Common Variation in NLRP3 Is Associated With Early Death and Elevated Inflammasome Biomarkers Among Advanced HIV/TB Co-infected Patients in Botswana

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Background. Elevated inflammation is associated with early mortality among HIV/tuberculosis (TB) patients starting antiretroviral therapy (ART); however, the sources of immune activation are unclear. We hypothesized that common variation in innate immune genes contributes to excessive inflammation linked to death. As single nucleotide polymorphisms (SNPs) in inflammasome pathway genes can increase risk for inflammatory diseases, we investigated their association with early mortality among a previously described cohort of HIV/TB patients initiating ART in Botswana.

Methods. We genotyped 8 SNPs within 5 inflammasome pathway genes and determined their association with death. For adjusted analyses, we used a logistic regression model. For SNPs associated with mortality, we explored their relationship with levels of systemic inflammatory markers using a linear regression model.

Results. Ninety-four patients in the parent study had samples for genetic analysis. Of these, 82 (87%) were survivors and 12 (13%) died within 6 months of starting ART. In a logistic regression model, NLRP3 rs10754558 was independently associated with a 4.1-fold increased odds of death (95% confidence interval, 1.04–16.5). In adjusted linear regression models, the NLRP3 rs10754558-G allele was linked to elevated IL-18 at baseline (Beta, 0.23; SE, 0.10; P = .033) and week 4 post-ART (Beta, 0.24; SE, 0.11; P = .026). This allele was associated with increased MCP-1 at baseline (Beta, 0.24; SE, 0.10; P = .02) and IL-10 (Beta, 0.27; SE, 0.11; P = .013) at week 4 post-ART.

Conclusion. The NLRP3 rs10754558-G SNP is associated with an increased risk for early mortality in HIV/TB patients initiating ART. These patients may benefit from therapies that decrease inflammasome-mediated inflammation.

Keywords. genetic variation; HIV; inflammasome pathway; mortality; NLRP3; tuberculosis.

Tuberculosis (TB) is the leading cause of mortality in HIV-infected patients, with 35% of HIV deaths being attributed to TB in 2015 [1]. Earlier antiretroviral therapy (ART) initiation is associated with a significant survival benefit, particularly among advanced HIV/TB co-infected patients [2–4]. Yet, nearly 10%–20% of patients die in the initial months of starting ART (ie, early mortality) [5–9]. Clinical factors such as low body mass index, anemia, and low baseline CD4 count have been implicated as risks for death soon after ART initiation [5, 6]. Immunologically, elevated pre-ART levels of inflammatory biomarkers that rapidly increase in the absence of adaptive immune recovery following ART initiation have been linked to early mortality [10, 11]. The causes of high levels of immune activation and inflammation in these patients could include both HIV and TB, co-infections, and noninfectious comorbidities.

Common variation in host genes that modulate innate immunity could also lead to clinically important heterogeneity in inflammation and immune activation. The polymorphism in the leukotriene-A4 hydrolase (LTA4H) gene that regulates tumor necrosis factor–alpha (TNF-α) production has been associated with disease severity and treatment response in TB meningitis (TBM) patients [12]. This LTA4H variant also increased incidence of severe TB–immune reconstitution inflammatory syndrome (TB-IRIS), which is characterized by exuberant systemic inflammation, among HIV/TB co-infected patients in 1 study [13]. Other studies have demonstrated that interleukin (IL)-7 receptor alpha gene [14] and copy number variation in the CCL3L1 gene can influence ART-mediated immune recovery in HIV-infected patients [15, 16]. Several recent studies have specifically implicated the inflammasome pathway in driving TB- and TBM-associated IRIS [17–19], and polymorphisms...
in genes of this pathway have been linked to increased risk for infectious and autoimmune inflammatory diseases [20–22]. However, genetic variation in the inflammasome pathway has not been interrogated in the context of HIV/TB co-infected patients and risk of death.

The inflammasome pathway is of specific interest also because inflammasomes, such as NOD-like receptor pyrin containing-3 (NLRP3), recognize intracellular pathogens like MTB and trigger potent innate immune responses [23, 24]. Upon NLRP3 stimulation, a multiprotein inflammasome complex is formed and activates caspase-1 [23, 25]. Caspase-1 converts pro-IL-1β and pro-IL-18 to their active and highly inflammatory secreted forms [23, 25]. The inflammasome pathway is regulated by caspase recruitment domain-containing protein 8 (CARD8), which inhibits caspase-1 activity via a protein-protein interaction [26, 27]. From a therapeutic perspective, small molecules and monoclonal antibodies targeting various members of the inflammasome pathway are being developed to dampen inflammation in the setting of other diseases [28]. If indeed this pathway contributes to death in advanced HIV/TB co-infected patients, such therapies may be able to mitigate inflammation and possibly improve outcomes in these individuals.

In the present study, we investigated the association between single nucleotide polymorphisms (SNPs) in genes of the inflammasome pathway (NLRP3, CARD8, IL-1β, and P2X7R) and death, as well as their relationship with pro-inflammatory markers in advanced HIV/TB co-infected patients initiating ART. We utilized data from a previously well-characterized cohort of advanced HIV/TB patients to conduct this study [10, 11].

**METHODS**

**Ethics**

The Institutional Review Boards at the University of Pennsylvania and the Botswana Ministry of Health approved the targeted genotyping of SNPs listed in the DNA Extraction and Genotyping section, and to relate these specific SNPs to inflammation and adverse treatment outcomes among patients enrolled in the parent study [11].

**Study Design and Setting**

We conducted a secondary analysis of a prospective cohort study set in Gaborone, Botswana [10, 11]. HIV prevalence among adults in this setting is approximately 22%, while TB incidence is estimated at 356 cases per 100,000 [1, 29]. The aim of this study was to determine the association between common variation in genes of the inflammasome pathway (exposure) and the outcome of nontraumatic death within 6 months of ART initiation. We also explored the relationship between SNPs found to be associated with death and systemic levels of inflammatory biomarkers at baseline and week 4 post–ART initiation, given our previous studies indicating that excessive inflammation prior to and on ART is associated with early mortality [10, 11].

**Study Participants**

The parent study enrolled ART-naïve HIV-infected adults with a diagnosis of active pulmonary TB [10, 11]. TB diagnosis was based on being sputum smear–positive for acid-fast bacilli (AFB), GeneXpert MTB/RIF assay–positive (Cepheid), or meeting the World Health Organization criteria for AFB smear–negative pulmonary TB [30]. To be included in the parent study, patients had to have advanced HIV, which was defined in our study as having a pre-ART CD4 count ≤125 cells/μL [10, 11]. As ART is provided only to citizens of Botswana, this was an additional inclusion criterion. Given that the use of immunomodulatory agents, drug-resistant TB, and pregnancy could potentially alter relationships that were of interest, patients were excluded based on these factors. To qualify for this subanalysis, patients needed to have peripheral blood mononuclear cells (PBMCs) or whole blood available for DNA extraction, as well as an outcome at the end of the study.

**Data and Sample Collection**

Patients were followed monthly for 6 months post–ART initiation, as previously described [10, 11]. Clinical data collected as part of the parent study were extracted from the database for analysis [10, 11]. The main study also collected and cryopreserved whole blood and PBMCs [10, 11].

**DNA Extraction and Genotyping**

DNA was extracted from PBMCs or whole blood using the QIAamp DNA Blood Mini Kit (Qiagen). DNA quantity and quality were measured on the Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific). For genotyping, commercially available, validated TaqMan primers and probe sets (Life Technologies, ThermoFisher Scientific) were used to determine SNPs in CARD8 (rs2043211, rs6590365), NLRP3 (rs10925026, rs10754558), IL-1β (rs1946518, rs549908), IL-1β (rs1143627), and P2RX7 (rs2220911). Prior to genotyping, target SNP sequences were enriched by specific target amplification (STA) to improve call rates [31, 32]. This multiplex PCR reaction was conducted on a PTC-200 Thermal Cycler (Bio-Rad). Next, SNP genotyping was carried out on a Fluidigm 96.96 Dynamic Array Integrated Fluidic Circuit (IFC), as per the manufacturer’s instructions (Fluidigm Corporation). The genotyping PCR reactions were run in triplicate, with end point fluorescence measured on a BioMark HD System (Fluidigm Corporation). Outcomes were blinded during the genotyping process.

**Inflammatory Biomarker Concentration**

In this study, we specifically analyzed levels of IL-1β, IL-1Ra, IL-18, IL-10, TNF-α, monocyte chemoattractant protein (MCP)–1, and IL-6, as they are either secreted following inflammasome activation or have been previously linked to death [11, 23, 25]. The parent study determined levels of these biomarkers at baseline and week 4 post–ART initiation using the Luminex assay (EMD Millipore) [11]. As the main study did
not quantitate IL-18, here we used a commercially available enzyme-linked immunosorbent assay kit (R&D Systems) following a 1:5 or 1:10 plasma dilution to determine levels of this cytokine before and after ART initiation.

**Statistical Analysis**

The Fluidigm SNP Genotyping Analysis software was used to analyze the Dynamic Array reads and obtain genotype calls. If a patient had an indeterminate genotype call, the patient was excluded from the analysis specific to that genetic locus. The minor allele frequencies at each locus of interest were calculated. Observed genotype frequencies were compared with expected frequencies by \( \chi^2 \) tests to detect any deviation from the Hardy-Weinberg equilibrium. In the unadjusted primary analysis, the association between genotype (exposure) and early mortality (outcome) was determined assuming a dominant model of inheritance. For this analysis, we combined patients who were homozygous (mm) and heterozygous (Mm) for the minor allele and compared them with those who were homozygous for the major allele (MM) with respect to outcomes using \( \chi^2 \) tests. Our sample size was not sufficient to evaluate other modes of inheritance. SNPs associated with the outcome in the unadjusted analysis were further evaluated in a logistic regression model. Here, clinical factors associated with outcomes at \( P < .2 \) were considered potential confounders and tested in the model 1-by-1 to avoid overadjustment [33]. Variables that changed the unadjusted odds ratio (OR) by >10% were considered true confounders.

SNPs found to be associated with death in the adjusted logistic regression analysis were examined in relation to inflammatory biomarker levels pre- and post-ART initiation. Biomarker levels were \( \log_{10} \)-transformed for approximate normal distribution. Cytokine/chemokine levels in MM patients were compared with the Mm and mm patients using 2-sample \( t \) tests. We also used a linear regression model to evaluate this relationship and adjusted for baseline CD4 counts, pre-ART HIV viral load, and time between TB treatment and ART initiation. For linear regression, we report effect size as standardized coefficient (Beta) and standard error, which are in units of standard deviation [34]. We also report unstandardized coefficients with 95% confidence intervals (CIs), which are in units of \( \log_{10} \) pg/mL.

Associations with a \( P \) value of less than .05 were considered statistically significant. We did not correct for multiple comparisons, given the focused candidate pathway approach used in this study. Stata Software, version 14.0 (StataCorp), was used for analysis. GraphPad Prism, version 7.0c (GraphPad Software, Inc.), was used for graphical representations of biomarker data.

**RESULTS**

**Baseline Clinical Characteristics**

We have previously described the 170 advanced HIV/TB co-infected patients enrolled in the parent study [11]. Of these individuals, 94 had DNA for genotyping and outcome data to qualify for the subanalysis. Baseline clinical characteristics were similar, comparing patients who were included with those not included in this subanalysis (Supplementary Table 1). With respect to outcomes, 82 of 94 (87%) were survivors (controls), and 12 (13%) died within 6 months of starting ART. Median time to death (interquartile range [IQR]) was 49 (31–84) days. The median CD4 count and HIV viral load among those included (IQR) were 62 (30–94) cells/μL and 5.3 (4.9–6.1) \( \log_{10} \) copies/mL, respectively (Supplementary Table 1). Baseline clinical characteristics of patients stratified by outcome are shown in Supplementary Table 2. As in previously published work on this cohort [11], those who died were more likely to have initiated a nevirapine (NVP)-based ART regimen compared with survivors (\( P = .001 \)) (Supplementary Table 2). Overall, the clinical manifestation of TB was similar between those who died and those who survived. Four of 82 (5%) survivors vs 1 of 12 (8%) deaths had extrapulmonary TB (\( P = .62 \)). None of the patients were diagnosed with central nervous system–associated TB.

**SNPs in the Inflammasome Pathway and Association With Early Mortality**

Genotype frequencies associated with the 8 SNPs assessed were in Hardy-Weinberg equilibrium (\( P > .1 \)) (Table 1). One patient had an indeterminate genotype for each of the NLRP3 rs10754558 (n = 93) and rs10925026 (n = 93) SNPs and was excluded from the primary analysis for these loci. In the primary unadjusted analysis, we compared patients who were homozygous for the major allele with those who were heterozygous or homozygous for the minor allele with respect to the outcome of death within 6 months of starting ART. Only the NLRP3 rs10754558 SNP was associated with early mortality, as 9 (75%) of 12 deaths vs 34 (42%) of 81 controls were carriers of the minor allele (G; \( P = .032 \)) (Table 2). In an unadjusted logistic regression analysis, this polymorphism was associated with a 4.1-fold increased odds of early mortality (95% CI, 1.04–16.5) (Table 3). Adjusting for confounding factors, such as baseline HIV viral load, NVP-based ART, and non-TB opportunistic infections (OIs), improved the model fit. Specifically, NLRP3 rs10754558-G was associated with a 4.8-fold (95% CI, 1.1–20.3) increased odds of death after adjusting for baseline non-TB OI. Similarly, adjusting for pre-ART HIV viral load and NVP-based ART increased the odds of death by 4.9-fold (95% CI, 1.2–20.1) and 7.0-fold (95% CI, 1.4–34.9), respectively (Table 3). Under these conditions, point estimates for the ORs, as well as the lower limit of the 95% CI, were greater than 1. Female sex was collinear with NVP-based ART treatment in this cohort [11]. Thus, we did not adjust for this variable.

**NLRP3 rs10754558-G Allele and Inflammatory Biomarker Levels**

While we have demonstrated an association between excessive inflammatory cytokines and chemokines, including MCP-1, IL-10, and TNF-α, with early mortality in this cohort, we have not previously investigated the relationship between the
Table 1. Observed and Expected Genotype Frequencies Associated With Inflammasome SNPs of Interest Among Advanced HIV/TB Co-infected Patients Initiating ART

| Gene     | RS ID      | Major Allele | Minor Allele | Observed MAF % | MM, No. | Mm, No. | mm, No. | χ² | P Value |
|----------|------------|--------------|--------------|----------------|---------|---------|---------|-----|---------|
| CARD8    | rs2043211  | A            | T            | 16             | 67      | 24      | 3       |     |         |
| CARD8    | rs6509365  | A            | G            | 25             | 53      | 35      | 6       |     |         |
| NLRP3    | rs10925026 | A            | C            | 32             | 45      | 37      | 11      |     |         |
| NLRP3    | rs10754558 | C            | G            | 26             | 50      | 37      | 6       |     |         |
| IL18     | rs1946518  | G            | T            | 35             | 37      | 48      | 9       |     |         |
| IL18     | rs549906   | G            | T            | 9              | 77      | 17      | 0       |     |         |
| IL18     | rs1143627  | G            | A            | 32             | 46      | 36      | 12      |     |         |
| P2RX7    | rs2230911  | C            | G            | 19             | 62      | 29      | 3       |     |         |

Abbreviations: ART; antiretroviral therapy; m, minor allele; M, major allele; MAF, minor allele frequency; SNP, single nucleotide polymorphism; RS ID, reference SNP ID; TB, tuberculosis.

Table 2. Unadjusted Analysis of SNPs in Inflammasome Pathway Genes and Association With Early Mortality in Advanced HIV/TB Co-infected Patients Following ART Initiation

| Gene     | RS ID      | Survivor vs Death | P Value |
|----------|------------|--------------------|---------|
| CARD8    | rs2043211  | .71                |         |
| CARD8    | rs6509365  | .63                |         |
| NLRP3    | rs10925026 | .91                |         |
| NLRP3    | rs10754558 | .032               |         |
| IL18     | rs1946518  | .28                |         |
| IL18     | rs549908   | .89                |         |
| IL18     | rs1143627  | .19                |         |
| P2RX7    | rs2230911  | .55                |         |

P values are from χ² test comparing survivors (n = 82) with those who experienced early mortality (n = 12) within 6 months of starting antiretroviral therapy with respect to single nucleotide polymorphisms in indicated inflammasome pathway genes. Comparisons were carried out assuming a dominant model, where patients homozygous for the major allele were compared with those heterozygous or homozygous for the minor allele.

Abbreviations: ART, antiretroviral therapy; SNP, single nucleotide polymorphism; TB, tuberculosis.

Table 3. Expected and Observed Genotype Frequencies Associated With Inflammasome SNPs of Interest Among Advanced HIV/TB Co-infected Patients Initiating ART

| Gene     | RS ID      | Major Allele | Minor Allele | Observed Genotype | Expected Genotype | χ²a | P Value |
|----------|------------|--------------|--------------|-------------------|------------------|-----|---------|
| CARD8    | rs2043211  | A            | T            | 16                | 67               | 3   | .27     |
| CARD8    | rs6509365  | A            | G            | 25                | 53               | 3   | .21     |
| NLRP3    | rs10925026 | A            | C            | 32                | 45               | 11  | .043    |
| NLRP3    | rs10754558 | C            | G            | 26                | 50               | 6   | .007    |
| IL18     | rs1946518  | G            | T            | 35                | 37               | 9   | .035    |
| IL18     | rs549906   | G            | T            | 9                 | 77               | 0   | .007    |
| IL18     | rs1143627  | G            | A            | 32                | 46               | 12  | .035    |
| P2RX7    | rs2230911  | C            | G            | 19                | 62               | 3   | .035    |

Abbreviations: ART, antiretroviral therapy; m, minor allele; M, major allele; MAF, minor allele frequency; SNP, single nucleotide polymorphism; RS ID, reference SNP ID; TB, tuberculosis.

As the NLRP3 rs10754558-G allele was also associated with early mortality, we next explored the relationship between this variant and levels of biomarkers that are downstream of the inflammasome pathway and those linked to death in this cohort [11]. This SNP was linked to elevated IL-18 (Beta, 0.21; SE, 0.10; P = 0.043) and IL-10 (Beta, 0.28; SE, 0.10; P = 0.007) levels at week 4 post–ART initiation. The model fits improved after adjusting for baseline CD4 count, HIV viral load, MCP-1 levels at baseline (Beta, 0.24; SE, 0.10; P = 0.013) (Figure 1B). As the NLRP3 rs10754558-G allele was also associated with early mortality, we next explored the relationship between this variant and levels of biomarkers that are downstream of the inflammasome pathway and those linked to death in this cohort [11]. This SNP was linked to elevated IL-18 (Beta, 0.21; SE, 0.10; P = 0.043) and IL-10 (Beta, 0.28; SE, 0.10; P = 0.007) levels at week 4 post–ART initiation. The model fits improved after adjusting for baseline CD4 count, HIV viral load, and time between TB treatment and ART initiation in the regression analysis of NLRP3 rs10754558-G allele and pre–ART (Beta, 0.23; SE, 0.10; P = 0.033) and post–ART (Beta, 0.24; SE, 0.11; P = 0.026) IL-18 levels (Table 4). Including these variables similarly improved the model fit in the regression analysis between the SNP and MCP-1 levels at baseline (Beta, 0.24; SE, 0.10; P = 0.02). The relationship between the SNP and IL-10 levels at week 4 post-ART persisted after adjusting for these factors (Beta, 0.27; SE, 0.11; P = 0.013). Table 4 also shows unstandardized coefficients with 95% CIs for the above relationships.

DISCUSSION

In this study, variation in the NLRP3 rs10754558 was independently associated with early mortality in HIV/TB co-infected patients initiating ART. Additionally, the genotype at NLRP3 rs10754558 influenced the extent of systemic inflammation as assessed by IL-18, MCP-1, and IL-10 levels pre– and/ or post–ART initiation in these patients.

We found that elevated IL-18 levels at week 4 post-ART were associated with early mortality. This is consistent with a recent
study demonstrating that persistently high levels of IL-18 are associated with clinical failure despite ART initiation in HIV-infected patients (20% of whom also had active TB) [35]. Notably, the NLRP3 rs10754558-G allele was also linked to elevated IL-18 levels at week 4 post–ART initiation in our cohort. Taken together, these data suggest that the SNP at the NLRP3 rs10754558 locus may modulate inflammasome activation and contribute to adverse inflammatory outcomes. Increased levels of IL-10 following ART initiation among the CG/GG NLRP3 rs10754558 patients also implicate excessive inflammasome stimulation. IL-10 is an anti-inflammatory cytokine [36] and has been identified as an inhibitor of NLRP3 activation [37]. Several studies have demonstrated that IL-10 secretion increases after the inflammasome pathway is triggered, perhaps as a way to downregulate hyperinflammation [37–40]. Based on these studies, we hypothesize that elevated IL-10 levels following 4 weeks of ART may partly be in response to exaggerated inflammasome activity, especially in the HIV/TB patients carrying the NLRP3 rs10754558-G allele. ART may have also aided in restoring this physiologic response to persistent inflammasome activation.

Carriers of the NLRP3 rs10754558-G allele also had significantly elevated MCP-1 levels compared with the CC patients prior to ART initiation. In the context of TB, MCP-1 can prime monocyte trafficking to the site of infection, further perpetuating immune activation [41]. Moreover, we have previously demonstrated that elevated pre-ART MCP-1 is independently associated with early mortality in this cohort [11]. Of note, MCP-1 is induced following inflammasome activation [42–44]. Specifically,

### Table 3. Logistic Regression Analysis of the Association Between NLRP3 rs10754558 and Early Mortality in Advanced HIV/TB Co-infected Patients Initiating ART

| Gene   | RS ID (Exposure) | Outcome | Factors | Odds Ratio (95% CI) | P Value |
|--------|------------------|---------|---------|---------------------|---------|
| NLRP3  | rs10754558       | Death   | Base model | 4.1 (1.04–16.5)    | .043    |
|        |                  |         | Baseline CD4 | 4.3 (1.1–17.1)    | .041    |
|        |                  |         | TB smear/Xpert status | 4.0 (1.0–16.1)    | .051    |
|        |                  |         | ATT-Art interval | 4.2 (1.0–17.7)    | .049    |
|        |                  |         | Baseline OI | 4.8 (1.1–20.3)b | .032    |
|        |                  |         | Baseline HIV/VL | 4.9 (1.2–20.1)b | .029    |
|        |                  |         | NVP       | 70 (1.4–34.9)b     | .018    |

Abbreviations: ART, antiretroviral therapy; ATT, antituberculosis therapy; CI, confidence interval; NVP, nevirapine based ART regimen; OI, opportunistic infection; RS ID, reference SNP ID; TB, tuberculosis; VL, viral load; Xpert, GeneXpert.

aPotential confounders.

bOdds ratio changed >10% after including this variable in the model.

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Figure 1. Advanced HIV/tuberculosis (TB) co-infected patients carrying the minor allele (G) at the rs10754558 locus in NLRP3 have elevated levels of inflammatory cytokines consistent with inflammasome activation. Levels of circulating cytokines and chemokines that are expressed downstream of the inflammasome pathway or previously associated with early death in this cohort (A) pre–antiretroviral therapy (ART; baseline) and (B) at week 4 post–ART initiation were compared between patients with the CC genotype (gray) and those with the CG or GG genotype (pink) at the rs10754558 locus in NLRP3. Inflammatory markers were measured using plasma from patients and quantitated by Luminex. Shown are the mean levels of log-transformed cytokines/chemokines, with errors bars indicating standard deviation. P values correspond to 2-sample t tests comparing inflammatory biomarker levels between the 2 groups.

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Table 4. Linear Regression Analysis of the Association Between the NLRP3 rs10754558 SNP and IL-18, MCP-1, and IL-10 Levels in HIV/TB Co-infected Patients Initiating ART

| Time Point          | Mean (SD), log10 pg/mL | Standardized Coefficient (SD) | Unstandardized Coefficient, log10 pg/mL | R² | P     | Standardized Coefficient (SD)a | Unstandardized Coefficient, log10 pg/mL a | R²a | P      |
|---------------------|------------------------|------------------------------|-----------------------------------------|-----|-------|------------------------------|------------------------------------------|-----|--------|
|                      |                        | Beta | SE  | Coeff | 95% CI | Beta | SE  | Coeff | 95% CI | Beta | SE  | Coeff | 95% CI |
| IL-10 Pre-ART        | 1.26 (0.45)            | 0.14 | 0.10 | 0.13 | -0.06 to 0.31 | 0.021 | 0.168 | 0.15 | 0.11 | 0.14 | -0.06 to 0.33 | 0.035 | 0.166 |
| (93, 91)             |                        |      |     |      |         |      |     |      |         |      |     |      |         |
| MCP-1 Pre-ART        | 2.74 (0.35)            | 0.22 | 0.10 | 0.15 | 0.01 to 0.30 | 0.035 | 0.048 | 0.24 | 0.10 | 0.17 | 0.03 to 0.32 | 0.10 | 0.20  |
| (93, 91)             |                        |      |     |      |         |      |     |      |         |      |     |      |         |
| Week 4 post-ART (90, 88)a | 3.05 (0.33) | 0.20 | 0.10 | 0.13 | 0 to 0.27 | 0.039 | 0.058 | 0.23 | 0.10 | 0.15 | 0.01 to 0.29 | 0.086 | 0.033 |
|                      |                        |      |     |      |         |      |     |      |         |      |     |      |         |
|                      |                        |      |     |      |         |      |     |      |         |      |     |      |         |

Overall mean (standard deviation) IL-18, MCP-1, and IL10, before and after ART initiation, are shown for the cohort. Two patients were missing HIV viral loads at week 4 post-ART initiation. Cytokine levels were log10-transformed for approximate normalization for linear regression model.

Abbreviations: ART, antiretroviral therapy; Beta, standardized beta coefficient; CI, confidence interval; Coeff, unstandardized coefficient; n, number of patients with complete data for adjusted analysis; SNP, single nucleotide polymorphism; TB, tuberculosis.

aAdjusted for CD4 count, HIV viral load, and time between antituberculosis therapy and ART initiation.

IL-18 can upregulate MCP-1 production in macrophages via the PI3K/Akt and MEK/ERK1/2 pathways [44]. This IL-18–mediated induction of MCP-1 was observed to be independent of auto-crine IFN-γ and TNF-α [44]. Another mechanistic study using a murine model for experimental autoimmune encephalomyelitis demonstrated that IL-1β and IL-18 increase expression of chemokines such as MCP-1 by antigen-presenting cells and their cognate receptors on T cells [43]. These events are critical for orchestrating immune cell chemotaxis. Patients carrying the NLRP3 rs10754558-G allele in our study also tended to have elevated IL-18 prior to ART. Although this relationship was statistically significant at baseline only after adjusting for potential confounders in a linear regression model, it is conceivable that elevated IL-18 levels triggered greater MCP-1 production, especially among those harboring the NLRP3 rs10754558-G allele.

IL-1β is also a potent inflammasome maturing cytokine; however, we did not find an association between IL-1β and NLRP3 rs10754558. This may, in part, be explained by differences in the regulation of IL-1β and IL-18 expression. IL-18 has been shown to be constitutively expressed and exist as pro-IL-18 in human PBMCs [45]. In contrast, IL-1β is not constitutively expressed [45]. Furthermore, a recent study in mice demonstrated that IL-18 expression is induced and elevated levels of IL-18 are maintained following chronic simulation [46]. In contrast, IL-1β expression decreased after reaching peak expression during chronic stimulation [46]. It is possible that we did not observe an association between IL-1β and the NLRP3 rs10754558 SNP due the highly dynamic nature of IL-1β expression.

A major limitation of our study was that we were unable to evaluate the functional consequences of the G allele at the NLRP3 rs10754558 locus. Nevertheless, one study demonstrated that this 3′-untranslated region (UTR) SNP increases NLRP3 mRNA stability [47]. In in vitro assays, the NLRP3 rs10754558-G allele–containing construct had 1.3-fold higher activity than the C allele construct [47]. Consistently, NLRP3 mRNA expression was elevated by 1.3- and nearly 2-fold in PBMCs isolated from CG and GG patients, respectively, compared with CC patients [48]. Considering these findings, it is plausible that HIV/TB patients harboring the gain-of-function G allele had persistent NLRP3 mRNA that was available for activation by MTB. Chronic NLRP3 expression and stimulation by this mechanism perhaps explains the relationship between the NLRP3 rs10754558-G allele and association with elevated IL-18, IL-10, and MCP-1 levels pre- and/or post-ART initiation.

As several factors likely play a role in driving excessive inflammation and early mortality in advanced HIV/TB, dissecting the individual contribution of each variable can be challenging. While the relatively low number of events in our study precluded us from adjusting for all confounding variables in a single logistic regression model [49], our data suggest that the NLRP3 rs10754558 variant may have an effect that is independent of obvious known confounders, including baseline CD4 count and time between initiation of TB treatment and ART; both of which have been previously linked to increased risk of death [50, 51]. NVP was another confounding variable in this study, as in our previous reports [10, 11]. Recently, NVP-derived reactive metabolites have been implicated in inducing cellular damage and subsequent release of damage-associated molecular patterns, which in turn can activate inflammasomes [52, 53]. Although we do not know how nevirapine-based ART may be related to risk of death in our cohort, adjusting for NVP in the logistic regression model increased the odds of mortality from 4- to 7-fold.
Other possible sources of inflammasome activation in advanced HIV/TB co-infected patients include non-TB opportunistic pathogens and HIV-1 [23, 54]. Furthermore, nearly half those who survived and a third of those who died in the parent study did not have samples available for genotyping, as PBMCs from these patients were used for other assays [11, 55]. Missing data, particularly from sicker patients, may have biased our effect size estimates. Additionally, patients may have had undiagnosed OIs at baseline or after ART initiation that we did not account for in our analyses. Although the NLRP3 rs10754558 SNP explained only a small proportion of the variability in IL-18, MCP-1, and IL-10 levels, our observations are overall consistent with genome-wide association studies that have investigated the relationship between SNPs and systemic levels of inflammatory biomarkers in other disease states [34, 56]. The small number of deaths available for analysis was perhaps the greatest limitation of this study. Thus, we urge future studies to evaluate the associations presented here in larger cohorts and determine if hyper-activation of the inflammasome pathway, from both a genetic and nongenetic standpoint, contributes to death among HIV/TB co-infected patients initiating ART.

In conclusion, we provide preliminary evidence for immunogenetic variation in modulating inflammatory response and contributing to adverse treatment outcomes in HIV/TB co-infected patients initiating ART. This study demonstrates a role for the inflammasome pathway in driving immune activation in advanced HIV/TB patients. In light of our observations, small molecules and monoclonal antibodies targeting various mediators of the inflammasome pathway [57] may have potential as adjunctive host-directed therapy in advanced HIV/TB co-infected patients to decrease excessive systemic inflammation and prevent disease progression.

**Supplementary Data**

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

**Acknowledgments**

*Financial support.* This study was supported by the National Institutes of Health (grant number R01AI080337), the National Center for Advancing Translational Sciences of the National Institutes of Health (grant number KL2TR001879), and the Penn Center for AIDS Research (grant number R01AI080337), the National Center for Advancing Translational Sciences of the National Institutes of Health (grant number R01AI080337). We are grateful to Dr. Caroline J. Rinaldo, MD, PhD, for her advice on the manuscript.

*Potential conflicts of interest.* All authors have reported conflicts of interest. 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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