Title: TCA cycle remodeling is associated with IL-1β-mediated proinflammatory eicosanoid signaling in humans with pulmonary tuberculosis

Authors: Jeffrey M. Colins¹, Dean P. Jones¹, Ashish Sharma², Manoj Khadka³, Ken Liu¹, Russell R. Kempker¹, Kristal Maner-Smith³, Nestani Tukvadze⁴, N. Sarita Shah⁵, James C.M. Brust⁶, Rafick P. Sekaly², Neel R. Gandhi¹,⁵, Henry M. Blumberg¹,⁵, Eric Ortlund³*, Tom R. Ziegler¹,⁷*

¹Department of Medicine, Emory University School of Medicine, Atlanta, GA
²Department of Pathology, Emory University School of Medicine, Atlanta, GA
³Department of Biochemistry, Emory University, Atlanta, GA
⁴National Center for Tuberculosis and Lung Diseases, Tbilisi, Georgia
⁵Department of Epidemiology and Global Health, Emory University Rollins School of Public Health, Atlanta, GA
⁶Divisions of General Internal Medicine and Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY
⁷Section of Endocrinology, Atlanta Veterans Affairs Medical Center, Atlanta GA

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.
Abstract:

The metabolic signaling pathways that drive pathologic tissue inflammation and damage in humans with pulmonary tuberculosis (TB) are not well understood. Using combined methods in plasma high-resolution metabolomics, lipidomics and cytokine profiling from a multicohort study of humans with pulmonary TB disease, we discovered that IL-1β-mediated inflammatory signaling was closely associated with TCA cycle remodeling, characterized by accumulation of TCA cycle intermediates such as succinate and decreases in itaconate. This inflammatory metabolic network was particularly active in persons with multidrug-resistant (MDR)-TB after receiving 2 months of ineffective treatment and was only reversed after 1 year of appropriate anti-TB chemotherapy. Both succinate and IL-1β were closely associated with increases in proinflammatory lipid signaling, including increases in the products of phospholipase A2, increased arachidonic acid formation, and metabolism of arachidonic acid to proinflammatory eicosanoids. Together, these results indicate that decreased itaconate and accumulation of succinate and other TCA cycle intermediates are important drivers of IL-1β-mediated proinflammatory eicosanoid signaling in humans with pulmonary TB disease. Host-directed therapies that mitigate such metabolic reprogramming may have potential to limit excessive pulmonary inflammation and tissue damage.
Introduction

Tuberculosis (TB) remains the leading global cause of infectious disease mortality, accounting for ~1.4 million deaths each year (1). Treatment outcomes are particularly poor for persons with multidrug resistant (MDR)-TB, who achieve treatment success in an estimated 57% of cases (1). Even those successfully completing MDR-TB treatment demonstrate high rates of post-TB obstructive lung disease (2, 3). While poor treatment outcomes among persons with MDR-TB are multifactorial, propagation of pathologic inflammatory responses during delays in initiation of appropriate anti-TB chemotherapy may play an important role. Even when available, sputum culture with drug susceptibility testing can take up to 3 months to perform, often resulting in treatment with ineffective antibiotics for several months. Prompt diagnosis of MDR-TB using newer molecular diagnostic tests leads to more rapid sputum culture conversion (4) and higher rates of cure (4, 5), suggesting treatment delays meaningfully contribute to long-term adverse outcomes.

Elucidating host response pathways that promote pathologic inflammation and tissue damage in persons with MDR-TB will be critical to identify those at greatest risk for adverse pulmonary outcomes as well as targets for host-directed therapeutics. Animal models of pulmonary TB disease show increases in proinflammatory eicosanoid signaling lead to greater neutrophilic infiltration, bacterial burden and tissue damage (6, 7). While early IL-1 production following infection with *Mycobacterium tuberculosis* (*Mtb*) is critical for control of bacterial replication (7), ongoing IL-1 signaling can lead to upregulation of proinflammatory eicosanoids and an influx of neutrophils, which promote further bacterial replication and tissue destruction (6). IL-1 blockade in macaques with
pulmonary TB disease limits tissue inflammation and damage, further supporting a pathologic role for dysregulated IL-1 signaling in pulmonary TB (8).

Recent studies in macrophage biology show tricarboxylic acid (TCA) cycle remodeling may play an important role in regulating IL-1 signaling (9). Inflammatory macrophage activation leads to accumulation of TCA cycle intermediates such as succinate, which, in turn, results in upregulation of the proinflammatory IL-1β-HIF-1α axis (9). This metabolic remodeling is regulated by the metabolite itaconate, which inhibits succinate dehydrogenase thereby limiting this proinflammatory cascade. However, the contribution of this host metabolic response pathway to IL-1-mediated inflammation in human pulmonary TB has not been previously described.

Using combined approaches in targeted and untargeted high-resolution metabolomics (HRM) and lipidomics, we sought to determine whether host metabolic phenotypes in persons with MDR-TB receiving ineffective therapy differed from persons with drug susceptible (DS)-TB at the time of diagnosis. We further sought to determine whether differences in host metabolism contribute to pathologic inflammatory responses and poor treatment outcomes in MDR-TB. We found that after 2-3 months of ineffective antibiotic therapy, persons with MDR-TB exhibit significant increases in arachidonic acid (AA) metabolism characterized by increased phospholipase A2 activity and increased conversion of AA to proinflammatory eicosanoids. We show that such changes are strongly associated with increased plasma concentrations of IL-1β, as well as TCA cycle intermediates succinate, fumarate and malate and negatively correlated with plasma concentrations of itaconate. Finally, we show TCA cycle remodeling is reversed only after prolonged treatment with effective anti-MDR-TB chemotherapy. These findings
suggest decreased itaconate and increases in TCA cycle intermediates are important drivers of IL-1β-mediated proinflammatory eicosanoid signaling in pulmonary TB that could be targeted by host-directed therapeutics to limit inflammation and tissue damage.

**Results:**

**Metabolic Pathway Regulation in MDR-TB**

We performed HRM on plasma samples of a well-characterized population of persons with pulmonary MDR-TB from KwaZulu-Natal province in South Africa (n = 85) (Table 1; described in Methods) (10). South African participants had a high prevalence of HIV co-infection (75%) and were enrolled after a sputum culture for *Mtb* demonstrated MDR-TB using phenotypic drug susceptibility testing (2–3 months after pulmonary TB was first diagnosed). HRM was used to compare plasma samples from South African patients at the time of MDR-TB diagnosis to a group of control participants (n = 57), which included asymptomatic individuals with LTBI (n = 20) and those without evidence of *Mtb* infection (n = 37). We also compared the MDR-TB cohort to persons with DS-pulmonary TB from the country of Georgia (n = 89) (11). All persons in the Georgia cohort were HIV negative, had culture-confirmed DS-TB, and were enrolled within 7 days of initiation of anti-TB treatment.
| DS-TB cohort, Tbilisi, Georgia (n=89) |  |
|--------------------------------------|-------------------------------|
| Female sex, n (%)                    | 31 (34.8)                     |
| Age, years (median [IQR])            | 31 (24-43)                    |
| HIV positive, n (%)                  | 0 (0)                         |
| AFB sputum smear positive at enrollment, n (%) | 89 (100)            |
| AFB sputum smear positive at first study visit, n (%) | 67 (75)            |
| Sputum culture positive for *M. tuberculosis* at enrollment, n (%) | 89 (100)            |
| Time to sputum culture conversion, days (median [IQR]) | 27.5 (21.5-46.5) |

| MDR-TB cohort, KwaZulu-Natal, South Africa (n=85) |  |
|-----------------------------------------------|-------------------------------|
| Female sex, n (%)                             | 53 (62.4)                     |
| Age, years (median [IQR])                     | 34 (27-42)                    |
| HIV positive, n (%)                           | 64 (75.3)                     |
| AFB sputum smear positive at enrollment, n (%) | 30 (45.5)*                    |
| Sputum culture positive for *M. tuberculosis* at enrollment, n (%) | 56 (78.9)**                  |
| Time to sputum culture conversion, days (median [IQR]) | 62 (51-87)$§$               |

| Controls without active TB disease (n=57)     |  |
|-----------------------------------------------|-------------------------------|
| Female sex, n (%)                             | 40 (70.2)                     |
| Age, median (IQR)                             | 46 (37-54)                    |
| Positive test for latent TB infection, n (%)  | 20 (35.1)                     |
| HIV positive, n (%)                           | 1 (1.8)                       |

*AFB sputum smear results were not available for 19 participants (22.4%)
**AFB sputum culture results were not available for 14 participants (16.5%)
§Data on sputum culture conversion was missing for 7 participants (12.5%) with a positive sputum culture at baseline

HRM analysis using HILIC positive chromatography yielded 7498 mass/charge (m/z) features in plasma samples. We first sought to determine which metabolic pathways were significantly regulated in persons with MDR-TB disease versus controls and persons with DS-TB (12). We found that prostaglandin formation from arachidonate and AA metabolism were among the most regulated pathways in South Africans with MDR-TB versus controls (Figure 1A) and Georgians with DS-TB (Figure 1B).
Figure 1: (A) Unbiased pathway analysis using plasma high-resolution metabolomics with HILIC positive chromatography to compare the metabolome of South Africans with multidrug resistant (MDR)-TB (n=85) to asymptomatic controls with and without LTBI (n=57) shows two pathways involving metabolism of arachidonic acid (AA) are the most regulated. (B) AA metabolism was also highly regulated in persons with MDR-TB compared to persons with drug susceptible (DS)-TB from the country of Georgia (n=89). The y-axis shows significantly regulated metabolic pathways and the x-axis shows the \(-\log p\)-value for pathway enrichment. All comparisons were adjusted for age, sex and HIV status. The green dashed line indicates statistical significance at \(p<0.05\).

The first step in AA metabolism occurs when phospholipase A2 hydrolyzes the sn-2 acyl bond of phospholipids, releasing AA and lysophosphatidic acid (13). Therefore, we reasoned that increases in AA metabolism in the MDR-TB cohort would be accompanied by increases in phospholipase A2 activity. To more fully characterize lipid metabolism, we performed untargeted lipidomics on plasma samples from a subset of the above population including HIV-positive persons with MDR-TB (n=29), HIV-
negative persons with MDR-TB (n=21), persons with DS-TB (n=30) and controls with and without LTBI (n=40). Of plasma lipids with confirmed chemical identities, persons with MDR-TB with or without HIV demonstrated significant upregulation of multiple species of lyso-phospholipids and significant decreases in phospholipids with two acyl chains compared to persons with LTBI (Figure 2A) and persons with DS-TB (Figure 2B), consistent with significant increases in phospholipase A2 activity.

**Figure 2**: Volcano plots of plasma lipids with confirmed chemical identities shows significant increases of lyso-phospholipids and significant decreases in phospholipids with 2 acyl chains in South Africans with MDR-TB (n=50) versus (A) asymptomatic controls with LTBI (n=20) and (B) Georgians with drug susceptible (DS)-TB (n=30), consistent with upregulation of phospholipase A2 activity. The x-axis shows the log2 fold change in expression in MDR-TB versus controls and DS-TB for each lipid and the y-axis shows the -log p-value for each comparison after adjustment for age, sex and HIV status. Lipids upregulated in MDR-TB with >1 log2 fold change at q<0.05 are labelled in blue and those downregulated are labelled in red.
Following formation from phospholipase A2, AA is further metabolized through one of three metabolic pathways to regulate inflammation: cyclooxygenases (COX) to form anti-inflammatory prostaglandins, lipoxygenases (LOX) to form proinflammatory eicosanoids and CYP450 enzymes to form the less biologically active dihydroxyeicosatrienoic acids (DHETs) (14). Prior studies have shown that proinflammatory eicosanoids formed through the LOX pathway are significantly upregulated in persons with pulmonary TB disease and associated with more severe clinical disease (7, 15) and cavity formation (16). We therefore hypothesized that increased phospholipase A2 activity and AA metabolism in MDR-TB patients were driving increased production of proinflammatory eicosanoids. To further evaluate which AA metabolites were upregulated in South Africans with MDR-TB versus Georgians with DS-TB and asymptomatic controls, we performed a targeted oxylipin assay to identify and quantify relevant eicosanoids in plasma samples (17). All persons with pulmonary TB demonstrated significant increases in 11,12-DHET and 14,15-DHET versus controls (Figure 3A and 3B), consistent with increases in AA metabolism. However, while concentrations of the less biologically active DHET molecules did not differ between TB disease groups, both HIV-positive and HIV-negative persons with MDR-TB demonstrated significant increases in the proinflammatory eicosanoids 5-HETE and 12-HETE compared to both persons with DS-TB and controls (Figure 3D and 3E). This indicates AA metabolism in persons with MDR-TB was both increased and more likely to produce proinflammatory eicosanoids metabolized via LOX pathways compared to persons with DS-TB. Examination of linoleic acid (LA) metabolites provided further
evidence of increased LOX activity in persons with MDR-TB. Whereas the LA metabolites formed by CYP450, such as 9,10-Dihydroxy-12-octadecenoic acid (DIHOME), did not differ between MDR-TB and DS-TB groups (Figure 3C), LA metabolites formed by LOX enzymes such as 9-Hydroxyoctadecadienoic acid (HODE), were significantly elevated in MDR-TB (Figure 3F) (14).

Figure 3: Compared to asymptomatic controls without TB disease (red; n=39), HIV positive (grey; n=29) and HIV-negative (green; n=21) South Africans with multidrug resistant (MDR)-TB as well as persons from the country of Georgia with drug susceptible (DS)-TB (blue; n=30) demonstrated significant increases in plasma
concentrations of the arachidonic acid (AA) metabolites (A) 11,12-DHET and (B) 14,15-DHET as well as (C) the linoleic acid (LA) metabolite 9,10-DIHOME metabolized by the CYP450 system. Proinflammatory eicosanoids including (D) 5-HETE and (E) 12-HETE produced by lipoxygenase metabolism of AA were significantly increased in persons with MDR-TB versus persons with DS-TB and asymptomatic controls. (F) The metabolite 9-HODE, a product of LA metabolism by LOX enzymes, was also upregulated in persons with MDR-TB versus persons with DS-TB and controls. Groups were compared using the Wilcoxon rank sum test: * p<0.05, ** p<0.01, *** p<0.001.

Prior studies have shown that IL-1 plays a critical role in regulating the balance of proinflammatory eicosanoids and anti-inflammatory prostaglandins following infection with *Mtb* (7). While IL-1 is necessary for initial control of *Mtb* replication (7), dysregulated IL-1β signaling in later stages of TB disease is associated with eicosanoid-mediated inflammation and tissue damage (6), which is ameliorated with IL-1 blockade (8). Thus, we posited that increased arachidonic acid metabolism and proinflammatory eicosanoid signaling in MDR-TB was due to increased IL-1β signaling. Indeed, we found that South Africans with MDR-TB had significantly higher plasma concentrations IL-1β versus Georgians with DS-TB and controls without TB disease (Figure 4A). Furthermore, we found plasma concentrations of IL-1β were significantly and positively correlated with plasma concentrations of pro-inflammatory eicosanoids 5-HETE (Figure 4B) and 12-HETE (Figure 4C).
Figure 4: (A) In a subset of South Africans with MDR-TB both with (gray; n=31) and without (green; n=6) HIV co-infection, plasma concentrations of IL-1β were significantly greater than those with DS-TB from Georgia (light blue; n=29) and controls without TB disease (red; n=20). Plasma concentrations of IL-1β were significantly and positively correlated with plasma concentrations of proinflammatory eicosanoids (B) 5-HETE and (C) 12-HETE. Groups were compared using the Wilcoxon rank sum test: * p<0.05, ** p<0.01, *** p<0.001.

Accumulation of TCA cycle intermediates has been described as a potent driver of IL-1β-mediated inflammatory responses in multiple disease states (9, 18, 19). We therefore hypothesized that increased IL-1β-mediated proinflammatory eicosanoid signaling in MDR-TB may be driven by TCA cycle remodeling and accumulation of TCA cycle intermediates such as succinate. To test this hypothesis, we analyzed plasma HRM data run in parallel using a C18 negative LC system to better identify and quantify organic acids.
We detected 9,787 m/z features in C18 negative ionization mode. Unbiased pathway analysis (12) revealed the TCA cycle was one of the most significantly regulated metabolic pathway in persons with MDR-TB versus controls without TB disease (Figure 5A) and versus persons with DS-TB (Figure 5B). In the subset of MDR-TB patients where plasma samples were available over the course of the 2-year treatment period (n=17), we also found the TCA cycle was also the most significantly regulated metabolic pathway during MDR-TB treatment (Figure 5C).

Figure 5: (A) Unbiased pathway analysis using plasma HRM with C18 negative chromatography to compare the metabolome of South Africans with multidrug resistant (MDR)-TB (n=85) to (A) asymptomatic controls with and without LTBI (n=57) and (B) Georgians with drug susceptible-TB shows TCA cycle metabolism is among the most significantly regulated pathways. (C) Analyzing changes to the plasma metabolome after 2-4 months, 12-15 months, and 21-27 months after initiation MDR-TB therapy (n=17) reveals the TCA cycle is also significantly regulated during effective treatment. The y-axis shows significantly regulated metabolic pathways and the x-axis shows the -log p-value for pathway enrichment. All comparisons were adjusted for age, sex and HIV status. The green dashed line indicates statistical significance at p<0.05.
TCA cycle metabolites that significantly differed between groups were confirmed and quantified by accurate mass, MS/MS and retention time relative to authentic standards (20). TCA cycle remodeling in MDR-TB was characterized by significant increases in plasma concentrations of succinate, fumarate and malate versus persons with DS-TB and asymptomatic controls (Figure 6 A-C), consistent with accumulation of TCA cycle intermediates. Conversely, plasma concentrations of itaconate, which has been shown to negatively regulate TCA cycle flux by inhibiting succinate dehydrogenase (9), were significantly decreased in persons with MDR-TB compared to persons with DS-TB and controls (Figure 6D). Plasma concentrations of itaconate were significantly and negatively correlated with plasma concentrations of succinate (Figure 6E). Similarly, plasma itaconate concentrations were significantly and negatively correlated with plasma concentrations of fumarate and malate (r= -0.47 and -0.48 respectively, p<0.001 for both), consistent with a role in negatively regulating these metabolites. In persons with DS-TB from Georgia, itaconate concentrations were significantly lower in persons with a persistently positive AFB sputum smear at enrollment versus those who had converted to a negative AFB sputum smear (Figure 6F).
Figure 6: TCA cycle remodeling in South Africans with multidrug resistant (MDR)-TB is characterized by significant increases in plasma concentrations of (A) succinate, (B) fumarate and (C) malate versus Georgians with drug susceptible (DS)-TB (n=89) and asymptomatic controls with and without LTBI (n=57). The anti-inflammatory metabolite itaconate was significantly decreased in persons with MDR-TB versus persons with DS-TB and asymptomatic controls. (E) In all cohorts, plasma itaconate concentrations were significantly and negatively correlated with plasma concentrations of succinate. (F) In persons with DS-TB from Georgia, itaconate was significantly decreased in persons with a positive sputum smear for acid-fast bacilli (AFB) at study enrollment versus those
who had a negative AFB sputum smear. Groups were compared using the Wilcoxon rank sum test: * p<0.05, ** p<0.01, *** p<0.001.

We then sought to examine the relationship between plasma concentrations of TCA cycle metabolites and plasma concentrations of IL-1β and proinflammatory eicosanoids. We found plasma concentrations of succinate were significantly and positively correlated with concentrations of 5-HETE, 12-HETE and IL-1β, confirming a close association between TCA cycle remodeling and IL-1β-mediated proinflammatory eicosanoids signaling (Figure 7 A-C). Conversely, we found significant and negative correlations between plasma concentrations of itaconate and those of 5-HETE, 12-HETE and IL-1β (Figure 7 D-F).
**Figure 7:** In South Africans with MDR-TB with and without HIV co-infection as well as Georgians with drug susceptible (DS)-TB and asymptomatic controls without TB disease, plasma concentrations of succinate were significantly and positively correlated with plasma concentrations of the proinflammatory eicosanoids (A) 5-HETE and (B) 12-HETE, as well as (C) IL-1β. Conversely, plasma concentrations of itaconate were negatively correlated with plasma concentrations of (D) 5-HETE, (E) 12-HETE and (F) IL-1β.

Given the TCA cycle was also the most regulated pathway over the course of MDR-TB treatment, we sought to determine whether TCA cycle remodeling was reversed with effective anti-TB chemotherapy. While there was minimal change in plasma concentrations of succinate, fumarate and malate at 2-4 months (**Figure 8A-C**), plasma concentrations of these metabolites significantly declined after 1 year and 2 years of effective treatment, mirroring the 2-year treatment course of MDR-TB that was standard of care during the study (10). Interestingly, plasma itaconate concentrations significantly increased after 2-4 months of treatment, but this increase was transient, returning to baseline after 1 year and 2 years of anti-TB treatment (**Figure 8D**) and suggesting depletion of itaconate in advanced TB disease may be prolonged. The decline in plasma concentrations of TCA cycle intermediates was mirrored by declines in plasma concentrations of IL-1β, which also significantly declined after 1 year of MDR-TB treatment (**Figure 8E**).
**Figure 8**: In persons with MDR-TB treated with 2 years of effective TB chemotherapy (n=17), (A) there was a significant decline in plasma succinate concentration after 1 year of therapy and a non-significant trend towards decreased concentrations of succinate at 2 years. Plasma concentrations of (B) fumarate and (C) malate also significantly declined at the 1-year and 2-year time points. (D) Plasma concentrations of itaconate increased significantly after 2-4 months of treatment but returned to baseline at the 1-year and 2-year time points. (E) Similarly, plasma concentrations of IL-1β significantly declined after 1 year of MDR-TB treatment. Groups were compared using the Wilcoxon signed rank test: * p<0.05, ** p<0.01, *** p<0.001.

**Discussion**
In this multicohort study, we used an unbiased approach to plasma metabolic pathway analysis combined with targeted and untargeted lipidomics to discover that TCA cycle remodeling is strongly associated with IL-1β-mediated proinflammatory eicosanoid signaling in persons with pulmonary TB disease. We found this inflammatory cascade, characterized by increased plasma concentrations of TCA cycle intermediates succinate, fumarate and malate and decreased concentrations of itaconate, was particularly upregulated in persons with MDR-TB after 2-3 months of ineffective anti-TB chemotherapy and was reversed only after 1 year of efficacious treatment. Collectively, these results indicate that TCA cycle remodeling is an important driver of IL-1β-mediated proinflammatory eicosanoid signaling in pulmonary TB. Furthermore, our findings suggest prolonged delays in effective treatment allow this proinflammatory cascade to perpetuate, potentially contributing to poor treatment outcomes in MDR-TB. These findings also indicate that administration of host-directed therapies that limit TCA cycle flux may be an effective way to reduce proinflammatory eicosanoid signaling and limit tissue damage (6-9).

Accumulation of TCA cycle intermediates such as succinate are known to drive pathologic inflammation in macrophages through activation of the HIF-1α-IL-1 axis that is an important mediator of tissue damage in ischemic-reperfusion injury (18, 19). The strong association between TCA cycle remodeling and IL-1β-mediated proinflammatory eicosanoid signaling in our study suggests TCA cycle intermediates also play a critical role in regulating the human inflammatory response to pulmonary TB. One potential source of accumulating TCA cycle intermediates in persons with MDR-TB is the host microbiome (21). The microbiome produces a large proportion of succinate circulating in
plasma (21), thus providing a potential avenue through which microbial composition could regulate the inflammatory response in TB disease. Indeed, microbiome differences in rhesus macaques have been associated with TB disease severity and bacterial burden (22). Another potential explanation that is not mutually exclusive is the ability of \textit{Mtb} to induce aerobic glycolysis (23, 24). This process, also termed the “Warburg Effect”, involves a shift in cellular energy metabolism to increased glycolysis and reduced oxidative phosphorylation, thereby causing TCA cycle intermediates to accumulate (25). Indeed, \textit{in vitro} studies of human pulmonary macrophages show the ability of \textit{Mtb} to induce aerobic glycolysis is necessary to stimulate production of IL-1β (23, 26).

IL-1β is known to play an important role in regulating the products of AA metabolism, which evolves over the course of infection with \textit{Mtb} (6-9). Initially, IL-1 provides a counterbalance to type-1-IFN signaling (7, 27) by promoting metabolism of AA to anti-inflammatory prostaglandins rather than proinflammatory eicosanoids (7). This signaling pathway promotes early control of \textit{Mtb} replication, with IL-1 receptor blockade leading to greater abundance of proinflammatory eicosanoids and increased bacterial burden while IL-1 receptor agonism attenuates disease severity (7, 27). Similarly, early IL-1 signaling by pulmonary macrophages triggered by induction of aerobic glycolysis is necessary to control \textit{Mtb} growth (23). However, as TB disease progresses, IL-1β becomes a driver of proinflammatory eicosanoids signaling leading to an influx of neutrophilic inflammation that is permissive to bacterial growth (6). In later stages of TB disease in mice, IL-1 blockade is therapeutic and limits tissue damage (8). In macaques with TB disease, plasma concentrations of IL-1β are highly correlated with
levels of pulmonary inflammation as measured by PET-CT and IL-1R blockade limits inflammation and tissue damage (8). Thus, accumulating TCA cycle intermediates during later stages of TB disease may be another example of Mtb exploiting a host metabolic adaption aimed at controlling Mtb replication during the initial stages of infection (28, 29). While TCA cycle remodeling initially promotes control of bacterial replication, these results suggest that in later stages of human TB disease it increases AA metabolism and conversion to proinflammatory eicosanoids, potentially worsening tissue damage and increasing disease severity.

Clinical studies also indicate IL-1β primarily acts as a driver of inflammation and tissue damage in human TB disease. Independent studies consistently show elevated plasma concentrations of IL-1β in persons with pulmonary TB versus asymptomatic controls (30, 31) that correlate with markers of inflammation such as ESR and CRP (30). Further, elevated plasma concentrations of IL-1β in pulmonary TB are associated with greater extent of disease and cavitation on chest radiograph (31, 32). Our findings suggest such signaling may be caused by TCA cycle remodeling and that tissue damage associated with elevated IL-1β is mediated by increased AA metabolism and production of proinflammatory eicosanoids.

The observation that dysregulated IL-1β signaling occurs disproportionately in persons with MDR-TB has been reported previously (30). We hypothesize this is caused by delays in adequate treatment in persons with MDR-TB, which allows for propagation of a proinflammatory cascade. However, rifampin resistance mutations in Mtb isolates have been associated with differential metabolic responses and secretion of IL-1β in human macrophages (33). Thus, it is possible the different host responses
observed are related to differential induction of proinflammatory cascades by the organism itself.

This study is subject to several limitations. Though we demonstrate a strong association between plasma concentrations of TCA cycle intermediates and proinflammatory eicosanoids, the observational nature of the study precludes us from definitively establishing a causal relationship. All persons with MDR-TB were from a single study site, so it remains possible that metabolic differences in this group are driven by host genetic differences and differences in microbial composition rather than MDR-TB status. In future studies it will be important to evaluate the relative contribution of pulmonary macrophages and the host microbiome to plasma concentrations of TCA cycle intermediates in persons with pulmonary TB.

In summary, we demonstrate that IL-1β-mediated proinflammatory eicosanoid signaling is strongly associated with TCA cycle remodeling in humans with pulmonary TB. This remodeling is characterized by significant increases in plasma succinate concentrations and significant decreases in concentrations of itaconate. These findings provide evidence that pathologic eicosanoid signaling in pulmonary TB is driven by accumulation of TCA cycle intermediates. The TCA cycle may therefore represent a promising target for host-directed therapies aimed at limiting pulmonary inflammation and tissue damage.

Methods

Sample collection

For all cohorts, blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes and centrifuged; isolated plasma was immediately frozen and stored at
-80°C. Samples collected outside of the U.S. were subsequently shipped on dry ice to Emory University, Atlanta, GA, USA. All samples remained frozen during transit and were kept at -80°C prior to metabolomics analysis.

**South Africa MDR-TB cohort**

Persons with pulmonary TB from KwaZulu-Natal province, South Africa were enrolled as part of a study of MDR-TB and TB/HIV co-infection (10). All persons in the South African cohort had MDR-TB as demonstrated by a positive sputum culture for *Mtb* and phenotypic DST indicating resistance to at least both isoniazid and rifampin. Baseline plasma samples were collected within 7 days of starting conventional treatment for MDR-TB. Persons with HIV-co-infection were continued on conventional anti-retroviral therapy (ART) and those not previously on ART were started on treatment. All patients were referred to a dedicated MDR-TB treatment center and treated with a standardized drug regimen that included kanamycin (15 mg/kg, maximum 1 g daily), moxifloxacin (400 mg daily), ethionamide (15–20 mg/kg, maximum 750 mg daily), terizidone (15–20 mg/kg, maximum 750 mg daily), ethambutol (15–20 mg/kg, maximum 1200 mg daily), and pyrazinamide (20–30 mg/kg, maximum 1600 mg daily). All persons who completed the study were treated for a period of two years and serial plasma samples were obtained 2-4 months, 12-15 months, and 21-27 months after treatment initiation.

**Georgia pulmonary TB cohort**

Persons with pulmonary TB were selected from a randomized, double blind controlled trial of adjunctive high-dose cholecalciferol (vitamin D₃) for TB treatment conducted in the country of Georgia (clinicaltrials.gov identifier NCT00918086) (11). All
persons included in this metabolomics sub-study were HIV-negative and had DS-TB. Inclusion criteria for patients included age ≥ 18 years and newly diagnosed active TB disease, suggested by a positive AFB sputum smear and confirmed by positive sputum culture for *Mtb*. Baseline plasma samples for HRM were obtained from eligible subjects within 7 days of initiating therapy with conventional dosing of first-line anti-TB drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) (11). Phenotypic DST was performed on *Mtb* isolates recovered from all persons with pulmonary TB using the absolute concentration method (34).

**Controls without active TB disease**

Plasma from persons with and without LTBI was analyzed for cross-sectional comparison with pulmonary TB cases. Persons with LTBI were enrolled from the DeKalb County Board of Health in DeKalb County, GA, USA. All persons with LTBI had positive test results from at least two FDA-approved tests for LTBI (QFT, TSPOT.TB [TSPOT] and/or tuberculin skin test [TST]). All tests were interpreted according to the guidelines from the Centers for Disease Control and Prevention (35, 36). Controls without LTBI were U.S.-born adults at low risk for *Mtb* exposure and infection, who had at least one negative TST within the year prior to plasma collection documented in medical records (37).

**Plasma metabolomics analysis**

De-identified samples were randomized by a computer-generated list into blocks of 40 samples prior to transfer to the analytical laboratory where personnel were blinded to clinical and demographic data. Thawed plasma (65 μL) was treated with 130 μl acetonitrile (2:1, v/v) containing an internal isotopic standard mixture (3.5 μL/sample),
as previously described (38). The internal standard mix for quality control consisted of 14 stable isotopic chemicals covering a broad range of small molecules (38). Samples were mixed and placed on ice for 30 min prior to centrifugation to remove protein. The resulting supernatant was transferred to low-volume autosampler vials maintained at 4°C and analyzed in triplicate using an Orbitrap Fusion Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). Dual c18 negative and hydrophilic interaction chromatography (HILIC) were used (Higgins Analytical, Targa, Mountain View, CA, USA, 2.1 x 10 cm) with a formic acid/acetonitrile gradient. The high-resolution mass spectrometer was operated in positive electrospray ionization mode over scan range of 85 to 1275 mass/charge (m/z) and stored as .Raw files (39). Data were extracted and aligned using apLCMS (40) and xMSanalyzer (41) with each feature defined by specific m/z value, retention time and integrated ion intensity (39). Three technical replicates were performed for each plasma sample and median summarized (42).

*Metabolite identification and reference standardization*

Identities of metabolites of interest were confirmed using ion dissociation methods (tandem MS/MS). Fragmentation spectra were generated using a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer with parallel reaction monitoring mode using a targeted inclusion list. TCA cycle metabolites were confirmed and quantified by accurate mass, MS/MS and retention time relative to authentic standards (20).

*Untargeted lipidomics*

Lipids were extracted from each plasma sample using a high throughput methyl tbutyl ether (MtBE) extraction procedure with an automated and robust liquid handling
Extracted samples were dried under nitrogen and reconstituted in 200 µl 1:1 chloroform:methanol prior to injection into the LC/MS system and lipids were resolved using a Thermo Acclaim C18 reverse phase column on a Thermo Vanquish UPLC coupled to a Thermo Fusion IDX mass spectrometer (Thermo, Waltham, MA) (44). Data were acquired at a resolution of 240,000 FWHM and deep MS/MS data was collected on pooled samples using an iterative data dependent strategy at multiple collision energies. Data was processed using LipidSearch (Thermo Fisher, San Jose, CA). Lipids that contained a signal to noise ratio of greater than 10 and had high confidence (MS/MS) identifications with CVs less than 30% across pooled QCs were used for downstream analysis.

**Targeted measurement of eicosanoids**

We selectively targeted oxylipins and endocannabinoids (OXYs), which are highly regulated, resulting in low abundance in human plasma. To enrich these lipids from bulk membrane lipids, we performed solid phase extraction methods using the Biotage Extrahera liquid handling system as previously described (17, 45, 46). The resulting extracts were analyzed using a multiple reaction monitoring (MRM)-based LC/MS protocol on a QTrap 5500 (Sciex, Waltham, MA), whereby detected oxylipins and endocannabinoids were fragmented and quantified against external standard curves. Oxylipins and endocannabinoids detected in less than 60% of patient samples were excluded from this analysis. Missing values were imputed using half of the minimum detected value for each lipid.

**Plasma cytokine detection**
The U-PLEX assay (Meso Scale MULTI-ARRAY Technology) commercially available by Meso Scale Discovery (MSD) was used for plasma cytokine detection. This technology allows the evaluation of multiplexed biomarkers by using custom made U-PLEX sandwich antibodies with a SULFO-TAG™ conjugated antibody and next generation of electrochemiluminescence (ECL) detection. The assay was performed according to the manufacturer’s instructions (https://www.mesoscale.com/en/technical_resources/technical_literature/technical_notes_search). In summary, 25μL of plasma from each participant was combined with the biotinylated antibody plus the assigned linker and the SULFO-TAG™ conjugated detection antibody; in parallel a multi-analyte calibrator standard was prepared by doing 4-fold serial dilutions. Both samples and calibrators were mixed with the Read buffer and loaded in a 10-spot U-PLEX plate, which was read by the MESO QuickPlex SQ 120. The plasma cytokines values (pg/mL) were extrapolated from the standard curve of each specific analyte.

**Statistical analysis**

Statistical comparisons of metabolite and lipid intensity values (abundance) and concentrations were performed in R version 3.5.0. For untargeted metabolomics and lipidomics analyses, metabolite intensity values were log2 transformed and compared between groups using linear regression, controlling for age, sex and HIV status (47). Metabolic pathway enrichment analysis was performed using *mummichog*, a Python-based informatics tool that leverages the organization of metabolic networks to predict functional changes in metabolic pathway activity (12, 28, 48). Following quantification of selected metabolites and lipids, cross-sectional comparison of plasma concentrations...
between groups was made using the Wilcoxon Rank Sum test. Changes relative to baseline during treatment of active TB were tested using a Wilcoxon Signed-Rank test. A p-value less than or equal to 0.05 was considered statistically significant.

Study Approval

All studies were approved by the Institutional Review Board (IRB) of Emory University (Atlanta, GA, USA), and by the individual IRBs associated with the original cohort studies: the Ethics Committee of the National Center for Tuberculosis and Lung Diseases of Georgia (Tbilisi, Georgia), the University of KwaZulu-Natal IRB (Durban, South Africa), and the Georgia Department of Public Health IRB (Atlanta, GA, USA), respectively, depending on the site of participant enrollment. All subjects provided written informed consent.
References

1. WHO. Global Tuberculosis Report 2019. World Health Organization; 2019 2015-10-28 18:36:54.
2. Vashakidze SA, Kempker JA, Jakobia NA, Gogishvili SG, Nikolaishvili KA, Goginashvili LM, et al. Pulmonary function and respiratory health after successful treatment of drug-resistant tuberculosis. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases. 2019;82:66-72.
3. Byrne AL, Marais BJ, Mitnick CD, Garden FL, Lecca L, Contreras C, et al. Chronic airflow obstruction after successful treatment of multidrug-resistant tuberculosis. ERJ Open Res. 2017;3(3).
4. Kipiani M, Mirtskhulava V, Tukvadze N, Magee M, Blumberg HM, Kempker RR. Significant clinical impact of a rapid molecular diagnostic test (Genotype MTBDRplus assay) to detect multidrug-resistant tuberculosis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2014;59(11):1559-66.
5. Padayatchi N, Naidu N, Yende-Zuma N, O'Donnell MR, Naidoo K, Augustine S, et al. Implementation and Operational Research: Clinical Impact of the Xpert MTB/RIF Assay in Patients With Multidrug-Resistant Tuberculosis. J Acquir Immune Defic Syndr. 2016;73(1):e1-7.
6. Mishra BB, Lovewell RR, Olive AJ, Zhang G, Wang W, Eugenin E, et al. Nitric oxide prevents a pathogen-permissive granulocytic inflammation during tuberculosis. Nat Microbiol. 2017;2:17072.
7. Mayer-Barber KD, Andrade BB, Oland SD, Amaral EP, Barber DL, Gonzales J, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. Nature. 2014;511(7507):99-103.
8. Winchell CG, Mishra BB, Phuah JY, Saqib M, Nelson SJ, Maiello P, et al. Evaluation of IL-1 Blockade as an Adjunct to Linezolid Therapy for Tuberculosis in Mice and Macaques. Front Immunol. 2020;11:891.
9. Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, Loginicheva E, et al. Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. Cell Metab. 2016;24(1):158-66.
10. Brust JCM, Shah NS, Mlisana K, Moodley P, Allana S, Campbell A, et al. Improved Survival and Cure Rates With Concurrent Treatment for Multidrug-Resistant Tuberculosis-Human Immunodeficiency Virus Coinfection in South Africa. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2018;66(8):1246-53.
11. Tukvadze N, Sanikidze E, Kipiani M, Hebbar G, Easley KA, Shevni N, et al. High-dose vitamin D3 in adults with pulmonary tuberculosis: a double-blind randomized controlled trial. The American journal of clinical nutrition. 2015.
12. Li S, Park Y, Duraisingham S, Strobel FH, Khan N, Soltow QA, et al. Predicting network activity from high throughput metabolomics. PLoS computational biology. 2013;9(7):e1003123.
13. Murakami M, Nakatani Y, Asumi GI, Inoue K, Kudo I. Regulatory Functions of Phospholipase A2. Crit Rev Immunol. 2017;37(2-6):127-95.
14. Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic acids research. 1999;27(1):29-34.
15. Lau SK, Lee KC, Curreem SO, Chow WN, To KK, Hung IF, et al. Metabolomic Profiling of Plasma from Patients with Tuberculosis by Use of Untargeted Mass Spectrometry Reveals Novel Biomarkers for Diagnosis. Journal of clinical microbiology. 2015;53(12):3750-9.
16. Marakalala MJ, Raju RM, Sharma K, Zhang YJ, Eugenin EA, Prideaux B, et al. Inflammatory signaling in human tuberculosis granulomas is spatially organized. Nat Med. 2016;22(5):531-8.
17. Maner-Smith KM, Goll JB, Khadka M, Jensen TL, Colucci JK, Gelber CE, et al. Alterations in the Human Plasma Lipidome in Response to Tularemia Vaccination. Vaccines (Basel). 2020;8(3).
18. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature. 2013;496(7444):238-42.
19. Chouchani ET, Pell VR, Gaude E, Aksentijević D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature. 2014;515(7527):431-5.
20. Liu KH, Nellis M, Uppal K, Ma C, Tran V, Liang Y, et al. Reference Standardization for Quantification and Harmonization of Large-Scale Metabolomics. Analytical chemistry. 2020;92(13):8836-44.
21. Fernández-Veledo S, Vendrell J. Gut microbiota-derived succinate: Friend or foe in human metabolic diseases? Rev Endocr Metab Disord. 2019;20(4):439-47.
22. Kauffman KD, Sakai S, Lora NE, Namasivayam S, Baker PJ, Kamenyeva O, et al. PD-1 blockade exacerbates Mycobacterium tuberculosis infection in rhesus macaques. bioRxiv. 2020:2020.08.05.237883.
23. Gleeson LE, Sheedy FJ, Palsson-McDermott EM, Triglia D, O'Leary SM, O'Sullivan MP, et al. Cutting Edge: Mycobacterium tuberculosis Induces Aerobic Glycolysis in Human Alveolar Macrophages That Is Required for Control of Intracellular Bacillary Replication. Journal of immunology (Baltimore, Md : 1950). 2016;196(6):2444-9.
24. Shi L, Salamon H, Eugenin EA, Pine R, Cooper A, Gennaro ML. Infection with Mycobacterium tuberculosis induces the Warburg effect in mouse lungs. Scientific reports. 2015;5:18176.
25. Cumming BM, Pacl HT, Steyn AJC. Relevance of the Warburg Effect in Tuberculosis for Host-Directed Therapy. Frontiers in cellular and infection microbiology. 2020;10:576596.
26. Cox DJ, Coleman AM, Gogan KM, Phelan JJ, C ÓM, Dunne PJ, et al. Inhibiting Histone Deacetylases in Human MacrophagesPromotes Glycolysis, IL-1β, and T Helper Cell Responses to Mycobacterium tuberculosis. Front Immunol. 2020;11:1609.
27. Ji DX, Yamashiro LH, Chen KJ, Mukaida N, Kramnik I, Darwin KH, et al. Type I interferon-driven susceptibility to Mycobacterium tuberculosis is mediated by IL-1Ra. Nat Microbiol. 2019;4(12):2128-35.
28. Collins JM, Siddiqa A, Jones DP, Liu K, Kempker RR, Nizam A, et al. Tryptophan catabolism reflects disease activity in human tuberculosis. JCI Insight. 2020;5(10).
29. Zhang YJ, Reddy MC, Ioerger TR, Rothchild AC, Dartois V, Schuster BM, et al. Tryptophan biosynthesis protects mycobacteria from CD4 T-cell-mediated killing. Cell. 2013;155(6):1296-308.
30. Wang Y, Hu C, Wang Z, Kong H, Xie W, Wang H. Serum IL-1β and IL-18 correlate with ESR and CRP in multidrug-resistant tuberculosis patients. J Biomed Res. 2015;29(5):426-8.
31. Kumar NP, Moideen K, Banurekha VV, Nair D, Babu S. Plasma Proinflammatory Cytokines Are Markers of Disease Severity and Bacterial Burden in Pulmonary Tuberculosis. Open forum infectious diseases. 2019;6(7):ofz257.
32. Sigal GB, Segal MR, Mathew A, Jarlsberg L, Wang M, Barbero S, et al. Biomarkers of Tuberculosis Severity and Treatment Effect: A Directed Screen of 70 Host Markers in a Randomized Clinical Trial. EBioMedicine. 2017;25:112-21.
33. Howard NC, Marin ND, Ahmed M, Rosa BA, Martin J, Bambouskova M, et al. Mycobacterium tuberculosis carrying a rifampicin drug resistance mutation reprograms macrophage metabolism through cell wall lipid changes. Nat Microbiol. 2018;3(10):1099-108.
34. Lomtadze N, Aspindzelashvili R, Janjgava M, Mirtskhulava V, Wright A, Blumberg HM, et al. Prevalence and risk factors for multidrug-resistant tuberculosis in the Republic of Georgia: a population-based study. The international journal of tuberculosis and lung disease: the official journal of the International Union against Tuberculosis and Lung Disease. 2009;13(1):68-73.
35. American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: controlling tuberculosis in the United States. American journal of respiratory and critical care medicine. 2005;172:1169-227.
36. Mazurek GH, Jereb J, Vernon A, LoBue P, Goldberg S, Castro K. Updated guidelines for using Interferon Gamma Release Assays to detect Mycobacterium tuberculosis infection - United States, 2010. MMWR Recomm Rep. 2010;59(Rr-5):1-25.
37. Al Mheid I, Kelli HM, Ko YA, Hammadah M, Ahmed H, Hayek S, et al. Effects of a Health-Partner Intervention on Cardiovascular Risk. J Am Heart Assoc. 2016;5(10).
38. Soltow QA, Strobel FH, Mansfield KG, Wachtman L, Park Y, Jones DP. High-performance metabolic profiling with dual chromatography-Fourier-transform mass spectrometry (DC-FTMS) for study of the exposome. Metabolomics. 2013;9(1 Suppl):S132-43.
39. Go YM, Walker DI, Liang Y, Uppal K, Soltow QA, Tran V, et al. Reference Standardization for Mass Spectrometry and High-Resolution Metabolomics Applications to Exposome Research. Toxicological sciences. 2015;148(2):531-42.
40. Yu T, Park Y, Johnson JM, Jones DP. apLCMS--adaptive processing of high-resolution LC/MS data. Bioinformatics (Oxford, England). 2009;25(15):1930-6.
41. Uppal K, Soltow QA, Strobel FH, Pittard WS, Gernert KM, Yu T, et al. xMSanalyzer: automated pipeline for improved feature detection and downstream analysis of large-scale, non-targeted metabolomics data. BMC bioinformatics. 2013;14:15.
42. Collins JM, Kempker RR, Ziegler TR, Blumberg HM, Jones DP. Metabolomics and Mycobacterial Disease: Don't Forget the Bioinformatics. Annals of the American Thoracic Society. 2016;13(1):141-2.
43. Collins JM, Walker DI, Jones DP, Tukvadze N, Liu KH, Tran VT, et al. High-resolution plasma metabolomics analysis to detect Mycobacterium tuberculosis-associated metabolites that distinguish active pulmonary tuberculosis in humans. PLoS one. 2018;13(10):e0205398.

44. Khadka M, Todor A, Maner-Smith KM, Colucci JK, Tran V, Gaul DA, et al. The Effect of Anticoagulants, Temperature, and Time on the Human Plasma Metabolome and Lipidome from Healthy Donors as Determined by Liquid Chromatography-Mass Spectrometry. Biomolecules. 2019;9(5).

45. Strassburg K, Huijbrechts AM, Kortekaas KA, Lindeman JH, Pedersen TL, Dane A, et al. Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery. Anal Bioanal Chem. 2012;404(5):1413-26.

46. Gouveia-Figueira S, Spath J, Zivkovic AM, Nording ML. Profiling the Oxylipin and Endocannabinoid Metabolome by UPLC-ESI-MS/MS in Human Plasma to Monitor Postprandial Inflammation. PloS one. 2015;10(7):e0132042.

47. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic acids research. 2015;43(7):e47.

48. Li S, Sullivan NL, Rouphael N, Yu T, Banton S, Maddur MS, et al. Metabolic Phenotypes of Response to Vaccination in Humans. Cell. 2017;169(5):862-77.e17.