

DNA hypermethylation during tuberculosis dampens host immune responsiveness

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Mycobacterium tuberculosis (*M. tuberculosis*) has coevolved with humans for millennia and developed multiple mechanisms to evade host immunity. Restoring host immunity in order to improve outcomes and potentially shorten existing therapy will require identification of the full complement by which host immunity is inhibited. Perturbation of host DNA methylation is a mechanism induced by chronic infections such as HIV, HPV, lymphocytic choriomeningitis virus (LCMV), and schistosomiasis to evade host immunity. Here, we evaluated the DNA methylation status of patients with tuberculosis (TB) and their asymptomatic household contacts and found that the patients with TB have DNA hypermethylation of the IL-2/STAT5, TNF/NF- κ B, and IFN- γ signaling pathways. We performed methylation-sensitive restriction enzyme-quantitative PCR (MSRE-qPCR) and observed that multiple genes of the IL-12/IFN- γ signaling pathway (*IL12B*, *IL12RB2*, *TYK2*, *IFNGR1*, *JAK1*, and *JAK2*) were hypermethylated in patients with TB. The DNA hypermethylation of these pathways was associated with decreased immune responsiveness with decreased mitogen-induced upregulation of IFN- γ , TNF, IL-6, CXCL9, CXCL10, and IL-1 β production. The DNA hypermethylation of the IL-12/IFN- γ pathway was associated with decreased IFN- γ -induced gene expression and decreased IL-12-inducible upregulation of IFN- γ . This study demonstrates that immune cells from patients with TB are characterized by DNA hypermethylation of genes critical to mycobacterial immunity resulting in decreased mycobacteria-specific and nonspecific immune responsiveness.

Introduction

Tuberculosis (TB) continues to be a global problem, with 10 million cases and over 1 million deaths each year (1). Anti-TB therapy (ATT) requires 6 months of antibiotics, despite the fact that the majority of individuals become culture negative within the first 2 months of therapy. Although multiple mechanisms of TB-induced immunosuppression have been identified, it is unknown why ATT must be continued for months after an individual becomes culture negative. Further, after successful TB therapy, patients have a 13-fold increased risk of disease recurrence compared with the general population (2). Along with strain virulence and environmental risk factors, persistent perturbation of host immunity is one of the likely causes of recurrent TB disease. It is therefore critical to identify mechanisms of host immunosuppression that persist despite successful TB therapy.

TB has been recognized as an archetypical chronic infection since at least 1882. Since 1984, the clone 13 strain of murine

lymphocytic choriomeningitis virus (LCMV) has emerged as the prototypical animal model of chronic infection (3). Chronic viral infections with viruses such as HIV, hepatitis B and C, and partially with EBV and CMV, and epitomized by clone 13 LCMV, induce immune exhaustion, which is defined by decreased antigen-induced immune cell proliferation, decreased immune effector function, and increased expression of immune checkpoint inhibitors (4–7). Immune exhaustion is mediated by epigenetic alterations, conformational changes in immune cell DNA methylation, and a chromatin architecture that inhibits robust cell-mediated immunity (8). These epigenetic changes are mediated by DNA methyltransferase (DNMT) and the enhancer of zeste 2 polycomb-repressive (EZH2) complex that imbues a repressive chromatin state, resulting in an immune-nonresponsive state (9–12). DNA methylation is a common and stable epigenetic alteration that blocks transcription factor binding and inhibits gene expression and mediates immune exhaustion (10). Once exhausted, the epigenetic perturbations persist, and the immune phenotype remains subdued even if cells are rested for 3 weeks, or if they are transferred into healthy, noninfected mice (11, 13). Similarly, patients with chronic HIV infection or schistosomiasis have DNA methylation and immune exhaustion phenotypes that persist for months to years after successful therapy (9, 14, 15).

Given that chronic antigen stimulation from HIV, schistosomiasis, or LCMV induces long-lasting epigenetic-mediated immune

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exhaustion (9, 11, 14–16), we were concerned that TB disease may also result in persistent, detrimental epigenetic changes. Since both TB and LCMV are chronic infections characterized by decreased immune function, we hypothesized that patients with TB would have a DNA methylation landscape similar to that described in chronic LCMV and after schistosomiasis. Mouse models of chronic LCMV have shown persistent epigenetic perturbations, with hypomethylating agents capable of restoring host immunity (10, 11, 13). We thought it pertinent to investigate the longitudinal DNA methylation of host immune cells before and after successful ATT. In a cohort of adults with microbiologically confirmed pulmonary TB from Eswatini, patients with TB exhibited a hypo-responsive immune phenotype with decreased immune proliferation and decreased mycobacterium-induced and mitogen-induced cytokine production in association with DNA hypermethylation of multiple critical immune genes and pathways. These DNA methylation perturbations persisted 6 months after successful ATT and identify a plausible mechanisms of suppressed host immunity.

Results

We previously described a cohort of asymptomatic, TB-exposed children from Eswatini (formerly known as Swaziland) with schistosomiasis-induced DNA hypermethylation and associated inhibition of bacillus Calmette–Guérin (BCG) and *Mycobacterium tuberculosis* (*M. tuberculosis*) immunity (15). Here, to evaluate whether TB induces similar DNA methylation perturbations that correspond with immune exhaustion, we assessed adults with TB symptoms and microbiologically confirmed pulmonary TB (by culture and/or GeneXpert). We compared their immune phenotype and DNA methylation status with their asymptomatic, healthy household contacts who remained asymptomatic for 12 months after initial exposure (Figure 1 and Supplemental Table 1; supplemental material available online with this article; <https://doi.org/10.1172/JCI134622DS1>). All controls were negative for ascaris and schistosomiasis by urine and stool microscopy and quantitative PCR (qPCR) as previously described (15). All study participants were BCG vaccinated, as determined via either vaccine records and/or a BCG scar. An HIV screen was performed at the time of the study's initiation and then annually thereafter. All included participants had TB treatment success as defined by both WHO criteria and a simplified clinically relevant definition (17).

Patients with TB demonstrate immune exhaustion phenotypes. To evaluate the cell-specific and mycobacterium-specific immunity of patients with TB, PBMCs were stimulated with ESAT-6 and CFP-10 (*M. tuberculosis*-specific antigens) or BCG sonicate followed by flow cytometry-based multidimensional immune profiling (MDIP). Cells were stained subsequently for viability and cell surface and intracellular markers. Multidimensional immune changes were evaluated using CITRUS (clustering identification, characterization, and regression) (18). Clustering was implemented via CITRUS using the surface markers CD3, CD4, CD8, and CD56 to identify subsets of Th cells (CD3⁺CD4⁺), cytotoxic T lymphocytes (CTLs) (CD3⁺CD8⁺), and NK cells (CD3⁺CD56⁺) (Figure 1). The median fluorescence intensity (MFI) of each node was characterized between healthy controls (HCs) and individuals with TB. CITRUS revealed that, compared with controls, participants with TB had Th cells, CTLs, and NK cells with decreased

IFN- γ and proliferative capacity in response to both *M. tuberculosis*-specific antigens (ESAT-6 and CFP-10) and BCG (Figure 1A, Supplemental Figure 1A, and Supplemental Table 1). Compared with controls, individuals with TB were more likely to be coinfecting with HIV, however, after excluding people living with HIV (PLWH), participants with TB still had lymphocytes (CD4⁺, CD8⁺, and CD3⁺CD56⁺) with decreased IFN- γ and proliferative capacity (Figure 1B and Supplemental Figure 1). Study participants with TB who were not HIV coinfecting had a 21% and 25% decrease in BCG-induced IFN- γ production compared with HIV-uninfected controls, respectively, for CD4⁺ and CD8⁺ T cell populations (Figure 1B). Similarly, participants with TB who were not infected with HIV had a 16% and 14% decrease in BCG-induced Ki-67 expression, respectively, for CD4⁺ and CD8⁺ T cell populations (Figure 1B). In response to the *M. tuberculosis*-specific peptides ESAT-6 and CFP-10, patients with TB had CD4⁺ and CD8⁺ T cell populations with 11% and 45% decreased IFN- γ production and 12% and 19% decreased Ki-67 expression, respectively (Supplemental Figure 1A).

The decrease in proliferative capacity was not mycobacterium-specific, as the superantigen staphylococcal enterotoxin B (SEB) also induced decreased Ki-67 upregulation (SEB; Supplemental Figure 1B). The decrease in SEB-induced IFN- γ was not statistically significant. Increased expression of immune checkpoint blockade, as measured by programmed cell death 1 (PD-1), cytotoxic T lymphocyte-associated protein 4 (CTLA4), and/or T cell immunoglobulin mucin-3 (TIM3), is another characteristic of immune exhaustion and similar to what has been described in previous reports (19, 20). Further, compared with asymptomatic HCs, we found that patients with TB had an increased abundance of PD-1-expressing NK cells (Supplemental Figure 1C).

The TB DNA methylome resembles the immune exhaustion epigenetic landscape. Epigenetic mechanisms mediate immune exhaustion (4, 5, 10, 21), and we therefore evaluated the DNA methylation status of participants with TB using the EPIC array. From bulk PBMCs, we determined cell-specific DNA methylation using epigenetic deconvolution (EDEC) (Figure 2A) (22). EDEC identified global differential methylation changes in Th cells, CTLs, NK cells, and monocytes both at baseline and 6 months after completion of successful ATT, 12 months after study enrollment (Figure 2D). Pathway enrichment (Figure 2B) indicated that the IL-2/STAT5 pathway, a critical component of cell proliferation, was differentially methylated at baseline and 6 months after successful therapy in all lymphocyte subsets from patients with TB compared with controls. The PI3K/AKT pathway, which modulates intracellular signaling of the immunometabolic pathway in both adaptive and innate cells, was differentially methylated in all cell types except CD4⁺ T cells. Similarly, the TNF/NF- κ B signaling pathway was differentially methylated in all evaluated cell types except Th cells. IFN- γ signaling, critical to antimycobacterial immunity, was differentially methylated in all cell types at baseline and 6 months after the completion of ATT (Figure 2B). Specifically, compared with HCs, patients with TB had DNA hypermethylation of the IFN- γ signaling pathway for all lymphocyte subsets (CD4⁺ Th cells, CD3⁺CD8⁺ CTLs, and CD3⁺CD56⁺ NK cells), whereas this pathway was hypomethylated in monocytes (Figure 3, Tables 1 and 2, and Supplemental Table 2).

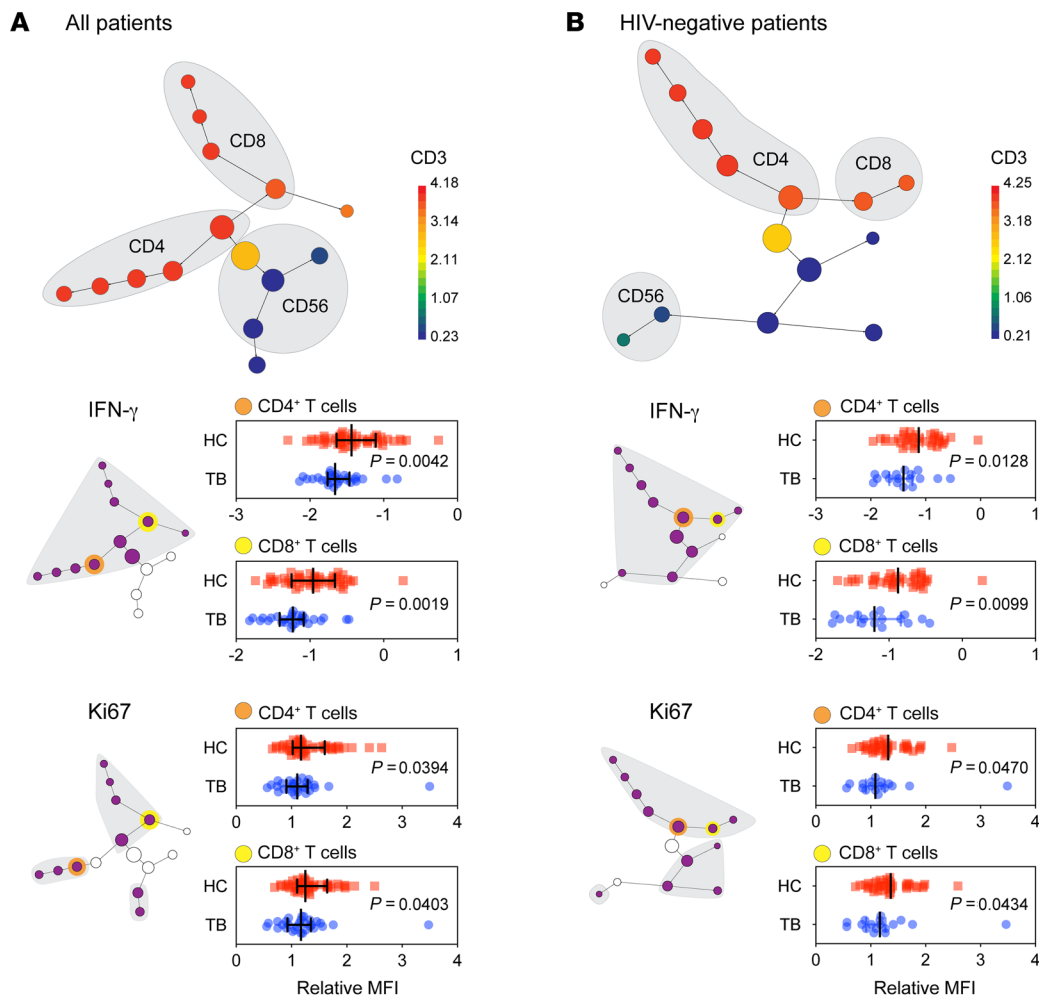


Figure 1. Patients with TB exhibit an exhausted immune phenotype. PBMCs from individuals with TB ($n = 29$) and their asymptomatic household contacts (HC) ($n = 49$) were subjected to antigenic stimulation with BCG sonicate followed by flow cytometry-based multidimensional immune profiling. CITRUS clustering was implemented on CD3, CD4, CD8, and CD56 surface markers with a minimum cluster size of 10% per node. Lines indicate the median and interquartile range, and P values were determined by nonparametric Mann-Whitney U test. (A) Citrus clustering identified Th (CD3⁺CD4⁺), CTL (CD3⁺CD8⁺), and NK (CD3⁺CD56⁺) cell populations, with CD3 signal depicted by a heatmap. Gray-shaded subclusters have an FDR of less than 1% and depict decreased IFN- γ and Ki-67 expression among Th cells (CD3⁺CD4⁺), CTLs (CD3⁺CD8⁺), and NK cells (CD3⁺CD56⁺). Representative dot plots of the MFI clusters are depicted from the yellow highlighted clusters. (B) Reanalysis of CITRUS, with exclusion of HIV-coinfected individuals, for patients with TB ($n = 20$) and controls ($n = 40$) shows CD4⁺, CD8⁺, and CD56⁺ lymphocyte subsets with decreased Ki-67 and IFN- γ (gray-shaded clusters have decreased IFN- γ and Ki-67, with an FDR <1%; representative dot plots of MFI clusters are depicted). Mann-Whitney U test P values describe MFI differences between controls and patients with TB.

To validate the EDEC results, we isolated CD4⁺ T cells and evaluated DNA methylation at baseline and 6 months after successful ATT. Of note, cell proliferation, intracellular calcium signaling, and IL-2/STAT5 and IFN- γ pathways were hypermethylated in both isolated CD4⁺ T cells and the deconvoluted CD4⁺ T cells (Figure 2C). Analysis of isolated CD4⁺ T cells confirmed the results from EDEC for 82% of hypermethylated genes (probability of overlap = 9.3×10^{-5}). Baseline DNA hypermethylation, as identified by EDEC and in isolated CD4⁺ T cells, was observed in *JAK1*, *IL12RB2*, *STAT1*, *FYN*, *GATA2*, *IKZF2*, and *TOX* (Figure 2D).

Previous studies of chronic LCMV, cancer, HIV, and schistosomiasis identified persistent epigenetic-mediated immune exhaustion long after removal of the chronic antigen stimulation (9, 11, 14, 15). DNA hypermethylation in patients with TB, both at baseline and 6 months after successful ATT, resembled the

closed chromatin confirmation induced by chronic LCMV infection models. We evaluated the similarities between the epigenetic landscape of murine LCMV and that of patients with TB by comparing the closed chromatin-accessible regions (ChARs) previously published by Sen et al. (4) and the DNA hypermethylated regions previously published by Ghoneim et al. (10) from chronic LCMV models with the CD8 DNA hypermethylated regions of TB patients from this study. In particular, we observed DNA hypermethylation and closed chromatin conformation of the IFN- γ pathway (*IL12RB2*, *JAK1*, *STAT4*) in both TB and chronic LCMV (Figure 3, A and B). We performed immunologic gene set enrichment analysis and found that CD8⁺ T cells from patients with TB were enriched ($P = 4.41 \times 10^{-11}$) to a degree similar to that of genes downregulated in LCMV-induced immune exhaustion (23). In addition to DNA methylation changes being induced by chronic

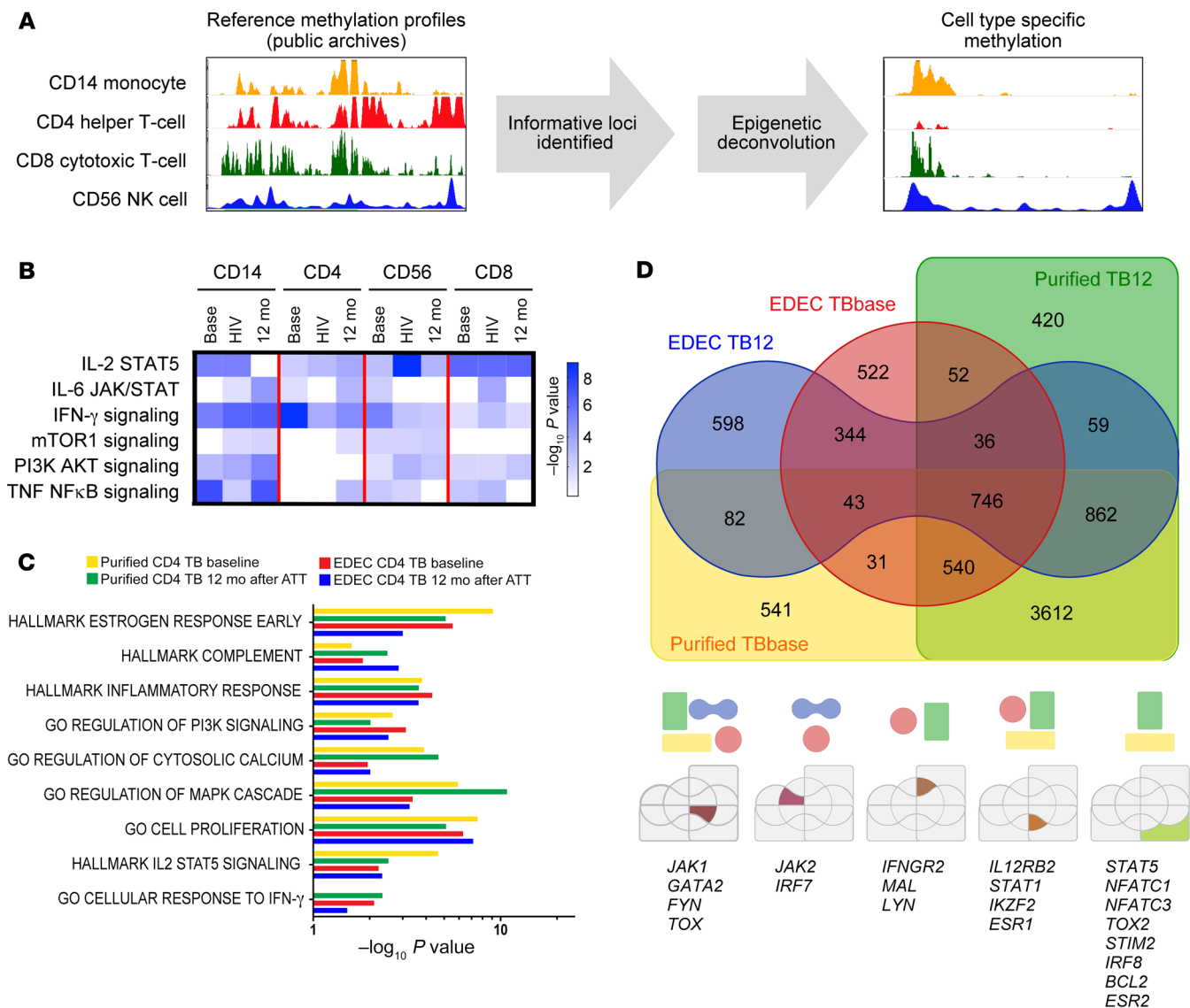


Figure 2. Global DNA methylation perturbations persist 6 months after successful ATT. The Illumina DNA Methylation EPIC array was performed on bulk PBMCs from asymptomatic household contacts ($n = 10$) and individuals with TB ($n = 15$; TB/HIV $^- = 7$; TB/HIV $^+ = 8$) at baseline. All individuals with TB had treatment success. For individuals without HIV coinfection, DNA methylation status was evaluated at baseline and 12 months later, 6 months after completion of successful ATT. (A) To ascertain the immune cell-specific DNA methylation changes, cell-specific DNA methylation reference profiles were downloaded from public archives, informative loci were identified, and cell type-specific methylation profiles were identified. (B) Cell-specific DNA methylation differences are shown for TB patients (base) and TB/HIV (HIV) patients at baseline and for TB patients 12 months after baseline (12 mo), 6 months after completion of successful ATT. All results were compared with HC data. GO pathway analysis for monocytes (CD14 $^+$), Th cells (CD3 $^+$ CD4 $^+$), NK cells (CD3 $^+$ CD56 $^+$), and CTLs (CD3 $^+$ CD8 $^+$). (C and D) Purified Th cells (CD3 $^+$ CD4 $^+$) ($n = 2$) and EDEC-identified Th cells (CD3 $^+$ CD4 $^+$) ($n = 8$ TB patients; $n = 10$ controls) were compared with cells from controls, and common hypermethylated pathways and genes were identified.

antigenic exposure, *M. tuberculosis* itself releases epigenetic-modifying enzymes that target host chromatin and DNA methylation. For example, Rv2966c is a DNMT that is secreted by *M. tuberculosis* and chaperoned into host immune cell nuclei (24). In in vitro models, Rv2966c induces DNA hypermethylation of host immune cells (25) in a manner similar to that seen in this cohort of TB patients, including hypermethylation of *IL12RB2*, *STAT4*, *IFNG*, *IRF1*, and *JAK1* (Supplemental Figure 2).

Patients with TB exhibit DNA hypermethylation of the IL-12/IFN- γ signaling pathway. To validate the DNA methylome results, we implemented targeted methylation-specific restriction endo-

nuclease qPCR (MSRE-qPCR) on genes previously relevant to mycobacterial biology (26). Although the EPIC array evaluates DNA methylation via bisulfite conversion, MSRE quantifies the methylation status using endonuclease isochizomers that degrade DNA on the basis of methylation status. MSRE-qPCR of isolated CD4 $^+$ T cells confirmed DNA hypermethylation of the IL-12/IFN- γ pathway (Figure 4). Specifically, *IL12B* demonstrated 6.1-fold increased methylation ($P = 0.007$); *IL12RB2* was hypermethylated 35.2-fold ($P < 0.03$); *TYK2* demonstrated 17.7-fold increased methylation ($P = 0.008$); *IFNGR1* was hypermethylated 24.0-fold ($P = 0.007$); and *JAK1* and *JAK2* demonstrated 33.3- and 11.7-fold

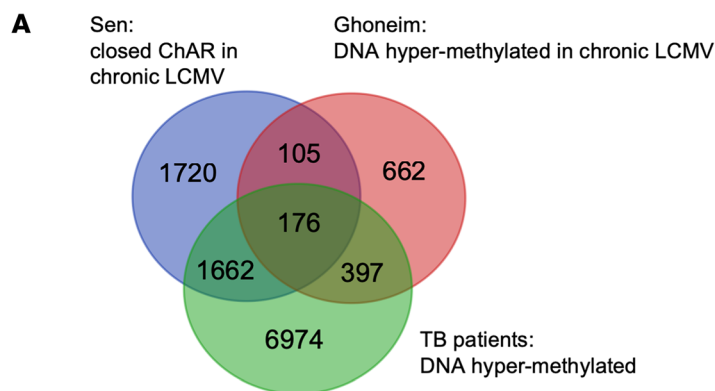


Figure 3. The TB epigenetic landscape is similar to LCMV-induced immune exhaustion. (A) DNA hyper-methylation of genes in CD3⁺CD8⁺ CTLs in TB patients (green), and that reported by Ghoneim et al. (10) (red) and Sen et al. (4) (blue). (B) Genes and GO pathways of CD8⁺ CTLs with DNA hypermethylation for patients with TB from this cohort were compared with genes with DNA hypermethylation, reported by Ghoneim et al. (10), and ChARs induced by chronic LCMV-induced immune exhaustion, reported by Sen et al. (4).

B

Sen CD8	Ghoneim CD8	TB CD8	Genes	Pathways
			STAT5A, IRF1, FYN	IFN- γ signaling, hypoxia, apoptosis
			IL12RB2, STAT4, JAK1, ESR1	IFN- γ signaling, glycolysis, ROS pathway, hypoxia
			TOX, IRF2, IRF8, LCK, IKZF3	Estrogen, IL2-STAT, hypoxia, PI3K-AKT-mTOR

increased DNA methylation ($P = 0.004$ and 0.03), respectively (Figure 4). *STAT1* and *IRF1* were not statistically hypermethylated ($P = 0.21$ and 0.15 , respectively; Figure 4B). Regulation of immunity is epigenetically controlled at specific pathways, but also by a network of transcription factors (27). MSRE-qPCR revealed that CD4⁺ T cells from patients with TB had DNA hypermethylation of the transcription factors *TOX*, *NFATC1*, *BATF3*, *ID2*, *PPARG*, *RUNX2*, *IRF5*, and *IKZF1* ($P < 0.001$; Figure 4B).

Patients with TB have decreased mitogen responsiveness. Patients with TB had DNA hypermethylation of IL-2, IL-6, TNF, and IFN- γ signaling pathways (Figures 2–4). Notably, the TNF/NF- κ B signaling pathway and the *TNF* gene were hypermethylated in CD14⁺, CD8⁺, and CD56⁺ cells from patients with TB (Figure 3). Given that these pathways result in activation of MAPK and are also downstream of mitogen activation, we hypothesized that patients with TB would have decreased mitogen responsiveness. Freshly collected whole blood was stimulated overnight with mitogen (phytohemagglutinin), and a multiplexed ELISA was performed on the supernatant. Although the baseline production of TNF was similar (3.7 vs 1.4 pg/mL; $P = 0.2$), after mitogen stimulation, the patients with TB exhibited 72% decreased upregulation of TNF by ELISA (80.5 vs. 293 pg/mL; $P = 0.01$; Figure 5). The IFN- γ signaling pathway was hypermethylated in all lymphocytes, and the *IFNG* gene was hypermethylated in CD8 and CD56 cells from patients with TB, and although patients with TB had increased IFN- γ at baseline by ELISA (1.31 vs. 0.58 pg/mL, $P = 0.007$), they had a 35.4% decreased capacity to upregulate IFN- γ upon mitogen activation (increase of 246.9 vs. 382.4 pg/mL; $P = 0.03$). The chemokines CXCL9 and CXCL10 are downstream of IFN- γ , and like IFN- γ , they were increased at baseline (42.5 vs. 10.6 and 238.3 vs. 117.4 pg/mL; $P < 0.01$) but had 30.7% and 30.1% decreased upregulation after mitogen-induced activation ($P = 0.01$ and 0.002 ,

respectively). IL-6 and IL-1 β levels were comparable at baseline, but patients with TB had 40.5% and 60.7% decreased mitogen-induced upregulation of IL-6 (8870 vs. 14,917 pg/mL; $P < 0.001$) and IL-1 β (1968 vs. 5974 pg/mL, $P = 0.0005$), respectively.

Patients with TB have decreased IFN- γ -induced gene upregulation. An intact IL-12/IFN- γ pathway is necessary, but not sufficient, for control of mycobacterial infections (26, 28). Since patients with TB exhibited DNA hypermethylation of the IL-12/IFN- γ signaling pathway in CD4⁺ Th cells, NK cells, and CTLs, we postulated that they would also exhibit decreased IFN- γ -inducible gene upregulation using a previously described 10-gene IFN- γ -inducible score (15, 29, 30). In healthy immune cells, IFN- γ stimulation induces gene expression of *CITTA*, *GBP1*, *STAT1*, *CXCL9*, *CXCL10*, *CXCL11*, *IL15*, *SERPING1*, *IDO1*, and *FCGR1A/B* (29); however, previous studies have demonstrated decreased IFN- γ -inducible gene expression in cancer (30) and chronic schistosomiasis infection (15). In this cohort, compared with controls, the PBMCs

Table 1. Genes hypermethylated in patients with TB

	CD4	CD8	CD14	CD56
<i>JAK2, STAT1</i>	x			
<i>IL12B, TYK2</i>		x		
<i>IL6</i>			x	
<i>IL12RB1</i>				x
<i>IL12A, TNF</i>			x	x
<i>IFNG, JAK1, NFKB1, STAT4</i>		x		x
<i>LCK</i>	x	x	x	
<i>IL12RB2</i>	x	x		x
<i>HLA-DPA1, FYN</i>	x	x	x	x

Table 2. Pathways hypermethylated in patients with TB

	CD4	CD8	CD14	CD56
PI3K/ATK/mTOR			x	
WNT/catenin	x			x
TNF signaling via NF-κB			x	x
IL-2/STAT5		x		x
Glycolysis, estrogen, KRAS	x		x	x
IFN-γ signaling	x	x		x

from patients with TB had 69.7% decreased IFN-γ-inducible gene upregulation (17.9- vs. 59.2-fold increase; $P = 0.02$; Figure 6).

Patients with TB have decreased IL-12-inducible IFN-γ production. Lymphocytes are primed with IL-12 for robust IFN-γ production (31). Since patients with TB showed DNA hypermethylation of the IL-12 pathway (*IL12A*, *IL12B*, *IL12RB1*, and *IL12RB2*), we evaluated the functionality of the IL-12/IFN-γ axis by stimulating PBMCs overnight with BCG, with and without IL-12. Although Th cell (CD3⁺CD4⁺) IFN-γ production was similar, patients with TB had a muted IL-12-induced production of IFN-γ in CTLs and NK cells. Specifically, NK cells had 62.5% decreased IL-12-inducible upregulation of IFN-γ ($P = 0.008$), whereas CTLs had a 43.4% decrease in IL-12-induced IFN-γ upregulation ($P = 0.03$; Figure 7 and Supplemental Figure 3).

Discussion

Despite becoming culture negative after 2 months of antituberculosis therapy, patients with TB require 6 months of antibiotics (32). However, even after these 6 months of antibiotics, patients with successful ATT have an increased risk of recurrent TB compared

with the general population (2). Epigenetic-mediated immune exhaustion has been demonstrated to impede immunotherapy in murine LCMV experimental models (10), but it has not previously been evaluated as a potential etiology of TB-induced immune exhaustion. Here we demonstrate that patients with TB have DNA hypermethylation in critical immune pathways including the IL-12/IFN-γ pathway and that this is associated with decreased mitogen, mycobacterial, IL-12, and IFN-γ immune responsiveness.

Depending on the definition applied for anergy, approximately 5%–25% of patients with TB fail to mount a positive skin reaction to tuberculin, or fail to release IFN-γ after exposure to mycobacterial antigens (33–39). Patients with TB who fail to produce *M. tuberculosis*-specific IFN-γ have increased mortality (40), potentially indicating more advanced disease. The TB-induced anergy is not specific to mycobacterial antigens, as patients with TB also exhibit decreased responsiveness to *Candida* and histoplasmin antigens (41). HIV increases the likelihood of anergy (36), but in some cohorts, up to 46% of HIV TB patients are anergic (42). Modern immune analysis has characterized TB-induced immune exhaustion demonstrating that patients with TB have decreased IFN-γ and IL-2 expression and proliferative capacity and increased expression of immune checkpoint inhibitors such as PD-1, TIM3, and CTLA4 (19, 20, 43, 44). In addition to limiting critical cytokines, previous studies have demonstrated that patients with TB have dysfunctional intracellular signaling downstream of IFN-γ with decreased upregulation of IFN-γ-inducible gene expression (45, 46). DNA hypermethylation has been shown to inhibit robust immunity in LCMV models, and here we demonstrate that DNA hypermethylation was associated with decreased immune responsiveness during TB.

Immune exhaustion after chronic antigen stimulation is not limited to TB and has been observed in many instances of per-

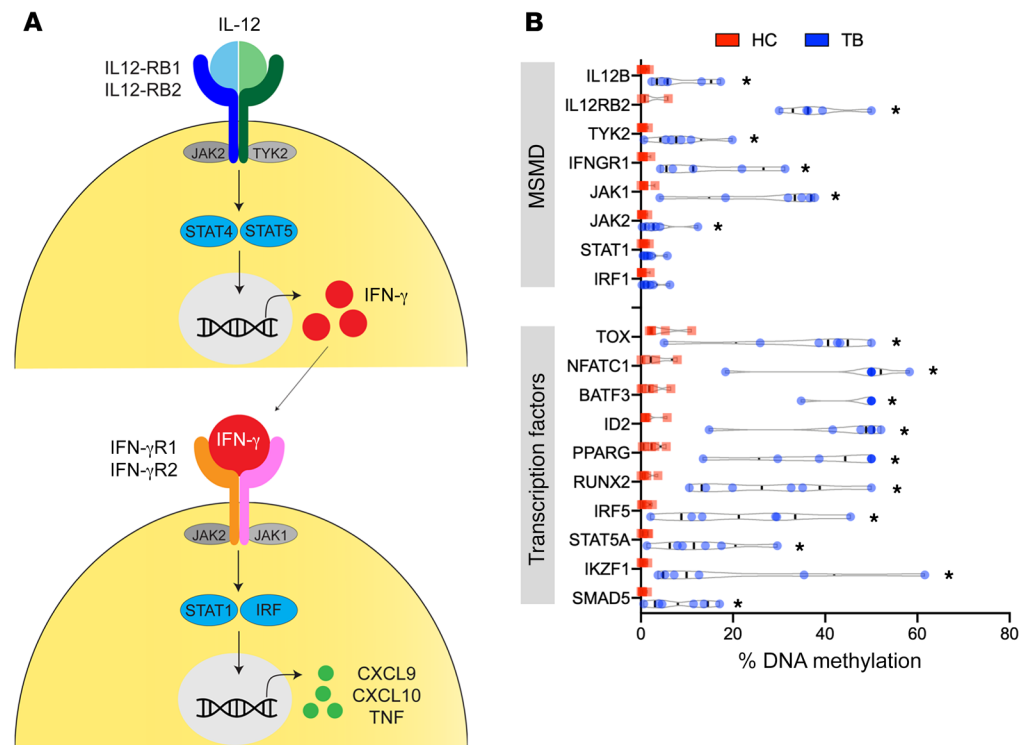


Figure 4. Targeted DNA methylation confirms DNA hypermethylation of the IL-12/IFN-γ pathway. (A) Graphic representation of the IL-12/IFN-γ pathway. (B) DNA methylation was evaluated using MSRE-qPCR in non-HIV-coinfected TB patients ($n = 6$) and their asymptomatic healthy household contacts ($n = 5$). $*P < 0.01$, by Mann-Whitney U test.

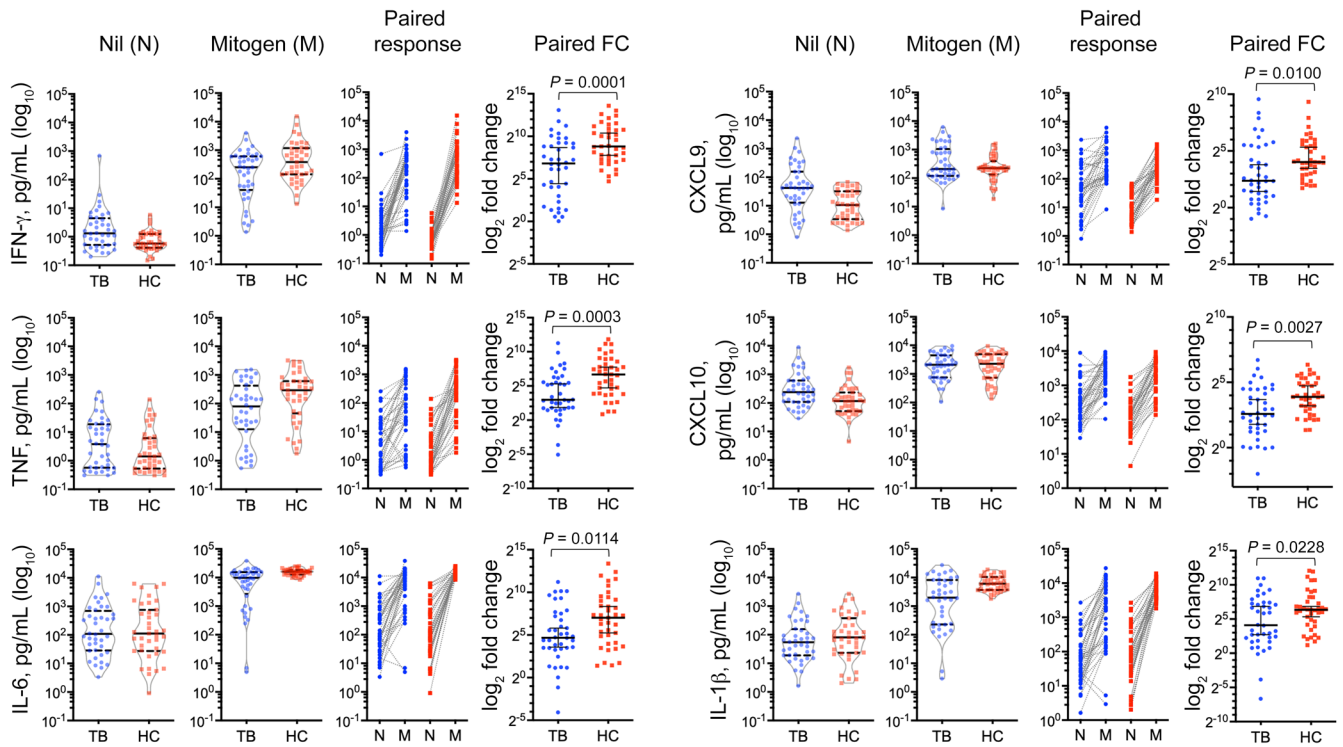


Figure 5. Decreased mitogen-induced responsiveness. Fresh whole blood (0.8–1.2 mL) from patients with TB ($n = 40$) and asymptomatic healthy household contacts ($n = 39$) was stimulated overnight with and without mitogen, and the resultant plasma was evaluated for cytokine and chemokine upregulation using a custom-designed, bead-based multiplex ELISA. P values were determined by nonparametric Mann-Whitney U test. FC, fold change.

sistent inflammation due to chronic infection, sepsis, or cancer (6, 47–49). Continuously expressed tumor antigens induce immune exhaustion via upregulation of DNMTs and the transcriptionally repressive EZH2 complex, both known moderators of epigenetic-mediated immune exhaustion (13, 21). Epigenetic-mediated immune exhaustion persists even if cells are rested for 3 weeks, or if they are transferred into healthy, noninfected mice (11, 13). In clinical cohorts, even 2 years after successful aviremia, patients infected with HIV retain detrimental epigenetic marks (9, 14). Similarly, 6 months after successful deworming, children previously infected with schistosomiasis retained detrimental DNA methylation

marks (15). In this report, 6 months after completing successful ATT, the immune system showed persistent DNA hypermethylation perturbations. Patients in this cohort were only followed 6 months after completion of ATT, and longitudinal studies will need to assess whether persistent detrimental DNA methylation marks correlate with recurrent TB disease.

Chronic LCMV models have demonstrated that immune exhaustion is moderated by broad remodeling of the epigenetic landscape and that these epigenetic perturbations encumber immune checkpoint blockade-based immunotherapy (4, 5). Ghoneim et al. demonstrated that immune exhaustion is mod-

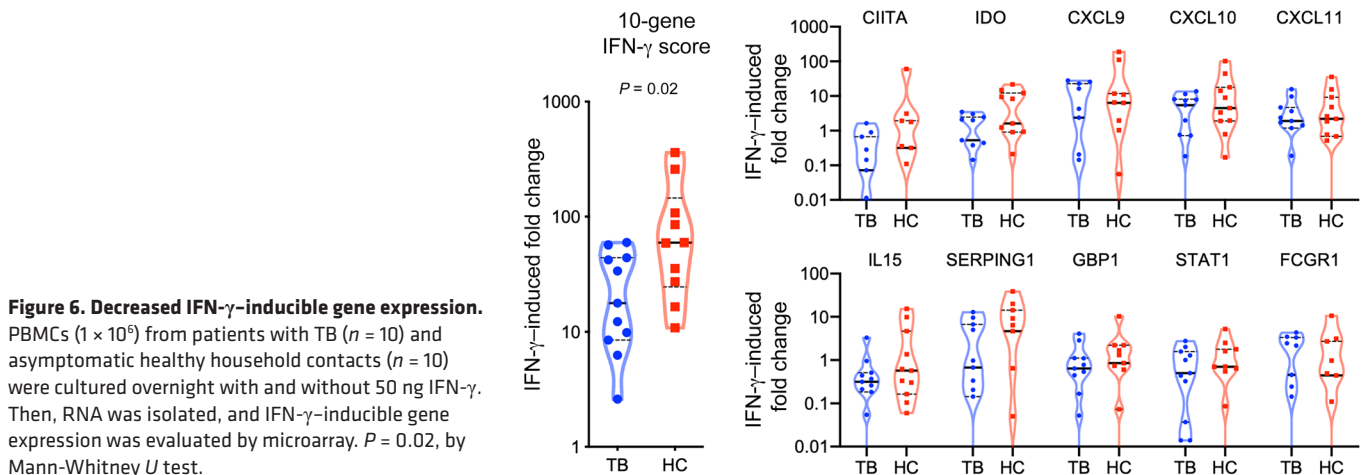


Figure 6. Decreased IFN- γ -inducible gene expression. PBMCs (1×10^6) from patients with TB ($n = 10$) and asymptomatic healthy household contacts ($n = 10$) were cultured overnight with and without 50 ng IFN- γ . Then, RNA was isolated, and IFN- γ -inducible gene expression was evaluated by microarray. $P = 0.02$, by Mann-Whitney U test.

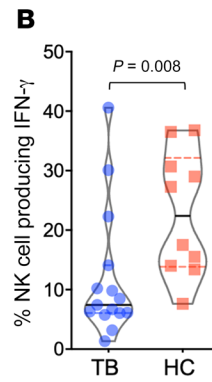
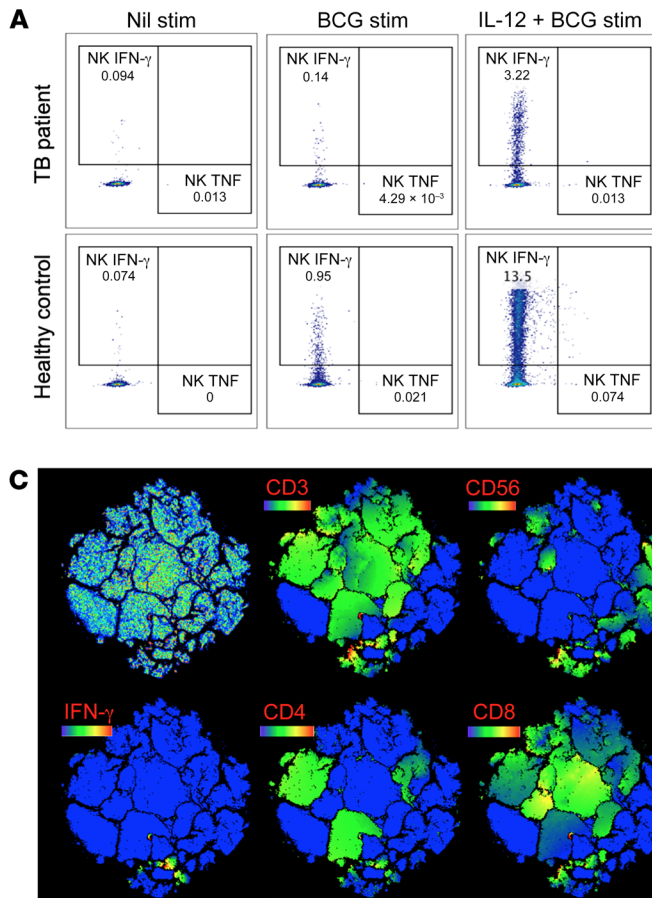
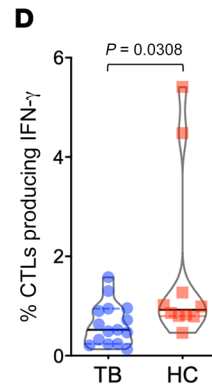


Figure 7. Decreased IL-12-inducible IFN- γ production. PBMCs from patients with TB ($n = 15$) and asymptomatic healthy household contacts ($n = 10$) were treated overnight with PBS media control (Nil stim), or were stimulated with BCG sonicate ($5 \mu\text{g}$) or IL-12 (20 ng/mL) and BCG (minimum of 0.5×10^6 PBMCs per condition) followed by multi-dimensional flow cytometry. See Supplemental Figure 3 for the gating strategy and representative dot plots of CD3 $^+$ CD4 $^+$ and CD3 $^+$ CD8 $^+$ T cells. **(A)** Representative dot plots of NK cell (CD3 $^+$ CD56 $^+$) production of IFN- γ and TNF- α after no stimulation or stimulation with BCG or with IL-12 plus BCG. **(B)** t -distributed stochastic neighbor embedding (tsNE) dimension reduction clustering of PBMCs from a control and from patients with TB demonstrated the cell-specific (CD3, CD4, CD8, and CD56) contribution of IFN- γ production after combined IL-12 and BCG stimulation, with blue indicating no protein expression, and green, yellow, and red indicating increasing protein expression. **(C and D)** Dot plots demonstrate the percentage of CTLs (CD3 $^+$ CD8 $^+$) and NK cells (CD3 $^-$ CD56 $^+$) that produced IFN- γ after stimulation with IL-12 and BCG. $P = 0.0308$ and 0.008 , by Mann-Whitney U test, for CTLs and NK cells, respectively.



erated by DNA methylation with hypomethylating agents dosed before immune checkpoint inhibitor therapy capable of reversing epigenetic-mediated immune exhaustion (10). These data demonstrate that the DNA hypermethylation landscape of patients with TB before and after successful ATT resembles murine LCMV-induced immune exhaustion regions of closed chromatin by chronic LCMV (45) and DNA hypermethylation (10). In addition to chronic antigen-induced epigenetic-mediated immune exhaustion, an alternative and nonmutually exclusive mechanism of host epigenetic perturbations is pathogen production of host epigenetic-modifying enzymes. *Leishmania*, HIV, HPV, and *M. tuberculosis* all produce epigenetic-modifying enzymes that target host epigenetic status (24, 25, 46, 50–54). *M. tuberculosis* produces a functional DNMT that is secreted and chaperoned into the nucleus, inducing host DNA methylation changes in the IL-12/IFN- γ signaling pathway (24, 25) similar to the DNA methylation changes observed in this cohort (Supplemental Figure 2). The current study did not evaluate the *M. tuberculosis* strain for DNMT production, but future studies should evaluate the effect of strain on host DNA methylation in clinical cohorts.

DNA hypermethylation of the IL-12/IFN- γ signaling pathway, especially among CD8 $^+$ T cell and NK cell populations, may partially explain the complexity of identifying IFN- γ -mediated immune correlates of protection. The Mendelian susceptibility to mycobacterial diseases (MSMDs) are a rare collection of gene mutations that increase an individual's risk for TB and other intracellular infections. The MSMDs highlight the critical nature

and complexity of this pathway. Upstream mutations of *IL12B*, *IL12RB*, or *NEMO* result in decreased IFN- γ , decreased IFN- γ -inducible gene expression, and decreased mycobacterial killing capacity. In contrast, mutations of *IFNGR1*, *IFNGR2*, or *STAT1* downstream of IFN- γ result in increased IFN- γ but decreased IFN- γ -inducible gene expression and decreased mycobacterial killing capacity. Here, we demonstrate that patients with TB have increased DNA methylation in the canonical IL-12/IFN- γ pathway (Figure 4A) both upstream (in *IL12*, *IL12RB2*, and *STAT4*) and downstream (in *IFNG*, *JAK1*, and *STAT1*) of IFN- γ (Figures 3 and 4). However, the *IFNG* gene expression response is dependent on both canonical and noncanonical signaling. Noncanonical modulators of IFN- γ -inducible gene expression include *FYN*, *MAL*, and the mTOR pathway, which were also hypermethylated in patients with TB. DNA hypermethylation of the canonical and noncanonical IL-12/IFN- γ pathways was associated with reduced IFN- γ -inducible gene expression (Figure 6), reduced mitogen-induced cytokine production (Figure 5), and reduced IL-12-inducible IFN- γ production (Figure 7).

Host-directed immunotherapy has been identified as a priority research area to improve TB treatment outcomes for the 10 million people afflicted each year. TB is an archetypical chronic infection known to inhibit host immunity, and here we demonstrate that decreased immune responsiveness to IL-12, IFN- γ , mitogen, and mycobacterial antigens is associated with DNA hypermethylation of critical immune transcription factors and the canonical and noncanonical IL-12/IFN- γ signaling pathways.

The persistent DNA methylation perturbations we have identified are a plausible explanation for why successful TB therapy must be continued for months after *M. tuberculosis* culture conversion. These data suggest that it is critical to evaluate whether modulating DNA methylation could effectively augment host antimycobacterial immunity.

Methods

Multidimensional immune profiling. PBMCs were isolated using Ficoll separation and cryopreserved in liquid nitrogen. Upon thawing, greater than 70% viability was determined by flow cytometry using trypan blue staining and lymphocyte amine reactive dye. PBMCs were stimulated under the following conditions: (a) DMSO vehicular control, (b) 2.5 μ g each of ESAT-6 and CFP-10 overlapping peptide pools, (c) 5 μ g BCG sonicate, and (d) 0.2 μ L staphylococcal enterotoxin B. Stimulations took place in the presence of costimulation (CD28 and CD49) for 18 hours with monensin and brefeldin A for the last 12 hours. Cells were stained with an amine reactive dye (Ghost Dye), surface antibodies (CD4, CD8, CD56, and PD-1), and intracellular antibodies (CD3, Ki-67, IFN- γ , TNF, IL-4, IL-10, IL-13, perforin, T-bet, and GATA-3) and acquired on a BD LSR II Fortessa. Complete antibody information can be found in Supplemental Table 3. The following reagents were obtained through BEI Resources, NIAID, NIH: peptide array, *M. tuberculosis* ESAT-6 protein, NR-50711; and peptide array, *M. tuberculosis* CFP-10 protein, NR-50712.

DNA methylation. Genomic DNA (gDNA) was isolated from 1×10^6 PBMCs or magnetic-bead isolated CD4⁺ T cells with a minimum of 80% viability using the DNeasy kit (QIAGEN). Nucleic acid quantity and quality were examined using a Qubit 3.0 fluorometer and an Agilent 4200 TapeStation System, and 500 ng gDNA was treated with bisulfite before running the Illumina Infinium MethyLEPIC array. Methylation IDAT files were preprocessed and normalized using Bioconductor's R statistical minfi package. Probes with greater than 2-fold or less than 0.5-fold differential methylation with *P* values of less than 0.05 were considered significant. Gene ontology (GO) and Gene Set Enrichment Analysis were implemented using the Molecular Signature Database (MSigDB) with hypergeometric distribution that accounted for multiple comparisons (55). Epigenetic deconvolution was performed as previously described (56). The EpiTect II DNA Methylation Enzyme kit (QIAGEN) was used to validate methylome results as previously described (15). In brief, gDNA underwent methylation-specific enzymatic digestion, and qPCR was performed to determine the percentage of methylated and unmethylated DNA relative to mock and double digestion (15, 57). The DNA MethyLEPIC data were deposited in the NCBI's Gene Expression Omnibus (GEO) database (GEO GSE145714).

ELISA. Fresh whole blood (0.4 mL per condition) from patients with TB and their asymptomatic, healthy household contacts was stimulated overnight with and without mitogen (phytohemagglutinin), and the resultant supernatant was evaluated for IFN- γ , TNF, IL-6, CXCL9, CXCL10, and IL-1 β using a customized bead-based multiplex ELISA (LEGENDplex kits, BioLegend).

Gene expression. PBMCs were stimulated overnight with IFN- γ (50 ng/mL) or media control (PBS) for 16 hours followed by RNA isolation, and gene expression was evaluated using the NanoString nCounter Human Immunology 2 probe set. Data were normalized using 8 negative controls and 10 housekeeping genes.

Statistics. Flow cytometric data were processed using FlowJo (Tree Star) and GraphPad Prism 6.0 (GraphPad Software). CITRUS was implemented using CytoBank (<http://www.cytobank.org>) using nearest shrunken centroid classification (PAMR) with a minimum cluster size of 10%, a cross validation fold of 10, and a FDR of 1%. DNA methyLEPIC probes were considered significant if they had greater than 2- or less than 0.5-fold differences compared with controls, with a *P* value of less than 0.05 considered significant. Gene expression, flow cytometry, ELISA, and targeted MSRE DNA methylation data were evaluated using a nonparametric Mann-Whitney *U* test, with a *P* value of less than 0.5 considered significant and violin plots showing the data distribution with the median and quartiles delineated.

Study approval. The study protocol was approved by the Baylor College of Medicine Children's Foundation-Swaziland and the Eswatini National Health Research and Review Board. All participation was voluntary and implemented in accordance with institutional and Declaration of Helsinki guidelines for the protection of human subjects, and written informed consent was obtained from all participants or their guardians. In this analysis, immune cells from patients with TB were analyzed if they had pulmonary disease as defined by symptoms of TB (cough, fevers, weight loss, etc.) and microbiologically confirmed disease by sputum Gene Xpert test and/or culture. All participants were screened for HIV. Individuals with helminth coinfection (by urine or stool microscopy and urine and stool qPCR) were excluded from this analysis. HCs were household contacts of the TB patients with a TB contact score (58) of 5 or greater and excluded if they progressed to active TB and did not remain asymptomatic for 12 months. All individuals were BCG vaccinated as confirmed by BCG scar and/or vaccine card.

Author contributions

ARD and AMM designed the study. G Mtetwa, G Maphalala, AK, ARD, QD, and JK implemented cohort enrollment and designed the clinical study. TN, ARD, QD, and JK performed the immunology and DNA methylation studies. SM, SLG, and ARD performed CITRUS analysis. KR, SLG, and CC conducted the bioinformatic and statistical analyses. ARD, AMM, G Makedonas, EMM, JDC, CC, MGN, and RVC assisted with data interpretation and designing of the biologic validation studies.

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