Amplification of the signal transducer and activator of transcription I signaling pathway and its association with apoptosis in monocytes from HIV-infected patients

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Background: Monocytes/macrophages play a major role in inflammation and pathogen clearance. However, chronic immune activation observed during HIV infection may also cause cellular dysfunction and tissue pathology. Indeed, several defects have been reported in these cells during HIV infections. As cytokine responsiveness via the signal transducer and activator of transcription (STAT1) signaling pathway is critical for these functions, we hypothesized that its activation in monocytes from HIV-positive patients may be disrupted.

Objectives: To evaluate cytokine-dependent STAT signaling in monocytes from HIV-positive patients and study the biological impact and molecular mechanisms responsible for the alterations in the interferon (IFN)-γ-induced STAT1 pathway observed.

Methods: Monocytes from chronically infected HIV-positive patients on and off antiretroviral therapy were assayed respectively for STAT activation, apoptosis, and other downstream effects by flow cytometry, real-time PCR and enzyme-linked immunosorbent assay.

Results: Unlike IFN-α, interleukin-10, granulocyte macrophage colony-stimulating factor, and interleukin-4, only IFN-γ-induced STAT1 activation was upregulated in monocytes from off-therapy patients compared with those on antiretroviral therapy and HIV-negative controls, correlating with increased total STAT1 expression. Among the IFN-γ responsive genes (IRF-1, CXCL9, CXCL10) studied, differential effects were observed, likely reflecting the more complex regulatory control over their expression. Interestingly, spontaneous monocyte apoptosis was elevated in HIV-positive patients off-therapy compared with HIV-negative controls and correlated with STAT1 expression. IFN-γ-induced apoptosis was also increased and persisted despite seemingly effective antiretroviral therapy.

Conclusion: Amplification of STAT1 signaling and apoptosis may reflect the chronic nature of immune activation in HIV-positive patients and contribute to the functional impairment observed in monocytes through the course of the disease.

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Introduction

Monocytes/macrophages (M/M) are key players in inflammation and pathogen clearance. However, chronic immune activation during HIV infection, regarded as an important viral pathogenic mechanism [1], may also contribute to M/M dysfunction. Several studies have suggested that HIV may impair M/M phagocytosis, antigen uptake and major histocompatibility complex (MHC) class II expression, and affect cytokine secretion [2–6]. The molecular mechanism by which HIV impairs M/M function is not clear but cytokine responsiveness is critical for these functions [7,8]. It has been suggested that HIV can impair T-cell and monocyte function, for example, by disrupting cytokine-dependent and antigen-dependent signal transduction. We showed previously that interleukin (IL)-2-induced signal transducer and activator of transcription (STAT)-5 activation was inhibited in CD8 T cells from a subset of chronically infected HIV-positive patients naive to therapy, but was restored after antiretroviral therapy (ART) [9]. Further, granulocyte macrophage colony-stimulating factor (GM-CSF)-induced STAT5 activation in macrophages was inhibited by HIV-1 infection *in vitro* [10]. Considering the biological importance of STAT signaling [11], we hypothesized that this pathway may be disrupted in monocytes from HIV-positive patients, thus contributing to the immune deficiency resulting from chronic HIV infection.

Methods

Patient characteristics

Chronically infected HIV-positive patients studied were from the Ottawa Hospital and included those on ART (>1 year; $n = 17$) and off-therapy (>6 months; $n = 10$). Plasma viral loads were $94(6–300) \times 10^3$ copies/ml [mean (range)] and less than 50 copies/ml in off-therapy and ART patients, respectively. CD4 cell counts were 603 (291–1002) cells/μl and 312 (9–733) cells/μl in patients on and off therapy, respectively.

Cytokines and antibodies

The recombinant human cytokines used for cell stimulation were interferon (IFN)-γ (Pierce, Rockford, Illinois, USA), IFN-α2a (PBL Biomedical Laboratories, New Brunswick, New Jersey, USA), GM-CSF, IL-10, and IL-4 (R&D Systems, Minneapolis, Minnesota, USA). The antihuman monoclonal antibodies (mAbs) used for flow cytometry were PerCp-CD14, Alexa488–labeled STAT1, STAT3, and STAT5, PE-labeled STAT1 and STAT6 [BD Biosciences (BD), Mississauga, Ontario, Canada], PE-IFN-γ-Receptor1 (IFN-γ-R1) (Biolegend, San Diego, California, USA), IFN-γ-R2 (Cell Science, Canton, Massachusetts, USA).

Monocyte isolation

CD14+ monocytes were purified (>90%, data not shown) from peripheral blood mononuclear cells (PBMC) by positive selection using CD14-microbeads, according to the manufacturer's protocol (AutoMACS; Miltenyi Biotec, Auburn, California, USA).

Flow cytometry

For surface staining, PBMC were stained with anti-IFN-γR1, R2, and anti-CD14 mAbs, as described [9,12]. For intracellular staining, PBMC were stimulated with the indicated cytokines and then stained, as described [13], using specific tyr-phosphorylated STAT (P-STAT)-specific or total STAT-specific and CD14-specific mAbs. For detection of apoptosis, purified monocytes were cultured for 24 h with IFN-γ, then fixed, permeabilized and stained with propidium iodide (PI)/RNase-containing buffer (BD).

Real time-PCR

Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen, Mississauga, Ontario, Canada) and reverse transcribed using the high capacity cDNA Archive kit [Applied Biosystems (ABI), Streetsville, Ontario, Canada]. cDNA was amplified using TaqMan Gene Expression Assays [interferon regulatory factor (IRF)-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] in a 7500 real-time PCR System (ABI). Relative mRNA expression was calculated by the comparative Ct method [14].

Enzyme-linked immunosorbent assay and cytometric bead array

Patient plasma or supernatant from cultured monocytes was assayed for TNF-related apoptosis-inducing ligand (TRAIL) or chemokine expression by Enzyme-linked immunosorbent assay (Diaclone, Stamford, Connecticut, USA) or the cytometric bead array (CBA) chemokine kit (BD Biosciences, San Diego, California, USA), respectively, following the manufacturer's instructions.

Statistical analysis

Pearson's r (two-tailed, $P \leq 0.05$) was used to calculate correlation between continuous variables. The Student's t-test was used for between group comparisons.

Results

Interferon-γ-dependent tyr-phosphorylated signal transducer and activator of transcription I upregulation in monocytes from HIV-positive patients off-therapy

In monocytes from all subjects, P-STAT1 was detected in response to IFN-γ and IFN-α, whereas P-STAT3 was induced in response to IL-10. P-STAT5 and P-STAT6 were activated in response to GM-CSF and IL-4, respectively (Fig. 1a and b). Interestingly, IFN-γ-induced

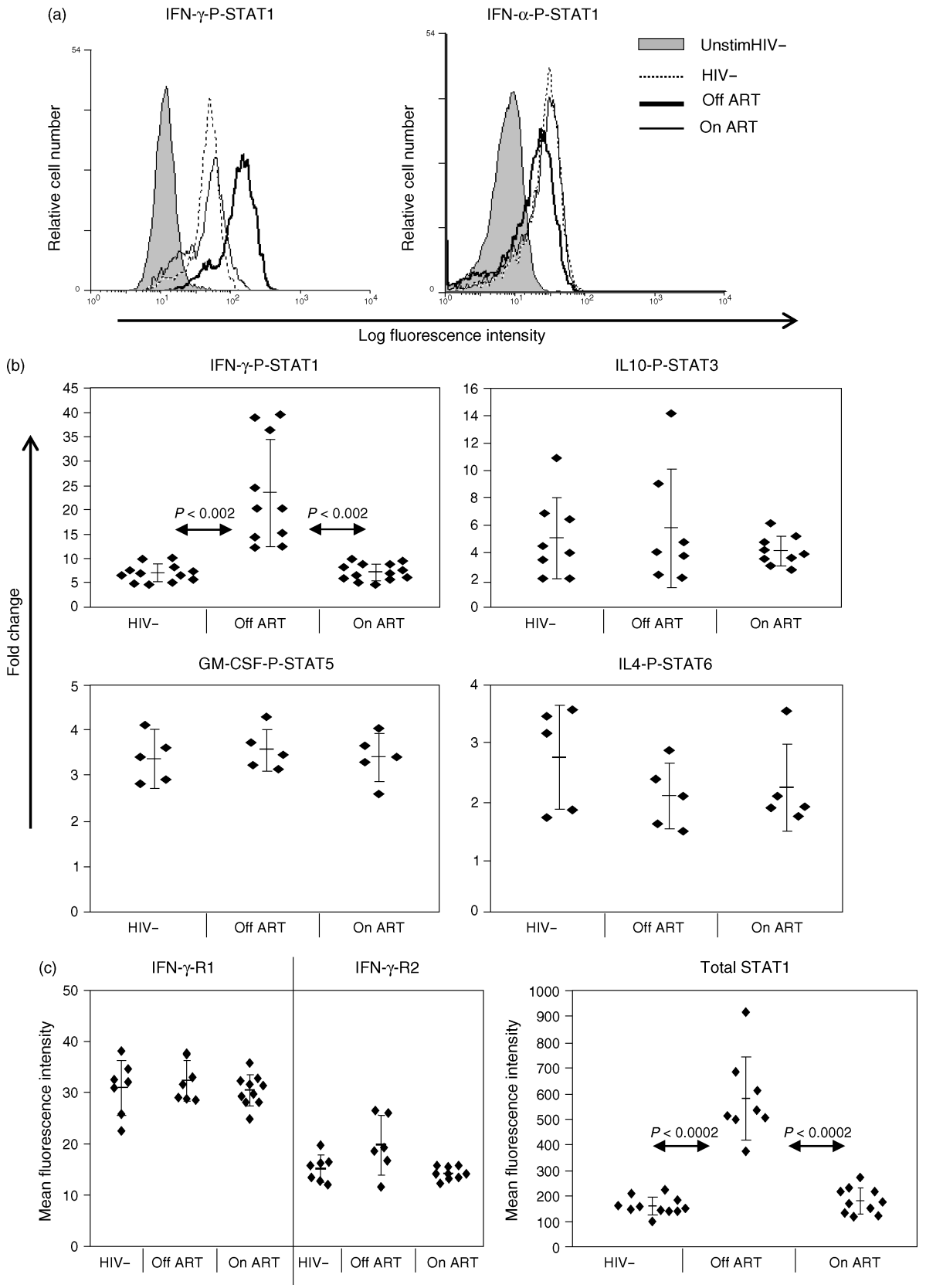

Fig. 1. Upregulation of tyr-phosphorylated STAT 1 (P-STAT1) and total signal transducer and activator of transcription 1 (STAT1) expression in monocytes from HIV-positive patients off therapy.

STAT1 activation was significantly upregulated in HIV-positive patients off-therapy compared with HIV-negative controls and HIV-positive patients on ART (Fig. 1b). There were no significant differences in IFN-α-induced P-STAT1 between the study groups (Fig. 1a). Similarly, there were no significant differences in respective P-STAT induction or basal levels of P-STAT between the patient groups in response to IL-10, GM-CSF, and IL-4 stimulation (Fig. 1b and data not shown).

Association of interferon-γ-induced tyr-phosphorylated signal transducer and activator of transcription I with total signal transducer and activator of transcription I expression

To elucidate the molecular mechanism underlying the amplification of IFN-γ-dependent STAT1 signaling, we evaluated IFN-γ-R and total STAT1 expression. There was no statistically significant difference in IFN-γ-R1 or IFN-γ-R2 subunit expression levels between patient groups (Fig. 1c). In contrast, total STAT1 expression was significantly elevated in monocytes from off-therapy patients compared with those on ART and HIV-negative controls (Fig. 1c), correlating with P-STAT1 expression ($r = 0.926$, $P < 0.001$). This suggested that the enhanced IFN-γ-induced STAT1 activation observed in off-therapy patients was downstream of IFN-γ-R expression and likely due to increased total STAT1 levels.

Elevated monocyte apoptosis, signal transducer and activator of transcription I and plasma TRAIL expression in patients

As it has been shown that IFN-γ and STAT1 can induce apoptosis in several studies [15–18], we hypothesized that upregulation of IFN-γ-induced STAT1 may predispose off-therapy patient monocytes to apoptosis. IFN-γ upregulated apoptosis compared with unstimulated cells in HIV-negative controls and ART patients (Fig. 2a). Despite a similar trend being noted in most off-therapy patients, this was not statistically significant. Interestingly, spontaneous apoptosis was elevated in monocytes from off-therapy patients compared with HIV-negative controls and correlated with total STAT1 expression levels (Fig. 2a; $r = 0.924$, $P = 0.025$). Also striking was that monocyte apoptosis in the presence of IFN-γ remained higher in ART patients compared with controls. Furthermore, we found that plasma levels of TRAIL were significantly increased in HIV-positive patients off-therapy compared with HIV-negative controls and patients on ART. Spontaneous apoptosis and plasma TRAIL exhibited a similar trend (Fig. 2b) but this was not statistically significant ($r = 0.542$, $P = 0.165$).

We hypothesized also that enhanced IFN-γ-induced STAT1 activation in off-therapy patients would upregulate IFN-γ-dependent STAT1-responsive gene expression [19]. However, expression of IRF-1, CXCL9/MIG, and CXCL10/IP10 was upregulated by IFN-γ compared with unstimulated monocytes in all patients studied (Fig. 2b, data not shown). Although IFN-γ-induced IRF-1 mRNA expression was further elevated in a few patients on ART compared with HIV-negative controls, this was not statistically significant (Fig. 2b). Similarly, CXCL9 was elevated in a few patients (on and off therapy) but the substantial variation within each HIV-positive patient group yielded a significant upregulation only in ART patients (Fig. 2b). IFN-γ-induced CXCL10 expression showed no significant differences between patient groups (Fig. 2b).

Discussion

We demonstrated for the first time that among the responses to cytokines tested (IFN-γ, IFN-α, IL-10, IL-4, and GM-CSF) in terms of STAT activation in monocytes, only IFN-γ showed a significant upregulation of P-STAT1 induction in HIV-positive patients off-therapy compared with HIV-negative controls and patients on ART. Furthermore, this potentiation of IFN-γ-induced P-STAT1 may

Fig. 1. (*Continued*).
Peripheral blood mononuclear cells (PBMC) (1×10^6/ml) were stimulated for 15 min with interferon (IFN)-γ (10 ng/ml), IFN-α (1000 unit/ml), interleukin (IL)-10 (10 ng/ml), granulocyte macrophage colony-stimulating factor (GM-CSF) (10 ng/ml), or IL-4 (4 ng/ml) in parallel with unstimulated controls. Cells were stained with each indicated anti-P-STAT monoclonal antibody (mAb) along with monocyte specific anti-CD14 mAb and analyzed by flow cytometry using a BD FACS Canto analyzer. Data was analyzed using FACSDiva (BD) and WinMDI 2.8 software (Joe Trotter, Scripps Institute, San Diego). (a) An overlay of histograms shows anti-P-STAT1 staining in unstimulated monocytes and in response to IFN-γ (left panel) and IFN-α (right panel) stimulation (one representative patient from each group). (b) P-STAT-1, P-STAT3, P-STAT5 and P-STAT6 induction following stimulation with IFN-γ, IL-10, GM-CSF, and IL-4, respectively, in monocytes from HIV-negative controls and patients on and off antiretroviral therapy (ART), was analyzed. The fold change in mean fluorescence intensity (MFI) in cytokine stimulated vs. unstimulated cells for each P-STAT was plotted. (c) IFN-γ-R1, IFN-γ-R2, and total STAT1 expression in monocytes from HIV-positive patients. PBMC from patients were stained with anti-IFN-γ-R1, anti-IFN-γ-R2, or anti-total STAT1, and anti-CD14 mAbs. MFI for IFN-γ-R1 and -R2 (left) and total STAT1 (right) expression was plotted. Each symbol represents data from one patient. The mean for each study group is represented by a horizontal dash and is plotted along with SD error bars. Statistical analysis was performed using the Student's *t*-test and *P* values are indicated where significant. Because of the limited number of cells obtained from some patients, it was not possible to conduct all experiments in all patient samples. This is reflected in the number of data points graphed for each HIV-positive patient group. Data analysis on monocytes gated by forward and side scatter properties or by CD14+ staining yielded similar results.

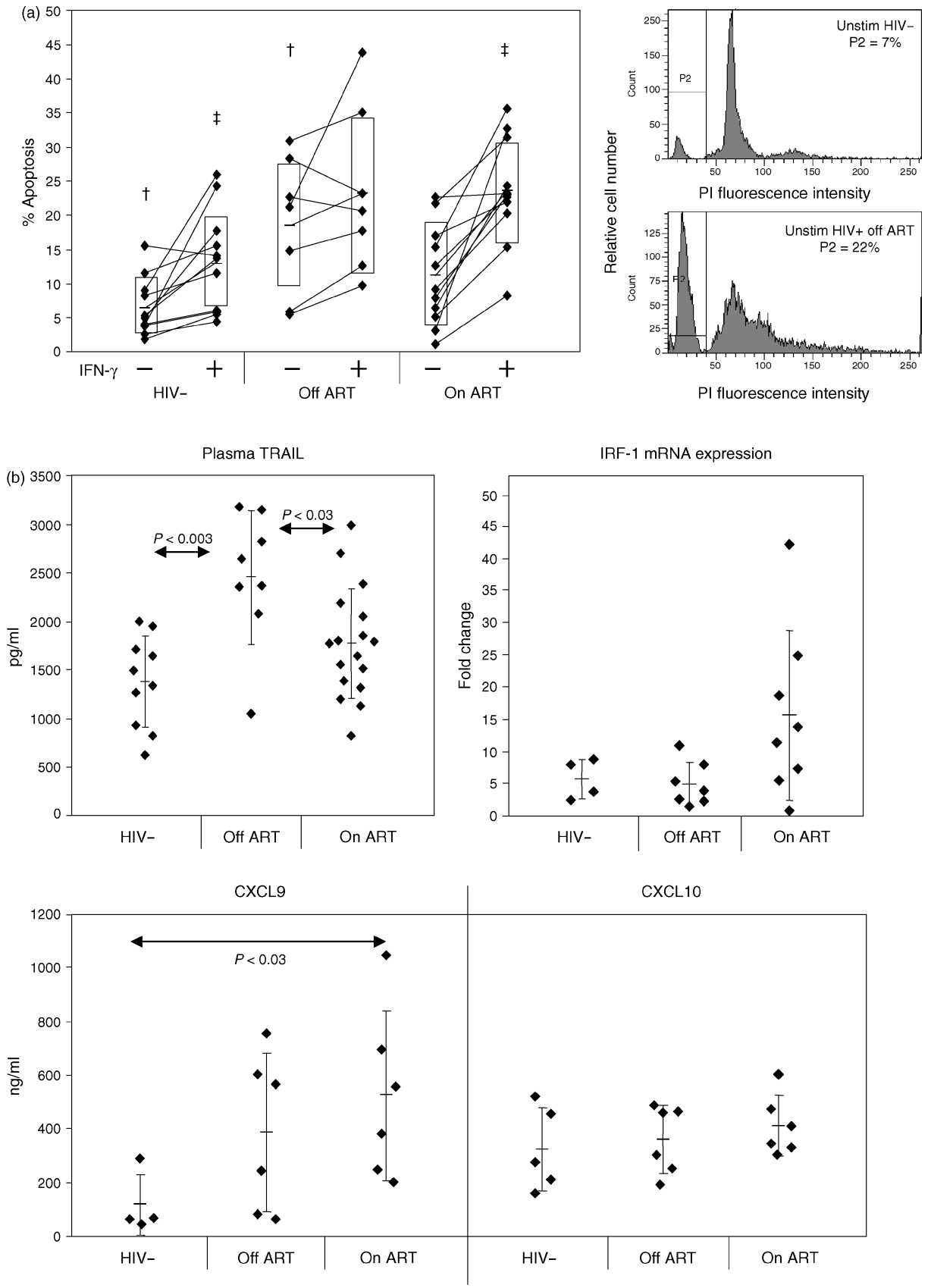

Fig. 2. Apoptosis and expression of interferon (IFN)-γ-activated signal transducer and activator of transcription 1 (STAT1) responsive genes in monocytes from HIV-positive patients.

result from increased STAT1 expression levels. Such observations are reminiscent of a priming phenomenon by which type I interferons may enhance innate antiviral immune responses [20,21]. Indeed, priming of macrophages with low doses of interferons (type I or II) enhanced subsequent IFN-γ signaling in these cells via a selective increase in total STAT1 expression [22,23]. We hypothesize that the amplification of IFN-γ-induced STAT1 activation observed in off-therapy patients may be a manifestation of in-vivo monocyte priming by cytokines like IFN-γ, known to be upregulated in the circulation of such patients [24]. Notably, a somewhat similar STAT1 amplification may also occur in chronic inflammatory disorders including ulcerative colitis, Crohn's disease, rheumatoid arthritis, and chronic hepatitis C virus-infected patient liver cells [22,25–28]. To date, other chronic viral infections have not been studied at this level in patient monocytes.

Upregulation of P-STAT1 appeared also to be unique to IFN-γ, as this was not observed in response to either IFN-α (Fig. 1a) or IL-10 (data not shown). Contributing to this selective effect may be that IFN-γ induces upstream Jak1 and Jak2 activation whereas IFN-α and IL-10 activate Jak1 and Tyk2 [29]. It is also possible that STAT1 may be preferentially recruited to the IFN-γ receptor, and both of these possibilities are being investigated further.

Interestingly, we demonstrated that spontaneous monocyte apoptosis was elevated in HIV-positive patient's off-therapy but not ART patients compared with HIV-negative controls. In contrast to T cells, apoptosis in monocytes from HIV-infected patients is not well established. One study found that spontaneous apoptosis was elevated in monocytes from patients included based on HIV-seropositivity [30]. In another report, such a fate could be averted by ex-vivo IL-13 stimulation, but this was not compared with uninfected controls [31]. We and others have shown that circulating levels of TRAIL are increased, particularly in viremic HIV-positive patients (Fig. 2b; [32]). Further analysis suggested that spontaneous monocyte apoptosis might be related to increased constitutive levels of total STAT1 but not tyr-P-STAT1 expression. Plasma TRAIL concentrations appeared also to parallel spontaneous apoptosis but this was not significant. However, additional studies are required to substantiate these associations directly. In support of a role for TRAIL is that in-vitro HIV-infected macrophages exhibited increased apoptosis in response to exogenous addition of this molecule [33,34]. Moreover, IFN-γ-induced monocyte apoptosis was elevated in HIV-positive patients compared with HIV-negative controls, irrespective of seemingly effective ART (undetectable viral load, stable CD4 counts). This may reflect the adverse effects of ART on cells of this lineage [35]. The mechanism by which IFN-γ enhanced apoptosis in patient monocytes is not known but appears to be unrelated to tyr-P-STAT1 induction (Fig. 1). IFN-γ did upregulate TRAIL secretion by monocytes but this was low compared with plasma and showed no significant differences between patient groups (data not shown). Since STAT1-ser-phosphorylation is also important for survival or apoptosis, depending on the cell type [36,37], future studies will investigate this in HIV-positive patient monocytes. Unfortunately, when this study was conducted, anti-P-ser-STAT1 Abs suitable for flow cytometry, were not commercially available.

The expression of STAT1-dependent genes IRF-1, CXCL9, and CXCL10 [19] was upregulated by IFN-γ but levels did not parallel differences in IFN-γ-induced P-STAT1 induction observed between patient groups. IFN-γ-induced CXCL9 was significantly elevated only in monocytes from ART patients compared with uninfected controls. However, such results need to be interpreted with caution, considering the limited sample number and large standard deviation obtained. We suggest that these genes are under complex regulatory control involving more than STAT1 alone, particularly in the context of HIV infection and ART.

Fig. 2. (*Continued*).
(a) Monocytes (1×106/ml) purified by positive selection were cultured with IFN-γ (10 ng/ml) for 24 h. Subsequently, permeabilized cells were analyzed for DNA content by propidium iodide (PI) staining and flow cytometry. The percentage values of apoptotic monocytes for each patient was plotted (left). Each joined pair of symbols represents data for a given patient's monocytes after a 24 h culture in the presence or absence of IFN-γ. $^{\dagger}P < 0.02$, comparing spontaneous apoptosis in monocytes from HIV-positive (HIV+) patients off therapy vs. HIV-negative (HIV−) controls; $^{\ddagger}P < 0.005$ comparing apoptosis after IFN-γ stimulation in HIV+ patients on antiretroviral therapy (ART) vs. HIV− controls. Histograms plotting the fluorescence intensity of intracellular PI staining (right panels) in unstimulated monocytes cultured for 24 h from one HIV− control and one HIV+ patient off ART are shown. Apoptotic cells were defined by their subdiploid DNA content and the percentage calculated by applying the P2 gate shown. (b) Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) expression was measured in patient plasma by enzyme-linked immunosorbant assay (upper left). Total RNA from IFN-γ-stimulated monocytes was assayed for interferon regulatory factor (IRF)-1 mRNA expression (upper right) by real-time PCR, and the fold change (IFN-γ-stimulated vs. unstimulated cells) was plotted. IFN-γ-induced CXCL9 and CXCL10 expression in monocytes supernatant (lower panel) was measured using the CBA chemokine kit and flow cytometry. Each symbol represents data from one patient. All data were analyzed by Student's *t*-test. *P* values are indicated where significant. The mean for each study group is represented by a horizontal dash and plotted along with the SD (vertical error bars or box). Similar results were obtained in monocytes purified by negative selection and apoptosis results were confirmed by annexin-V/PI staining (data not shown).

An interesting point is that a $CD16^{+}/CD14^{low}$ subset of blood monocytes is expanded in HIV+ patients [6,38,39], and recent observations suggest that this population likely represents an important viral reservoir even under antiretroviral therapy [40]. Although we did not analyze this marker specifically, our results could not be attributed solely to an expansion of the CD16+ subset. However, to rule out this possibility directly, further studies would be needed.

In conclusion, we demonstrated that monocytes from chronically infected HIV-positive patients exhibit reduced survival *ex vivo* compared with HIV-negative controls and that there are significant alterations in their expression of STAT1 and signaling capacity in response to IFN-γ These results may reflect the chronic nature of the immune activation observed, particularly in viremic HIV-positive patients, and may also be significant in view of the potential utility of IFN-γ as an adjunct immunotherapy for HIV-related opportunistic infections [41–43].

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Author contributions are as follows. A.A. played a major role in performance of experiments, data analysis, study design, and drafting and revision of the article. Y.Y., K.A., and N.S. made significant contributions to data acquisition, and provided intellectual input on study design, data interpretation and manuscript preparation. F.D.M. had a significant input on data acquisition and manuscript preparation. A.K. contributed intellectually to study design, data interpretation, and manuscript preparation and revision. M.K. conceived and designed the study, acquired funding, supervised the work, and played a principal role in the drafting and revision of the paper.

There are no conflicts of interests.

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