Desensitization to type-I interferon in HIV-1 infection correlates with markers of immune activation and disease progression

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Abbreviations: IFNα/β, type I IFN; IFNα/βR, IFNα/β receptor; ISG, interferon-stimulated gene; MxA, myxovirus resistance gene; OAS, 2', 5' oligoadenylate synthase-3; TLR, Toll-like receptor; IQR, interquartile range; sMFI, specific mean fluorescence intensity; pSTAT1, phosphorylated STAT1; SOCS, suppressor of cytokine synthesis; PIAS, protein inhibitor of activated STATs; pDC, plasmacytoid DC; mDC, myeloid DC.
Abstract

Type I interferon (IFNα/β) plays a complex role in HIV-1 infection and has been proposed alternately to have roles in either disease protection or progression. Although IFNα/β plays crucial roles in regulating monocytes and dendritic cells, responsiveness of these cells to IFNα/β in HIV-1 infection is poorly understood. We report significant defects in IFNα/β receptor (IFNα/βR) expression, IFNα signaling and IFNα-induced gene expression in monocytes from HIV-1-infected subjects. IFNα/βR expression correlated directly with CD4+ T cell count and inversely with HIV-1 RNA level and expression of CD38 by memory (CD45RO+) CD8+ T cells, a measure of pathological immune activation in HIV-1 infection associated with disease progression. In addition, monocytes from HIV-1-infected persons showed diminished responses to IFNα, including decreased induction of phosphorylated STAT1 and the classical interferon-stimulated genes MxA and OAS. These IFNα responses were decreased regardless of IFNα/βR expression, suggesting that regulation of intracellular signaling may contribute to unresponsiveness to IFNα/β in HIV-1 disease. Defective monocyte responses to IFNα/β may play an important role in the pathogenesis of HIV-1 infection, and decreased IFNα/βR expression may serve as a novel marker of disease progression.
Introduction

HIV-1 infection disrupts numerous elements of the innate immune system \(^1,^2\). At the interface between innate and acquired immune responses, antigen presenting cells (APCs) such as dendritic cells (DCs) and monocytes/macrophages recognize distinct microbial structures through Toll-like receptors (TLRs) and other pattern recognition receptors \(^3\). Signaling through these receptors induces expression of cytokines, such as type I IFN (IFN\(\alpha/\beta\)), that promote innate immunity and APC maturation \(^4,^5\). Our previous studies showed that TLR9 agonist stimulation of unfractionated PBMCs generates monocyte responses that are defective in HIV-1 infection \(^6\); these studies implicated both reduced TLR induction of IFN\(\alpha/\beta\) and reduced monocyte responsiveness to IFN\(\alpha/\beta\) as potential mechanisms in HIV-1 infection. Despite its potential significance to HIV-1 pathogenesis, knowledge of IFN\(\alpha/\beta\) signaling and its regulation in HIV-1 disease remains limited.

IFN\(\alpha/\beta\) comprises 13 different functional isoforms of IFN\(\alpha\) and one IFN\(\beta\), all of which signal through the same IFN\(\alpha/\beta\) receptor (IFN\(\alpha/\beta\)R), a heterodimer composed of IFNAR1 and IFNAR2 \(^7\). IFN\(\alpha/\beta\)R signaling activates tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1), which in turn phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2 \(^8\). Phosphorylated STAT1 and STAT2 heterodimerize and associate with interferon regulatory factor-9 (IRF-9) to form interferon-stimulated gene factor-3 (ISGF-3). ISGF-3 binds to interferon-stimulated response elements in the promoters of hundreds of interferon-stimulated genes (ISGs), which include the myxovirus resistance gene (MxA) and 2', 5' oligoadenylate synthase-3 (OAS)\(^8\). IFN\(\alpha/\beta\) is produced by a variety of cell types, particularly plasmacytoid DCs.
(pDCs) $^{9,10}$, which produce up to 1000-fold more IFNα/β than other cell types $^{11}$. IFNα/β has pleiotropic effects on many cell types, including direct anti-viral effects, differential promotion of cell survival and apoptosis, inflammatory effects and enhancement of differentiation and maturation of blood myeloid DCs (mDCs) and monocytes into potent T cell stimulators $^{12,13}$.

Despite its well-characterized antiviral activity, the role of IFNα/β in HIV-1 infection is controversial, with conflicting observations suggesting protective versus pathological roles. Administration of recombinant human IFNα may have beneficial effects during the asymptomatic phase of HIV-1 infection, stabilizing CD4 decline and reducing the incidence of AIDS-defining events $^{14}$, although these effects are not observed in more advanced disease $^{15}$. Transiently high levels of endogenous serum IFNα have been described primary HIV-1 infection $^{16}$ and acute simian immunodeficiency virus infection in macaques $^{17}$. During the asymptomatic phase of chronic HIV-1 infection, elevated serum IFNα levels are found at increasing frequency with advancing disease progression, reaching high levels in late stage HIV-1 infection $^{16}$ and correlating with poor outcomes in response to anti-retroviral therapy $^{18}$. IFNα/β may protect T cells from spontaneous apoptosis, but this effect is reduced in HIV-1 disease $^{19}$. Other studies suggest that IFNα/β may contribute to bystander apoptosis of uninfected CD4+ T cells $^{20}$ and that depletion of CD4+ T-cells in HIV-1 infection may be mediated by IFNα/β-induced activation $^{21}$. Alternatively, the late increase in IFNα/β may be a result, rather than a cause, of disease progression, and may reflect increasing pathological immune activation, driven by HIV-1 itself, opportunistic pathogens or other microbial stimuli $^{22}$. We propose that responses to IFNα/β may be desensitized in late
HIV-1 infection, potentially explaining the lack of efficacy of recombinant human IFNα therapy at that stage.

The studies presented here demonstrate a significant loss of monocyte responsiveness to IFNα/β in HIV-1 infection. We observed defects at multiple levels, including decreased expression of IFNα/βR, loss of IFNα/β signaling through phosphorylated STAT1 and loss of induction of ISGs. Moreover, these defects correlated significantly with markers of disease progression in HIV-1 infection, including decreased CD4+ T cell count and induction of CD38 on memory (CD45RO+) CD8+ T cells (expression of this activation marker on CD8+ T cells is an important predictor of HIV-1 disease progression). Diminished responsiveness to IFNα/β may decrease responses of HIV-1-infected individuals to immunotherapeutic agents or to vaccine adjuvants that act through induction of IFNα/β production, e.g. CpG DNA or imiquimod, and could account for the inconsistent effects observed in clinical trials of IFNα/β for treatment of HIV-1 infection. Defects in IFNα/β responsiveness may represent an important pathological mechanism in HIV-1-disease, leading to failure of innate immunity and impairment of acquired immune responses. Moreover, loss of IFN-α/βR expression by monocytes correlates with markers of disease progression and could potentially serve as a novel marker to help assess or predict disease progression in HIV-1 infection.
Methods

Study subjects and cell samples

Peripheral blood was obtained from HIV-1-infected and uninfected subjects in this Institutional Review Board-approved cross sectional study at Case Western Reserve University. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. HIV-1-infected participants were not receiving and had not received antiretroviral therapy or any immunotherapy in the last two years. HIV-1-infected and uninfected subjects donated 60 ml of blood on one occasion each (into lithium heparin tubes). Plasma was separated from each sample and stored at -80°C.

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation with endotoxin-free Ficoll-paque™ (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). PBMCs were analyzed by flow cytometry or used to prepare monocytes by negative selection at 4°C with the Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA). Monocyte purity (by flow cytometry with CD14 staining) was over 90%.

Flow cytometry

PBMCs were stained on ice with murine anti-human monoclonal antibodies (from BD, San Jose, CA, USA, if not specified otherwise): anti-CD14-PE (Miltenyi Biotec), anti-CD3-FITC, anti-CD4-PerCp, anti-CD8-PerCp, Lin-1 cocktail (anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, anti-CD56)-FITC, anti-HLA-DR-PerCP, anti-CD11c-PE, anti-CD3-APC, anti-CD45RO-FITC and anti-CD38-PE. Biotin-conjugated polyclonal goat anti-IFNα/βR (specific for the IFNAR1 chain) and control normal goat
IgG (R&D Systems, Minneapolis, MN, USA) were used with streptavidin-conjugated APC. For analysis of DCs, HLA-DR+, Lin-1- cells were gated according to their expression of CD11c. CD11c+ cells were considered to be mDCs; CD11c- cells were considered to be pDCs (this exclusionary gating definition may have included a small number of non-pDCs). Stained cells were washed in PBS/0.05% sodium azide, fixed in 1% formaldehyde and analyzed with a four-color FACS Caliber flow cytometer and Cell Quest™ software (BD) with an acquisition threshold of 20,000 gated events.

To detect phosphorylated STAT1, 10⁶ PBMCs were pre-incubated at 4°C for 30 min with anti-CD14-PE mAb (Miltenyi Biotec), washed, resuspended in 1 ml of RPMI with 10% FCS (Hyclone, Logan, UT, USA), and cultured for 15 min +/- IFNα2a (PBL Biomedical Labs, Piscataway, NJ, USA) at 1,000, 3,000 or 10,000 U/ml. Cells were washed, fixed with BD cytofixR buffer, permeabilized with BD Phosflow Perm Buffer III and stained with ALEXA-488 conjugated murine anti-human phosphorylated STAT1 mAb (BD Pharmingen) or isotype control MOPC-173 mAb (BD Pharmingen) for 30 min at room temperature.

**Real-time PCR**

Monocytes were resuspended in MACS Rinsing Solution (Miltenyi Biotec) supplemented with 10% FCS. Equal aliquots (1-2 x 10⁶ cells) were placed immediately into RLT lysis buffer (Qiagen, Valencia, CA, USA) and stored at -80°C (ex-vivo sample) or cultured in 24-well plates for 18 h at 37°C in RPMI with 2 mM L-glutamine, 10 mM penicillin and streptomycin, and 5% human male AB serum (Gemini Bio Products, West
Sacramento, CA, USA) +/- 1000 IU/ml IFNα2a (PBL Laboratories). Supernatants were removed, cells were lysed in situ using RLT lysis buffer, and lysates were stored at -80°C. Lysates were passed through QIAshredder columns (Qiagen), and mRNA was extracted following on-column DNase digestion using the RNeasy plus kit (Qiagen) and stored in RNase-free sterile water at -80°C.

Concentration of mRNA was determined by optical density; cDNA was reverse transcribed from mRNA using oligo(dT)-primer based Superscript II First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) and quantified by real-time qRT-PCR in triplicate with an iCycler (Bio-Rad, Hercules, CA) using SYBR green detection master mix (Abgene, Rockford, IL, USA). Absolute quantities of mRNA product were determined from a standard curve of serial dilutions of known quantities of each specific amplicon. Results were normalized to GAPDH. Primer pairs were as follows: GAPDH (sense 5’-GACCTGACCTGCCGTCTA-3’; antisense, 5’GTTGCTGTAGCCAAATTCGTT-3’), MxA (sense, 5’- AGAAGGAGCTGGAAGAAG-3’; antisense, 5’-CTGGAGCATGAAGAACTG-3’), OAS (sense, 5’-GACCACTGTAGCCGAATCAG-3’; antisense, 5’-TGGCACCCATTCAATCAT3’) and IFNAR2 (sense, 5’-AGTCAGAGGGAATTGTTAAGAAGCA-3’; antisense, 5’-TTTGAATTTGTTAAGAAGCA-3’). The IFNAR2 amplicon was representative of all three known variants of IFNAR2. Published primers were used for detection of IFNAR1 (sense, 5’-CCCAGTGTCTTTTCTCAAA-3’; antisense, 5’-AAGACTGGAGGAAAGTAGGAAGC-3’).

IFNα ELISA
Plasma was separated from blood taken into EDTA anti-coagulant. IFNα was detected by an ELISA (PBL) that detects 12 human IFNα types (detection limit of 12.5 pg/ml). IFNα standards and neat plasma samples were incubated in pre-coated 96-well ELISA plates for 1 h (all ELISA procedures were at room temperature). IFNα was detected using a biotinylated anti-IFNα antibody and streptavidin-horseradish peroxidase with tetramethyl-benzidine substrate. Optical density was measured at 450 nm using a Bio-Rad model 680 microplate reader.

**Statistical analysis**

We used conventional measures of central location and dispersion to describe the data. Pairs of variables were compared with Mann-Whitney U test or Wilcoxon signed ranks test, depending on the relations between the groups. To explore associations between pairs of continuous variables, we used correlation analysis or simple linear regression. Relationships of multiple predictors of interest with a continuous dependent variable were assessed by multiple regression with a stepwise approach to select significant covariates. Analyses were performed using SPSS, v. 16.01 (SPSS Inc, Chicago, IL, USA) and Stata MP, v. 10 (Stata Corp., College Station, TX, USA) without explicit correction for multiple comparisons. All tests were two-sided, and p-values ≤0.05 were considered statistically significant.
Results

*Expression of IFNα/βR is decreased on monocytes of HIV-1-infected individuals*

Since reduced sensitivity to IFNα/β may affect HIV-1 disease pathogenesis, we investigated monocyte expression of IFNα/βR and functional responses of monocytes to IFNα in 59 viremic HIV-1-infected subjects who were not receiving antiretroviral therapy and 32 uninfected individuals in a cross sectional study. The median age of HIV-1-infected individuals at time of sample collection was 45 years (interquartile range [IQR] 39 – 48) and 26% were female. The median age for uninfected-individuals was 38 years (IQR 31 – 47) and 53% were female. For HIV-1-infected subjects, the median absolute CD4+ T cell count was 383 cells/µl (IQR 315 – 544), and the median HIV-1 RNA level was 26,000 copies/ml (IQR 9,500 – 91,700). The same donor samples were used for multiple analyses to assess monocyte IFNα/βR expression (this section) and functional responses to exogenous IFNα (below), although we were not able to perform all analyses on every sample.

Flow cytometry was used to assess expression of IFNα/βR *ex vivo* (i.e. without *in vitro* incubation) on monocytes from 54 HIV-1-infected subjects and 32 uninfected persons. The monocyte gate was defined by CD14 expression and side scatter characteristics. Representative histograms of IFNα/βR expression are shown for uninfected (Fig. 1A) and HIV-1-infected (Fig. 1B) subjects, demonstrating a decrease in monocyte expression of IFNα/βR in HIV-1-infection. The median IFNα/βR specific mean fluorescence intensity (sMFI), i.e. MFI with anti-IFNα/βR Ab minus MFI with isotype control Ab, was 87.12 (IQR 53.98 - 159.44) for uninfected persons (n = 32) and 19.18 (IQR 8.45 – 47.0) for HIV-1-infected subjects (n = 54) (Fig. 1C). The median
percentage of monocytes expressing detectable IFNα/βR was 76.52% (IQR 19.76 – 94.77) in uninfected persons and only 14.91% (IQR 6.25 – 28.72) for HIV-1-infected subjects (data not shown). Thus, IFNα/βR expression was significantly reduced on monocytes from HIV-1-infected subjects as assessed by both sMFI (p <0.001) and the percentage of monocytes expressing detectable receptor (p <0.001).

Expression of IFNα/βR is decreased on DCs but not T cells in HIV-1 infection

To determine whether the loss of IFNα/βR was restricted to monocytes or affected other cell types, we assessed IFNα/βR expression ex vivo on DCs and T cells. IFNα/βR expression ex vivo on CD11c+ mDCs and CD11c- pDCs (Fig. 1D) was lower than on monocytes but showed a similar pattern of reduction in HIV-1-infected subjects relative to uninfected persons. The median sMFI for IFNα/βR on mDCs was 7.98 (IQR 0 – 12.44) for uninfected persons (n = 14) and 1.81 (IQR 0 – 5.41) for HIV-1-infected subjects (n = 33) (p = 0.052). Furthermore, the percentage of mDCs with positive staining for IFNα/βR was significantly lower in HIV-1-infected subjects (median 1.69% positive, IQR 0.01 – 3.64%) than in uninfected persons (median 16.81% positive, IQR 5.93 – 29.48%) (p < 0.001). The median sMFI for IFNα/βR on pDCs was 7.0 (IQR 3.0 – 19.0) for uninfected persons (n = 16) and 2.0 (IQR 0 – 9.0) for HIV-1-infected subjects (n = 32) (p = 0.046). The percentage of pDCs with positive staining for IFNα/βR was also higher in uninfected persons (median 5.82% positive, IQR 2.14 – 10.89%) than HIV-1-infected subjects (median 1.5% positive, IQR 0.03 – 2.6%) (p = 0.002). Thus, HIV-1 infection was associated with reduced expression of IFNα/βR on mDCs and pDCs.
IFNα/βR expression on CD4+ and CD8+ T cells was lower than on monocytes and was not significantly altered by HIV-1 infection (Fig. 1E). The median sMFI for IFNα/βR expression on CD4+ T cells was 14.4 (IQR 10.4 – 19) for uninfected persons (n = 12) versus 14.7 (IQR 5.9 – 21.9) for HIV-1-infected subjects (n = 27) (p = 0.584). The percentage of CD4+ T cells that expressed IFNα/βR was 15.85% (IQR 12.24-40.05%) for uninfected individuals versus 11.16% (IQR 3.75-28.43%) for HIV-1-infected individuals (p = 0.181) (data not shown). Similarly, the median sMFI on CD8+ T cells was 11.94 (IQR 8.6 – 32.2) for uninfected persons (n = 10) and 9.8 (IQR 7.3 – 20.1) for HIV-1-infected subjects (p = 0.321), and the percentage of CD8+ T cells that expressed IFNα/βR was 9.32% (IQR 4.36-46.48%) for uninfected individuals versus 5.51% (IQR 2.73-9.39%) for HIV-1-infected subjects (p = 0.141) (data not shown). Thus, we did not observe a statistically significant change in IFNα/βR expression by CD4+ or CD8+ T cells of HIV-1-infected persons. We conclude that decreased IFNα/βR expression in the setting of HIV-1 infection was detected on monocytes, mDCs and pDCs, but was not observed on T cells.

Monocyte IFNα/βR expression correlates directly with peripheral absolute CD4+ T cell count and inversely with plasma HIV-1 RNA levels and memory CD8+ T cell immune activation

We investigated the potential correlation of IFNα/βR expression with clinical indicators of HIV-1 disease progression. We focused these analyses on monocytes as the cell type with most robust baseline expression of IFNα/βR expression (allowing clearer detection of inhibition) and greatest numbers of cells available for other
investigations. Peripheral blood CD4+ T cell count was significantly associated with monocyte IFNα/βR sMFI in HIV-1-infected subjects \((r = 0.545, p < 0.001)\) (Fig. 2A), and plasma HIV-1 RNA level was inversely correlated with monocyte IFNα/βR sMFI \((r = -0.577, p < 0.001)\) (Fig. 2B). Since expression of CD38 on memory CD8+ T cells is a marker of chronic immune activation and strongly associated with HIV-1 disease progression \(^{23-25}\), we also analyzed the relationship of IFNα/βR expression on monocytes with expression of CD38 on memory (CD45RO+) CD8+ T cells (the memory subset is targeted as CD38 is expressed on naive CD8+ T cells in the absence of activation) \(^{27,28}\). We found a significant inverse relationship between sMFI for CD38 expression on memory CD8+ T cells and sMFI for IFNα/βR on monocytes \((r = -0.620, p < 0.001)\) (Fig. 2C). In uninfected subjects there was not a significant correlation between CD38 expression on memory CD8+ T cells and monocyte IFNα/βR expression \((r = 0.437, p = 0.119)\). Thus, monocyte IFNα/βR expression correlated with markers of HIV-1 disease stage and level of immune activation in HIV-1 infection.

Since CD4+ T cell count, HIV-1 RNA and memory CD8+ T cell CD38 expression were each associated with IFNα/βR expression, we examined whether these variables were independent predictors IFNα/βR expression level (Table 1). Multivariate regression analysis revealed that CD38 expression on memory CD8+ T cells independently predicted IFNα/βR expression on monocytes among HIV-1-infected subjects after controlling for CD4+ T cell count and HIV-1 RNA levels in plasma \((p = 0.013)\), whereas CD4+ T cell count and HIV-1 RNA levels in plasma were not independent predictors of IFNα/βR expression after accounting for CD38 expression on memory CD8+ T cells. The independent relationship between CD38 expression by
memory CD8+ T cells and IFNα/βR suggests that diminished IFNα/βR expression on monocytes may be an especially important marker of chronic immune activation and may be associated with subsequent disease progression, although this hypothesis remains to be tested.

*Monocyte IFNα/βR expression is regulated at the translational or post-translational level*

Processes that may lead to loss of IFNα/βR expression include a variety of mechanisms from transcriptional regulation to post-translational regulation, such as ligand-induced receptor internalization and degradation. We used quantitative real-time RT-PCR (qRT-PCR) to quantify mRNA for the two subunits of the IFNα/βR, IFNAR1 and IFNAR2, in purified monocytes (n = 17). Univariate linear regression analysis demonstrated that IFNα/βR protein expression did not correlate with IFNAR1 (r = 0.048, p = 0.866) or IFNAR2 mRNA (r = 0.074, p = 0.793) (Fig. 3). For example, some individuals with substantial loss of cell surface IFNα/βR protein had IFNAR1 and IFNAR2 mRNA levels similar to those of HIV-1-infected individuals with higher IFNα/βR protein expression. These data suggest that IFNα/βR expression is diminished by post-translational mechanisms in HIV-1 infection.

We considered the hypothesis that *in vivo* exposure to IFNα/β may contribute to loss of IFNα/βR expression, consistent with prior observations of ligand-induced post-translational degradation of IFNα/βR. Plasma IFNα levels were assessed by ELISA for 22 HIV-1-infected subjects and 6 uninfected persons. Plasma IFNα levels in uninfected persons (median 10.6 pg/ml, IQR 1.2 – 19.5) and HIV-1-infected subjects (median 5.4 pg/ml, IQR 2.5 – 10.8) were close to or below the threshold of detectability and were not
significantly different (p = 0.502) (data not shown). Although significant detection of plasma IFNα was not achieved in these assays, ligand-induced downregulation of IFNα/βR may still result from either exposure of cells to IFNα at different times or anatomical sites, or the effects of other type I IFN species (e.g. IFNβ or IFNω).

**IFNα/β-stimulated gene induction is significantly impaired by multiple mechanisms in monocytes of HIV-1-infected subjects**

In order to investigate the functional relevance of diminished monocyte IFNα/βR, we tested the ability of IFNα to induce expression of two classical ISGs, MxA and OAS, in monocytes from HIV-1-infected subjects and uninfected persons. ISG induction was assessed by qRT-PCR of mRNA isolated from purified monocytes either directly *ex vivo* or after incubation for 18 h +/- 1000 U/ml IFNα2a. Fold-induction was calculated as mRNA expression after stimulation divided by mean baseline mRNA expression in unstimulated monocytes (*ex vivo*). IFNα2a induced a substantial increase in expression of MxA in monocytes of uninfected persons (n = 9) (Fig. 4A), but this induction was significantly impaired in monocytes from HIV-1-infected subjects (n = 18) (Fig. 4A). The median fold induction of MxA mRNA in monocytes was 112.3 (IQR 37.3 – 149.8) for uninfected persons versus 2.6 (IQR 1.5 – 8.2) for HIV-1-infected subjects (p <0.001). A similar pattern was observed for induction of OAS mRNA (Fig. 4B). The median fold-induction of OAS in monocytes was 30.2 (IQR 9.6 – 38.8) in uninfected persons versus 1.1 (IQR 0.1 – 4.4) in HIV-1-infected subjects (p <0.001). Thus, the ability of IFNα2a to induce MxA and OAS was impaired significantly in monocytes of HIV-1-infected subjects.
Multiple regression analysis was used to assess the relationship between IFNα/βR expression and the induction of ISGs by IFNα. While HIV-1 status was strongly and independently associated with the magnitude of IFNα-induction of MxA (p = 0.008) and OAS (p = 0.002), linear regression analysis of HIV-1-infected subjects revealed no significant relationship between expression of IFNα/βR and magnitude of induction of MxA (Fig. 5C; n = 15, r = -0.023, p = 0.936) or OAS (Fig. 5D; n = 18, r = 0.258, p = 0.301). These results do not exclude IFNα/βR expression as a contributor to loss of IFNα/β responsiveness in HIV-1 infection, but they suggest that inhibition of post-IFNα/βR signaling mechanisms may contribute significantly to limiting induction of MxA and OAS. In uninfected individuals there was also a lack of correlation between IFNα/βR expression and induction of MxA (Fig. 5A; n = 8, r = 0.521, p = 0.186) or OAS (Fig 5B; n = 7, r = 0.209, p = 0.652), likely due to sufficient expression of receptors beyond a level that would limit responses to IFNα/β. We conclude that loss of IFNα/βR is associated with disease progression, but inhibition of post-receptor signaling mechanisms may also contribute to loss of specific IFN-induced effects.

IFN-induced STAT1 phosphorylation is impaired in monocytes of HIV-1-infected persons independent of IFNα/βR expression

Since factors other than decreased IFNα/βR expression were implicated in the diminished induction of MxA and OAS by IFNα in HIV-1-infection (Fig. 5), we assessed other steps in IFNα/β signaling, including STAT1 tyrosine phosphorylation. PBMCs from HIV-1-infected and uninfected persons were stimulated with IFNα2a at 0, 1,000, 3,000 and 10,000 U/ml for 15 min, fixed and permeabilized for intracellular staining and flow
cytometric detection of phosphorylated STAT1 (pSTAT1) \(^{29}\) in gated monocytes. IFN\(\alpha\)2a induced phosphorylation of STAT1 in monocytes of uninfected persons (Fig. 6A), but monocytes from HIV-1-infected subjects were substantially deficient in STAT1 phosphorylation at each concentration of IFN\(\alpha\)2a (Fig. 6B). The delta (\(\Delta\)) pSTAT1 sMFI was calculated as the difference in sMFI between cells incubated in the presence of IFN\(\alpha\)2a and cells incubated in medium alone. Substantial dose-dependent pSTAT1 responses were evident in monocytes from uninfected persons with median \(\Delta\) sMFI of 76 (IQR 44 – 100) at 1,000 U/ml IFN\(\alpha\)2a, 124 (IQR 97 – 132) at 3,000 U/ml IFN\(\alpha\)2a and 136 (IQR 128 – 144) at 10,000 U/ml IFN\(\alpha\)2a (Fig. 6C). In contrast, STAT1 phosphorylation was impaired in monocytes from HIV-1-infected subjects with median \(\Delta\) sMFI of 8 (IQR 2 – 29) at 1,000 U/ml, 20 (IQR 7 – 65) at 3,000 U/ml, and 57 (IQR 20 – 126) at 10,000 U/ml IFN\(\alpha\)2a (Fig. 6C). The difference in monocyte \(\Delta\) pSTAT1 sMFI between uninfected and HIV-1-infected subjects was statistically significant at concentrations of 1,000 U/ml (\(p = 0.012\)) and at 3,000 U/ml (\(p = 0.005\)), but not at 10,000 U/ml of IFN\(\alpha\)2a (\(p = 0.075\)). The greatest difference between HIV-1-infected and uninfected persons was at 3,000 U/ml IFN\(\alpha\)2a, a concentration on the rising phase of the dose-response curve. We conclude that IFN\(\alpha\)-induced phosphorylation of STAT1 was significantly impaired in monocytes from HIV-1-infected subjects.

We examined the relationship between induction of pSTAT1 and cell surface IFN\(\alpha\)/\(\beta\)R expression. The \(\Delta\) pSTAT1 sMFI at all three concentrations of IFN\(\alpha\)2a was compared with cell surface IFN\(\alpha\)/\(\beta\)R expression by linear regression. For uninfected individuals, monocytes demonstrated varying levels of IFN\(\alpha\)/\(\beta\)R expression and IFN\(\alpha\)2a-induced pSTAT1, but the levels of IFN\(\alpha\)/\(\beta\)R expression and STAT1 phosphorylation...
were not significantly correlated at 1,000 U/ml IFNα2a (r = 0.114, p = 0.789) (data not shown), 3,000 U/ml (r = -0.318, p = 0.443) (Fig. 7A) or 10,000 U/ml (r = 0.481, p = 0.275) (data not shown). Analysis of monocytes from HIV-1-infected subjects similarly showed that monocyte expression of IFNα/βR was not correlated with phosphorylation of STAT1 at 1,000 U/ml IFNα2a (r = 0.424, p = 0.131) (data not shown), 3,000 U/ml (r = 0.330, p = 0.249) (Fig. 7B) or 10,000 U/ml (r = 0.153, p = 0.618) (data not shown).

These data indicate that the magnitude of STAT1 phosphorylation was influenced by factors other than the level of IFNα/βR expression. For example, ablation of IFNα2a-induced STAT1 phosphorylation was observed in a subset of HIV-1-infected subjects despite intermediate or high level IFNα/βR expression. Therefore, failure of IFNα2a to induce tyrosine phosphorylation of STAT1 may stem at least in part from inhibitory signaling mechanisms other than loss of cell surface IFNα/βR. These data imply that impairment of monocyte responses to IFNα/β in HIV-1 infection results from defects at multiple signaling levels and is not completely explained by down-regulation of the IFNα/βR.
Discussion

In this study we demonstrate significant loss of IFNα/β responsiveness in monocytes from HIV-1-infected persons that is manifested at multiple levels of the IFNα/β signaling pathway, including loss of IFNα/βR (p < 0.001), IFNα-induced STAT1 phosphorylation (p = 0.005) and IFNα induction of MxA and OAS mRNA (both p < 0.001). Early in the course of the HIV pandemic, loss of IFNα/βR expression was noted on unfractionated PBMCs of HIV-1-infected persons, with symptomatic disease defined as AIDS or AIDS-related complex 30, but there is little recent information to assess cell type-specific mechanisms, provide functional data on IFNα/β responsiveness or determine associations with markers of disease progression. Our studies indicate that there is a profound impairment in IFNα/β signaling in monocytes obtained from HIV-1 infection even during the asymptomatic phase of infection. While defects at multiple stages of IFNα/β signaling may contribute, loss of IFNα/βR provides a potentially powerful and practical indicator of pathological immune activation in HIV-1 infection that correlates well with markers of disease progression.

Deficits in IFNα/βR expression, STAT1 phosphorylation and ISG induction were observed similarly in male and female individuals with HIV-1 infection. There were no significant gender differences for sMFI or percent positive expression of IFNα/βR on monocytes, mDCs or pDCs ex vivo. In addition, there were no significant gender differences for induction of induction of pSTAT1 or mRNA for MxA or OAS in monocytes exposed to IFNα2a.

Results from this study indicate a widespread loss of IFNα/β responsiveness in monocytes. We do not find evidence for dichotomous responses of different cell
subpopulations to IFNα/β that would suggest presence of both responsive and non-responsive monocyte subsets. In all flow cytometry based assays, IFNα/βR expression and induction of phosphorylated STAT1 were consistently represented by single populations, as can be seen in the flow histograms in Figs. 1 and 6.

Significant loss of IFNα/βR expression (sMFI) in HIV-1-infected individuals was detected in monocytes, mDCs and pDCs. We found no evidence for reduced IFNα/βR expression on T-cells. Monocytes provide a particularly robust system for analysis of IFNα/βR in HIV infection, as they express higher baseline levels of IFNα/βR (allowing clearer detection of inhibition), and their abundance allows a range of analyses. Thus, IFNα/βR expression on monocytes may be both important and pragmatic as a marker that correlates with disease progression.

Mechanisms other than receptor loss may contribute to impairment of IFNα/β responsiveness in HIV-1 infection. IFNα-induced STAT1 phosphorylation was impaired significantly in monocytes of HIV-1-infected subjects (Fig. 6), and these monocytes failed to increase expression of the IFN-stimulated genes MxA and OAS following exposure to exogenous IFNα (Fig. 4). Deficits in IFNα2a-induced STAT1 phosphorylation and induction of MxA and OAS were not significantly related to levels of IFNα/βR, indicating contributions of inhibitory mechanisms in addition to loss of cell surface IFNα/βR. Alhetheel et al. recently reported elevation of total STAT1 and IFN-gamma-induced STAT1 phosphorylation, but not IFNα-induced STAT1 phosphorylation, in monocytes from HIV-1-infected subjects, suggesting that STAT1 phosphorylation defects may be specific to the IFNα/β signaling pathway. Mechanisms that could reduce STAT1 signaling include induction or activation of suppressor of cytokine
synthesis (SOCs) molecules \(^{32}\) or protein inhibitor of activated STATs (PIAS) \(^{33}\), which inhibit induction of phosphorylated STAT1 or its activity, respectively. Thus, impairment of monocyte responses to IFN\(\alpha/\beta\) in HIV-1 infection may be multifactorial, resulting from cumulative deficits at more than one signaling level.

Since IFN\(\alpha/\beta\) is known to decrease expression of IFN\(\alpha/\beta\)R by ligand-induced receptor degradation, one hypothesis to explain the loss of IFN\(\alpha/\beta\) responsiveness in HIV-1 infection is that chronic exposure to IFN\(\alpha/\beta\) results in desensitization. A chronic period of exposure at potentially suboptimal levels of IFN\(\alpha/\beta\) may contribute to desensitization of the response. We did not detect plasma levels of IFN\(\alpha\) sufficient to determine any relationship with IFN\(\alpha/\beta\)R expression by monocytes, although these assays may lack sufficient sensitivity and may not detect IFN\(\alpha\) potentially expressed at different times or anatomical sites, or the effects of IFN\(\beta\) or IFN\(\omega\). Other reports provide evidence that chronic exposure to IFN\(\alpha/\beta\) occurs in HIV-1 infection, resulting in increased expression of ISGs \(^{34}\) and influencing turnover of uninfected CD4+ T-cells \(^{21}\). Thus, a role for ligand-induced receptor degradation is still possible. The finding that IFN\(\alpha/\beta\)R mRNA did not correlate with IFN\(\alpha/\beta\)R expression suggests a role for translational or post-translational regulation, consistent with the hypothesis that exposure to IFN\(\alpha/\beta\) induces degradation of monocyte IFN\(\alpha/\beta\)R in HIV-1 infection, but this remains to be tested.

HIV-1 infection is associated with increased levels of microbial ligands for innate immune receptors that may regulate IFN\(\alpha/\beta\) production and responsiveness. Systemic immune activation may result from exposure to HIV-1 RNA sequences that bind TLR7 or TLR8, or exposure to bacterial products such as the TLR4 agonist, LPS, that are
translocated across damaged gut epithelium\textsuperscript{22,35-37}. Chronic TLR signaling in HIV-1 infection may result in chronic expression of IFN\(\alpha/\beta\), which may induce some genes and proteins (e.g. CD38 expression on CD8\(^+\) T cells\textsuperscript{19}) but may also result in decreased expression of IFN\(\alpha/\beta\)R and desensitization to IFN\(\alpha/\beta\). In addition, TLR signaling may induce expression or activity of SOCS, PIAS or other negative regulators of IFN\(\alpha/\beta\) signaling, providing another mechanism for decreased IFN\(\alpha/\beta\) responsiveness. Thus, changes in IFN\(\alpha/\beta\)R expression and IFN\(\alpha/\beta\) responsiveness may correlate with TLR-dependent systemic immune activation in general and monocyte activation specifically. We propose that IFN\(\alpha/\beta\)R downregulation may be a powerful indicator of pathological immune activation and disease progression, although this hypothesis requires further study.

Our studies revealed significant correlations between IFN\(\alpha/\beta\)R expression level and markers of disease progression in HIV-1 infection. Monocyte IFN\(\alpha/\beta\)R correlated directly with CD4\(^+\) T cell count and inversely with plasma HIV-1 RNA levels and expression of CD38 on memory CD8\(^+\) T cells (a marker of pathological immune activation that is prognostic for disease progression\textsuperscript{24,25}). Although CD4\(^+\) T cell count, HIV-1 RNA and CD8\(^+\) T cell immune activation (as manifested by CD38 expression) correlated individually with IFN\(\alpha/\beta\)R expression, multivariate analysis indicated that only CD38 expression on CD8\(^+\) T cells was independently associated with IFN\(\alpha/\beta\)R expression (Table 1), suggesting that factors in HIV-1 infection that drive pathological immune activation may be more closely related to monocyte IFN\(\alpha/\beta\)R expression than to plasma HIV-1 RNA level or CD4\(^+\) T cell count. We have previously shown that IFN\(\alpha\) treatment upregulates CD38 expression, especially on CD8 T cells of HIV-1 infected
individuals \textsuperscript{19}, which suggests that IFN\textgreek{a}/\textgreek{b} may provide a common pathway to both monocyte IFN\textgreek{a}/\textgreek{b}R loss and expression of CD38 on memory CD8+ T cells. Therefore, we propose that IFN\textgreek{a}/\textgreek{b}R expression on monocytes may be a novel, highly predictive marker for disease progression (either alone or in combination with CD38 expression on CD8+ T cells), a hypothesis that must be addressed in future longitudinal studies. Impairment of IFN\textgreek{a}/\textgreek{b} signaling may contribute significantly to immunopathogenesis of HIV-1 infection in a number of ways, including potential attenuation of anti-viral defenses and antigen presenting cell maturation and activation.
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Authorship

G.A.D.H. contributed to experimental design and performed most of the experiments, data analysis and manuscript preparation. S.S. assisted in experimental design, assay development, data analysis and manuscript preparation. B.R. assisted in study design, statistical analysis and manuscript preparation. W.J. contributed preliminary data. R.A. assisted in selection and recruitment of donors and assessment of clinical data. M.M.L. and C.V.H. contributed to experimental design, data interpretation and manuscript preparation. All authors reviewed the manuscript prior to submission. The authors have no conflicts of interest.
References


Table 1. Monocyte IFNα/βR expression is independently associated with CD38 expression by memory CD8+ T cells.

<table>
<thead>
<tr>
<th>Control variable</th>
<th>Correlated variable</th>
<th>CD8+ T cell CD38 expression</th>
<th>HIV-1 RNA load</th>
<th>CD4 T cell count</th>
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<td></td>
<td></td>
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<tr>
<td>HIV-1 RNA</td>
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<td>-</td>
<td>0.494</td>
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<tr>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
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<tr>
<td>CD4 and HIV-1 RNA</td>
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<td>-</td>
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Multivariate analysis was used to determine associations between monocyte IFNα/βR expression and clinical markers of disease progression. Analysis was restricted to the subset of HIV-1-infected subjects for whom CD4+ T cell count, plasma HIV RNA level and CD38 expression by memory CD8+ T cells were all measured (n = 22). Data are shown as the partial correlation coefficients of IFNα/βR expression with each of the correlated variables shown in columns after controlling for each of the variables shown in rows. Corresponding p-values are shown under each coefficient. The association of IFNα/βR expression with CD38 expression by memory CD8+ T cells changes minimally after controlling for CD4+ T cell count, HIV-1 RNA level, or both.
Figure Legends

Figure 1. IFNα/βR expression is diminished on monocytes of HIV-1-infected individuals. Individual flow cytometry histograms show IFNα/βR expression (solid curve) with isotype control (dashed line) on CD14 gated monocytes from uninfected (A) and HIV-1-infected (B) individuals. IFNα/βR expression was significantly diminished on CD14+ monocytes of HIV-1-infected individuals compared to uninfected individuals (p < 0.001 (C)). IFNα/βR expression was also diminished on CD11c+ mDCs and CD11c-pDCs of HIV-1-infected individuals compared to uninfected subjects (p = 0.052 and p = 0.046, respectively) (D). DCs were defined according to the expression of CD11 on the gated HLA-DR bright, Lin1- population (see Methods). HIV-1 infection was not associated with significant reductions in IFNα/βR expression on CD3+/CD4+ or CD3+/CD8+ T cells (p = 0.584 and p = 0.321, respectively) (E).

Figure 2. Loss of IFNα/βR on monocytes correlates with markers of disease progression in HIV-1 infection. Univariate linear regression analysis was used to assess relationships of disease markers with IFNα/βR expression. In HIV-1-infected individuals, monocyte expression of IFNα/βR correlated with absolute CD4+ T cell count (A) (p <0.001), correlated inversely with HIV-1 RNA level (B) (p <0.001) and correlated inversely with CD38 expression on memory (CD45RO+) CD8+ T cells (C) (p <0.001). In panel C, there was no significant correlation between monocyte IFNα/βR and CD38 expression on memory CD8 T cells in uninfected individuals.

Figure 3. Loss of IFNα/βR protein expression at the cell surface is not explained by diminished IFNα/βR mRNA. RNA was extracted from purified monocytes from
uninfected and HIV-1-infected individuals ex vivo, and mRNA for IFNAR1 and IFNAR2 (both chains of the IFNα/βR) was quantified by qRT-PCR and normalized to GAPDH. Cell surface IFNα/βR expression, as determined by flow cytometry, did not correlate with mRNA for IFNAR1 (r = 0.048, p = 0.866) or IFNAR2 (r = 0.074, 0.793).

Figure 4. Impaired induction of MxA and OAS in monocytes from HIV-1-infected individuals. RNA was extracted from purified monocytes from uninfected and HIV-1-infected individuals ex vivo or after culture for 18 h with 1,000 U/ml IFNα2a or without IFNα2a (“medium”), and mRNA for MxA or OAS was quantified by qRT-PCR and normalized to GAPDH. Results are expressed as fold induction (expression after 18 h incubation versus ex-vivo). Fold induction was significantly reduced for MxA mRNA (p < 0.001) (A) and OAS mRNA (p < 0.001) (B) in monocytes of HIV-1-infected individuals in comparison to uninfected subjects.

Figure 5. Deficits in ISG induction are not significantly correlated with deficits in IFNα/βR expression. Univariate linear regression analysis was used to assess relationships of ISG gene induction (Fig. 4) with IFNα/βR expression. For uninfected subjects, induction of MxA (A) and OAS (B) was not significantly related to monocyte IFNα/βR expression (p = 0.186 and p = 0.652, respectively). Similarly, for HIV-1-infected subjects, induction of MxA (C) and OAS (D) was not significantly related to IFNα/βR expression (p = 0.936 and p = 0.301, respectively).

Figure 6. Induction of phosphorylated STAT1 by IFNα2a is inhibited in monocytes from HIV-1-infected individuals. Monocytes were incubated for 15 min with IFNα2a at 0, 1,000, 3,000 or 10,000 U/ml. Individual histograms are shown for uninfected (A) and HIV-1-infected (B) subjects. Dose response induction of phosphorylated STAT1 is
evident in uninfected individuals but is inhibited in HIV-1-infected individuals (C). The difference in phosphorylated STAT1 response between uninfected and HIV-1-infected subjects is significant at 1,000 U IFNα2a (p = 0.012), and 3,000 U IFNα2a (p = 0.005), but not at 10,000 U IFNα2a (p = 0.075). Results are expressed as Δ sMFI (sMFI of stimulated monocytes - sMFI of unstimulated monocytes).

**Figure 7.** Induction of phosphorylated STAT1 by IFNα2a is not related to IFNα/βR expression in monocytes. Univariate linear regression analysis was used to assess the relationship between IFNα/βR expression and capacity to induce STAT1 phosphorylation. Significant correlation was not observed between induction of phosphorylated STAT1 and expression of IFNα/βR for uninfected individuals (r = -0.318, p = 0.443) (A) or HIV-1-infected individuals (r = 0.330, p = 0.249) (B).
Hardy et al, Figure 1
Hardy et al, Figure 2
Hardy et al, Figure 3
A) MxA induction by IFNα2a

B) OAS induction by IFNα2a

Hardy et al, Figure 4
Hardy et al, Figure 5
A) Phospho-STAT1 induction, uninfected individuals

B) Phospho-STAT1 induction, HIV-1-infected individuals

C) Phospho-STAT1 induction

Hardy et al, Figure 6
A) Phospho-STAT1 induction
(3,000 U/ml IFNα2a)
Uninfected individuals

B) Phospho-STAT1 induction
(3,000 U/ml IFNα2a)
HIV-1-infected individuals

Hardy et al, Figure 7
Desensitization to type-I interferon in HIV-1 infection correlates with markers of immune activation and disease progression

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