MAJOR ARTICLE

HIV-1 Infection of Macrophages Dysregulates Innate Immune Responses to *Mycobacterium tuberculosis* by Inhibition of Interleukin-10

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Human immunodeficiency virus (HIV)–1 and *Mycobacterium tuberculosis* (*M. tuberculosis*) both target macrophages, which are key cells in inflammatory responses and their resolution. Therefore, we tested the hypothesis that HIV-1 may modulate macrophage responses to coinfection with *M. tuberculosis*. HIV-1 caused exaggerated proinflammatory responses to *M. tuberculosis* that supported enhanced virus replication, and were associated with deficient stimulus-specific induction of anti-inflammatory interleukin (IL)–10 and attenuation of mitogen-activated kinase signaling downstream of Toll-like receptor 2 and dectin-1 stimulation. Our in vitro data were mirrored by lower IL-10 and higher proinflammatory IL-1β in airway samples from HIV-1–infected patients with pulmonary tuberculosis compared with those with non-tuberculous respiratory tract infections. Single-round infection of macrophages with HIV-1 was sufficient to attenuate IL-10 responses, and antiretroviral treatment of replicative virus did not affect this phenotype. We propose that deficient homeostatic IL-10 responses may contribute to the immunopathogenesis of active tuberculosis and propagation of virus infection in HIV-1/*M. tuberculosis* coinfection.

Keywords. HIV-1; inflammation; interleukin-10; macrophage; tuberculosis.

The coincident global distribution of *Mycobacterium tuberculosis* (*M. tuberculosis*) and human immunodeficiency virus (HIV)–1 pandemics has generated high rates of *M. tuberculosis*/HIV-1 coinfection, associated with up to 40-fold greater risk of active tuberculosis and with increased HIV-1 replication [1, 2]. Both of these pathogens have successfully established ecological niches within macrophages. HIV-1 evades innate immune detection by macrophages to establish a foothold in the host from which the virus can efficiently spread to T cells and may contribute to the pathogenesis of AIDS [3, 4]. *M. tuberculosis* subverts intracellular killing mechanisms to survive and grow within macrophages [5], and to drive inflammatory responses that contribute to tissue destruction in the pathogenesis of active tuberculosis [6, 7].

Macrophages are tissue-resident cells that generate potent inflammatory responses to innate immune stimulation, and regulate anti-inflammatory homeostatic responses to maintain tissue integrity and function [8]. They are the predominant sentinel immune cells within the respiratory tract, which is the principal site of active tuberculosis and the route for acquisition and transmission of *M. tuberculosis*. Alveolar macrophages are permissive to HIV-1 infection in vitro [9, 10], and HIV-1 can be detected in bronchoalveolar lavage samples and in alveolar macrophages obtained from HIV-1–infected patients [11–13].

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These suggest that HIV/*M. tuberculosis* coinfection of macrophages may take place in vivo. This subject has therefore attracted extensive research interest [14–18], although many questions remain unresolved.

Productive HIV-1 infection of macrophages causes negligible changes to the host cell transcriptome and exerts no cytopathic effect [3]. We have previously shown that HIV-1–infected macrophages exhibit attenuation of classical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) innate immune signaling pathways in response to specific Toll-like receptor (TLR)4 or TLR2 stimulation but only modest attenuation of downstream transcriptional responses [19]. Herein, we extend the study of this model to test the hypothesis that HIV-1 infection of macrophages modulates host responses to coinfection with *M. tuberculosis* in such a way that may contribute to the pathogenesis of tuberculosis in HIV-1–infected patients.

MATERIALS AND METHODS

Monocyte-Derived Macrophages

Blood samples were obtained from healthy volunteers or single-donor buffy coats (National Blood Transfusion Service) for production of monocyte-derived macrophages (MDMs) as described previously [19, 20] and in supplementary methods. The study was approved by the University College London Research Ethics Committee, and written informed consent was obtained from participants.

HIV-1 Strains and Cell Culture Infections

Macrophage-tropic HIV-1 strains Ba-L and Yu2 were used to establish uniformly infected MDMs as previously described [3]. Single-round vesicular stomatitis virus G glycoprotein (VSV-G)–pseudotyped HIV-1 was derived from the R9 Ba-L molecular clone [21] by truncating *env*, and used for cotransfection of producer cell lines with a plasmid-encoding VSV-G envelope. VSV-G–psuedotyped HIV-1Δ *env* was cotransduced with virus-like particles containing the simian immunodeficiency virus (SIV) accessory protein, Vpx, to increase macrophage permissivity in a single-round infection as previously described [22]. For protease inhibitor experiments, 10 μM indinavir sulphate (Centre for AIDS Reagents, NIBSC) was added to HIV-1–infected macrophages for 3 days before stimulation.

Detection of Extracellular and Intracellular HIV-1 p24

Cell-free HIV-1 p24 concentrations were quantified by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (kit v9.2, AIDS and Cancer Virus Program, National Cancer Institute, Frederick, MD). Intracellular p24 staining was performed as previously described [19].

M. tuberculosis and *Streptococcus pneumoniae* Culture

M. tuberculosis H37Rv was cultured in Middlebrook 7H9 medium (BD Bioscience) with 10% albumin/dextrose/catalase enrichment medium, 0.2% glycerol and 0.02% Tween 80, and used at mid-log growth (optical density $[OD]_{600\ nm}$ 0.6), representing 10^8 colony forming units (CFU)/mL. *M. tuberculosis* culture filtrate was generated by centrifugation of *M. tuberculosis* at 13 000 rpm for 5 minutes followed by filtration through a 0.2-μm filter (Whatman). The *S. pneumoniae* strain, TIGR4, was cultured in Todd-Hewitt broth with 0.5% yeast extract to $OD_{600\ nm}$0.4 (approximately 10^8 CFU/mL) and stored at −80°C in 10% glycerol as single-use aliquots.

Stimulation of MDM

Zymosan, lipopolysaccharide (LPS), synthetic diacylated lipopeptide Pam_2CSK_4, and curdlan were purchased from Invivogen. MDM were stimulated for 4–72 hours with *M. tuberculosis* (H37Rv) at a multiplicity of infection (MOI) of 1, *M. tuberculosis* culture filtrate for 4–24 hours or *S. pneumoniae* (TIGR4) for 4 hours at an MOI of 10. Chemical inhibition of intracellular signaling pathways was performed by preincubation of MDM with inhibitors (10 μM) for 2 hours. The pyridinyl imidazole inhibitor SB203580 was used to inhibit p38 mitogen-activated protein kinase (MAPK). A nonselective mitogen-activated protein kinase kinase (MEK) inhibitor (PD98059) or selective MEK1 inhibitor (U0126) was used to inhibit extracellular-signal-regulated kinases 1/2 (ERK1/2) signaling. The protein tyrosine kinase inhibitor [(3,5-Di-tert-butyl-4-hydroxybenzylidene)-malononitrile] (AG17) was used to inhibit activation of Pyk2 (all from Calbiochem).

Transcriptional Profiling by cDNA Microarray

Total RNA was purified from MDM lysates collected in RLT buffer (Qiagen) or TRIzol (Invitrogen) using the RNeasy Mini kit (Qiagen), and processed for Agilent microarrays as previously described [23]. Principal component analysis was used to compare global gene expression profiles as previously described [24] and paired *t* tests with Welch approximation and >2 fold-change filter were used to identify significant gene expression differences ($P < .05$) between samples using the MultiExperiment Viewer v4.6.0 application [25]. DAVID functional annotation clustering (http://david.abcc.ncifcrf.gov) was used to annotate gene lists of interest by gene ontology associations. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-TABM-1163.

Quantitative Polymerase Chain Reaction Detection of Gene Transcription

First-strand cDNA was synthesized using the qScript cDNA Supermix kit (Quanta BioSciences) and quantitative polymerase chain reaction reaction (qPCR) of selected genes was performed using TaqMan inventoried assays (Supplementary Table 1) (Applied Biosystems) according to the manufacturer's instructions. Expression levels of target genes were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [3] or hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Supplementary Table 1).

Cytokine and Matrix Metalloproteinase Measurements

MDM culture supernatant cytokine and matrix metalloproteinase (MMP) concentrations were quantified by ELISA (eBioscience) or by Luminex array (R&D Systems) using Luminex beads (Biorad) according to the manufacturer's instructions.

Western Immunoblotting Analysis of HIV-1 gag Expression and Innate Immune Signaling

Cell lysates from MDM cultures were collected in sodium dodecyl sulfate sample buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich) for polyacrylamide gel electrophoresis and immunoblotting as previously described [19]. Primary antibodies used were mouse anti-HIV-1 gag (p24) (E365/366, NIBSC), rabbit antiphosphorylated and antitotal p38 MAPK, rabbit antiphosphorylated and antitotal ERK1/2, rabbit antiphosphorylated Pyk2 (all from Cell Signaling Technology) and mouse antiactin (Abcam).

Induced Sputum and Bronchoalveolar Lavage Fluid Analysis

Induced sputum or bronchoalveolar lavage (BAL) was obtained from HIV-1–positive patients with tuberculosis (n = 18) or other respiratory infections (4 *Pneumocystis jirovecii* pneumonia and 11 other lower-respiratory-tract infections) (n = 15), median blood CD4 lymphocyte count 259 cells/μL (range, 11–705) and HIV plasma load 4.56 $\log_{10}$ copies/mL (range, 1.70–6.00). Samples were processed as described previously [26, 27] and in Supplementary Methods, and analyzed by Luminex array according to the manufacturer's instructions. Data were normalized to total protein concentrations in each sample. The study was approved by the Royal Free Hospital Ethics committee and written informed consent was obtained from all participants.

Statistical analysis

Parametric data were analyzed by *t* test or ANOVA, and nonparametric data were analyzed by the Mann–Whitney *U* test or Spearman's rank correlation, as indicated.

RESULTS

HIV-1 Infection of Macrophages Augments Proinflammatory Responses to *M. tuberculosis*

We first studied the effect of HIV-1 infection of MDMs on transcriptional responses to 24 hours coinfection with *M. tuberculosis*. Gene expression differences in HIV-1–infected and uninfected MDM following *M. tuberculosis* stimulation were subjected to functional annotation clustering by gene ontology associations and found to be significantly enriched for genes involved in immune and inflammatory responses and for genes related to cytokine and chemokine activity (Table 1). Comparison of relative expression levels for this gene list, in HIV-1–infected and uninfected MDM cultures before and after 24 hour coinfection with *M. tuberculosis*, showed that many proinflammatory responses to *M. tuberculosis* were augmented in HIV-1 coinfected cells (Figure 1*A*). HIV-1 infection alone did not significantly influence their expression. Enhanced expression of IL-23 in response to *M. tuberculosis* in HIV-1–infected cells was confirmed by qPCR and correlated with protein secretion in cell culture supernatants. These findings were replicated using an alternative HIV-1 strain (Yu2) to confirm that the effect was not virus-strain specific (Supplementary Figure 1*A* and 1*B*). Enhanced expression of proinflammatory mediators in HIV-1–infected cells compared with uninfected MDM was still evident at 72 hours after infection of MDM cultures with *M. tuberculosis* (Figure 1*B*). We have previously shown that *M. tuberculosis* also stimulates production of MMP1, implicated in the pathogenesis of tissue destruction in tuberculosis [7]. Like the other proinflammatory molecules assessed here, greater MMP1 production was evident in HIV-1/*M. tuberculosis*–coinfected macrophages (Figure 1*B*).

Table 1. Functional Annotation Clustering Analysis by Gene Ontology Classification[a] of Significant Gene Expression Differences Identified by Transcriptional Profiling of HIV-1–Infected and Control MDM Stimulated With *M. tuberculosis* for 24 hours (in 3 Separate Experiments), Showing the Top 10 Gene Ontology Terms

Term	% of Gene List	*P* Value	Fold Enrichment
GO:0006955~immune response	32.7	3.8×10^{-11}	7.5
GO:0005125~cytokine activity	21.8	4.3×10^{-11}	17.4
GO:0042330~taxis	20.0	1.1×10^{-10}	19.8
GO:0006935~chemotaxis	20.0	1.1×10^{-10}	19.8
GO:0005615~extracellular space	30.9	1.2×10^{-09}	6.6
GO:0044421~extracellular region part	34.5	2.8×10^{-09}	5.3
GO:0006954~inflammatory response	21.8	8.2×10^{-09}	10.6
GO:0006952~defense response	27.3	8.7×10^{-09}	7.0
GO:0008009~chemokine activity	12.7	1.0×10^{-08}	42.9
GO:0042379~chemokine receptor binding	12.7	1.5×10^{-08}	40.3

Abbreviations: GO, gene oncology; HIV, human immunodeficiency virus; MDM, monocyte-derived macrophages; Mtb, M. tuberculosis.

[a] http://amigo.geneontology.org

HIV-1 Infection of Macrophages is Associated With Diminished IL-10 Responses to *M. tuberculosis*

To explore the mechanism leading to enhanced proinflammatory responses to *M. tuberculosis* in HIV-1–coinfected macrophages, we investigated the effect of HIV-1 infection of MDM on the primary transcriptional response to *M. tuberculosis* by

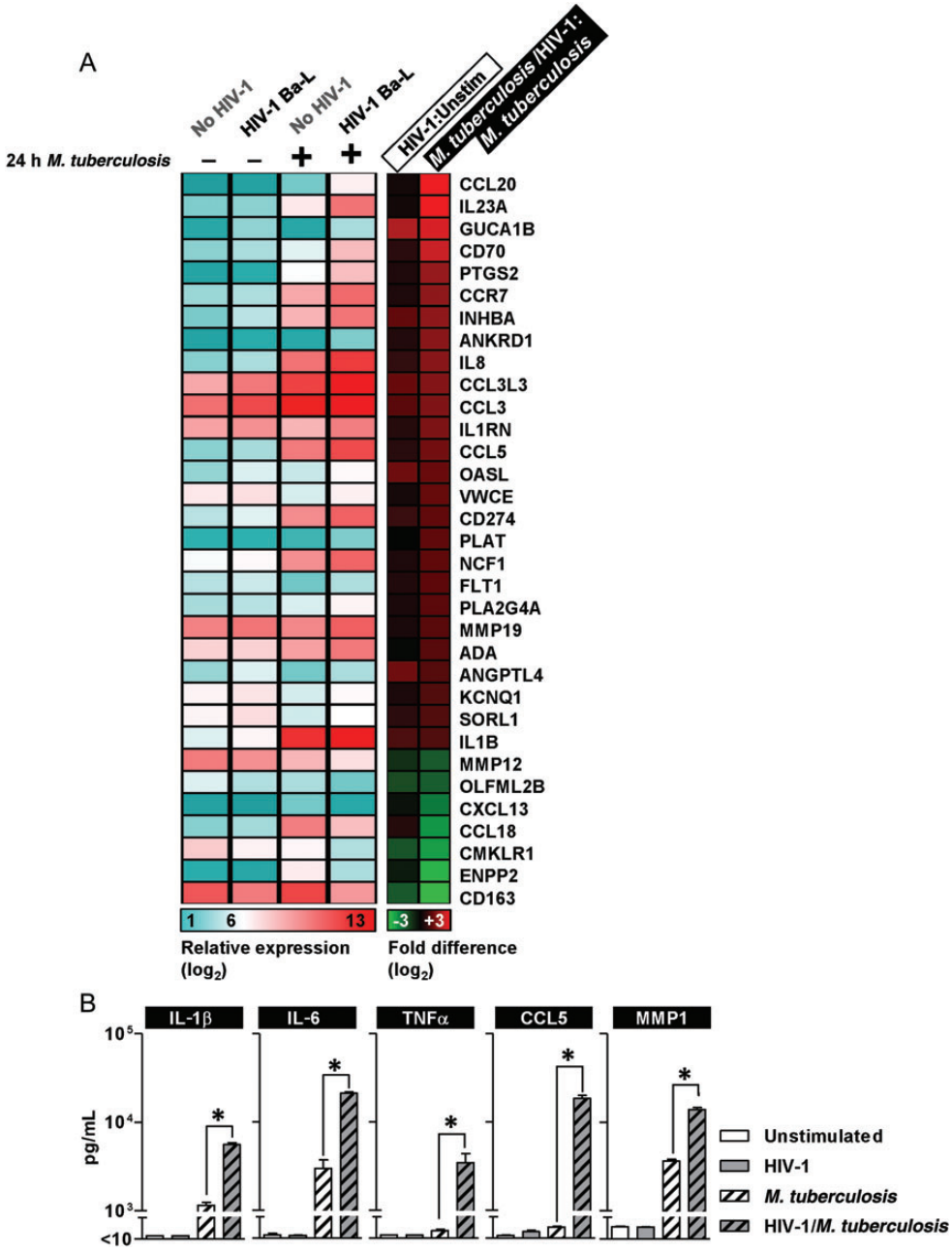

Figure 1. Augmented proinflammatory responses to *M. tuberculosis* in HIV-1–infected macrophages. *A*, Mean gene expression and fold difference matrices are shown for the most highly enriched gene ontology cluster (Table 1) with differential gene expression in HIV-1–infected and uninfected MDMs after stimulation with *M. tuberculosis* for 24 hours in 3 independent experiments. *B*, Luminex analysis of HIV-1–infected MDM culture supernatants also showed significantly higher levels of proinflammatory cytokines and chemokines as well as MMP1 after 72-hour stimulation with *M. tuberculosis*, compared with HIV-1–uninfected cells. Bars represent mean ± SEM for at least 3 separate experiments (*denotes $P < .01$, *t* test). Abbreviations: CCL5, Chemokine (C-C motif) ligand 5; HIV, human immunodeficiency virus; IL, interleukin; MDMs, monocyte-derived macrophages; MMP1, matrix metalloproteinase 1; *M. tuberculosis*, *Mycobacterium tuberculosis*; SEM, standard error of measurement; TNFα, tumor necrosis factor α.

genome-wide expression arrays, 4 hours after coinfection. Principal component (PC) analysis to visualize changes to gene expression profiles showed similar gene expression profiles in HIV-1–infected and uninfected MDM cultures before and after coinfection with *M. tuberculosis*, suggesting that the primary transcriptional response to *M. tuberculosis* was broadly unaffected by HIV-1 (Supplementary Figure 1*C*). However, paired *t* tests identified 14 genes whose response to *M. tuberculosis* at 4 hours was significantly attenuated in HIV-1–infected MDM (Figure 2*A*). Because HIV-1–infected MDM generate

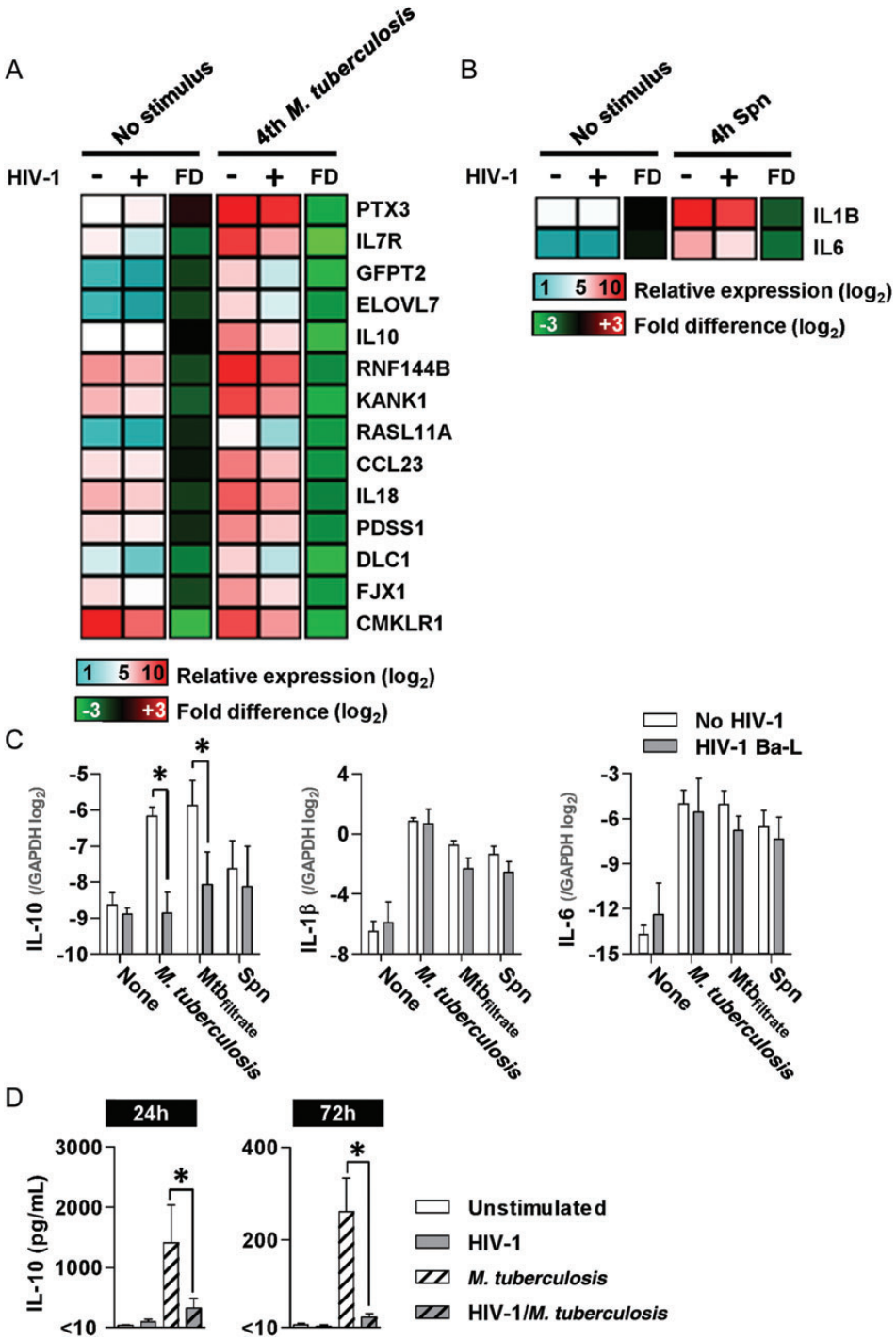

Figure 2. Deficient IL-10 responses to *M. tuberculosis* in HIV-1 infection. *A* and *B*, Heat maps show mean gene expression and mean FD in HIV-1 infected and uninfected MDM cultures following 4-hour stimulation with either *M. tuberculosis* or Spn in at least 3 separate experiments. Gene-by-gene analysis showed significantly attenuated responses (>2-fold and $P < .05$, *t* test) in HIV-1–infected MDM for 14 genes following *M. tuberculosis* stimulation (*A*) and 2 genes following Spn stimulation (*B*). *C*, Inhibition of IL-10 responses to 4-hour stimulation with *M. tuberculosis* , and cell-free filtrate from *M. tuberculosis* cultures ($Mtb_{filtrate}$) in HIV-1–infected MDMs, compared with HIV-1–uninfected cells, is confirmed by qPCR. HIV-1 infection did not affect IL-10 responses to Spn or proinflammatory cytokine (IL-1β and IL-6) responses to any stimulus at 4 hours. *D*, Deficient IL-10 production in HIV-1–infected cells was also evident in cell culture supernatants (quantified by Luminex array) after 24 and 72 hours' stimulation with *M. tuberculosis*. Bars represent mean ± SEM for at least 3 separate experiments (*denotes $P < .01$, *t* test). Abbreviations: FD, fold differences; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIV, human immunodeficiency virus; IL, interleukin; MDMs, monocyte-derived macrophages; MMP1, matrix metalloproteinase 1; *M. tuberculosis*, *Mycobacterium tuberculosis*; qPCR, quantitative polymerase chain reaction; SEM, standard error of measurement; Spn, S. pneumoniae.

augmented proinflammatory responses to *M. tuberculosis* at later time points, significant attenuation of IL-10 induction in the early response to *M. tuberculosis* was of particular interest, given its role in homeostatic regulation of inflammatory responses [28].

To assess whether early downregulation of macrophage IL-10 was specific to HIV-1/*M. tuberculosis* coinfection, we made similar comparisons in HIV-1–infected and control MDM cultures after 4 hour stimulation with live *S. pneumoniae*. The primary transcriptional response to *S. pneumoniae* generated differences in PC1 only and was comparable in HIV-1–infected and uninfected MDM (Supplementary Figure 1*D*). Direct comparison of these gene expression profiles revealed statistically significant, albeit modest attenuation, in 2 genes only, IL-1β and IL-6 (Figure 2*B*). These findings suggest that the effects of HIV-1 infection of MDM on innate immune transcriptional responses to coinfecting pathogens may be stimulus specific.

We validated our array findings by qPCR (Figure 2*C*), confirming attenuation of IL-10 responses to *M. tuberculosis* in HIV-1 coinfected MDM cultures, and that the same effect was not evident in HIV-1/*S. pneumoniae* coinfection. Proinflammatory transcriptional responses to *M. tuberculosis*, represented by IL-1β and IL-6, were not affected by HIV-1 infection at this time point (Figure 2*C*).

Innate immune induction of IL-10 by *M. tuberculosis* stimulation of macrophages is well documented [29–31]. The cell-free filtrate of *M. tuberculosis* cultures also induced increased IL-10 expression in MDM, which was attenuated in HIV-1–infected cells, suggesting that this effect was independent of live bacterial infection (Figure 2*C*). Furthermore, IL-10 secretion remained significantly diminished at 24 and 72 hours after stimulation with *M. tuberculosis* in HIV-1–infected cells (Figure 2*D*), in contrast to increased production of proinflammatory mediators at these time points (Figure 1*B*). In order to confirm the anti-inflammatory effects of IL-10 in our model, we showed that the addition of exogenous IL-10 to MDM inhibited selected proinflammatory responses to *M. tuberculosis*, either at the transcriptional level (measured by qPCR at 24 hours) or the protein level (measured by ELISA at 72 hours) (Supplementary Figure 1*E* and 1*F*).

The in vitro experiments detailed above suggested that HIV/*M. tuberculosis* coinfection in vivo might be associated with diminished IL-10 levels and consequently, enhanced inflammatory cytokine responses. To evaluate whether similar findings may occur in vivo, we measured IL-1β, as a prototypic proinflammatory marker, and IL-10 in induced sputum and BAL samples from HIV-infected patients with tuberculosis or other lower-respiratory-tract infections as previously described [26, 27]. In keeping with our in vitro data, we found that in HIV-infected patients with intercurrent respiratory-tract infections, pulmonary tuberculosis was associated with significantly higher IL-1β levels (Figure 3*A*) and lower IL-10 levels (Figure 3*B*). There were no significant differences in total protein concentration in respiratory samples or plasma HIV-1 viral load between tuberculosis and nontuberculosis groups and no correlation between cytokine measurements in respiratory samples and plasma HIV-1 viral load (Figure 3*C*–*E*).

M. tuberculosis–induced p38 and ERK1/2 Activation is Attenuated in HIV-1–Infected Macrophages

Innate immune induction of IL-10 expression in macrophages is principally attributed to Toll-like receptor 2 (TLR2) or dectin-1–mediated signaling pathways that involve activation of p38 and ERK1/2 MAPK [32–34], summarized in Figure 4*A*. We confirmed activation of these MAPK pathways in MDM stimulated with *M. tuberculosis* by Western blot detection of phosphorylated p38 and ERK1/2 (Figure 4*B*). By comparison, activation of these signaling pathways was attenuated in HIV-1–infected MDM. Total p38 and ERK1/2 levels were unaffected by HIV-1 infection as was *M. tuberculosis*-stimulated phosphorylation of Pyk2, an intermediate in the spleen tyrosine kinase (SYK) signaling pathway, downstream of dectin-1 stimulation [33]. TLR2 and dectin-1 can also be stimulated by the fungal cell wall derivative zymosan [33]. We therefore tested IL-10 responses to zymosan stimulation in our model and also found them to be attenuated in HIV-1 infected MDM (Figure 4*C*). These data suggest that the effect of HIV-1 is mediated by inhibition of conserved innate immune signaling events in IL-10–induction pathways in response to different stimuli, but lower levels of IL-10 induction by stimulation of specific receptors—dectin-1 by curdlan, TLR2 by Pam_2CSK_4, and TLR4 by LPS—showed less marked attenuation by HIV-1 (Figure 4*C*). As previously, early IL-6 responses to these stimuli were unaffected by HIV-1.

In order to confirm the functional role of these pathways in the induction of IL-10 responses within our model, we tested the effect of chemical inhibition of components of these pathways on *M. tuberculosis* and zymosan-induced production of IL-10 (Figure 4*D*). Inhibition of p38 significantly attenuated IL-10 production by both stimuli. Targeting ERK1/2 signaling alone did not attenuate IL-10 production, but dual p38/ERK1/2 inhibition modestly enhanced the effect of p38 inhibition. Pyk2 inhibition also significantly attenuated IL-10 production. Taken together, our data suggest that HIV-1 attenuation of innate immune IL-10 responses by MDM may be mediated by inhibition of the p38 MAPK pathway with modest additional effect of inhibition of the ERK1/2-dependent pathway.

The Consequence of Attenuated IL-10 Responses to *M. tuberculosis* on HIV-1 Replication in MDM

Stimulation of HIV-1–infected macrophages with *M. tuberculosis* has been reported to inhibit HIV-1 replication as a result of induction of the inhibitory isoform of the C/EBPβ transcription factor [35]. Pro-inflammatory cytokines such as tumor

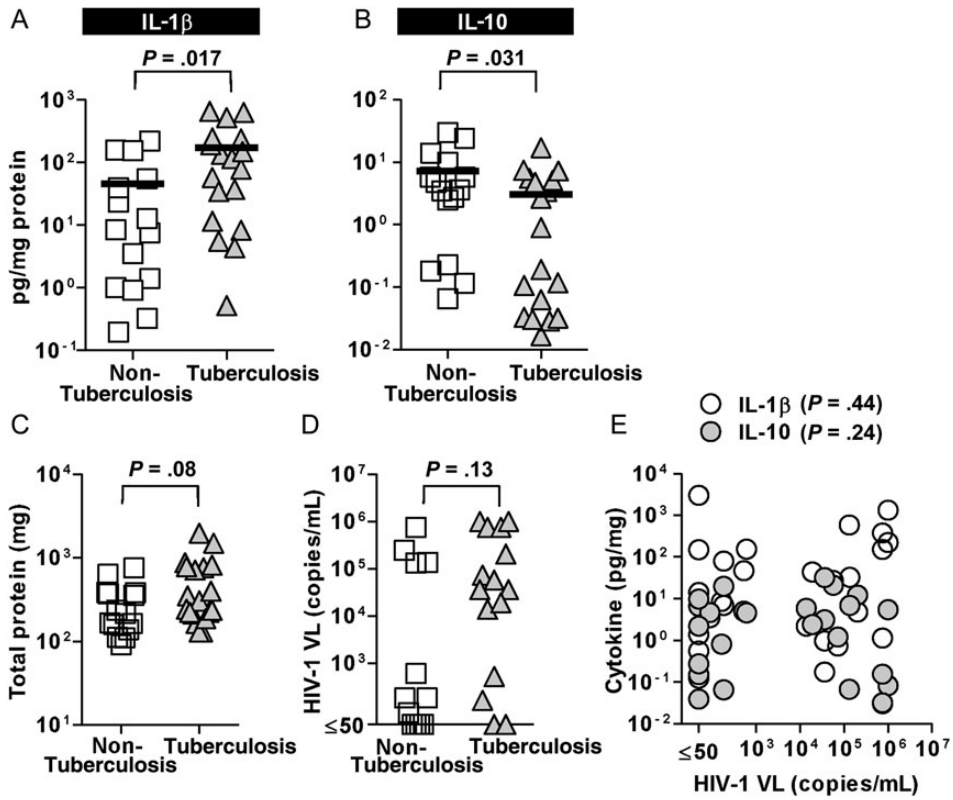

Figure 3. Lower IL-10 and higher IL-1β levels are evident in respiratory samples from HIV-1–infected patients with pulmonary tuberculosis, compared with nontuberculous infections. Induced sputum and BAL fluid samples from HIV-1–infected patients with tuberculosis (n = 18) showed significantly greater IL-1β (*A*) and lower IL-10 levels (*B*) (measured by Luminex and normalized to total protein concentration) compared with samples from patients with nontuberculosis respiratory-tract infections (n = 15) (Mann–Whitney *U* test). Total protein in respiratory samples (*C*) and plasma HIV-1 viral load were similar (*D*) in both study groups and there was no significant correlation (Spearman's rank test) between plasma viral load and IL-1β or IL-10 concentrations in respiratory samples (*E*). Data points indicate individual measurements, and lines indicate the median values. Abbreviations: BAL, bronchoalveolar lavage; HIV, human immunodeficiency virus; IL, interleukin; VL, viral load.

necrosis factor (TNF)α, IL-1β and IL-6 that are induced by *M. tuberculosis* are known to increase HIV-1 replication by activation of NF-κB pathways and consequent transcriptional activation of the HIV-1 long terminal repeat [36, 37]. We found that HIV-1 release by macrophages, quantified by p24 concentrations in cell-culture supernatants, was significantly attenuated after 24 hours coinfection with *M. tuberculosis*. However, by 72 hours, p24 levels recovered, and in 5 of 8 experimental replicates exceeded equivalent HIV-1 infected macrophage cultures that were not coinfected with *M. tuberculosis* (Figure 5*A*). These data suggest that early suppression of HIV-1 replication, as a result of cellular coinfection with *M. tuberculosis*, may be reversed at later time points that are associated with enhanced proinflammatory cytokine responses. To extend these observations, we confirmed that stimulation of HIV-1–infected cells with zymosan also led to significantly increased transcription of the viral genome and p24 levels in the cell culture supernatants at 72 hours, and that complementation of deficient IL-10 responses by the addition of recombinant IL-10 suppressed virus production (Figure 5*B*). Hence, in HIV-1–infected macrophages, inhibition of IL-10 responses to coinfecting pathogens may serve to increase virus replication and propagation.

HIV-1 Attenuation of Innate Immune IL-10 Induction in MDM Is Dependent on Infection With Replication-Competent Virus but not On-going Virus Propagation

We assessed whether integration or replication were necessary for HIV-1 to inhibit macrophage IL-10 production. Infection of MDM with an envelope-deficient pseudotyped HIV-1, which is only capable of a single round of infection, was sufficient to attenuate IL-10 responses to zymosan. However, UV-inactivated virus, which cannot reverse transcribe and integrate, failed to attenuate IL-10 responses to zymosan (Figure 5*C*). Inhibition of HIV-1 propagation in MDM by the addition of protease inhibitor (Figure 5*D*) after infection had no effect on attenuation of IL-10 responses (Figure 5*E*). These data suggest that HIV-1 integration and transcription, but not on-going virus propagation, mediate the effect on IL-10, and importantly, that this effect of HIV-1 within macrophages may persist in the face of effective antiretroviral therapy that blocks virus propagation.

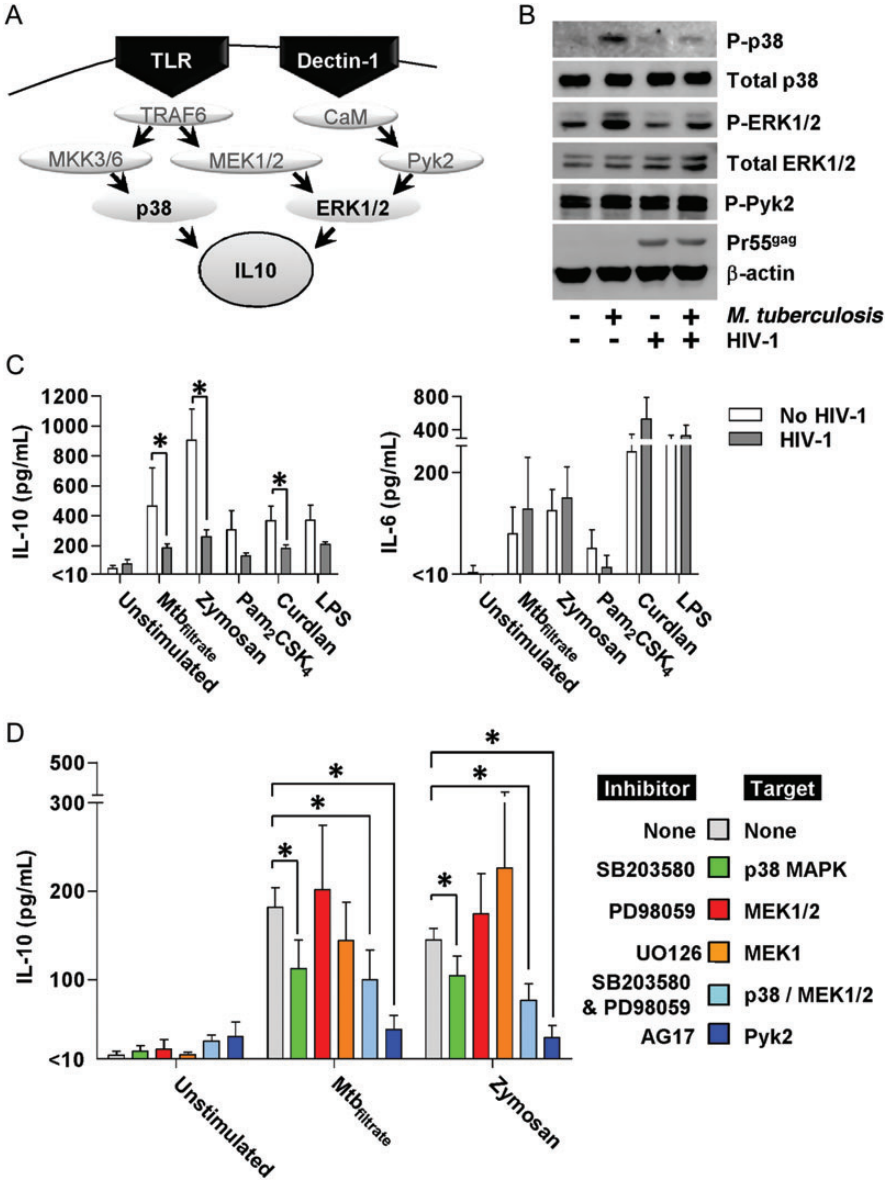

Figure 4. HIV-1 inhibition of IL-10 responses is associated with attenuated p38 MAPK signaling. *A*, Signaling pathways involved in macrophage IL-10 production in response to innate immune activation via TLRs and dectin-1. *B*, Phosphorylation of p38 and ERK1/2, following 2-hour stimulation with *M. tuberculosis*, was attenuated in HIV-1–infected MDMs compared with HIV-1–uninfected cells. Total p38 and ERK levels were unaffected by HIV-1 infection. Detection of precursor HIV-1 gag protein confirmed productive virus infection, and β-actin levels show equivalent sample loading. Representative Western blots are shown of experiments using 3 separate donors. *C*, IL-10 and IL-6 production by MDM cultures ± HIV-1 infection were quantified by ELISA after 4-hour stimulation with $Mtb_{filtrate}$, zymosan (0.4 mg/mL), Pam_2CSK_4 (100 ng/mL), curdlan (0.1 mg/mL), and LPS (100 ng/mL). *D*, Preincubation of MDMs with inhibitors of p38 signaling (SB203580) and Pyk2 activation (AG17) for 2 hours attenuated IL-10 production in response to 4-hour stimulation with $Mtb_{filtrate}$ or zymosan, detected by ELISA measurement of IL-10 levels in cell-culture supernatants. Bars represent mean ± SEM of 4 separate experiments.*Denotes significant differences by paired *t* tests ($P < .05$). Abbreviations: ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular-signal-regulated kinases 1/2; HIV, human immunodeficiency virus; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; MDMs, monocyte-derived macrophages; *M. tuberculosis*, *Mycobacterium tuberculosis*; SEM, standard error of measurement; TLRs, Toll-like receptors.

DISCUSSION

The pathogenesis of active tuberculosis is mediated by excessive proinflammatory responses, highlighted by paradoxical reactions following antituberculous therapy, or immune reconstitution inflammatory syndrome (IRIS) in HIV-1–infected patients following antiretroviral therapy [38]. We sought to evaluate the potential contribution of HIV-1 infection of macrophages to the immunopathogenesis of coinfection with *M. tuberculosis*. Infection of macrophages with HIV-1 and *M. tuberculosis* has

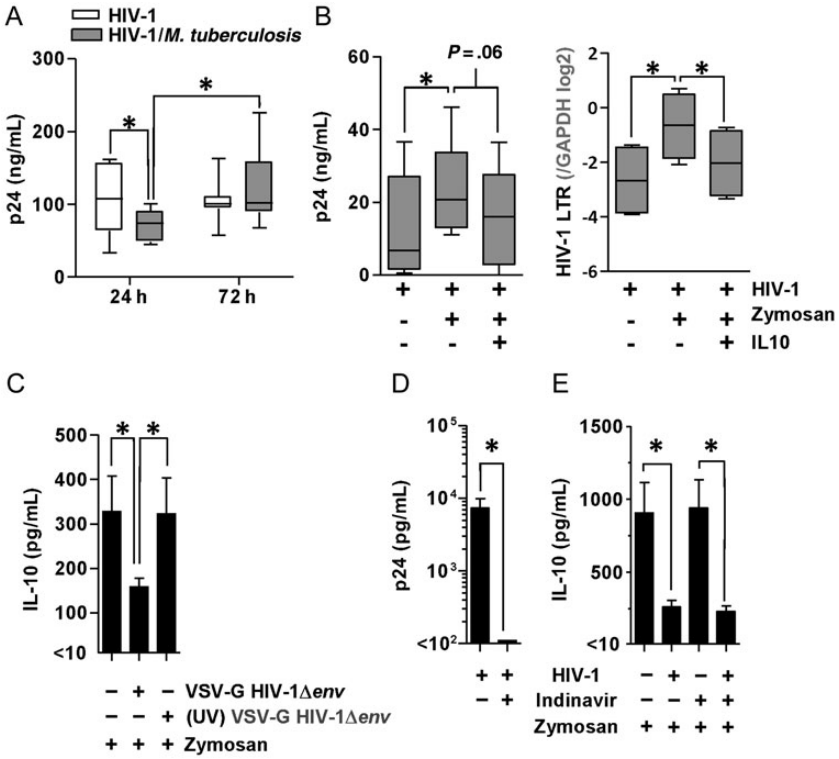

Figure 5. HIV-1 replication is sensitive to IL-10 and attenuation of IL-10 responses requires live virus but not virus propagation. *A*, HIV-1 replication (quantified by HIV-1 p24 ELISA of cell culture supernatants) was initially inhibited in MDMs ($P = .034$, paired *t* test) stimulated with *M. tuberculosis* for 24 hours, but subsequently recovered by 72 hours, in comparison to viral replication in cells that were not co-infected with *M. tuberculosis*. *B*, HIV-1 transcription quantified by qPCR of the HIV-1 LTR and p24 levels in cell culture supernatants also increased significantly after 72 hours stimulation with zymosan, but this effect was abrogated by the addition of recombinant IL-10 (10 ng/mL) 4 hours after stimulation to complement the deficient IL-10 responses in HIV-1–infected cells. *C*, Infection of MDMs with single-round envelope-deficient virus (HIV-1Δ*env*) pseudotyped with the VSV-G envelope was sufficient to attenuate IL-10 responses to 4-hour zymosan stimulation, but this effect was not evident when using ultraviolet-inactivated virus. *D*, In MDMs infected with full-length replication-competent HIV-1, treatment with the protease inhibitor Indinavir (10 μM) for 3 days before zymosan stimulation completely abolished the release of any mature virus, reflected in the absence of any detectable p24 in the cell-culture supernatant. *E*, Early (4-hour) innate immune IL-10 responses were similarly attenuated in the presence and absence of protease inhibitor. Box-and-whisker plots represent the median and range of 5 to 8 experiments in each case. Bars represent mean ± SEM of 5 separate experiments (*denotes $P < .05$, *t* test). Abbreviations: ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; IL, interleukin; LTR, long terminal repeat; MDMs, monocyte-derived macrophages; *M. tuberculosis*, *Mycobacterium tuberculosis*; qPCR, quantitative polymerase chain reaction; SEM, standard error of measurement; VSV-G, vesicular stomatitis virus G glycoprotein.

previously been reported to be dominated by transcriptional responses to *M. tuberculosis* [17], consistent with our findings that HIV-1 itself does not induce innate immune cellular activation or any significant changes to the host-cell transcriptome [3]. Another study to test the effect of HIV-1 infection in macrophages on responses to *M. tuberculosis* reported enhanced proinflammatory responses by the measurement of selected cytokines [18]. We found that the early proinflammatory response to *M. tuberculosis*, like the response to *S. pneumoniae* and LPS [19], is comparable in HIV-1–infected and uninfected macrophages. However, HIV-1–infected macrophages showed an early attenuation of anti-inflammatory IL-10 transcription, and a more striking reduction in IL-10 protein secretion for up to 72 hours. Diminished early IL-10 responses were associated with exaggerated proinflammatory responses at later time points. Attenuation of IL-10 by HIV-1 is clearly contingent on stimuli that induce IL-10 responses in macrophages. In contrast to *M. tuberculosis*, infection of MDM with *S. pneumoniae* did not induce a significant IL-10 response and consequently no effect of HIV-1 coinfection. Therefore, this phenotype would be expected to exhibit pathogen specificity. In keeping with this hypothesis and the possibility that this phenomenon may contribute to in vivo pathogenesis, we found significantly lower IL-10 and higher IL-1β in lung secretions from HIV-1–positive patients with tuberculosis compared with other respiratory-tract infections. Others have reported preserved IL-10 responses to TLR4 stimulation in the U1 leukemia cell line harboring integrated HIV-1 virus [39], increased IL-10 levels in BAL fluid from asymptomatic HIV-infected patients [16], and higher plasma IL-10 levels in patients with HIV/*M. tuberculosis* coinfection and CD4 lymphocyte counts <200 [40]. The discrepancies with our data are likely to reflect differences in experimental paradigms

or context, but merit further consideration, as do potential differences in the biology of distinct macrophage populations.

M. tuberculosis induction of IL-10 requires MAP kinase activity [29, 32, 41]. HIV-1 infection of macrophages was observed to attenuate p38 and ERK1/2 MAPK phosphorylation induced by *M. tuberculosis*. Alveolar macrophages from HIV-1–infected patients have also been reported to show reduced ERK1/2 phosphorylation [42]. In our model, inhibition of p38 had a greater effect on IL-10 production than selective inhibition of ERK1/2, consistent with the existing literature on a dominant role for p38 in IL-10 production by mononuclear phagocytic cells [30, 41, 43]. We found that HIV-1 infection of macrophages also attenuated IL-10 responses to the fungal cell wall derivative, zymosan, suggesting that the effect of HIV-1 is to inhibit common signaling pathways upstream of IL-10 transcription, although the mycobacterial components that are involved require further investigation.

Interestingly, *M. tuberculosis* and fungal pathogens are most commonly associated with IRIS in HIV-1–infected patients [38, 44]. Pathogenesis studies of IRIS have focused principally on the role of T cells [45, 46], although a potential role for macrophages has been postulated [47] and supported by recent data that highlight the role of inflammatory cytokines commonly associated with myeloid cells [48]. We show, by using a model of single-round virus replication or protease inhibitors, that ongoing virus propagation is not necessary to inhibit IL-10 responses in macrophages. Therefore, given that macrophages are relatively long-lived, HIV-1 attenuation of IL-10 and dysregulation of inflammatory responses may persist despite effective antiretroviral therapy. These responses may be further compounded by lymphocyte recovery and recruitment following antiretroviral therapy and suggest a mechanism by which macrophages may contribute to the pathogenesis of IRIS.

In the absence of antiretrovirals, we initially observed diminished virus production after coinfection with *M. tuberculosis*, as previously reported [35]. However, this effect was transient and stimulation of HIV-1–infected macrophages with *M. tuberculosis* or zymosan led to sustained increases in virus production at later time points. Increased virus production was sensitive to complementation of the deficient IL-10 response by addition of recombinant IL-10. These data are in keeping with previous reports that IL-10 can inhibit HIV-1 replication [49, 50], and that proinflammatory cytokines can enhance HIV-1 replication [36, 37]. Therefore, we propose that attenuated IL-10 responses and exaggerated proinflammatory responses to *M. tuberculosis* may contribute to a cell-autonomous mechanism for increased HIV-1 viral load during coinfection [1]. This effect may also enhance viral propagation by the transfer of HIV-1 to permissive T cells, for which recruitment to the site of infection may be increased as a result of exaggerated proinflammatory cytokine and chemokine responses in HIV-1/*M. tuberculosis*–coinfected macrophages.

In conclusion, we propose that in vivo, even low-frequency HIV-1–infected macrophages can provide a nidus of exaggerated inflammatory responses to coinfecting pathogens, as a consequence of insufficient induction of homeostatic IL-10. This may contribute to the immunopathogenesis of active tuberculosis in HIV-1–infected patients, and viral propagation as a result of increased virus replication and recruitment of HIV-1–permissive T cells. The pathogenic role of aberrant IL-10 responses in active tuberculosis, and the potential for therapeutic intervention in this pathway, merit further assessment.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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