Mycobacterium tuberculosis WhiB3 Responds to Vacuolar pH-induced Changes in Mycothiol Redox Potential to Modulate Phagosomal Maturation and Virulence*§

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The ability of Mycobacterium tuberculosis to resist intraphagosomal stresses, such as oxygen radicals and low pH, is critical for its persistence. Here, we show that a cytoplasmic redox sensor, WhiB3, and the major M. tuberculosis thiol, mycothiol (MSH), are required to resist acidic stress during infection. WhiB3 regulates the expression of genes involved in lipid anabolism, secretion, and redox metabolism, in response to acidic pH. Furthermore, inactivation of the MSH pathway subverted the expression of whiB3 along with other pH-specific genes in M. tuberculosis. Using a genetic biosensor of mycothiol redox potential (EMSH), we demonstrated that a modest decrease in phagosomal pH is sufficient to generate redox heterogeneity in EMSH of the M. tuberculosis population in a WhiB3-dependent manner. Data indicate that M. tuberculosis needs low pH as a signal to alter cytoplasmic EMSH which activates WhiB3-mediated gene expression and acid resistance. Importantly, WhiB3 regulates intraphagosomal pH by down-regulating the expression of innate immune genes and blocking phagosomal maturation. We show that this block in phagosomal maturation is in part due to WhiB3-dependent production of polyketide lipids. Consistent with these observations, MtbΔwhiB3 displayed intramacrophage survival defect, which can be rescued by pharmacological inhibition of phagosomal acidification. Last, MtbΔwhiB3 displayed marked attenuation in the lungs of guinea pigs. Altogether, our study revealed an intimate link between vacuolar acidification, redox physiology, and virulence in M. tuberculosis and discovered WhiB3 as a crucial mediator of phagosomal maturation arrest and acid resistance in M. tuberculosis.

Mycobacterium tuberculosis causes a chronic persistent infection that affects one-third of the world’s population (see the World Health Organization website). Macrophage is the major human host cell for growth, survival, and persistence of M. tuberculosis. Studies indicate that M. tuberculosis continuously senses the phagosomal environment and modulates genetic pathways to regulate intramacrophage growth for long term persistence (1–3). In this regard, acidic pH has recently been appreciated as an important intraphagosomal signal sensed by M. tuberculosis to regulate gene expression and establish chronic infection (3). The importance of pH emerged from several studies showing that pathogenic mycobacteria successfully restrict fusion of phagosomes with acidic lysosomes and therefore multiply in a growth-permissive vacuolar compartment with a pH of ~6.2 (4–6). However, upon activation of macrophages by interferon-γ (IFN-γ) and Escherichia coli-derived lipopolysaccharide (LPS), the pH of the M. tuberculosis phagosome drops to <5.0, resulting in M. tuberculosis growth restriction (6–8). Hence, the inhibition of phagosomal maturation during early stages of infection and induction of acid resistance mechanisms later during immune-activation are considered major virulence strategies adopted by M. tuberculosis to establish chronic infection (9). Despite the recognized role of acidic pH in regulating M. tuberculosis pathogenesis, the mechanisms by which M. tuberculosis responds to fluctuations in phagosomal pH and calibrates its gene expression for intracellular growth and persistence remain poorly characterized.

Recent studies indicate that M. tuberculosis maintains a neutral intrabacterial pH (~7.2) after exposure to a range of acidic pH levels (from 6.2 to 4.5) in vitro, and inside the acidic phagosomal milieu of resting or activated macrophage (10). This indicates that M. tuberculosis stably maintains intrabacterial pH homeostasis during infection and, therefore, that the intrabacterial pH per se is unlikely to be the signal that triggers alterations in M. tuberculosis gene expression in response to changes in phagosomal acidity. Therefore, novel insights are needed to discover which aspects of mycobacterial physiology are modulated by phagosomal acidity and what are the bacterial sensors of phagosomal pH. In this context, an Fe-S cluster containing putative transcription factor WhiB3 is induced in response to acidic pH in medium, inside macrophages, and in the lungs of infected animals (11, 12). In fact, whiB3 was the only transcription factor whose expression was found to be pH-responsive inside macrophages. In vitro studies have shown that WhiB3 responds to dormancy signals, such as O2 and nitric oxide (NO), via its 4Fe-4S cluster (13). However, phenotypic experiments revealed no role of WhiB3 in controlling mycobacterial survival in response to NO or hypoxia (13). Therefore, how

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WhiB3 regulates mycobacterial persistence remained uncharacterized. Consistent induction of whiB3 in acidic environments in vitro and inside macrophages (12) implicated WhiB3 in regulating adaptation of M. tuberculosis in response to phagosomal pH. A fundamentally important question remains unanswered. What is the mechanism by which WhiB3 mediates the acid response of M. tuberculosis during infection?

In this study, we performed global microarray analysis to identify genes regulated by WhiB3 in response to acid stress. More importantly, we measured the dynamic changes in mycothiol redox potential ($E_{\text{MSSH}}$) of WT M. tuberculosis and $\text{Mtb}^{\Delta\text{whiB3}}$ in response to acidic pH in vitro and inside naive and activated macrophages. Confocal studies and host microarray studies were performed to examine the function of WhiB3 in regulating phagosomal maturation. Last, the physiological importance of whiB3-mediated effects on gene expression, redox homeostasis, and phagosomal maturation were investigated by performing survival studies in macrophages and guinea pigs. Our study, for the first time, demonstrates how M. tuberculosis recalibrates its redox physiology in response to vacuolar pH during infection and identifies a major role of WhiB3 in responding to acid stress.

### Experimental Procedures

**Bacterial Strains and Growth Conditions**—Wild type M. tuberculosis H37Rv (WT M. tuberculosis), Mtb<sup>ΔwhiB3</sup>, and whiB3-comp strains were cultivated as described (13). E. coli cultures were grown in LB medium. When required, culture medium was supplemented with kanamycin (25 $\mu$g/ml) or hygromycin (50 $\mu$g/ml). For acid stress, the pH of 7H9 broth was adjusted using hydrochloric acid (HCl) and buffered using 100 mM MES. Approximately 1 $\times$ 10<sup>7</sup> cells/ml were exposed to various pH-adjusted media, and survival was monitored at day 0 and day 4 by serially diluting the culture and enumerating colony-forming units (cfu). For carbonyl cyanide $m$-chlorophenylhydrazone (CCCP) treatment, bacteria were treated with 500 $\mu$m CCCP for 4 h.

**Generation of Mtb<sup>ΔwhiB3</sup> and whiB3 Complemented Strains**—For constructing Mtb<sup>ΔwhiB3</sup>, 1-kb left flanking and right flanking regions of whiB3 (Rv3416) were cloned upstream and downstream of the loxP-gfp-hygromycin-loxP cassette in a mycobacterial sacB-based suicide vector, pML523 (a kind gift from Michael Neiderwies, University of Alabama at Birmingham). The construct was digested with SspI and NsiI to release the mycobacterial sacB-based suicide vector, pML523. The construct was electroporated into unmarked M. tuberculosis strain. Expression of whiB3-comp strain was confirmed by qRT-PCR.

**Antibodies and Reagents**—Antibodies for mammalian markers (CD63 and vacuolar H<sup>+</sup>-ATPase (V-ATPase)) were purchased from Santa Cruz Biotechnology. Lysotracker<sup>®</sup> DND-99 was purchased from Thermo Fisher Scientific. Secondary antibody Alexa Fluor 568 was purchased from Thermo Fisher Scientific. Fluorescein 5(6)-isothiocyanate (FITC) was purchased from Sigma-Aldrich. Bafilomycin A1 was purchased from Invivogen, and stocks were made in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20°C.

**Growth Conditions for qRT-PCR and Microarray Analysis**—For analyzing the influence of acid stress on gene expression, M. tuberculosis strains (M. tuberculosis, Mtb<sup>ΔwhiB3</sup>, and Mtb<sup>ΔmshA</sup>) were grown until an $A_{600\text{\ nm}}$ of 0.3–0.4 and exposed to 7H9 medium adjusted to a range of acidic pH 4.5, 5.5, 6.2, and 7.0 and normal 7H9 (pH 6.6) for 2 h at 150 rpm at 37°C. Total RNA from WT M. tuberculosis and Mtb<sup>ΔwhiB3</sup> (pH 4.5 and 6.6) was processed and hybridized to the M. tuberculosis whole genome gene expression profiling microarray G2509F (AMADID: G2509F_034585, Agilent Technologies PLC). DNA microarrays were provided by the University of Delhi South Campus MicroArray Centre. RNA amplification, cDNA labeling, microarray hybridization, scanning, and data analysis were performed at the University of Delhi South Campus MicroArray Centre as described (16). Slides were scanned on a microarray scanner (Agilent Technologies) and analyzed using GeneSpring software. Results were analyzed in MeV with significance analysis of microarrays considered significant at p ≤ 0.05. The normalized data from the microarray gene expression experiment have been submitted to the NCBI Gene Expression Omnibus and can be queried via Gene Expression Omnibus series accession number GSE61579.

**qRT-PCR**—First-strand cDNA synthesis was performed using 500 ng of the total RNA with the iScript Select cDNA synthesis kit (Bio-Rad) using random oligonucleotide primers. PCR was performed using gene-specific primers (Table 1). Gene expression was analyzed with real-time PCR using iQTM SYBR Green Supermix (Bio-Rad) and a CFX96 RT-PCR system (Bio-Rad). Data analysis was performed with CFX Manager<sup>TM</sup> software (Bio-Rad). For comparison between WT M. tuberculosis
Redox-mediated Acid Resistance in *M. tuberculosis*

### TABLE 1

| Oligonucleotides used in the study | Target Primer sequence |
|-----------------------------------|-----------------------|
| whiB3 RT F | 5'-AACGACAGACATCTGGAACTG-3' |
| whiB3 RT R | 5'-TCAGCTCAGACGCTTCAAG-3' |
| pk2 RT F | 5'-ATCATCTGCTGATGATGATG-3' |
| pk2 RT R | 5'-CGAAGTAAATGCTAATGATG-3' |
| pk3 RT F | 5'-GCTGAAAGATGAGCTAAGAC-3' |
| pk3 RT R | 5'-FACCCGACATCTCCATCAAG-3' |
| papA1 RT F | 5'-ATCCGCCGCTACGGAGTGAG-3' |
| papA1 RT R | 5'-AACCGCTAATTTGCTCCTAC-3' |
| Rv361e RT F | 5'-GAACAGCTGCTTCCATCTCG-3' |
| Rv361e RTR | 5'-GCCAGCTAAACGCTAATAAA-3' |
| Rv399e RT F | 5'-TGACACATCGACACCTAC-3' |
| Rv399e RT R | 5'-TGACACATTGAGCTGACAC-3' |
| 16S rRNA RT F | 5'-GCCCATCTAACGACGCTAAT-3' |
| 16S rRNA RT R | 5'-GCCGCTAATTTGCTCCTAC-3' |
| whiB3-comp clone check R1 | 5'-GGACTCTTGCTACGCTGAC-3' |

Isolation of WT *M. tuberculosis* Surface-exposed Polyketide Lipids—50 ml of WT *M. tuberculosis* was grown in a 37°C shaker incubator until mid-log phase (*A*<sub>600 nm</sub> = 0.5–0.8). Surface-exposed extractable total lipids were isolated as described previously (17). For lipid complementation assays, 100 µg/ml lipids were coated on the coverslips, and THP-1 cells were seeded, PMA-differentiated, and infected with *Mtb*<sub>ΔwhiB3</sub> as done above.

**Confocal Microscopy**—Logarithmically grown *M. tuberculosis* strains were stained with FITC as described earlier (18). PMA-differentiated THP-1 cells (0.25 × 10⁵) were seeded, and infection was done with WT *M. tuberculosis*, *Mtb*<sub>ΔwhiB3</sub>, *whiB3-comp* strains at an MOI of 10 as described elsewhere (19). 1 h prior to the time point, the medium of the cells was replaced with complete medium containing 100 nM Lyso-tracker<sup>®</sup> Red DND-99. Staining for CD63 and V-ATPase was started with fixative treatment (4% paraformaldehyde in 1× PBS) for 15 min, followed by permeabilization (0.2% Triton X-100 in 1× PBS) and blocking (3% BSA and 0.5% TWEEN 80 in 1× PBS). Samples were stained with primary antibodies (anti-CD63 and anti-V-ATPase) followed by secondary antibodies, followed by mounting using ProLong<sup>®</sup> antifade reagent (Thermo Fisher Scientific). The stained cells were visualized under a Leica TCS SP5 confocal microscope. z stacks were taken, collapsed into a two-dimensional image, and analyzed by LAS AF version 2.6.0, build 7266. All of the z stacks were taken at 63× oil immersion objective (with zoom). A minimum of five fields were captured, and at least 50 macrophages were analyzed for all of the phagosomal markers, accounting for an approximate analysis of ~100–200 bacteria/well. For each experimental group, a minimum of three replicates were scored.

**THP-1 Microarrays**—The infection of THP-1 monocytic cells, followed by RNA isolation, sample processing, and hybridization (Illumina Human-Ht-12 BeadChip), was performed as described earlier (19). Array data processing was performed on Illumina Bead Studio software. Expression analysis was done using a volcano plot-based approach using fold change >1.5 and *p* value >0.05 as cut-off. GO-Elite (version 1.2.5) was used for significant biological analysis of differentially expressed genes with z-score > 1.96 and *p* value < 0.05 set as cut-offs. The normalized data from the microarray gene expression experiment can be queried via Gene Expression Omnibus series accession number GSE65714.

**Aerosol Infection of Guinea Pigs**—Outbred Hartley guinea pigs (~300–400-g body weight) were given a low dose of logarithmic phase-grown cultures of WT *M. tuberculosis*, *Mtb*<sub>ΔwhiB3</sub>, and *whiB3-comp*, using a Madison chamber aerosol generation instrument calibrated to deliver 50–100 cfu/animal lung. Animals were sacrificed (*n* = 5) at 1, 30, and 60 days postinfection for determination of organ bacterial burden and histopathology analysis. Histopathology analysis was performed as described previously (14). A blinded examination of at least three serial sections from each guinea pig was carried out.

**Statistical Analysis**—Statistical analyses were conducted using GraphPad Prism software, and values were presented as mean ± S.D. The statistical significance of the differences

**Measurement of Intramycobacterial *E*<sub>MSH</sub> in Vitro and during Infection**—For intramycobacterial *E*<sub>MSH</sub> determination, various strains expressing Mxr1-roGFP2 were cultured and exposed to pH stress as indicated above. At the indicated time points, cells were treated with 10 mM N-ethylmaleimide for 5 min at room temperature and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing three times with 1× phosphate-buffered saline (PBS), bacilli were analyzed using a FACS Verse Flow cytometer (BD Biosciences). The biosensor response was measured by analyzing the ratio at a fixed emission (510 nm) after excitation at 405 and 488 nm. Data were analyzed using FACSuite software. Intramycobacterial *E*<sub>MSH</sub> was measured using the Nernst equation as described earlier (15).

For measuring intramycobacterial *E*<sub>MSH</sub> during infection, PMA-differentiated THP-1 cells were infected with WT *M. tuberculosis*, *Mtb*<sub>ΔwhiB3</sub>, and *whiB3-comp* strains expressing Mxr1-roGFP2 at a multiplicity of infection (MOI) of 10. Infected macrophages were treated with N-ethylmaleimide/paraformaldehyde, washed with 1× PBS, and analyzed by flow cytometry as described previously (15). In the case of bafilomycin A1 (BafA1) treatment, THP-1 cells were treated with 10 nM BafA1 (Invivogen) or DMSO (vehicle control) 1 h before infection and processed for infection and sample preparation as described above.

**Survival of *M. tuberculosis* Strains in Macrophages**—PMA-differentiated THP-1 monocytes and IFNγ + LPS-activated RAW 264.7 macrophages were infected with WT *M. tuberculosis*, *Mtb*<sub>ΔwhiB3</sub>, and *whiB3-comp* strains at an MOI of 2 for 4 h, followed by treatment with 200 µg/ml amikacin to remove extracellular bacteria. For BafA1 experiments, THP-1 cells were treated with 10 nM BafA1 or DMSO (vehicle control) 1 h prior to infection. Cells were maintained in BafA1 throughout the experiment. After infection, cells were washed thoroughly with warm RPMI medium and resuspended in 10% RPMI medium containing BafA1 or DMSO as per requirements. Samples were collected at the indicated time points, lysed using 0.06% SDS-7H9, serially diluted in 7H9, plated on OADC-7H11 agar medium, and incubated at 37°C incubator. Colonies were counted 3 weeks after plating.

**Lipids**—Logarithmically-grown *M. tuberculosis* logarithmic phase-grown cultures of WT *M. tuberculosis*, *Mtb*<sub>ΔwhiB3</sub>, and *whiB3-comp*, using a Madison chamber aero-sol generation instrument calibrated to deliver 50–100 cfu/animal lung. Animals were sacrificed (*n* = 5) at 1, 30, and 60 days postinfection for determination of organ bacterial burden and histopathology analysis. Histopathology analysis was performed as described previously (14). A blinded examination of at least three serial sections from each guinea pig was carried out.
between experimental groups was determined by two-tailed, unpaired Student’s t test. Differences with a p value of <0.05 were considered significant.

Results

*M. tuberculosis* WhiB3 Regulates Survival and Gene Expression in Response to Acidic pH—Recent studies indicated that intraphagosomal pH might be one of the earliest cues to which *M. tuberculosis* responds and realigns its transcriptional programming (12). Microarray studies have revealed that the expression of *whiB3* was induced early in macrophages in a pH-dependent manner (12). We also found an ~2-, 4-, and 35-fold increased expression of *whiB3* as compared with 16S rRNA at pH 6.2, 5.5, and 4.5, respectively.

Given the sustained induction of *whiB3* in response to acidic pH, we reasoned that WhiB3 might play an important role in tolerating acid stress. We generated an unmarked strain of *M. tuberculosis* lacking the entire open reading frame (ORF) of *whiB3*, and putative *Mtb*Δ*whiB3* clones were verified by PCR (Fig. 1, A–C). We monitored the survival of WT *M. tuberculosis*, *Mtb*Δ*whiB3*, and *whiB3-comp* in 7H9-tyloxapol medium adjusted to pH 6.6 (normal 7H9), 5.5, and 4.5. At day 4 post-treatment, survival at various pH stress conditions was monitored by enumerating cfu. We observed that *Mtb*Δ*whiB3* survived to a level comparable with WT *M. tuberculosis* at pH 6.6 and 5.5 (Fig. 1D). However, it displayed an ~55-fold reduction in its survival at pH 4.5 as compared with WT *M. tuberculosis* (p = 0.007; Fig. 1D). Stable expression of *whiB3* in *Mtb*Δ*whiB3* resulted in a significant complementation of this survival defect (Fig. 1D). These observations confirmed that not only is *whiB3* expression induced by acidic pH; it is also required for growth in acidic environments in vitro.

Next, we sought to determine the role of WhiB3 in controlling pH-specific gene expression. We minimized any influence of pH-induced cell death on gene expression by performing microarrays at an early time point (2 h) after exposure to pH 4.5. Expression data revealed differential regulation of several genes involved in secretion, central metabolism oxidative phosphorylation, lipid metabolism, amino acid metabolism, cell wall biosynthesis/membrane transporters, and gene regulation at pH 4.5 as compared with pH 6.6 (Fig. 2, A and B, and supplemental Table S1).

Surprisingly, genes directly implicated in mitigating redox stress in *M. tuberculosis* were also influenced by acid stress. For example, expression of major antioxidant genes, including thioredoxins (*txrB1*, *txrB2*, and *txrC*), superoxide dismutase (*sodA*), MSH synthesis (*mshB* and *mca*), and rubredoxin (*rubA* and *rubB*), was elevated by acidic conditions (supplemental Table S1). Genes associated with biosynthesis of redox-active amino acids (cysteine and methionine), DNA repair (*recR*, *dnak*, *dnal*, and *ogt*), and NAD+/NADH balance (*ndh*) were also up-regulated (Fig. 2B and supplemental Table S1). Noticeably, the expression of *whiB3* was induced to a higher degree than other transcriptional regulators (supplemental Table S1). To understand the physiological basis of our findings, we performed a comparative gene expression analysis at pH 4.5 in vitro with the transcriptional changes in WT *M. tuberculosis* in response to early phagosomal acidity (12). We observed that 22 genes that were induced by early phagosomal acidity (12), 17 were also induced by pH 4.5 (~2-fold, p < 0.05) (Fig. 2C).

Subsequently, we examined the role of WhiB3 in regulating pH-induced changes in gene expression. In *Mtb*Δ*whiB3*, the expression of 70 pH-induced and 27 pH-repressed genes showed 2-fold (p < 0.05) differential expression as compared with WT *M. tuberculosis* (supplemental Table S1). Several genes exhibited pH-specific induction only in WT *M. tuberculosis*, whereas they were constitutively expressed in acidic environments.
**FIGURE 2.** WhiB3 regulates gene expression in response to acidic pH in vitro. WT *M. tuberculosis* and *MtbΔwhiB3* strains were grown to an \( A_{600 \text{ nm}} \) of 0.3 and exposed to acidic pH of 4.5 for 2 h at 37 °C. Total RNA was isolated and subjected to microarray analysis as described under “Experimental Procedures.” A, genes with >2-fold (\( p < 0.05 \)) up- or down-regulation by acid stress relative to neutral pH were classified in 14 classes based on the annotation given in TubercuList. The pie chart represents the relative fraction of various pathways affected by acid stress in WT *M. tuberculosis*. B, heat map comparing WT *M. tuberculosis* and *MtbΔwhiB3* genes induced or repressed significantly (>2-fold, \( p < 0.05 \)) at pH 4.5 relative to neutral pH. C, heat map showing the overlap of genes differentially regulated in WT *M. tuberculosis* and *MtbΔwhiB3* by in vitro acid stress (pH 4.5) with concanamycin A-sensitive phagosome-induced genes (12). Highlighted areas represent genes affected by phagosomal acidification in WT *M. tuberculosis* in a WhiB3-dependent manner at pH 4.5 in vitro (1.5-fold up- and down-regulated, \( p < 0.05 \)).
**TABLE 2**

qRT-PCR analysis of a select set of pH-specific genes regulated by WhiB3 and mycothiol under acidic stress (pH 4.5)

| Gene       | WT M. tuberculosis pH 4.5 | WT M. tuberculosis pH 6.6 | MtbΔwhiB3 pH 4.5 | WT M. tuberculosis pH 4.5 | WT M. tuberculosis pH 7.0 | MtbΔ whiB3 pH 7.0 |
|------------|--------------------------|--------------------------|------------------|--------------------------|--------------------------|------------------|
| whiB3      | 26 ± 1.6                 | ND                       | ND               | –1.1 ± 0.87              | –17.36 ± 13.11          | –17.36 ± 13.11   |
| pks2       | 12 ± 0.8                 | 1.5 ± 0.24               | 2 ± 0.31         | –1.66 ± 0.805            | –4.9 ± 1.91             | –4.9 ± 1.91      |
| pks3       | 5.17 ± 2.8               | 0.55 ± 0.16              | 9.3 ± 0.62       | –1.15 ± 0.17             | –5.11 ± 2.46            | –5.11 ± 2.46     |
| papaA1     | 4.6 ± 2.25               | 0.71 ± 0.07              | 6.1 ± 0.53       | –0.2 ± 1.93              | –12.43 ± 4.76           | –12.43 ± 4.76   |
| Rv3616c    | 14.8 ± 4.1               | 2.3 ± 1                  | 8 ± 0.52         | –1.46 ± 2.48             | 1.96 ± 0.26             | 1.96 ± 0.26      |
| Rv2390c    | 54 ± 26                  | 14.6 ± 3.1               | 3.7 ± 0.26       | 1.19 ± 0.36              | 3.03 ± 0.75             | 3.03 ± 0.75      |

*MtbΔwhiB3*. This includes genes involved in the biosynthesis of complex polyketylic lipids (sulfolipid-1 (pks2), polycylctrehalose/diacylctrehalose (pks3-pks4)), and cysteine metabolism (cysW, cysN, cysA1, and metZ) (Fig. 2B and supplemental Table S1). Genes involved in amino acid biosynthesis (metH, metK, and sahi), ESM-1 secretion (Rv3614c-Rv3616c), MSH antioxidant system (mca and mtr), nitrite transport (narK1), and leucine biosynthesis (leuB) were up-regulated significantly more in WT *M. tuberculosis* compared with *MtbΔwhiB3* at pH 4.5 (Fig. 2B and supplemental Table S1). A large subset of genes was up-regulated to a higher degree in *MtbΔwhiB3* as compared with WT *M. tuberculosis* at acidic pH, indicating the role of WhiB3 in fine tuning the expression of pH-inducible genes. This includes the PE-PPE family (PE-24, PE-8, PE-32, PPE-65, and PPE-31), ribosomal proteins (rpmH, rpmU, and rpmY), and transcriptional regulators (whiB7, Rv0827c, and Rv3183) (supplemental Table S1). Because induction of whiB3 was responsive to early phagosomal acidic pH (12), we checked the expression status of other phagosomal pH-responsive genes in *MtbΔwhiB3*. We discovered that 22 phagosomal pH-responsive genes, expression of 16 was controlled by WhiB3 (Fig. 2C, ±1.5-fold, p ≤ 0.05). Last, microarray data were validated by measuring the expression of a selected set of pH- and whiB3-dependent genes by qRT-PCR (Table 2). Taken together, our results implicate WhiB3 of a selected set of pH- and MSH is the most abundant low molecular weight thiol protein in *M. tuberculosis* strains at neutral pH to be approximately –275 mV (Fig. 3A). The comparable E_MSH of *MtbΔwhiB3* and WT *M. tuberculosis* indicates that WhiB3 is not required for maintaining ambient E_MSH of *M. tuberculosis* at neutral pH. Interestingly, 24-h exposure of WT *M. tuberculosis* to either pH 6.2, pH 5.5, or pH 4.5 resulted in a significant decrease in intramyococobacterial E_MSH (approximately –305 ± 0.7 mV), indicating that a transition from neutral to either milder or harsher acidic pH conditions uniformly induces reductive *E_MSH* in *M. tuberculosis* (Fig. 3, B–D). Moreover, pH-exposed WT *M. tuberculosis* largely maintained *E_MSH* reduced throughout the course of the experiment. We noted a very modest recovery from reductive *E_MSH* (i.e. approximately –295 mV) at pH 6.2 and 5.5 at later time points, whereas no such effect was observed at pH 4.5 (Fig. 3, B–D). Importantly, whereas changes in intramyococobacterial *E_MSH* for *MtbΔwhiB3* mostly followed the WT *M. tuberculosis* pattern at pH 6.2 (Fig. 3B), distinct redox deviations were observed at pH 5.5 and 4.5. For example, at pH 5.5, *MtbΔwhiB3* displayed a relatively lesser decrease in intramyococobacterial *E_MSH* (–297 ± 0.7 mV) at 24 h followed by a slightly better recovery at 48–72 h (approximately –287 ± 0.7 mV) as compared with WT *M. tuberculosis* (Fig. 3C). Noticeably, exposure of *MtbΔwhiB3* to pH 4.5 displayed only a marginal decrease in *E_MSH* (–287 ± 1.5 mV) at 24 h, followed by a significant increase in intramyococobacterial *E_MSH* at 48 h (–260 ± 3.5 mV) and at 72 h (–266 ± 0.7 mV) as compared with WT *M. tuberculosis*, suggesting an overall oxidative shift in *E_MSH* of *MtbΔwhiB3* (Fig. 3D). The complemented strain showed changes in intramyococobacterial *E_MSH* that were comparable with WT *M. tuberculosis* (Fig. 3, A–D). These results indicate that acidic pH perturbs the redox physiology of *M. tuberculosis* by inducing a reductive shift in intramyococobacterial *E_MSH* and that the loss of WhiB3 impaired the ability of *M. tuberculosis* to orchestrate an efficient and dynamic MSH-specific reductive response upon acid stress.

The above results point toward a pH-mediated reductive shift in *E_MSH* of *M. tuberculosis* that might act as a signal for WhiB3 to regulate gene expression. We reasoned that disruption of the MSH reductive pathway would provide an ideal opportunity to study, in parallel, the effect of reductive *E_MSH* on pH-specific gene expression. Therefore, we expressed Mrx1-rogFP2 in an MSH-deficient *M. tuberculosis* strain

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*Redox-mediated Acid Resistance in M. tuberculosis*
(MtbΔmshA) and found that $E_{\text{MSH}}$ of the strain remained oxidized ($\sim -240 \text{ mV}$) at various pH conditions (7.0, 6.2, 5.5, and 4.5). Next, we analyzed the expression of a set of pH-inducible genes in a MSH-deficient M. tuberculosis strain (MtbΔmshA) by qRT-PCR. At pH 7.0, expression of pH-inducible genes in MtbΔmshA is comparable with WT M. tuberculosis (Table 2). In contrast, expression of pH-inducible genes did not show any up-regulation in MtbΔmshA at pH 4.5 (Table 2). More importantly, the expression of whiB3 was $\sim 37$-fold down-regulated in MtbΔmshA at pH 4.5 (Table 2). These results, along with our microarray data showing whiB3-dependent expression of MSH biosynthetic genes, suggest that MSH and WhiB3 are the components of a regulatory circuit mediating gene expression upon acid stress.

Dissipation of pH Gradient Perturbs pH-specific Induction of Reductive $E_{\text{MSH}}$ in M. tuberculosis—Excess increase in intracellular acidity can damage DNA, proteins, and lipids to exert bacterial killing. However, whether acid is the main effector of bacterial killing in response to pH stress is not clear. Because M. tuberculosis maintains cytoplasmic pH homeostasis under severe pH stress, how an increase in internal acidity will affect mycobacterial redoxphysiology and survival has not been studied. To examine the influence of elevated cytoplasmic acidity on intramycobacterial redox potential, we cultured M. tuberculosis strains at various pH levels for 72 h, as described above, and disrupted pH homeostasis by discharging the pH gradient (ΔpH) using a well known protonophore, CCCP. Treatment with 500 $\mu$M CCCP for 4 h is sufficient to permeate the cell wall of mycobacteria and equilibrates cytoplasmic pH with the external pH (20). We followed intramycobacterial $E_{\text{MSH}}$ and viability of M. tuberculosis strains at 4 h post-treatment with 500 $\mu$M CCCP. Expectedly, at pH 7.0, treatment with CCCP has no effect on either intramycobacterial $E_{\text{MSH}}$ or viability of M. tuberculosis (Fig. 3, A and E). At pH 6.2, CCCP treatment triggers a moderate recovery from reductive shift in $E_{\text{MSH}}$ of WT M. tuberculosis ($\sim -248 \text{ mV}$),
As expected, *M. tuberculosis* strains displayed subpopulations with oxidized (−240 ± 3 mV), reduced (−300 ± 6 mV), and basal (−275 ± 5 mV) *E*$_{MSH}$ inside THP-1 cells (15). The WT *M. tuberculosis* displayed a gradual increase in population with *E*$_{MSH}$ reduced (−30–65%) at initial time points (0–24 h postinfection), followed by an increase in *E*$_{MSH}$ oxidized population (−20%) at the intermediate period (48 h postinfection) and recovery from oxidative stress at 72 h postinfection. (Fig. 4A). In contrast to what was observed with *whiB3*-sufficient strains (WT *M. tuberculosis* and *whiB3-comp*), *MtbΔwhiB3* showed negligible proportions of *E*$_{MSH}$ reduced bacteria and a greater fraction of bacteria with *E*$_{MSH}$ oxidized, at each time point examined (Fig. 4, A–C). The defective ability of *MtbΔwhiB3* to maintain reductive *E*$_{MSH}$ correlated with a significant growth defect in its survival inside THP-1 macrophages as compared with WT *M. tuberculosis* and *whiB3-comp* (Fig. 4D).

Activated murine macrophages are known to control mycobacterial survival by generating excessive acid, ROS, and RNS stress (1). To investigate the role of WhiB3 in controlling intramycobacterial *E*$_{MSH}$ upon stimulation of antimycobacterial stresses, we infected IFN-γ- and LPS-treated (activated) RAW264.7 macrophages with *M. tuberculosis* expressing Mrx1-roGFP2 expressing WT *M. tuberculosis* (E), *MtbΔwhiB3* (F), and *whiB3-comp* strains (MOI of 10) (G). At the indicated time points, intramycobacterial *E*$_{MSH}$ subpopulations were measured as described for A–C. H, activated RAW264.7 macrophages were infected with WT *M. tuberculosis*, *MtbΔwhiB3*, and *whiB3-comp* strains (MOI of 2), and intramacrophage survival was monitored as described for D. The data in all panels are representative of three independent experiments performed in quadruplicate. Error bars, S.D. *, *p < 0.05 (*E*$_{MSH}$-oxidized subpopulation of *MtbΔwhiB3* as compared with WT *M. tuberculosis*); **, *p < 0.01; ***, *p < 0.005 (as compared with WT *M. tuberculosis*); ++, *p < 0.01; +++, *p < 0.005 (as compared with *whiB3-comp*) in intramacrophage survival experiments.
Redox-mediated Acid Resistance in M. tuberculosis

An oxidative shift in the $E_{MSH}$ of WT M. tuberculosis inside activated macrophages, where phagolysosomal pH drops to 4.5, is in contrast with the reductive shift in intramacrophobacterial $E_{MSH}$ at pH 4.5 in 7H9 culture medium. However, these counterintuitive findings can be reconciled by the synergistic effect of vacuolar acidification with host ROS and RNS production. In particular, low pH of phagolysosomes allows dismutation of nitrous acid (generated via autoxidation of NO) to generate highly toxic nitrogen dioxide (21). Hence, the increase in $E_{MSH}$ oxidized subpopulations is most likely due to composite response of M. tuberculosis toward multiple stresses encountered inside activated macrophages. Altogether, using in vitro and macrophage-based assays, we demonstrated the role of WhiB3 in mounting an efficient antioxidant response to promote intramacrophage survival.

Effect of Phagosomal Acificationization on Intramacrophobacterial $E_{MSH}$ and Survival—Because phagosomal pH synergizes with multiple intramacrophage cues, such as ROS and RNS, determination of the specific effect of acidic pH on mycobacterial physiology, gene expression, and survival remains challenging. This has been extremely difficult inside activated macrophages, where several mycobactericidal mechanisms (e.g. lysosomal hydrolases, ROS, and RNS) are likely to be pH-dependent (21). Therefore, to begin delineating the role of vacuolar acidification on intramacrophobacterial $E_{MSH}$ we decided to examine the effect of limited acidification encountered inside THP-1 cells (22). To block phagosomal acidification, we treated THP-1 macrophages with a specific inhibitor of V-ATPase, BafA1, and then infected them with M. tuberculosis strains expressing Mrx1-roGFP2 at an MOI of 10. Treatment with 10 nM BafA1 is known to effectively block phagosomal acidification without affecting macrophage viability during infection with M. tuberculosis (23). We also observed no influence of 10 nM BafA1 on THP-1 viability (data not shown). Given that M. tuberculosis phagosomes acidify to pH 6.2–6.4 within minutes of infection (24), we measured intramacrophobacterial $E_{MSH}$ at initial time points (0 and 24 h postinfection).

In case of WT M. tuberculosis, BafA1 treatment significantly diminished the proportion of bacilli with $E_{MSH}$ reduced as compared with untreated macrophages at 0 and 24 h postinfection (Fig. 5, A and B). The consequent increase in M. tuberculosis subpopulations with basal $E_{MSH}$ (similar to 7H9, pH 7.0, grown bacteria) indicates that cells are experiencing nearly neutral pH in vacuoles upon BafA1 treatment (Fig. 5, A and B). Importantly, these results indicate that limited acidification encoun-
moter inside macrophages is sufficient to induce reductive shift and heterogeneity in $E_{\text{MSH}}$ of $M. tuberculosis$ during infection. In contrast to WT $M. tuberculosis$, $Mtb$whiB3 showed no significant changes in subpopulations with basal, oxidized, and reduced $E_{\text{MSH}}$ at 0 and 24 h postinfection upon BafA1 treatment (Fig. 5, A and B). The whiB3-comp strain showed intramycobacterial $E_{\text{MSH}}$ changes comparable with WT $M. tuberculosis$ upon BafA1 treatment (Fig. 5, A and B). These results suggest that the lack of WhiB3 impaired the ability of $M. tuberculosis$ to dynamically modulate cytoplasmic $E_{\text{MSH}}$ in response to vacuolar pH.

Next, we examined whether the inability of $Mtb$whiB3 to maintain mycothiol redox homeostasis in response to vacuolar acidification was the reason underlying the intramacrophage survival defect of the mutant. To do this, we monitored the survival of WT $M. tuberculosis$, $Mtb$whiB3, and whiB3-comp strains inside untreated and BafA1-treated THP-1 cells by enumerating cfu at 48 and 96 h postinfection. In contrast to detectable intramacrophage growth of $Mtb$whiB3 observed earlier, treatment with BafA1 completely rescued its survival to WT $M. tuberculosis$ and whiB3-comp levels (Fig. 5C). Together, these results indicate the importance of mycothiol redox buffer and WhiB3 in responding to phagosomal acidification and maintaining intramacrophage survival of $M. tuberculosis$.

**WhiB3 Is Required to Subvert Phagosomal Acidification—** One of the main mechanisms exploited by $M. tuberculosis$ to resist acid stress is by blocking the normal process of phagosomal maturation to acidified phagolysosomes (4). In this regard, we have shown that the majority of bacilli within acidified phagolysosomal fractions display oxidative $E_{\text{MSH}}$ (15). Because a relatively higher proportion of $E_{\text{MSH}}$-oxidized subpopulations of $Mtb$whiB3 were detected inside THP-1 cells, we hypothesized that increased fusion of phagosomes containing $Mtb$whiB3 with acidified lysosomes may be one of the factors that underlies the observed redox variability and intramacrophage survival defect. We therefore sought to determine the acidification status of phagosomes containing $Mtb$whiB3. To do this, THP-1 macrophages were infected with $M. tuberculosis$ strains labeled with FITC at an MOI of 10, and localization of $M. tuberculosis$ bacilli was assessed using well-established markers of phagosomal maturation. Localization of $M. tuberculosis$ bacilli was examined using confocal microscopy. For measurements, a minimum of five fields/well were captured, and $\sim 100–200$ bacterium-containing phagosomes were scored per well. For each test group, three replicate wells were scored per experiment.

Staining with Lysotracker revealed that WT $M. tuberculosis$ largely remained in non-acidified phagosomes, with only 25 ± 4% of bacilli colocalized to acidified phagosomes at 24 h postinfection (Fig. 6A). In contrast, a significantly greater fraction of $Mtb$whiB3 ($\sim 70\%$, $p = 0.0026$) was found in acidified phagosomes, and this phenotype was significantly reversed in the complemented strain (Fig. 6A). It has been shown that $M. tuberculosis$ actively inhibits phagosome acidification by preventing recruitment and/or inducing degradation of a molecular proton motor, V-ATPase (24, 25). Therefore, we questioned whether the increased association of $Mtb$whiB3 with acidified phagosomes correlates with greater V-ATPase association. Infected THP-1 macrophages were immunostained for human V-ATPase, and colocalization was measured at 24 h postinfection. A significantly greater percentage of phagosomes containing $Mtb$whiB3 were found to be positive for V-ATPase ($\sim 43 \pm 10\%$) as compared with WT $M. tuberculosis$ ($\sim 11 \pm 12\%$) or whiB3-comp ($\sim 25 \pm 10\%$) (Fig. 6B). Because phagosome acidification is a relatively early step in phagosomal maturation, we next analyzed the status of $Mtb$whiB3-containing phagosomes for the late endosome-lysosome fusion marker, CD63. It has been reported that WT $M. tuberculosis$ prevents phagosomes from maturing into the CD63-positive state (26). Consistent with our earlier results, $\sim 53 \pm 2\%$ of $Mtb$whiB3-containing phagosomes acquired CD63 as compared with $\sim 8 \pm 3\%$ and $31 \pm 4\%$ in the case of WT $M. tuberculosis$ and whiB3-comp strains, respectively (Fig. 6C). The observed differences in the phagosomes of $Mtb$whiB3 were confirmed by repeating experiments at least three times in triplicate.

Our data show that WhiB3 positively regulates the pH-specific expression of various genes involved in producing secretory proteins and lipids (e.g. sulfolipid-1 (pks2, papA1, and mmpL8), TDM (fabD, acpP, and kasA), and ESX-1 system (Rv3615c, Rv3870, and Rv3871)), which are well known to restrict phagosomal maturation (27–29). Hence, the inability of $Mtb$whiB3 to block phagosomal maturation could be a consequence of defective polyketide surface lipid anabolism. To investigate this possibility, total surface exposed lipids were extracted from WT $M. tuberculosis$ as described earlier (17). Moreover, pretreatment of macrophages with the surface-exposed lipids of mycobacteria has been shown to modulate cellular processes, such as phagosomal maturation and autophagy (30, 31). Based on these studies, THP-1 cells were pretreated with the surface lipids and subsequently infected with WT $M. tuberculosis$ or $Mtb$whiB3 at an MOI of 10. Infected macrophages were assessed for the colocalization of LysoTracker with $Mtb$whiB3 at 0 and 24 h postinfection. Assessment of more than 100 phagosomes revealed that pretreatment with the WT $M. tuberculosis$ lipids resulted in a significant inhibition of Lysotracker staining of $Mtb$whiB3-containing phagosomes as compared with untreated controls at each time point tested (Fig. 7, A–C). Importantly, the percentage of Lysotracker-positive phagosomes containing $Mtb$whiB3 upon pretreatment with total lipids was comparable with WT $M. tuberculosis$ at 0 h postinfection (Fig. 7, A and C). These results suggest that the altered composition of surface-associated polyketide lipids is likely to be one of the factors responsible for the defective ability of $Mtb$whiB3 to block phagosomal maturation. In sum, the data generated from macrophages and in vitro experiments clearly suggest that WhiB3 protects $M. tuberculosis$ from acid stress by regulating gene expression, dynamic changes in $E_{\text{MSH}}$ of $M. tuberculosis$, and polyketide-mediated restriction of phagosomal acidification.

**WhiB3 Modulates the Expression of Host Innate Response Genes—** We examined whether WhiB3-mediated regulation of bioactive lipids and secretory proteins modulates expression of host transcriptome. Using microarrays, we compared the expression of THP-1 cells infected with WT $M. tuberculosis$ and $Mtb$whiB3 at various time points. We found that major
innate immune mechanisms normally suppressed by pathogenic *M. tuberculosis* strains, such as phagosomal maturation/endocytosis, apoptosis, and TLR signaling, were up-regulated in *Mtb*\(\Delta\)whiB3 (1.5-fold, \(p < 0.05\)) (Fig. 8 and supplemental Table S2). In agreement with the role of WhiB3 in responding to early increase in phagosomal acidity, we observed a significantly greater impact of WhiB3 loss on host transcriptome at an early time point (i.e. 12 h postinfection) (Fig. 8). Finally, using qRT-PCR, we validated microarray data by measuring the expression of a selected set of genes involved in phagosomal maturation in a whiB3-dependent manner (data not shown). Altogether, the data indicate that WhiB3 plays an important role in influencing expression of host-directed mechanisms associated with controlling intraphagosomal survival of *M. tuberculosis*.

**WhiB3 Regulates Survival of *M. tuberculosis* in Vivo**—Given that WhiB3 regulates pH-dependent modulation of expression and redox signaling, resulting in differential intracellular trafficking and survival inside macrophages, we hypothesize that WhiB3 plays an important role during *M. tuberculosis* infection. To examine this, we assessed the in vivo phenotype of *Mtb*\(\Delta\)whiB3 in guinea pigs. Aerosol infection of out-bred Hartley guinea pigs showed a clear growth attenuation of *Mtb*\(\Delta\)whiB3 as compared with WT *M. tuberculosis* in the lungs.
of animals. At day 1 postinfection, cfu analysis showed that nearly identical numbers of bacteria were implanted in the lungs of guinea pigs infected with WT M. tuberculosis, MtbΔwhiB3, and whiB3-comp strain (Fig. 9A). At days 30 and 60 postinfection, the number of bacteria present in lungs of animals infected with MtbΔwhiB3 was ~70 (p = 0.0073) and ~200-fold (p = 0.0007) lower than in those infected with WT M. tuberculosis, respectively (Fig. 9A). Interestingly, in contrast to our lung data, bacterial numbers in the spleen at days 30 and 60 postinfection were comparable in WT M. tuberculosis and whiB3-comp strains (Fig. 9C).

**Discussion**

In 1905, Metchnikoff (32) reported the presence of acidic milieu within the phagosomes of macrophages infected with pathogens. Despite this early observation, how M. tuberculosis bacilli respond, resist, and persist in response to a gradient of acidic pH during infection remains poorly characterized. Here, we identified acidic pH as a physiological stimulus to which WhiB3 regulates (i) gene expression, (ii) mycothiol redox homeostasis, (iii) phagosomal maturation, and (iv) virulence.

Our microarray data highlight the role of WhiB3 in regulating acid stress response in M. tuberculosis. Differential regulation of several genes involved in redox metabolism of M. tuberculosis in response to acid stress in a WhiB3-dependent manner and acute sensitivity displayed by MtbΔwhiB3 at pH 4.5 suggest that WhiB3 facilitates M. tuberculosis persistence in response to acid stress by maintaining intramycobacterial redox homeostasis. Until now, direct evidence linking acidic stress encountered in phagosomes to internal redox balance of M. tuberculosis was lacking. A recent in vitro study, using a genetically encoded redox biosensor (roGFP-R12), has demonstrated that the cytoplasmic redox state of M. tuberculosis shifts to reductive when bacilli are cultured under specific carbon sources at pH 5.5 (33). Because conventional roGFPs, such as roGFP-R12, predominantly interact with glutathione redox buffer (34, 35), which is absent in mycobacteria, the utility of roGFPs in M. tuberculosis is limited by unknown specificity and poor response to changes in redox potential (15). Moreover, reliable measurement range for roGFPs (i.e. between 10 and 90% of sensor oxidation) covers about ±30 mV from the standard midpoint potential (34). Therefore, roGFP-R12 with a less negative midpoint potential (~265 mV) cannot accurately measure a reductive shift in redox potential beyond ~295 mV. In this context, Mrx1-roGFP2 with a midpoint potential of ~280 mV allowed dynamic and precise imaging of the EMPH of M. tuberculosis, in response to both oxidative and reductive stresses, with high sensitivity and temporal resolution (15, 36). Using this bioprobe, we provide accurate numerical evidence that acidic pH promotes reductive shift in EMPH of M. tuberculosis in vitro and inside phagosomes in a WhiB3-dependent manner. The reductive shift in EMPH at initial phases of intramacrophage growth is consistent with the rapid drop in vacuolar pH within...
minutes of infection with *M. tuberculosis* (24), which also serves as an early cue to induce expression of genes linked to reductive stress (e.g. *whiB3*, *whiB7*, and *dosR*) (29, 37). More importantly, inhibition of vacuolar acidification resulted in both the loss of gene expression (28) and a substantial decrease in the *M. tuberculosis* subpopulations with *E*$_{MSH}$ oxidized. 

In contrast to WT *M. tuberculosis*, which preferentially resides in early endosomes, we observed that *MtbΔwhiB3* mainly localized to acidified lysosomes and displayed a higher proportion of *E*$_{MSH}$-oxidized bacilli. These findings support our earlier observations that lysosomes enrich *E*$_{MSH}$-oxidized bacteria, whereas phagosomes with limited acidity (early endosomes) induce a reductive shift in *E*$_{MSH}$ of *M. tuberculosis* (15). Interestingly, whereas treatment with BaFAl prevented acidification of *MtbΔwhiB3*-containing phagosomes and reversed intramacrophage survival defect, the proportion of mutant bacilli with *E*$_{MSH}$ oxidized remained uninfluenced. One likely possibility is that the loss of WhiB3 compromised the ability of the mutant to respond to changes in the phagosomal environment via the mycothiol redox system. In line with this, several components of the mycothiol pathway, including MSH disulfide reductase involved in recycling MSSM to MSH, are down-regulated in *MtbΔwhiB3* upon acid stress. Alternatively, *MtbΔwhiB3* may exploit another antioxidant system, such as ergothionine (ERG), to respond to the intraphagosomal environment. A compensatory protective role of ERG has already been established in mycothiol-defective mycobacterial strains (38). Because Mrx1-roGFP2 does not respond to ERG (15), further work testing the role of ERG redox potential will allow more precise determination of relative contributions of ERG and MSH pathways in responding to the phagosomal milieu.

The acidic pH-induced reductive *E*$_{MSH}$ in WT *M. tuberculosis* most likely resulted from increased synthesis of MSH or a higher rate of MSSM reduction to MSH via the activity of NADPH-dependent MSH disulfide reductase. Expression data indicated a significant up-regulation of MSH-biosynthetic genes in response to acidic pH. Additionally, a decreased expression of respiratory genes involved in regenerating NAD$^+$ cofactor (e.g. *nuo* operon), along with the up-regulation of fatty acid catabolic genes, which generate excessive NADH through $\beta$-oxidation, may further lead to accumulation of NADH/NADPH cofactors during acidity. Because excessive accumulation of NADH/NADPH is known to elevate endogenous ROS levels through autoxidation or via Fenton reaction (39, 40), a reductive shift in *E*$_{MSH}$ by NADPH-dependent conversion of MSSM to MSH via MSH disulfide reductase could be a mechanism to dispose of excess reductants. In this context, studies in *M. tuberculosis* have indicated only a marginal increase in the NADPH pool at acidic pH, whereas levels of cytoplasmic thiols, such as MSH and CoA-SH, were substantially elevated (33, 41), agreeing with our findings that the MSH pathway can function as a reductive sink to reduce toxicity associated with low pH.

Further strengthening this connection is our finding showing the inability of *MtbΔwhiB3* to maintain *E*$_{MSH}$ reduced, along with a previous report demonstrating massive accumulation of NADH/NADPH in *MtbΔwhiB3* inside macrophages (17). The mycothiol redox system has recently been shown to interact with a major antioxidant enzyme; superoxide dismutase, to
exert an efficient adaptive response during infection (42). In light of this, an early elevation in reductive capacity of mycothiol ($EMSH_{\text{reduced}}$), in response to vacuolar pH, might be important to activate additional mechanisms to detoxify a range of hostile radicals that $M. \text{tuberculosis}$ encounters later in the infection cycle (e.g. during immune activation). Altogether, these observations, along with the fact that external acidity does not increase $H^+_{/H_{1001}}$ concentration inside $M. \text{tuberculosis}$ (10), serve to implicate intrabacterial reductive $EMSH$ as an internal physiological signal to which $M. \text{tuberculosis}$ responds through WhiB3 to coordinate gene expression and survival. These findings were further strengthened by our data showing a complete loss of $\text{whiB3}$ induction along with other pH-responsive genes in an $M. \text{tuberculosis}$ strain lacking mycothiol ($\text{Mtb}/H_{9004}mshA$) at acidic pH. Because WhiB3 is cytoplasmically located and implicated in responding to changes in intracellular redox conditions through redox-sensitive $[4\text{Fe-4S}]$ cluster (13, 17), we hypothesize that the intramycobacterial reductive $E_{\text{MSH}}$ under acidic stress can promote accumulation and/or stabilization of the reduced form of the $4\text{Fe}-4\text{S}$ cluster ($[[4\text{Fe-4S}]^{1+}]_{/\text{WhiB3}}$), which directly or indirectly activates pH-responsive genes in $M. \text{tuberculosis}$. Alternatively, because the DNA binding activity of apo-WhiB3 (without an Fe-S cluster) is modulated by the redox state of its cysteine thiols (17), an interesting possibility is that the reversible $S$-mycothiolation of WhiB3 thiols in response to pH-mediated changes in $E_{\text{MSH}}$ may function as a redox-regulatory switch to modulate gene expression. The functional linkage between MSH and WhiB3, as revealed in this study, can now be exploited to understand the molecular mechanism(s) of how mycothiol exerts its influence on the redox behavior and gene regulatory properties of WhiB3.

We found that $\text{Mtb}/\text{whiB3}$ has an impaired ability to arrest phagosomal maturation, which can be rescued by supplementation of surface-associated polyketide lipids from WT $M. \text{tuberculosis}$. These discoveries have significant implications in understanding mycobacterial pathogenesis, which suggests an intertwined association between immunomodulatory virulence factors and the core metabolic processes in $M. \text{tuberculosis}$. We propose that WhiB3-mediated synthesis of virulence factors, including secretory lipids and proteins (ESX-1 system), in response to intracellular redox changes associated with acidic pH will play a larger part, in both redox maintenance and in counteracting phagosomal acidification, to ensure long term persistence of $M. \text{tuberculosis}$ (Fig. 10). Although our results indicate that WhiB3 is a major regulator of pH and redox homeostasis, other regulators, such as PhoP, that similarly modulate the expression of virulence factors to influence phagosomal maturation and intraphagosomal survival can provide overlap-
Redox-mediated Acid Resistance in M. tuberculosis

FIGURE 10. Model depicting the role of $E_{\text{MSH}}$ and WhiB3 in responding to acid stress during infection. In resting macrophages, M. tuberculosis impairs phagosome maturation and preferentially resides in a mildly acidic environment. Activation with IFN-$\gamma$ induces phagosomal-lysosomal fusion to elevate the levels of proton (pH 4.5), reactive oxygen intermediates (ROI), and reactive nitrogen intermediates (RNI) in the microenvironment. Despite these changes in external pH, the internal pH of M. tuberculosis remains close to neutral (pH 7.2). Importantly, these variations in phagosomal pH induce dynamic changes in the $E_{\text{MSH}}$ of M. tuberculosis. Limited acidification inside resting macrophages induces a reductive shift in $E_{\text{MSH}}$ of M. tuberculosis (approximately $-305$ mV), whereas activation of macrophages induces oxidative shift ($-240$ mV). Pharmacological inhibition of phagosomal acidification by BaF1 neutralizes acidic pH to prevent reductive shift in $E_{\text{MSH}}$ of M. tuberculosis. M. tuberculosis responds to phagosomal acidification with the help of a putative redox-sensitive transcription factor, WhiB3. WhiB3 can sense changes in intrabacterial $E_{\text{MSH}}$ via its Fe-S cluster or cysteine thiols (S-mycophylation) to modulate the expression of virulence genes involved in blocking phagosomal maturation (e.g., polyketides, secretory antigens) and redox homeostasis. Impaired ability of Mtb$\Delta$whiB3 to maintain mycothiol balance and block phagosomal maturation, along with the survival defect in vivo, suggests a central role of WhiB3 in regulating mycobacterial persistence in response to acid stress. The exact mechanisms by which WhiB3 senses pH-induced changes in internal $E_{\text{MSH}}$ via 4Fe-4S and/or cysteine thiols remain to be identified.

In summary, we have identified a new mechanism exploited by M. tuberculosis to respond to phagosomal pH. Our study provides a unique example of the tight connections forged between core redox machinery and virulence in mycobacterial pathogenesis. Furthermore, pH-induced redox signalling and its connection with gene expression and virulence may be relevant to other intracellular pathogens. For example, acidification is required for virulence expression in Salmonella (46), phagosomal escape of Listeria monocytogenes (47), and efficient replication of Legionella pneumophila and Coxiella burnetii (48, 49). Thus, our findings have broad implications for several intracellular pathogens for which phagosomal pH plays a critical role in modulating virulence and long-term persistence.

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