Open Forum Infectious Diseases

MAJOR ARTICLE

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

Shruthi Ravimohan,[1,2] Kebatshabile Nfanyana,[2] Neo Tamuhla,[2] Caroline T. Tiemessen,[3] Drew Weissman,[1,2] and Gregory P. Bisson[1,2,4]

[1]Division of Infectious Diseases, Department of Medicine, and [4]Department of Biostatistics, Epidemiology, and Informatics, Center for Clinical Epidemiology and Biostatistics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania; [2]Botswana-UPenn Partnership, Gaborone, Botswana; [3]Centre for HIV-1 and STIs, National Institute for Communicable Diseases, and Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

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

Received 2 January 2018; editorial decision 4 April 2018; accepted 6 April 2018.

Correspondence: S. Ravimohan, PhD, University of Pennsylvania, Perelman School of Medicine, 423 Guardian Drive, 529 Blockley Hall, Philadelphia, PA 19104 (shruthir@pennmedicine.upenn.edu).

Open Forum Infectious Diseases®

DOI: 10.1093/ofid/ofy075

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-18*, *IL-1*β, 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, rs6509365), *NLRP3* (rs10925026, rs10754558), *IL-18* (rs1946518, rs549908), *IL-1*β (rs1143627), and *P2RX7* (rs2230911). 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 χ^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 χ^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, %	Observed Genotype			Expected Genotype			χ^{2a}	*P* Value
					MM, No.	Mm, No.	mm, No.	MM, No.	Mm, No.	mm, No.		
CARD8	rs2043211	A	T	16	67	24	3	66.4	25.2	2.4	0.22	.64
CARD8	rs6509365	A	G	25	53	35	6	52.9	35.3	5.9	0	1.0
NLRP3	rs10925026	A	C	32	45	37	11	43.4	40.3	9.4	0.62	.43
NLRP3	rs10754558	C	G	26	50	37	6	50.5	36.1	6.5	0.02	.89
IL18	rs1946518	G	T	35	37	48	9	39.6	42.8	11.6	1.37	.24
IL18	rs549908	T	G	9	77	17	0	77.8	15.5	0.8	0.93	.33
IL1B	rs1143627	G	A	32	46	36	12	43.6	40.9	9.6	1.33	.25
P2RX7	rs2230911	C	G	19	62	29	3	62.3	28.5	3.3	0.03	.86

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

[a]Expected and observed frequencies of genotypes did not deviate significantly from Hardy-Weinberg equilibrium. One patient had an indeterminate genotype for each of the *NLRP3* rs10754558 (n = 93) and rs10925026 (n = 93) SNPs and was excluded from the analysis for those specific SNPs.

inflammasome maturing cytokine IL-18 and death [11]. In unadjusted analysis, IL-18 levels at baseline were not related to death ($P = .25$). However, those who died had significantly higher levels of IL-18 levels vs survivors at week 4 post-ART ($P = .04$) (Supplementary Table 3). In logistic regression analysis, pre-ART levels of this inflammasome cytokine were not associated with death (odds ratio [OR], 2.8; 95% CI, 0.49–16.0). In contrast, elevated IL-18 levels at week 4 post-ART increased the odds of death by 9.1-fold (95% CI, 1.03–80.1), after adjusting for pre-ART HIV viral load, NVP-based ART, and baseline non-TB OI.

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]. CG/GG patients had significantly elevated levels of MCP-1 (mean [SD], 2.82 [0.23] $\log_{10}$ pg/mL) vs the CC patients (mean [SD], 2.67 [0.42] $\log_{10}$ pg/mL; $P = .035$) (Figure 1A) before ART initiation. Pre-ART IL-18 levels tended to be slightly higher among the CG/GG patients (mean [SD], 3.13 [0.33] $\log_{10}$ pg/mL) compared with the CC patients (mean [SD], 2.9 [0.34] $\log_{10}$ pg/mL; $P = .058$) (Figure 1A). Following 4 weeks of ART, CG/GG patients had significantly elevated IL-18 (mean [SD], 3.12 [0.41] $\log_{10}$ pg/mL) compared with CC patients (mean [SD], 2.95 [0.34] $\log_{10}$ pg/mL; $P = .043$) (Figure 1B). CG/GG patients also had elevated levels of IL-10 (mean [SD], 1.47 [0.48] $\log_{10}$ pg/mL) compared with CC patients (mean [SD], 1.2 [0.44] $\log_{10}$ pg/mL; $P = .008$) (Figure 1B).

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
IL1B	rs1143627	.19
P2RX7	rs2230911	.55

P values are from χ^2 test comparing survivors (n = 82) with those who experienced early morality (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.

In a linear regression model, carriage of the *NLRP3* rs10754558-G allele was associated with elevated MCP-1 prior to ART initiation (Beta, 0.22; SE, 0.10; $P = .048$) (Table 4). This SNP was linked to elevated IL-18 (Beta, 0.21; SE, 0.10; $P = .043$) and IL-10 (Beta, 0.28; SE, 0.10; $P = .007$) 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; $P = .033$) and post-ART (Beta, 0.24; SE, 0.11; $P = .026$) 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; $P = .02$). 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; $P = .013$). 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

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[a]	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	7.0 (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.

[a]Potential confounders.

[b]Odds ratio changed >10% after including this variable in the model.

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,

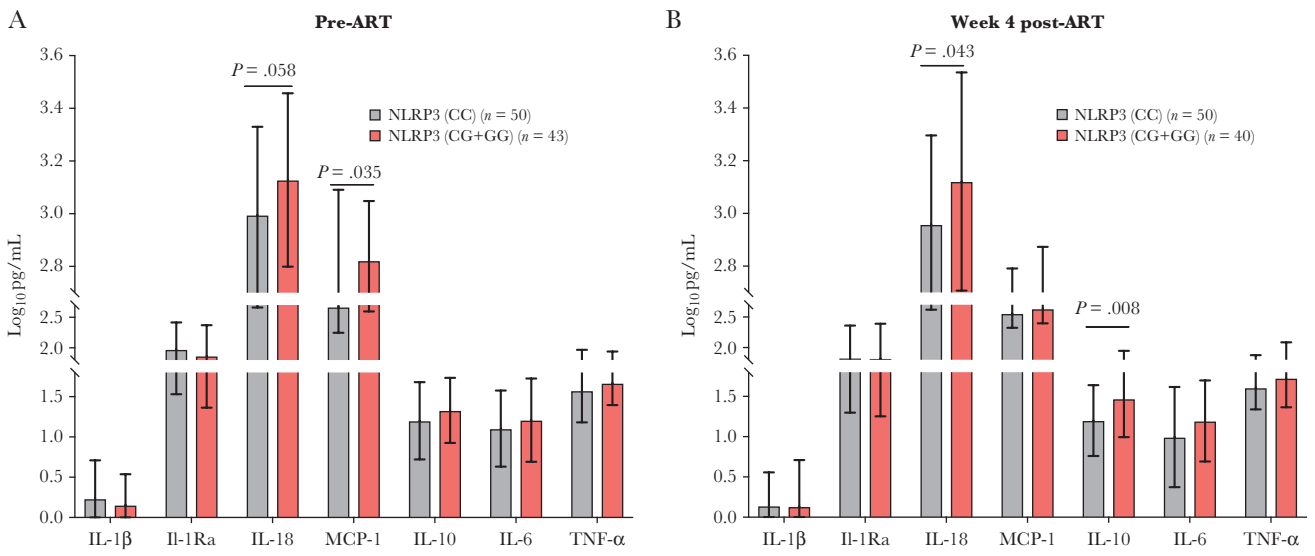

Figure 1. Advanced HIV/tuberculosis (TB) co-infected patients carrying the minor allele (G) at the rs10754558 locus in *NLRP3* have elevated levels of inflammatory cytokines consistent with inflammasome activation. Levels of circulating cytokines and chemokines that are expressed downstream of the inflammasome 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 $\log_{10}$-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.

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 (n, n[a])	Mean (SD), log_{10} pg/mL	Standardized Coefficient (SD)		Unstandardized Coefficient, log_{10} pg/mL				Standardized Coefficient (SD)[a]		Unstandardized Coefficient, log_{10} pg/mL[a]			
			Beta	SE	Coeff	95% CI	R^2	*P*	Beta[a]	SE[a]	Coeff[a]	95% CI[a]	R^{2a}	P^a
IL-18	Pre-ART (93, 91[a])	3.05 (0.33)	0.20	0.10	0.13	0 to 0.27	0.039	.058	0.23	0.10	0.15	0.01 to 0.29	0.086	.033
	Week 4 post-ART (90, 88[a])	3.03 (0.38)	0.21	0.10	0.16	0.01 to 0.32	0.046	.043	0.24	0.11	0.18	0.02 to 0.35	0.111	.026
MCP-1	Pre-ART (93, 91[a])	2.74 (0.35)	0.22	0.10	0.15	0.01 to 0.30	0.035	.048	0.24	0.10	0.17	0.03 to 0.32	0.10	.020
	Week 4 post-ART (90, 88[a])	2.59 (0.23)	0.16	0.11	0.08	–0.02 to 0.18	0.027	.122	0.18	0.11	0.08	–0.02 to 0.18	0.076	.107
IL-10	Pre-ART (93, 91[a])	1.26 (0.45)	0.14	0.10	0.13	–0.06 to 0.31	0.021	.168	0.15	0.11	0.14	–0.06 to 0.33	0.035	.166
	Week 4 post-ART (90, 88[a])	1.32 (0.47)	0.28	0.10	0.26	0.07 to 0.46	0.079	.007	0.27	0.11	0.25	0.05 to 0.45	0.112	.013

Overall mean (standard deviation) IL-18, MCP-1, and IL-10, 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 log_{10}-transformed for approximate normalization for linear regression model.

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

[a]Adjusted 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 autocrine 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 hyperactivation 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 P30 AI045008).

Potential conflicts of interest. All authors: no 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.

References

1. WHO. Global Tuberculosis Report 2016. Geneva, Switzerland: WHO Press, World Health Organization. http://apps.who.int/medicinedocs/documents/s23098en/s23098en.pdf.
2. Abdool Karim SS, Naidoo K, Grobler A, et al. Timing of initiation of antiretroviral drugs during tuberculosis therapy. N Engl J Med **2010**; 362:697–706.
3. Havlir DV, Kendall MA, Ive P, et al; AIDS Clinical Trials Group Study A5221. Timing of antiretroviral therapy for HIV-1 infection and tuberculosis. N Engl J Med **2011**; 365:1482–91.
4. Uthman OA, Okwundu C, Gbenga K, et al. Optimal timing of antiretroviral therapy initiation for HIV-infected adults with newly diagnosed pulmonary tuberculosis: a systematic review and meta-analysis. Ann Intern Med **2015**; 163:32–9.
5. Gupta A, Nadkarni G, Yang WT, et al. Early mortality in adults initiating antiretroviral therapy (ART) in low- and middle-income countries (LMIC): a systematic review and meta-analysis. PLoS One **2011**; 6:e28691.
6. Bisson GP, Ramchandani R, Miyahara S, et al. Risk factors for early mortality on antiretroviral therapy in advanced HIV-infected adults. AIDS **2017**; 31:2217–25.
7. Lawn SD, Harries AD, Anglaret X, et al. Early mortality among adults accessing antiretroviral treatment programmes in sub-Saharan Africa. AIDS **2008**; 22:1897–908.
8. Odone A, Amadasi S, White RG, et al. The impact of antiretroviral therapy on mortality in HIV positive people during tuberculosis treatment: a systematic review and meta-analysis. PLoS One **2014**; 9:e112017.
9. Janssen S, Schutz C, Ward A, et al. Mortality in severe human immunodeficiency virus-tuberculosis associates with innate immune activation and dysfunction of monocytes. Clin Infect Dis **2017**; 65:73–82.
10. Ravimohan S, Tamuhla N, Steenhoff AP, et al. Early immunologic failure is associated with early mortality among advanced HIV-infected adults initiating antiretroviral therapy with active tuberculosis. J Infect Dis **2013**; 208:1784–93.
11. Ravimohan S, Tamuhla N, Steenhoff AP, et al. Immunological profiling of tuberculosis-associated immune reconstitution inflammatory syndrome and non-immune reconstitution inflammatory syndrome death in HIV-infected adults with pulmonary tuberculosis starting antiretroviral therapy: a prospective observational cohort study. Lancet Infect Dis **2015**; 15:429–38.
12. Tobin DM, Vary JC Jr, Ray JP, et al. The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. Cell **2010**; 140:717–30.
13. Narendran G, Kavitha D, Karunaianantham R, et al. Role of LTA4H polymorphism in tuberculosis-associated immune reconstitution inflammatory syndrome occurrence and clinical severity in patients infected with HIV. PLoS One **2016**; 11:e0163298.
14. Rajasuriar R, Booth D, Solomon A, et al. Biological determinants of immune reconstitution in HIV-infected patients receiving antiretroviral therapy: the role of interleukin 7 and interleukin 7 receptor α and microbial translocation. J Infect Dis **2010**; 202:1254–64.
15. Ahuja SK, Kulkarni H, Catano G, et al. CCL3L1-CCR5 genotype influences durability of immune recovery during antiretroviral therapy of HIV-1-infected individuals. Nat Med **2008**; 14:413–20.
16. Shalekoff S, Meddows-Taylor S, Schramm DB, et al. Host CCL3L1 gene copy number in relation to HIV-1-specific CD4+ and CD8+ T-cell responses and viral load in South African women. J Acquir Immune Defic Syndr **2008**; 48:245–54.
17. Lai RP, Meintjes G, Wilkinson KA, et al. HIV-tuberculosis-associated immune reconstitution inflammatory syndrome is characterized by Toll-like receptor and inflammasome signalling. Nat Commun **2015**; 6:8451.
18. Tan HY, Yong YK, Andrade BB, et al. Plasma interleukin-18 levels are a biomarker of innate immune responses that predict and characterize tuberculosis-associated immune reconstitution inflammatory syndrome. AIDS **2015**; 29:421–31.
19. Marais S, Lai RPJ, Wilkinson KA, et al. Inflammasome activation underlying central nervous system deterioration in HIV-associated tuberculosis. J Infect Dis **2017**; 215:677–86.
20. Verma D, Lerm M, Blomgran Julinder R, et al. Gene polymorphisms in the NALP3 inflammasome are associated with interleukin-1 production and severe inflammation: relation to common inflammatory diseases? Arthritis Rheum **2008**; 58:888–94.
21. Asfaw Idosa B, Sahdo B, Balcha E, et al. C10X polymorphism in the CARD8 gene is associated with bacteraemia. Immun Inflamm Dis **2014**; 2:13–20.
22. Ko DC, Shukla KP, Fong C, et al. A genome-wide in vitro bacterial-infection screen reveals human variation in the host response associated with inflammatory disease. Am J Hum Genet **2009**; 85:214–27.
23. Gross O, Thomas CJ, Guarda G, Tschopp J. The inflammasome: an integrated view. Immunol Rev **2011**; 243:136–51.
24. Mishra BB, Moura-Alves P, Sonawane A, et al. *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell Microbiol **2010**; 12:1046–63.
25. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell **2002**; 10:417–26.
26. Razmara M, Srinivasula SM, Wang L, et al. CARD-8 protein, a new CARD family member that regulates caspase-1 activation and apoptosis. J Biol Chem **2002**; 277:13952–8.

27. Agostini L, Martinon F, Burns K, et al. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity **2004**; 20:319–25.
28. Coll RC, Robertson AA, Chae JJ, et al. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. Nat Med **2015**; 21:248–55.
29. UNAIDS. HIV and AIDS Estimates. **2015**. Geneva, Switzerland: UNAIDS. http://www.unaids.org/en/regionscountries/countries/botswana/.
30. WHO. *Treatment of Tuberculosis: Guidelines*. 4th ed. **2010**. http://whqlibdoc.who.int/publications/2010/9789241547833_eng.pdf.
31. Qin J, Jones RC, Ramakrishnan R. Studying copy number variations using a nanofluidic platform. Nucleic Acids Res **2008**; 36:e116.
32. Wang J, Lin M, Crenshaw A, et al. High-throughput single nucleotide polymorphism genotyping using nanofluidic dynamic arrays. BMC Genomics **2009**; 10:561.
33. Cepeda MS, Boston R, Farrar JT, Strom BL. Comparison of logistic regression versus propensity score when the number of events is low and there are multiple confounders. Am J Epidemiol **2003**; 158:280–7.
34. Zeller T, Haase T, Müller C, et al. Molecular characterization of the NLRC4 expression in relation to interleukin-18 levels. Circ Cardiovasc Genet **2015**; 8:717–26.
35. Balagopal A, Gupte N, Shivakoti R, et al. Continued elevation of interleukin-18 and interferon-γ after initiation of antiretroviral therapy and clinical failure in a diverse multicountry human immunodeficiency virus cohort. Open Forum Infect Dis **2016**; 3:ofw118.
36. Grütz G. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. J Leukoc Biol **2005**; 77:3–15.
37. Gurung P, Li B, Subbarao Malireddi RK, et al. Chronic TLR stimulation controls NLRP3 inflammasome activation through IL-10 mediated regulation of NLRP3 expression and caspase-8 activation. Sci Rep **2015**; 5:14488.
38. Yao Y, Vent-Schmidt J, McGeough MD, et al. Tr1 cells, but not Foxp3+ regulatory T cells, suppress NLRP3 inflammasome activation via an IL-10-dependent mechanism. J Immunol **2015**; 195:488–97.
39. Barranco C. Experimental arthritis: link between IL-10 and activation of the NLRP3 inflammasome in the synovium. Nat Rev Rheumatol **2014**; 10:576.
40. Greenhill CJ, Jones GW, Nowell MA, et al. Interleukin-10 regulates the inflammasome-driven augmentation of inflammatory arthritis and joint destruction. Arthritis Res Ther **2014**; 16:419.
41. Ansari AW, Kamarulzaman A, Schmidt RE. Multifaceted impact of host C-C chemokine CCL2 in the immuno-pathogenesis of HIV-1/M. Tuberculosis co-infection. Front Immunol **2013**; 4:312.
42. Petrasek J, Bala S, Csak T, et al. IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. J Clin Invest **2012**; 122:3476–89.
43. Inoue M, Williams KL, Gunn MD, Shinohara ML. NLRP3 inflammasome induces chemotactic immune cell migration to the CNS in experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A **2012**; 109:10480–5.
44. Yoo JK, Kwon H, Khil LY, et al. IL-18 induces monocyte chemotactic protein-1 production in macrophages through the phosphatidylinositol 3-kinase/Akt and MEK/ERK1/2 pathways. J Immunol **2005**; 175:8280–6.
45. Puren AJ, Fantuzzi G, Dinarello CA. Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells. Proc Natl Acad Sci U S A **1999**; 96:2256–61.
46. Zhu Q, Kanneganti TD. Cutting edge: distinct regulatory mechanisms control proinflammatory cytokines IL-18 and IL-1β. J Immunol **2017**; 198:4210–5.
47. Hitomi Y, Ebisawa M, Tomikawa M, et al. Associations of functional NLRP3 polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma. J Allergy Clin Immunol **2009**; 124:779–85.e6.
48. Zhu Z, Yan J, Geng C, et al. A polymorphism within the 3'UTR of NLRP3 is associated with susceptibility for ischemic stroke in chinese population. Cell Mol Neurobiol **2016**; 36:981–8.
49. Vittinghoff E, McCulloch CE. Relaxing the rule of ten events per variable in logistic and Cox regression. Am J Epidemiol **2007**; 165:710–8.
50. Laureillard D, Marcy O, Madec Y, et al; CAMELIA (ANRS 1295–CIPRA KH001) Study Team. Paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome after early initiation of antiretroviral therapy in a randomized clinical trial. AIDS **2013**; 27:2577–86.
51. Naidoo K, Yende-Zuma N, Padayatchi N, et al. The immune reconstitution inflammatory syndrome after antiretroviral therapy initiation in patients with tuberculosis: findings from the SAPiT trial. Ann Intern Med **2012**; 157:313–24.
52. Kato R, Uetrecht J. Supernatant from hepatocyte cultures with drugs that cause idiosyncratic liver injury activates macrophage inflammasomes. Chem Res Toxicol **2017**; 30:1327–32.
53. Zhang X, Sharma AM, Uetrecht J. Identification of danger signals in nevirapine-induced skin rash. Chem Res Toxicol **2013**; 26:1378–83.
54. Chattergoon MA, Latanich R, Quinn J, et al. HIV and HCV activate the inflammasome in monocytes and macrophages via endosomal Toll-like receptors without induction of type 1 interferon. PLoS Pathog **2014**; 10:e1004082.
55. Ravimohan S, Tamuhla N, Nfanyana K, et al. Robust reconstitution of tuberculosis-specific polyfunctional CD4+ T-cell responses and rising systemic interleukin 6 in paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome. Clin Infect Dis **2016**; 62:795–803.
56. Ahola-Olli AV, Würtz P, Havulinna AS, et al. Genome-wide association study identifies 27 loci influencing concentrations of circulating cytokines and growth factors. Am J Hum Genet **2017**; 100:40–50.
57. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med **2015**; 21:677–87.