).

After 72 hours in culture, proportions of CD4<sup>+</sup> (median [interquartile range], 1.0% [0.6–2.0] vs 0.8% [0.3–1.4];  $P = .3$ ) and CD8<sup>+</sup> (0.8% [0.4–1.3] vs 0.9% [0.5–1.6];  $P = .5$ ) T cells in apoptosis (Annexin V<sup>+</sup>, 7-AAD<sup>+</sup>) or preapoptotic CD4<sup>+</sup> (4.0% [1.9–7.2] vs 4.0% [2.9–6.4];  $P = .5$ ) and CD8<sup>+</sup> (3.9% [2.6–5.8] vs 4.3% [2.4–5.4];  $P = .8$ ) T cells (Annexin V<sup>+</sup>, 7-AAD<sup>-</sup>) were similar in patients with low or high CD4<sup>+</sup> T-cell counts. In the patient cohort assessed as a whole, no correlations were observed between proportions of CD4<sup>+</sup> T cells expressing HLA-DR, Fas, or CD57, and the proportion of apoptotic (Annexin V<sup>+</sup>, 7-AAD<sup>+/−</sup>) CD4<sup>+</sup> T cells (Figure 1G–I).



**Figure 2.** Expression of IF16-16, ISG56, and IF127 was higher in patients with human immunodeficiency virus with low CD4<sup>+</sup> T-cell counts (●) than in patients with high CD4<sup>+</sup> T-cell counts (○) and healthy controls (▲) (A–C). Expression of IF16-16, ISG56, and IF127 in purified CD8<sup>+</sup> T cells was similar in patients with low and high CD4<sup>+</sup> T cells but higher than in healthy controls (D–F). Data are presented for the patients when divided by current total and naive CD4<sup>+</sup> T cells and estimated total CD4<sup>+</sup> T-cell counts at 10 years. \* $P \leq .05$ , \*\* $P \leq .005$ , \*\*\* $P \leq .0005$ .

#### Interferon-Stimulated Gene mRNA Levels in CD4<sup>+</sup> T-Cells Was Higher in HIV Patients With Low CD4<sup>+</sup> T-Cell Counts on ART

To evaluate IFN- $\alpha$  activity as a determinant of low CD4<sup>+</sup> T-cell counts, we quantitated mRNA of 3 interferon-stimulated gene products (IF16-16, ISG56, and IF127) in purified CD4<sup>+</sup> T cells of patients and healthy controls. Levels of IF16-16, ISG56, and IF127 mRNA were higher in patients with low total or naive CD4<sup>+</sup> T-cell counts than in patients with high total or naive CD4<sup>+</sup> T-cell counts ( $P < .0001$ ,  $P < .0001$ , and  $P = .02$ , respectively) and healthy controls ( $P < .0001$ ,  $P < .0001$ , and  $P < .0001$ , respectively). Levels of interferon-stimulated gene transcripts in patients with high CD4<sup>+</sup> T-cell counts were similar to those in healthy controls (Figure 2A–C). Levels of IF16-16, ISG56, and IF127 mRNA in purified CD8<sup>+</sup> T cells were similar in patients with low and high CD4<sup>+</sup> T cells but higher than in healthy controls (Figure 2D–F).

When levels of each of the interferon-stimulated gene transcripts were compared in patients with low or high estimated CD4<sup>+</sup> T-cell counts 10 years after ART, the results were similar to those observed with current total or naive CD4<sup>+</sup> T-cell counts (Figure 2).

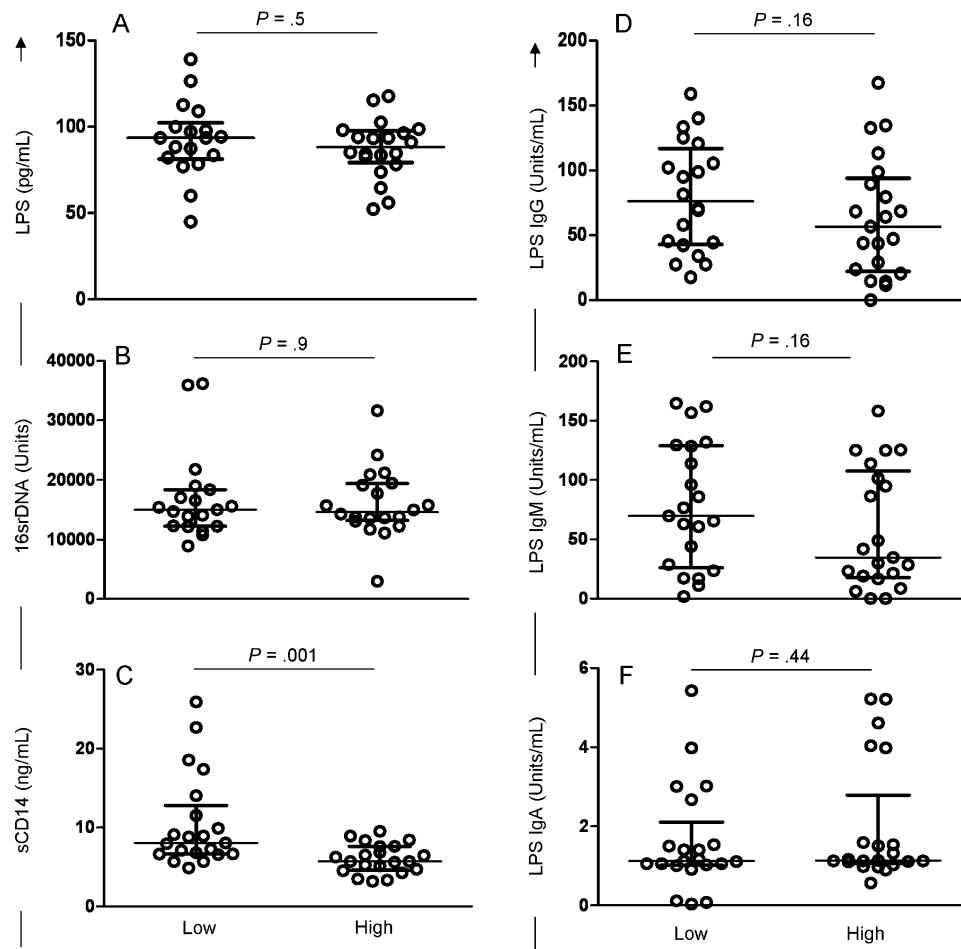
No statistically significant relationship was observed between the expression of any interferon-stimulated gene transcripts and markers of T-cell activation or apoptosis ( $-0.28 < r < 0.33$ ;  $.08 < P < .9$ ). However, marginally significant associations were observed between the level of IF127 mRNA in purified CD4<sup>+</sup>

T cells and the proportion of CD4<sup>+</sup> T cells expressing Fas ( $r = 0.32$ ;  $P = .08$ ); the level of ISG56 mRNA in purified CD4<sup>+</sup> T cells and the proportion of CD4<sup>+</sup> T cells expressing CD57 ( $r = 0.31$ ;  $P = .09$ ); and the level of ISG56 mRNA in purified CD8<sup>+</sup> T cells and the proportion of CD8<sup>+</sup> T cells expressing HLA-DR ( $r = 0.33$ ;  $P = .09$ ).

#### Higher Plasma Levels of sCD14 Were Associated With Low CD4<sup>+</sup> T-Cell Counts After Long-term ART

We assessed microbial translocation directly through plasma levels of microbial products (LPS and 16S rDNA) and indirectly using sCD14 (LPS receptor and monocyte activation marker) and IgG, IgM, and IgA antibodies to LPS, bearing in mind that LPS may be bound by sCD14 or antibodies to LPS [24]. Plasma levels of LPS were similar in patients with low or high CD4<sup>+</sup> T-cell counts ( $P = .5$ ; Figure 3A) and did not correlate with CD4<sup>+</sup> T-cell counts when the patient cohort was combined ( $r = -0.1$ ;  $P = .5$ ). Similarly, plasma levels of 16S rDNA were similar in patients with low or high CD4<sup>+</sup> T cells ( $P = .9$ ; Figure 3B) and did not correlate with CD4<sup>+</sup> T-cell counts ( $r = 0.2$ ;  $P = .2$ ).

In contrast, plasma levels of sCD14 were higher in patients with low CD4<sup>+</sup> T cells ( $P = .001$ ; Figure 3C) and correlated inversely with CD4<sup>+</sup> T-cell counts ( $r = -0.48$ ;  $P = .0012$ ). Serum IgG, IgM, and IgA anti-LPS levels were similar between patients with low and high CD4<sup>+</sup> T-cell counts ( $P = .16$ ,  $P = .16$ , and  $P = .44$ , respectively; Figure 3D–F).



**Figure 3.** Plasma levels of LPS (A, median [interquartile range], 94 pg/mL [81–102 pg/mL] vs 88 pg/mL [79–98 pg/mL]) and 16SrDNA (B, 14 990 units [12 210–18 360 units] vs 14 593 units [13 173–19 369 units]) were similar in patients with low or high CD4<sup>+</sup> T cells. Plasma levels of sCD14 were significantly higher in patients with low CD4<sup>+</sup> T cells (C, 8.1 ng/mL [6.6–12.8 ng/mL] vs 5.7 ng/mL [4.6–7.6 ng/mL]). Levels of IgG anti-LPS (D, 76 units/mL [43–117 units/mL] vs 57 units/mL [22–94 units/mL]), IgM anti-LPS (E, 70 units/mL [26–129 units/mL] vs 35 units/mL [18–107 units/mL]), and IgA anti-LPS (F, 1.1 units/mL [1.0–2.1 units/mL] vs 1.1 units/mL [1.1–2.8 units/mL]).

When each of the indicators of microbial translocation was examined in relation to the estimated 10-year CD4<sup>+</sup> T-cell count, only high plasma levels of sCD14 were found to be associated with low CD4<sup>+</sup> T-cell counts ( $P = .02$ , data not shown).

Finally, we examined the relationship between immune activation, senescence, and apoptosis markers expressed by CD4<sup>+</sup> T cells (HLA-DR, CD57, or Fas) and the indicators of microbial translocation. Plasma levels of sCD14 correlated with the proportion of CD4<sup>+</sup> T cells expressing HLA-DR ( $r = 0.39$ ;  $P = .01$ ), serum IgG anti-LPS correlated with the proportion of CD4<sup>+</sup> T cells expressing Fas ( $r = 0.35$ ;  $P = .02$ ), and serum IgM anti-LPS correlated with the proportion of CD4<sup>+</sup> T cells expressing CD57 ( $r = 0.58$ ;  $P < .0001$ ). No other correlations between indicators of microbial translocation and markers of T-cell immune activation or apoptosis were evident. There was also no relationship between the indicators of microbial translocation and interferon-stimulated gene transcript levels in purified CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells.

## DISCUSSION

We and others have demonstrated that immune activation is elevated in HIV patients who have persistently low CD4<sup>+</sup> T-cell counts despite virologically effective ART [16, 18], but the underlying mechanisms remain unclear. Here, we examined the association of CD4<sup>+</sup> T-cell recovery with markers of microbial translocation and interferon-stimulated gene mRNA levels. As the effects of immune activation on circulating CD4<sup>+</sup> T cells are most evident within the naive CD4<sup>+</sup> T-cell population [18], we compared patients with low or high naive CD4<sup>+</sup> T-cell counts as well as low or high total CD4<sup>+</sup> T-cell counts after at least 3 years of effective ART. Mathematical curve fitting demonstrated that current CD4<sup>+</sup> T-cell counts reflected total CD4<sup>+</sup> T-cell recovery capacity over 10 years.

We have demonstrated for the first time elevated expression of 3 interferon-stimulated gene transcripts in purified CD4<sup>+</sup> T cells from patients on long-term ART with low total or

naive CD4<sup>+</sup> T-cell counts (Figure 2), whereas expression in the patients with high CD4<sup>+</sup> T-cell counts was similar to that in non-HIV controls. Furthermore, elevated levels of interferon-stimulated gene transcripts were also associated with low estimated 10-year CD4<sup>+</sup> T-cell counts. In contrast, expression of the interferon-stimulated gene transcripts in purified CD8<sup>+</sup> T cells was not related to CD4<sup>+</sup> T-cell counts but was elevated compared with healthy controls (Figure 2). Sedaghat et al [33] demonstrated that interferon-stimulated gene transcripts were increased in activated CD4<sup>+</sup> T cells from untreated HIV patients and suggested that immune activation may be a consequence of IFN- $\alpha$  activity. Our data suggest that normalization of interferon-stimulated gene expression in HIV patients receiving ART is associated with good recovery of naive and total CD4<sup>+</sup> T-cell counts. These findings provide a rationale for the use of therapies that inhibit IFN- $\alpha$  production, such as chloroquine or hydroxychloroquine, which suppress HIV-induced production of IFN- $\alpha$  by pDC in vitro and in vivo [41, 42] and immune activation in ART-naive HIV patients [43].

We have also shown that patients with low CD4<sup>+</sup> T-cell counts, despite effective ART, had higher proportions of CD4<sup>+</sup> T cells expressing HLA-DR (a marker of activation), CD57 (a marker of senescence), and Fas (a proapoptotic molecule) than did patients with high CD4<sup>+</sup> T-cell counts (Figure 1). However, there was no correlation between expression of these markers and mRNA levels of the interferon-stimulated genes, suggesting that increased interferon activity is associated with low CD4<sup>+</sup> T-cell counts via a pathway that does not involve T-cell activation or Fas-induced apoptosis. A series of studies by Herbeuval et al [30–32] demonstrated that the downstream effects of IFN- $\alpha$  production by pDCs include increased production of soluble TRAIL, upregulation of membrane-bound TRAIL, and induction of death receptor 5 expression on CD4<sup>+</sup> T cells but not CD8<sup>+</sup> T cells. It is therefore possible that T-cell survival is decreased by activation of the TRAIL/death receptor 5 pathway.

Although expression of Fas on CD4<sup>+</sup> T cells was elevated in patients with low CD4<sup>+</sup> T-cell counts, rates of spontaneous CD4<sup>+</sup> T-cell apoptosis in culture were not higher in patients with low CD4<sup>+</sup> T-cell counts, and expression of Fas (or HLA-DR and CD57) did not predict apoptosis. Our results contrast with previous studies that found increased apoptosis of CD4<sup>+</sup> T cells in patients with low CD4<sup>+</sup> T-cell counts after long-term ART [17, 44–46]. It is possible that this reflects the use of a 72-hour culture in our study, which is longer than cultures used in most other studies.

Although CD4<sup>+</sup> T cells from patients with low CD4<sup>+</sup> T cells had elevated expression of HLA-DR, CD57, and Fas, these markers of T-cell activation did not correlate with direct indicators of microbial translocation (though they did correlate

with levels of sCD14). Furthermore, there was no relationship between direct or indirect markers of microbial translocation and interferon-stimulated gene transcript levels in CD4<sup>+</sup> T cells. Our previous longitudinal study showed that plasma LPS and sCD14 levels fluctuated widely and were not associated with CD4<sup>+</sup> T-cell recovery on ART [36]. This fluctuation may explain variable findings from cross-sectional studies. Our findings agree with a cross-sectional study where sCD14, but not LPS levels, was associated with poor CD4<sup>+</sup> T-cell recovery [47]. However, Piconi et al [44] and Marchetti et al [48] demonstrated elevated plasma LPS levels in patients with CD4<sup>+</sup> T-cell counts <500 or <200 cells/ $\mu$ L, respectively, after long-term ART, whereas Jiang et al [25] demonstrated associations between microbial translocation (plasma LPS and 16S rDNA levels), high levels of immune activation, and blunted CD4<sup>+</sup> T-cell gains on ART. In a separate cross-sectional study, we have shown that plasma LPS levels correlate negatively with CD4<sup>+</sup> T-cell counts in HIV patients receiving ART [38]. Overall, we conclude that microbial translocation varies over time and between patients. It may contribute to T-cell deficiency, or the reverse may be true: microbial translocation may be a consequence of poor immune reconstitution.

Several causes of increased interferon-stimulated gene mRNA levels in HIV patients with low CD4<sup>+</sup> T-cell recovery on ART are possible and should be examined in future studies. These include nonreplicating HIV virions [19], reactivation of HIV infection from cellular reservoirs [20], and reactivation of other persistent virus infections. Asymptomatic reactivation of cytomegalovirus (CMV) infection has been proposed as a cause of immune activation associated with limited CD4<sup>+</sup> T-cell recovery in HIV patients on ART [49]. A recent report demonstrated a reduction in CMV DNA that coincided with a decline in the frequency of activated CD8<sup>+</sup> T cells following treatment of such patients with valganciclovir [50].

In assessing our findings, limitations of the experimental method should be considered. This was a cross-sectional study, so it is only possible to associate increased interferon-stimulated gene expression with CD4<sup>+</sup> T-cell deficiency. Longitudinal studies will be needed to investigate causality. Patients had received ART for different lengths of time, but all patients had received at least 3 years of therapy, and mathematical modeling confirmed that the current CD4<sup>+</sup> T-cell count was concordant with CD4<sup>+</sup> T-cell recovery capacity over 10 years.

In conclusion, we have provided evidence that increased production and/or activity of IFN- $\alpha$  may adversely affect recovery of total and naive CD4<sup>+</sup> T cells in HIV patients receiving ART but not via mechanisms of immune activation that result in increased T-cell expression of HLA-DR, CD57, and Fas. We also demonstrated that low CD4<sup>+</sup> T-cell counts on ART are associated with increased plasma levels of sCD14, which appears to be distinct from the mechanism associated with increased interferon-stimulated gene expression by CD4<sup>+</sup> T cells.

## Notes

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