Published in final edited form as:

AIDS. 2014 March 13; 28(5): 679–687. doi:10.1097/QAD.0000000000000192.

Role of protease inhibitor 9 in survival and replication of *Mycobacterium tuberculosis* in mononuclear phagocytes from HIV-1-infected patients

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Abstract

Objective and design—Predisposition to opportunistic infections by *Mycobacterium tuberculosis* (MTB) is a concomitant of HIV-1 infection and occurrence of tuberculosis is independent of circulating CD4⁺ T-cell count in HIV-1-infected patients. Infection of mononuclear phagocytes from healthy individuals by virulent MTB is associated with expression of the antiapoptotic molecule protease inhibitor 9 (PI-9), and PI-9 contributes to successful parasitism of macrophages by MTB. Here we studied the contribution of PI-9 to successful MTB infection of monocytes from HIV-1-infected patients.

Methods—Blood monocytes obtained from HAART-treated HIV-1-infected patients (HIV+) and healthy controls were assessed for support of MTB H37Rv growth by assessment of MTB 16S ribosomal (r)RNA in cell lysates on day 1 and day 7 by real-time reverse transcription-PCR. PI-9 expression in monocyte cell lysates was assessed by ELISA and by reverse transcription-PCR. Inhibition of intracellular PI-9 was achieved by siRNA to PI-9 and compared to control constructs.

Results—Monocytes from HIV-infected patients supported higher MTB growth [MTB 16S rRNA (d7/d1)] as compared with monocytes from healthy controls. Both PI-9 protein and mRNA were significantly higher in monocytes from HIV-infected patients as compared with healthy controls. PI-9 protein levels prior to MTB infection correlated with MTB replication on day 7, and with plasma soluble CD14 levels. Silencing of PI-9 by transfection of monocytes from HIV-1-infected patients with PI-9-specific siRNA prior to infection improved intracellular containment of MTB.

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Conflicts of interest

The authors have no conflicts of interest.

Conclusion—Increased intracellular PI-9 activity in mononuclear phagocytes from HIV-infected patients contributes to successful intracellular infection by virulent MTB.

Keywords

HIV-1 infection; monocyte; *Mycobacterium tuberculosis*; protease inhibitor 9

Introduction

Tuberculosis (TB) remains the most common coinfection in individuals with HIV-1 infection [1], and is associated with significant mortality and morbidity in this population [2]. Increased risk of development of active TB occurs shortly after HIV-1 seroconversion [3]; however, it remains sustained over time [4]. Increased susceptibility to TB in HIV-1-infected patients is observed regardless of levels of circulating CD4⁺ T-cell count [5]. Specifically, up to 50% of cases of pulmonary TB in HIV-1-infected patients occur at CD4⁺ cell counts over 250 cells/μl and up to 15% at CD4⁺ T-cell count more than 500 cells/μl [6]. Recent evidence using RFLP analysis has led to the conclusion that progressive primary TB, rather than reactivation of *Mycobacterium tuberculosis* (MTB) infection, accounts for the majority of cases of TB in HIV-1-infected patients [3,7]. These data implicate defects in macrophage functionality as a predisposing factor to TB in HIV-infected patients. However, HIV-associated alterations in specific macrophage pathways associated with TB remain as yet undefined.

Following aerosol MTB infection in the lung, initial containment of replication is dependent on terminally differentiated alveolar macrophages, which are continuously replenished by newly arrived blood monocytes to sites of infection. As such, the functional profile of mononuclear phagocytes is critical to containment of MTB infection. It is well established that apoptosis of MTB-infected mononuclear phagocytes is critical to control of MTB growth [8], and that virulent MTB evades apoptosis of infected macrophages [9]. It has also been shown that in-vitro HIV-1 infection of macrophages from healthy individuals impairs their capability to undergo apoptosis [10]. Further, alveolar macrophages obtained from HIV-infected patients display decreased capacity to undergo apoptosis following exposure to heat-inactivated MTB H37Rv [11]. In a recent microarray analysis, we identified significant induction of the antiapoptotic molecule protease inhibitor 9 (PI-9), as one of six apoptosis-related genes upregulated following infection of alveolar macrophages from healthy individuals with live MTB H37Rv [12]. Induction of PI-9 expression was specific to virulent MTB H37Rv, and not observed upon infection of alveolar macrophages by avirulent MTB H37Ra. We subsequently demonstrated that expression of PI-9 in alveolar macrophages was associated with reduced apoptosis as measured by low caspase-3 and increased Bcl-2 expression [13]. In this latter study, inhibition of PI-9 mRNA in MTB H37Rv-infected alveolar macrophages by siRNA was associated with improvement of MTB containment as assessed by MTB 16S ribosomal (r) RNA [13]. However, the role of PI-9 in success of MTB infection in mononuclear phagocytes from HIV-infected patients is not known.

Other studies have related the defect in apoptosis of macrophages from HIV-1-infected patients exposed to heat-inactivated MTB to low induction and/or release of tumor necrosis factor- α (TNF- α), in addition to heightened interleukin 10 (IL-10)-mediated deactivation of MTB-infected macrophages [11]. Further, HIV-1 Nef has been found to inhibit MTB-induced TNF- α production and macrophage apoptosis [14], implicating a possible role for HIV-1 components in undermining macrophage function against MTB. However, these studies have not assessed the relationship of defective MTB-induced TNF- α with intracellular MTB growth in mononuclear phagocytes. Defective phagosome-lysosome fusion of alveolar macrophages, and phagosome acidification of in-vitro monocyte differentiated macrophages (MDMs) from HIV-1-positive patients upon MTB infection have also been described [15, 16]. Whether these macrophage defects converge on a reduced apoptotic profile of MTB-infected mononuclear phagocytes in HIV-1 disease is unclear.

The goal of the current study was to examine the role of PI-9 in prevention of apoptosis of mononuclear phagocytes from HIV-infected patients in promotion of virulent MTB intracellular growth. We found higher expression of PI-9 mRNA and protein in monocytes isolated from HIV-infected patients as compared with healthy individuals prior to MTB infection. PI-9 protein activity in monocytes correlated with intracellular MTB replication. Inhibition of PI-9 by specific short interfering (si) RNA inhibited MTB growth in monocytes from HIV-positive patients. These studies identify excessive baseline PI-9 activity in monocytes from HIV-1-infected patients as a predisposing factor for impaired containment of intracellular growth of MTB.

Methods

Patients ($n = 18$) with HIV-1 infection attending Special Immunology Unit at University Hospitals/Case Medical Center in Cleveland were recruited and underwent blood draw after IRB-approved consent. Characteristics of HIV-infected patients at the time of study included a median age of 49.5 years (range: 32–65), a median CD4⁺ T-cell count of 537 cells/ μ l (range: 104–1310), a median duration of HAART treatment of 9 years (range: 2–20), and viral loads of less than 50 copies/ μ l at the time of study in all but two patients.

Healthy individuals ($n = 18$) were research personnel in the laboratory, and were recruited to encompass the age range of the HIV-1-infected patients; age was a median of 45.4 years (range: 28–65).

Isolation of peripheral blood mononuclear cells and blood monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (GE Healthcare Biosciences, Uppsala, Sweden). Monocytes were isolated from PBMCs by negative immunomagnetic selection using the monocyte isolation kit II (Miltenyi Biotech, Auburn, California, USA). The purity of sorted monocytes was more than 90% based on immunostaining and FACS analysis for CD14.

Infection of monocytes with *Mycobacterium tuberculosis*

MTB strain H37Rv (ATCC, Manassas, Virginia, USA) was cultured and prepared for infection as previously described [17]. Cell monolayers were incubated with the organism at

multiplicities of infection (MOI) of 0.1: 1 to 10: 1 (bacteria/cell) for 90 min in 30% unheated pooled human serum (PHS; Gemini-Bioproducts, Sacramento, California, USA) in RPMI-containing penicillin. Then, cultures were washed extensively and received RPMI with 5% PHS. Cultures were harvested on day 1 and day 7 after infection.

Detection and analysis of gene expression

Following total RNA extraction and reverse transcription [18], quantitative real-time PCR of cDNA was performed using the ABI StepONE Plus Detection System.

Primers and probes for PI-9, Bcl2, BAX, TNF- α , and ribosomal 18S (R18; Applied Biosystems Primer Express software, Foster City, California, USA) were as before [13]. Expression of mRNA copies was normalized to R18 copy numbers in the same sample.

Assessment of *Mycobacterium tuberculosis* replication

Total RNA isolated from MTB H37Rv-infected monocyte lysates was also assessed for MTB 16S ribosomal (r) RNA as before [13]. MTB intracellular replication was expressed as fold increase in MTB 16S rRNA [MTB 16S rRNA in monocyte cultures on day 7 (d7) over that on day 1 (d1) after MTB infection]. Previously, we have shown that MTB 16S rRNA (d7/d1) correlated with MTB growth by colony-forming unit assay [13].

Inhibition of protease inhibitor 9 expression by small inhibitory RNA

Target-specific PI-9 and nontargeting (control) siRNA constructs were purchased from Dharmacon Inc. (Lafayette, Colorado, USA). PI-9-specific and control siRNAs were introduced by incubation of monocytes (0.5×10^6) in Accell medium (Dharmacon) for 24 h. Then, siRNA constructs were added [13]. After 24 h, monocyte monolayers were washed and underwent MTB infection.

ELISA for intracellular protease inhibitor 9 and soluble CD14

Monocyte cell lysates were prepared in Passive Lysis Buffer (Promega, Madison, Wisconsin, USA) and assessed for PI-9 protein by ELISA as before [13]. Polyclonal (coating) and conjugated monoclonal (detecting) anti-PI-9 antibodies were obtained from Sanquin Foundation (Amsterdam, Netherlands) [19]. Soluble CD14 (sCD14) in plasma was assessed using commercially available ELISA kit for sCD14 with a detection limit of 125 pg/ml (R&D Systems, Minneapolis, Minnesota, USA).

Statistical analysis

Comparisons of findings from HIV-1-infected and healthy individuals were evaluated by unpaired *t*-test. Comparisons of measures using cells from the same group of patients were done by paired *t*-tests. *P* values of <0.05 were considered significant. Correlation between parameters was assessed by Spearman's rank analysis.

Results

***Mycobacterium tuberculosis* growth in monocytes from HIV-1-infected patients**

First, monocytes isolated from PBMCs of HIV-1-infected ($n = 18$) and healthy individuals ($n = 18$) were infected with MTB H37Rv (MOI 1: 1) and assessed for MTB 16S rRNA on day 1 and 7 after infection. As shown in Fig. 1a, fold increase in MTB 16S rRNA (d7/d1) was significantly higher in monocytes from HIV-1-infected patients as compared with healthy controls ($P < 0.05$).

Next, the correlation of increased intracellular MTB growth [MTB 16S rRNA (d7/d1)] in monocytes from HIV-infected patients with their clinical parameters was assessed. There was no correlation of increase in growth of MTB H37Rv, that is, 16S rRNA (d7/d1), with CD4⁺ T-cell count at the time of study. Nadir CD4⁺ T-cell count and maximum viral load were available in 13 of 18 patients studied. There was a positive correlation ($R = 0.771$, $P = 0.006$) of nadir CD4⁺ T-cell count and fold increase in MTB 16S rRNA (d7/d1; Fig. 1b). No significant correlation was observed between maximum viral load and fold increase in MTB H37RV growth (data not shown).

Expression of protease inhibitor 9 in monocytes from HIV-1-infected patients

Recently, we have reported on the role of PI-9 in undermining containment of MTB H37Rv in human mononuclear phagocytes [13]. To assess whether PI-9 activity was different in monocytes from HIV-infected patients, we assessed PI-9 protein levels in lysates of monocytes obtained both at the time of isolation of monocytes from PBMCs (t0), and 24 h later, which was the time point immediately prior to infection with MTB (day 1). Levels of PI-9 protein at both t0 and day 1 were higher in monocytes from HIV-infected patients as compared with healthy controls ($P < 0.05$ and 0.005 , respectively; Fig. 2a). Levels of PI-9 protein in monocytes from HIV-1-infected patients on day 1 (i.e. immediately prior to MTB H37Rv infection) correlated significantly with MTB 16S rRNA expression at day 7 ($r = 0.636$, $P < 0.02$). Interestingly, PI-9 protein activity in monocytes at day 1 correlated with nadir CD4⁺ T-cell count ($r = 0.60$, $P < 0.05$), but not to CD4⁺ T-cell count at the time of study.

Persistent systemic immune activation is a well established feature of HIV-1 infection that responds to HAART incompletely. Systemic immune activation has been shown to predict progressive HIV-1 disease [20]. To assess whether excessive PI-9 expression in monocytes from HIV-1-infected patients is related to augmented systemic immune activation, we assessed sCD14 in plasma samples from patients ($n = 13$) both at the time of nadir CD4⁺ T-cell count and at the time of study. HAART did not affect plasma sCD14 levels significantly [3384 ± 424 (nadir) compared with 3581 ± 254 (time of study), $P = 0.6$]. Interestingly, plasma sCD14 levels at the time of study correlated significantly to PI-9 levels in monocytes both at t0 ($R = 0.586$, $P < 0.05$; data not shown) and on day 1 (immediately prior to MTB infection; $R = 0.723$, $P < 0.01$; Fig. 2b).

Basis of excessive protease inhibitor 9 expression in monocytes from HIV-1-infected patients

To understand whether heightened PI-9 immunoreactivity in monocytes from HIV-1-infected patients was a result of reduced degradation of PI-9 protein, we assessed whether the kinetics of PI-9 protein decay were different in HIV-1-infected patients as compared with healthy individuals. For this purpose, monocytes were cultured in medium for up to 5 days. Monocyte lysates from different time points of culture (day 1, 3, 5) were assessed for PI-9 by ELISA. Intracellular PI-9 protein levels remained significantly higher in monocytes from HIV-infected patients as compared with healthy individuals at all time points: on day 1 ($P < 0.005$), on day 3 ($P < 0.05$), and on day 5 ($P < 0.02$; Fig. 3a). However, the rate of decay in PI-9 protein over time (calculated as percentage drop of PI-9 at each time point compared to previous time point) was not lower in monocytes from HIV-1-infected patients as compared with healthy individuals (data not shown).

We then assessed whether higher constitutive PI-9 protein levels in monocytes from HIV-1-infected patients may be based on higher transcriptional activity of PI-9. Therefore, we assessed PI-9 mRNA on day 1 (prior to MTB infection) in monocytes from a second cohort of HIV-1-infected patients ($n = 10$) and healthy individuals ($n = 10$). In these experiments, monocytes were prepared by adherence to tissue culture plates. Monocytes were cultured for 24 h in RPMI and 2% PHS, and then assessed for PI-9 mRNA expression. Results are shown in Fig. 3b.

At 24 h, levels of PI-9 mRNA were significantly higher in monocytes from HIV-1-infected individuals ($P < 0.05$). We also assessed TNF- α mRNA in the same samples. Although TNF- α mRNA was somewhat higher in HIV-1-infected ($6.85 \times 10^5 \pm 2.38$) as compared with healthy individuals ($1.3 \times 10^5 \pm 0.3$), these differences were not significant (data not shown). Expression of BcL-2 and BAX mRNA likewise were not different in samples from HIV-1-infected patients and those of healthy controls. Therefore, monocytes from HIV-1-infected patients displayed constitutively higher gene expression for PI-9, but not TNF- α , BcL-2, or BAX.

Inhibition of protease inhibitor 9 reduces intracellular growth of *Mycobacterium tuberculosis*

To understand the contribution of higher PI-9 mRNA and protein activity prior to MTB infection in monocytes from HIV-1-infected patients to higher mycobacterial replication subsequent to MTB infection, we used gene silencing methodologies to inhibit PI-9 expression. For this purpose, 24-h precultured monocytes from HIV-1-infected patients received PI-9-specific or control siRNA by passive transfection [13]. Twenty-four hours later, monocytes were washed and infected with MTB H37Rv at MOI of 0.1: 1 to 10: 1. Cultures were harvested 24 h and 7 days after MTB infection. Replication of MTB was quantified by assessment of MTB 16S rRNA and reported as MTB 16S rRNA_{d7/d1}. Figure 4a illustrates MTB H37Rv growth [MTB 16S rRNA (d7/d1)] at all MOIs of MTB H37Rv infection for one donor. PI9-specific siRNA reduced MTB H37Rv growth when compared with control siRNA at all MOIs of MTB. Results of infection by MTB H37Rv at MOI of 1: 1 for 10 patients are shown in Fig. 4b. In these experiments, MTB 16S rRNA expression in

monocytes transfected by control siRNA was assigned a value of 100%, and effects of PI-9 siRNA were calculated as a percentage of that. PI-9 siRNA reduced MTB 16S rRNA by $60\% \pm 12$ ($P < 0.001$).

***Mycobacterium tuberculosis*-induced apoptosis/antiapoptosis genes in monocytes from HIV-1-infected patients**

To understand whether differences in expression of pro (BAX and TNF) or antiapoptotic (BCL-2 and PI-9) genes induced by MTB H37Rv infection contributed to survival of MTB in infected monocytes, 24-h precultured monocytes from HIV-1-infected patients and healthy individuals were infected with MTB H37Rv (1: 1). Twenty-four hours later, cells were harvested and assessed for Bcl-2, PI-9, BAX, and TNF- α mRNA. Results are shown in Fig. 5. Expression of BAX, PI-9, and Bcl-2 mRNA in MTB-infected monocytes from HIV-infected patients was similar to that in healthy patients. Expression of TNF- α mRNA was significantly lower in HIV-1-infected patients ($13.5 \times 10^4 \pm 3.7$) as compared with healthy individuals ($61.6 \times 10^4 \pm 13.9$; $P < 0.003$). However, we did not find any correlation of TNF- α mRNA expression in MTB H37Rv-infected monocytes with MTB H37Rv replication (MTB 16S rRNA d7/d1) or d7 MTB 16S rRNA levels.

We found a high correlation between expression of PI-9 and Bcl-2 mRNA ($R = 0.728$, $P < 0.005$) in MTB-infected monocytes from HIV-1-infected patients (data not shown). However, there was no correlation of BCL-2 or PI-9 mRNA expression subsequent to MTB H37Rv infection with MTB replication (MTB 16S rRNA d7/d1) or d7 MTB 16S rRNA expression.

Discussion

It is well established that HIV-1-infected patients are at increased risk to develop uncontrolled MTB infection [1–4]. Likewise, multiple studies have demonstrated that MTB promotes its own intracellular growth by inhibition of apoptosis of infected mononuclear phagocytes [9,21,22]. Our prior assessment of the profile of genes expressed by human lung macrophages upon infection with MTB H37Rv identified a class of antiapoptotic genes, most significant among which is PI-9 [12]. Here we found elevated constitutive intracellular expression of PI-9, at both protein and mRNA levels, in monocytes from HIV-1-infected patients as compared with monocytes from healthy individuals. Increased intracellular PI-9 protein activity correlated with subsequent intracellular MTB replication on day 7 after MTB infection. Importantly, inhibition of PI-9 by specific siRNA inhibited MTB 16S rRNA expression. Heightened constitutive intracellular activity of PI-9 in monocytes from HIV-1-infected patients prior to MTB H37Rv infection may be one mechanism by which macrophages from HIV-1-infected patients provide a favorable environment for intracellular survival and growth of virulent MTB.

Others have shown that HIV-1 infection of MDM decreased their capacity to contain MTB [23]. In the current study, we found significantly higher MTB growth (MTB 16S rRNA d7/d1) upon MTB H37Rv infection of monocytes from HIV-1-positive patients as compared with healthy controls. There was no correlation of increased intracellular MTB replication in monocytes from HIV-1-infected patients with CD4⁺ T-cell count or viral load at the time of

study. Unexpectedly, however, there was a strong positive correlation between inability of monocytes to contain MTB H37Rv growth and higher nadir CD4⁺ T-cell counts. These data imply a sustained effect of HIV-1 infection prior to institution of HAART (documented by nadir CD4⁺ T-cell count) on the capacity of mononuclear phagocytes to contain the replication of virulent MTB. Further, they implicate a to date unidentified process of loss of capacity of monocytes to contain intracellular MTB growth, in addition to CD4⁺ T-cell immunodeficiency, in predisposition to TB. Presumably, at higher nadir CD4⁺ T-cell counts, the immunologic status of HIV-1 disease supports defective monocytes function with regard to MTB. These data are intriguing however need to be expanded to understand the full implication of excessive PI-9 expression by monocytes in pathogenesis of MTB infection during HIV-1 disease.

Lung macrophages from HIV-1-infected patients show impairment of apoptotic cell death upon exposure to heat-inactivated virulent MTB [10,11]. Here, significantly higher baseline expression of the antiapoptotic molecule PI-9 protein activity in monocytes from HIV-1-infected patients prior to in-vitro infection with MTB H37Rv was found. Levels of PI-9 at the time of MTB infection correlated with bacterial replication (at day 7). Silencing of PI-9 gene in monocytes from HIV-infected patients led to reduced growth of MTB (MTB 16S rRNA d7/d1). Interestingly, intracellular monocytes PI-9 levels correlated with systemic immune activation as assessed by plasma sCD14. Higher PI-9 protein activity in monocytes from HIV-1-infected patients was found to be because of heightened transcriptional activity of PI-9. Higher protein levels of PI-9 in uninfected monocytes from HIV-1-infected patients persisted over time for up to 5 days, which may implicate an underlying mechanism for successful spread and survival of MTB in uninfected monocytes in culture.

The pro-inflammatory cytokine IL-1 β has been shown to induce PI-9 mRNA through induction of NF- κ B and AP-1 sites in the promoter region of PI-9 gene [24]. Of note, PI-9 itself binds IL-1 converting enzyme (caspase-1) [25] and modifies IL-1 release and thereby IL-1-mediated inflammation. These studies suggest an autoregulatory circuit in which PI-9 downregulates its own expression through modulation of pro-inflammatory cytokines. Higher PI-9 activity both at mRNA and protein levels in MTB-uninfected monocytes from HIV-1-infected patients suggests that this autoregulatory circuit may be defective.

Excess intracellular expression of PI-9, however, may not be particular to HIV-1 infection alone, and rather shared with other viral infections. In a study of acutely ill children, PI-9 was induced in blood monocytes during infection with Epstein–Barr virus, but not with bacterial pathogens [26]. Other studies indicate that expression of PI-9 is induced by triggering of viral dsRNA sensors such as TLR-3 and retinoic acid-inducible gene-1 (RIG-1) [27]. In this latter study, viral infection by cytomegalovirus, Epstein–Barr virus, or BK virus was associated with increased PI-9 expression. Whether excess of PI-9 expression in monocytes from HIV-1-infected patients indicates activation of dsRNA sensors is not known.

In addition to its direct role in prevention of apoptosis of MTB-infected mononuclear phagocytes [13], PI-9 is the only serine protease inhibitor that inhibits Granzyme B (product of cytotoxic T cells and NK cells). This is presumably because of the fact that PI-9's

reactive center cleaves acidic residues [28]. Inactivation of Granzyme B by PI-9 prevents this component of cytotoxic lymphocyte granules to target cell DNA degradation, resulting in apoptosis. Data presented here suggest that monocytes from HIV-1-infected patients contain excess PI-9 and therefore are well equipped to neutralize Granzyme B effectively upon interaction with cytotoxic T cells and NK cells. This scenario is dire in promotion of MTB infection in HIV-1-infected patients, and needs to be evaluated more directly in future studies.

Subsequent to MTB H37Rv infection of monocytes from HIV-1-infected patients, expression of PI-9, Bcl-2, and BAX were similar to that in monocytes from healthy individuals. In confirmation of data from Patel *et al.* [10] however, significantly lower TNF- α was found in MTB-infected monocytes from HIV-1-infected patients. However, expression of TNF- α mRNA in MTB-infected monocytes from HIV-1-infected patients did not correlate with increase in growth of MTB on day 7. Whether low TNF expression after MTB infection of monocytes synergizes with high intracellular PI-9 activity to diminish apoptosis of MTB-infected macrophages needs to be studied further.

In summary, in this study, we found higher baseline levels of PI-9 protein and mRNA activity in monocytes from HIV-1-infected patients prior to infection by MTB H37Rv. PI-9 protein levels correlated with both growth of MTB in monocytes subsequent to infection, and with systemic immune activation. Inhibition of PI-9 expression by siRNA led to decreased MTB H37Rv replication in monocytes. Whether excessive constitutive PI-9 activity underlies successful intracellular opportunistic infections, such as TB, in HIV-1-infected patients needs to be further studied.

Acknowledgments

Z.T. wrote the study protocols, assessed all measurements, and wrote the article. M.W. performed all reverse transcription-PCR measurements. S.L. performed all PI-9 measurements. C.H. performed all statistical analyses, prepared figures, and was involved in all stages of article preparation. J.W. performed all infections with *Mycobacterium tuberculosis*. R.S. supervised all work with *M. tuberculosis* and assisted in protocol writing and article writing.

Funding for these studies was provided by NHLBI (HL-05636) and NIAID (AI 080313), VA Merit Review grant, and Center for AIDS Research at Case Western Reserve University (AI036219).

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Fig. 1. *Mycobacterium tuberculosis* growth in monocytes from HIV-1-infected patients and uninfected individuals

Monocytes from HIV-1-infected patients and uninfected healthy individuals were infected with *Mycobacterium tuberculosis* (MTB) H37Rv at multiplicity of infection (MOI) of 1: 1 (bacteria/cell). Monocyte cultures were harvested on day 1 (D1) and day 7 (D7), and assessed for MTB 16S rRNA by real-time PCR. MTB replication was calculated as fold increase in MTB 16S rRNA (D7/D1). (a) Mean fold increase in MTB H37Rv 16S rRNA in HIV-1-infected patients (HIV+; $n = 18$; left) was significantly higher than in HIV-1-uninfected healthy controls ($n = 18$; right) $P < 0.05$ (unpaired t -test). (b) Nadir CD4⁺ T-cell count was available from 13 HIV-1-infected patients. MTB replication calculated as fold increase in MTB 16S rRNA (D7/D1) correlated with nadir CD4⁺ T-cell count ($R = 0.731$, $P = 0.006$). HC, healthy control.

Fig. 2. Baseline protease inhibitor 9 protein activity in monocytes from HIV-1-infected patients and uninfected individuals

Monocytes from HIV-1-infected patients (HIV+) and uninfected healthy controls were assessed for protease inhibitor 9 (PI-9) by ELISA. (a) PI-9 protein activity at T0 (time of isolation from blood), and at day 1 (D1; i.e., immediately prior to MTB infection) are shown as a dot blot. (b) Correlation of systemic immune activation measured as plasma soluble (s) CD14 correlated with intracellular PI-9 on D1 ($R = 0.723$, $P = 0.005$) is shown. HC, healthy control.

Fig. 3. Basis of excessive protease inhibitor 9 expression in monocytes from HIV-1-infected patients and uninfected individuals

Monocytes from HIV-1-infected patients (HIV+) and uninfected healthy controls were cultured [1, 3, and 5 day (D)]. (a) Monocyte lysates prepared on D1-D7 were assessed for protease inhibitor 9 (PI-9) by ELISA. Median and SEM of PI-9 activity in monocytes are shown. (b) PI-9 mRNA activity in monocytes from HIV-1-infected patients and healthy controls was assessed at D1 by quantitative reverse transcription-PCR. Expression of PI-9 mRNA was over six-fold higher in HIV-1-infected patients (HIV+) as compared with healthy controls, $P < 0.05$. HC, healthy control.

Fig. 4. Inhibition of protease inhibitor 9 in monocytes from HIV-1-infected patients by short interfering RNA

Twenty four-hour cultured monocytes from HIV-1-infected patients were transfected with protease inhibitor 9 (PI-9) or control (Ctrl) short interfering RNA (siRNA). After 24 h, monocytes were infected with *Mycobacterium tuberculosis* (MTB) H37Rv. MTB 16S rRNA was assessed in cell lysates after 24 h and 7 days. MTB replication is shown as fold increase of MTB 16S rRNA (D7/D1). (a) Results are shown for MTB H37Rv multiplicity of infection (MOI) of 0.1: 1, 1: 1, and 10: 1 bacteria/cell for a single donor. (b) Results of infection with MTB H37Rv at MOI of 1: 1 is shown for 10 patients. For each patient, MTB 16S rRNA expression in monocytes transfected by control siRNA was assigned a value of 100%, and that of PI-9 siRNA was calculated as a percentage of it. As compared to monocytes transfected with control siRNA, PI-9 siRNA reduced MTB 16S rRNA activity by $60\% \pm 12$ ($P < 0.001$).

Fig. 5. Expression of anti-apoptosis (protease inhibitor 9, Bcl-2) and pro-apoptosis (BAX, tumor necrosis factor) mRNA in *Mycobacterium tuberculosis*-infected monocytes

Twenty four hours after isolation, monocytes from HIV-1-infected patients (HIV+) and healthy controls were infected with *Mycobacterium tuberculosis* (MTB) H37Rv at an MOI of 1: 1. Expression of mRNA for Bcl-2, protease inhibitor 9 (PI-9), Bax, and tumor necrosis factor a (TNF-α) were assessed 24 h after MTB H37Rv infection. TNF-α was significantly lower in MTB-infected monocytes from HIV-1-infected patients as compared with healthy controls ($P < 0.003$). HC, healthy controls