

Heme Oxygenase-1-derived Carbon Monoxide Induces the *Mycobacterium tuberculosis* Dormancy Regulon^{*[S]}

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The mechanisms that allow *Mycobacterium tuberculosis* (*Mtb*) to persist in human tissue for decades and to then abruptly cause disease are not clearly understood. Regulatory elements thought to assist *Mtb* to enter such a state include the heme two-component sensor kinases DosS and DosT and the cognate response regulator DosR. We have demonstrated previously that O₂, nitric oxide (NO), and carbon monoxide (CO) are regulatory ligands of DosS and DosT. Here, we show that in addition to O₂ and NO, CO induces the complete *Mtb* dormancy (Dos) regulon. Notably, we demonstrate that CO is primarily sensed through DosS to induce the Dos regulon, whereas DosT plays a less prominent role. We also show that *Mtb* infection of macrophage cells significantly increases the expression, protein levels, and enzymatic activity of heme oxygenase-1 (HO-1, the enzyme that produces CO), in an NO-independent manner. Furthermore, exploiting HO-1^{+/+} and HO-1^{-/-} bone marrow-derived macrophages, we demonstrate that physiologically relevant levels of CO induce the Dos regulon. Finally, we demonstrate that increased HO-1 mRNA and protein levels are produced in the lungs of *Mtb*-infected mice. Our data suggest that during infection, O₂, NO, and CO are being sensed concurrently rather than independently via DosS and DosT. We conclude that CO, a previously unrecognized host factor, is a physiologically relevant *Mtb* signal capable of inducing the Dos regulon, which introduces a new paradigm for understanding the molecular basis of *Mtb* persistence.

Mycobacterium tuberculosis (*Mtb*)³ causes approximately two million deaths annually, and it is estimated that one

third of the world's population is latently infected with *Mtb* (1). This clinically latent state of disease can last for decades during which *Mtb* remains unresponsive to drug therapy. Evidence suggests roles for at least two signals, oxygen tension and nitric oxide (NO), in assisting *Mtb* to enter and maintain a latent state (2–4). Using the murine model for latent tuberculosis (TB), interferon γ (IFN- γ), tumor necrosis factor- α , inducible nitric-oxide synthase (iNOS), and, thus, NO were shown to be continuously expressed and necessary to prevent reactivation of TB (5).

Several studies have demonstrated a dramatic overlap between gene expression profiles of *Mtb* cultured under hypoxic conditions and upon treatment with NO (6–8). These findings led to the identification of the 48-member *Mtb* dormancy (Dos) regulon, which is under the control of the two heme sensor kinases DosS and DosT, and the response regulator DosR (9). Notably, mycobacterial *dosR* mutants were shown to be impaired for survival during hypoxia (8, 10), whereas the *Mtb* Beijing lineage was shown to constitutively express the Dos regulon 50-fold higher than non-Beijing strains (11). Intriguingly, the *Mtb dosR* mutant is attenuated in guinea pigs (12), but not mice (13).

Recently, we (14) and others (15–17) have shown that DosS and DosT are heme proteins that in addition to binding O₂ and NO are able to bind CO. The latter findings led us to hypothesize that CO could be a third physiologically relevant ligand whose binding might induce the Dos regulon. CO is produced *in vivo* by heme oxygenase (HO), which is a cytoprotective enzyme that degrades heme to generate CO (18). Notably, HO-1 confers protection against oxidative cellular stress through the anti-oxidative, anti-apoptotic, and anti-inflammatory actions of its byproducts (18–20). In addition, HO-1 has been shown to inhibit key components of innate immunity, the production of tumor necrosis factor- α and NO (18–20). Thus, HO activity in the context of inflammatory lesions caused by live bacteria may have important implications for both the host and the pathogen. Importantly, a recent seminal study described a protective role for HO-1-generated CO in the progression of experimental cerebral malaria (21).

CO has become the focus of a great deal of interest, especially when the therapeutic potential of CO and CO-generating molecules as novel gaseous therapy was recognized (20). However, prior to this study the role of HO-1, and therefore CO, had not been considered in *Mtb* pathogenesis. Herein, we hypothesize that host HO-1 generates CO, which induces the *Mtb* Dos regu-

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³ The abbreviations used are: *Mtb*, *Mycobacterium tuberculosis*; NO, nitric oxide; TB, tuberculosis; IFN- γ , interferon γ ; iNOS, inducible nitric-oxide synthase; CO, carbon monoxide; HO-1, heme oxygenase-1; BMM, bone marrow-derived macrophage; Q-PCR, quantitative PCR.

lon. To test this hypothesis, we examined the induction of the *Mtb* Dos regulon in response to CO exposure and assessed the independent contribution of DosS and DosT in relaying this signal to the *Mtb* Dos regulon. We also studied the expression of the *Mtb* Dos regulon in response to physiological levels of CO generated within macrophages. Lastly, we examined HO-1 levels in macrophages and in the lungs of *Mtb*-infected and uninfected mice.

EXPERIMENTAL PROCEDURES

Mice—C3HeB/FeJ mice were purchased from the Jackson Laboratory. HO-1^{-/-} mice and age-matched HO-1^{+/+} littermates (C57BL/6 × FVB background) and iNOS^{-/-} (C57BL/6 background) were used. The animal protocols were approved by the Institutional Care and Use Committees at the University of Alabama at Birmingham and Harvard School of Public Health. The mice were regularly tested and found to be free of common mouse pathogens and were used for experiments at 6–12 weeks of age.

***Mtb* Mutant Strains**—*Mtb dos* mutant strains were kind gifts from Dr. David Sherman (Seattle Biomedical Research Institute, WA). Construction of *MtbΔdosS* and *MtbΔdosR* is described by Sherman *et al.* (6), and *MtbΔdosT* and *MtbΔΔdosSΔdosT* are described by Roberts *et al.* (9). *MtbΔdosR* was found to be attenuated for growth in the Wayne model for *in vitro* dormancy (8, 13) and is not capable of inducing the *Mtb* Dos regulon under hypoxic conditions or in response to NO (8). The expression of *hspX* was reduced 40–45% in *MtbΔdosS* or *MtbΔdosT*, whereas *MtbΔdosSΔdosT* abolishes expression of *hspX* in response to hypoxia (9).

Bacterial Infection—Virulent *Mtb* (strain Erdman, TMC 107) was obtained from the Trudeau Mycobacterial Culture Collection (Trudeau Institute). Mice were intravenously infected with 1 × 10⁵ colony-forming units of *Mtb* in 100 μl of phosphate-buffered saline via tail vein. *Mtb* H37Rv was used in all other experiments.

Microarray Hybridization and Data Analysis—Microarrays used in this study were produced and processed at the Center for Applied Genomics at the Public Health Research Institute as described earlier (22). See supplemental information for details.

Expression Analysis of the *Mtb* Dos Regulon in Bone Marrow-derived Macrophages (BMM)—Four independent experiments were performed using BMM collected from 8–12-week-old HO-1^{-/-} mice and HO-1^{+/+} littermates (C57BL/6 × FVB background). 1–2 × 10⁶ macrophages were infected with a multiplicity of infection of 5–10. RNA was isolated and utilized in real-time PCR analysis according to previously described protocols (14, 23). The -fold expression of a specified gene transcript of bacilli residing in HO-1^{+/+} BMM was measured relative to the gene transcript of bacilli residing in HO-1^{-/-} BMM. Expression was normalized using *Mtb* 16 S rRNA as internal control.

Expression Analysis of HO-1—Experiments were performed using BMM collected from 8–12-week-old HO-1^{-/-} mice and HO-1^{+/+} littermates (C57BL/6 × FVB background). 1–2 × 10⁶ macrophages were infected with a multiplicity of infection of 5–10. Extracellular bacteria were removed by washing with cell culture medium prewarmed to 37 °C after 12–14 h of infection.

Macrophages were lysed with guanidine thiocyanate, and bacteria were harvested by centrifugation. Approximately 1–2 μg of bacterial RNA was isolated from four independent biological samples and analyzed via quantitative PCR (Q-PCR).

Western Blot Analysis—Approximately 2 × 10⁶ cells (murine macrophage cell line or BMM) were grown in a six-well plate and infected with *Mtb* (multiplicity of infection 5–10). For Western blot analysis, cells were harvested and lysed in radio-immune precipitation buffer, samples were sonicated and centrifuged, and the protein concentration of the lysate was determined using the BCA protein assay. Western blot analysis was performed as described earlier (24).

HO Enzymatic Activity Assay—RAW cells grown in 175-cm² tissue culture flasks (5 × 10⁷ cells) were infected with *Mtb* (multiplicity of infection 5–10). Uninfected cells were used to serve as controls. HO activity was determined as described earlier (25).

Immunohistochemistry—Tissue sections from infected animals (*n* = 4) of each group were treated with 10% formalin in neutral buffer and cut into sections to 5 μm. These sections were deparaffinated and rehydrated in graded ethanol. Sections were washed in phosphate-buffered saline followed by endogenous peroxidase inactivation in 0.3% H₂O₂, labeled with primary antibody against HO-1 (Stressgen, SPA-896), stained with anti-rabbit peroxidase (Jackson Laboratories), and developed with the 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories) to produce a characteristic brown color. Control reactions were performed using rabbit IgG instead of the primary antibody, and the secondary antibody alone. Sections were also stained with hematoxylin and counterstained with eosin according to previously described protocols (26).

Statistical Analysis—Results are expressed as mean ± S.E. and are derived from at least three independent experiments. Student's *t*-test or analysis of variance with Student-Newman-Keuls post-test was used for comparisons.

RESULTS

CO Elicits the Induction of the Complete *Mtb* Dos Regulon—We have recently reported that the redox state of the DosS and DosT heme irons, as well as the ligands O₂, NO, and CO, modulates DosS and DosT autokinase activity (14). Because the effect of CO on the complete Dos regulon is not yet established, we sought to determine whether CO could selectively induce the *Mtb* Dos “fingerprint,” consisting of ~48 genes. We used microarray expression profiling and captured the transcriptional response of *Mtb* cells exposed to 50 μM CO. The results demonstrate that CO rapidly induced the complete Dos regulon (Fig. 1). We repeated the microarray experiment using the *MtbΔdosR* mutant strain and found that induction of the Dos regulon by CO was mediated via DosR (Fig. 1). Thus, the induction of the Dos regulon by hypoxia (6), NO (7, 8), and now CO is mediated by the DosR/S/T system. We next tested a series of CO concentrations (5 nM–100 μM) and examined *fdxA* expression via Q-PCR as an indicator (9) for Dos regulon expression. We observed that full induction of *fdxA* occurred at ≥5 μM CO (Fig. 2A). Similarly, we analyzed the time course for the induction of *fdxA* expression after exposure to 50 μM CO and found that maximal expression occurred within 30 min (results not

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Rv No	Fold change		Gene	Protein function
	Rv	Δ dosR		
↑Rv0079	3.3	0.98		HP
↑Rv0080	2.9	0.98		CHP
↓Rv0081	2.3	1.09		Probable transcription factor
↓Rv0569	6.0	1.02		CHP
↓Rv0570	3.2	1.03	<i>nrdZ</i>	Probable ribonucleoside diphosphate reductase
↑Rv0571c	2.3	0.97		CHP
↑Rv0572c	2.8	1.07		HP
↑Rv0573c	1.5	1.01		CHP
↑Rv0574c	1.7	1.03		CHP
↑Rv1733c	2.3	0.92		Probable conserved transmembrane protein
↑Rv1734c	2.0	1.03		CHP
↑Rv1735c	1.6	1.02		Hypothetical membrane protein
↑Rv1736c	2.4	1.06	<i>narX</i>	Probable nitrate reductase
↑Rv1737c	2.1	0.99	<i>narK2</i>	Probable nitrite/nitrate transporter
↓Rv1738	5.8	0.98		CHP
↑Rv1812c	1.9	1.09		probable dehydrogenase
↑Rv1813c	5.1	1.01		CHP
↑Rv1996	2.4	1.04		CHP
↓Rv1997	6.7	1.00	<i>ctpF</i>	Probable metal cation transporter
↑Rv2003c	3.3	1.03		CHP
↑Rv2004c	4.8	1.07		CHP
↑Rv2005c	6.7	0.93		CHP
↓Rv2006	2.6	1.08	<i>otsB1</i>	Probable trehalose-6-phosphate phosphatase
↑Rv2007c	7.1	0.91	<i>fdxA</i>	Probable ferredoxin
↑Rv2028c	1.9	0.91		CHP
↑Rv2029c	6.6	1.00	<i>pfkB</i>	Probable phosphofructokinaseB
↑Rv2030c	9.2	0.92		CHP
↑Rv2031c	5.2	0.87	<i>acr</i>	Alpha-crystalline
↓Rv2032	8.4	0.82	<i>acg</i>	acr-coregulated gene
↓Rv2623	6.8	1.05		CHP
↑Rv2624c	7.1	0.96		CHP
↑Rv2625c	2.2	1.01		Probable conserved transmembrane protein
↑Rv2626c	10	1.01		CHP
↑Rv2627c	4.2	1.03		CHP
↑Rv2628	6.8	0.95		HP
↑Rv2629	2.6	0.96		CHP
↑Rv2630	5.2	0.92		HP
↓Rv2631	2.7	1.10		CHP
↑Rv3126c	1.8	0.93		HP
↓Rv3127	2.7	0.99		CHP
↑Rv3130c	11.0	0.92		CHP
↓Rv3131	3.3	0.98		CHP
↑Rv3132c	3.1	1.05	<i>dosS</i>	Two component sensor histidine kinase
↑Rv3133c	3.0	1.15	<i>dosR</i>	Two component response regulated
↑Rv3134c	7.1	1.02		CHP

FIGURE 1. Up-regulation of the complete *Mtb* Dos regulon upon exposure to CO. Microarray expression analysis was performed using RNA isolated from wild-type *Mtb* and *Mtb* Δ dosR cells treated/untreated with 50 μ M CO for 3 h. Arrows indicate the direction of transcription according to Tuberculist. CHP, conserved hypothetical protein; HP, hypothetical protein.

shown). By 24 h post treatment, expression of *fdxA* declined to basal levels but rapidly increased upon treatment with a second dose of CO (results not shown).

In sum, these data demonstrate that, similar to hypoxia and NO, exposure to low, non-toxic concentrations of CO specifically induces the complete *Mtb* Dos regulon in a rapid and coordinated fashion. Thus, we have identified CO as a third signal that induces the *Mtb* Dos regulon.

***Mtb* DosS Is the Primary Sensor of CO**—Because *dosT*, as opposed to *dosS*, is not up-regulated in response to any condition tested to date, including hypoxia (6), NO (7, 8), or CO (Fig. 1), we sought to examine the independent contributions of DosS and DosT in modulating the Dos regulon. First, we exposed *Mtb* Δ dosS Δ dosT cells to CO and found that similar to the *Mtb* Δ dosR strain, this strain was impaired in its ability to induce the Dos regulon (Fig. 2B). We also utilized the single knock-out strains, *Mtb* Δ dosS and *Mtb* Δ dosT, and found that *Mtb* Δ dosS was severely attenuated in its ability to induce the Dos regulon compared with wild-type *Mtb*, whereas *Mtb* Δ dosT was moderately affected (Fig. 2B). We conclude that CO is sensed through the *Mtb* DosR/S/T system and that DosS is the preferred, but not the sole, sensor of CO.

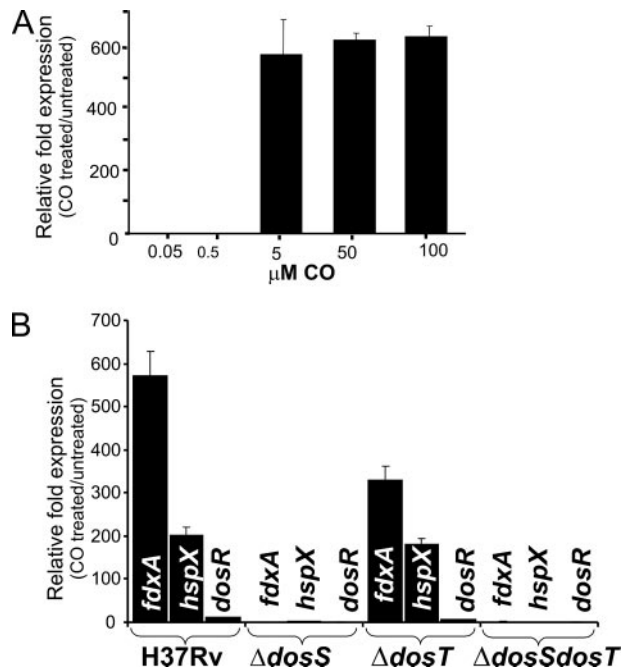


FIGURE 2. Regulation of the *Mtb* Dos regulon in response to CO. A, using Q-PCR, *fdxA* expression was examined after exposing *Mtb* cells to different concentrations of CO. B, *Mtb* DosS is the preferred sensor of CO. Wild-type *Mtb*, *Mtb* Δ dosS, *Mtb* Δ dosT, and *Mtb* Δ dosS Δ dosT cells were independently exposed to CO followed by *dosR*, *hspX*, and *fdxA* expression analysis using Q-PCR. Results are expressed as mean \pm S.D. ($n = 3$ in triplicate).

Induction of the *Mtb* Dos Regulon in Macrophages Is Modulated by HO-1—We next sought to determine whether HO-1-generated CO could be a physiologically relevant ligand of *Mtb* DosS and DosT to ultimately modulate the Dos regulon. We utilized BMM isolated from HO-1^{+/+} and HO-1^{-/-} mouse strains rather than chemical inhibitors or inducers of HO, which have nonspecific effects, including inhibition of guanylate cyclase activity (27), inhibition (28) or stimulation of NO synthase activity (29), and inactivation of caspase 3 and 8 (30). To analyze the effect of *Mtb* infection on HO-1 expression, we first infected BMM from 8–12-week-old HO-1^{+/+} mice with *Mtb* and subsequently used Western blot analysis to examine HO-1 levels. *Mtb*-infected BMM showed significantly increased HO-1 levels compared with uninfected BMM (Fig. 3A), whereas the constitutive isoform of HO-2 was unchanged. Similar to previous iNOS studies (31), we infected BMM from HO-1^{+/+} and HO-1^{-/-} mice with *Mtb* and performed Q-PCR using primers specific for *fdxA*, *hspX*, and *dosR*. Prior studies have shown that these genes are highly responsive to hypoxia and NO (6–8). We observed a 3- to 68-fold up-regulation of these genes in bacilli recovered from HO-1^{+/+} cells relative to HO-1^{-/-} cells (Fig. 3B), supporting a role for HO-1 in the induction of the Dos regulon. In sum, these data suggest that *Mtb*-infected macrophages specifically up-regulate HO-1 to increase CO, which is sensed by the *Mtb* sensor kinases DosS and DosT and then relayed to DosR to induce the Dos regulon.

Up-regulation of HO-1 Is Independent of the NO Pathway—It is well known that during *Mtb* infection iNOS is induced via IFN- γ to generate NO (32). Because NO is an inducer of HO-1 (see supplemental note 1 and supplemental Fig. S1) and because the above results demonstrate up-regulation of HO-1 in

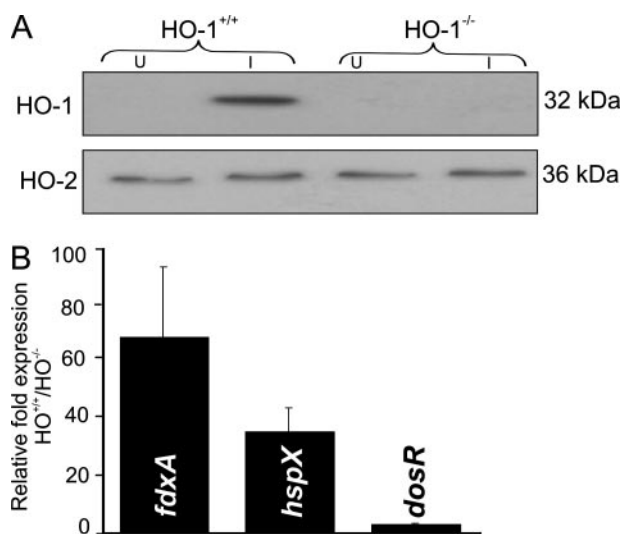


FIGURE 3. Induction of the *Mtb* Dos regulon in macrophages is modulated by HO-1. *A*, HO-1 expression in HO-1^{+/+} and HO-1^{-/-} BMM. BMM were collected from HO-1^{-/-} and HO-1^{+/+} littermates and infected with *Mtb*. HO-1 protein levels were analyzed using Western blotting. Note that levels of HO-2, the constitutive isoform of HO, are unchanged. *U*, uninfected; *I*, infected. *B*, HO-1-generated CO induces the *Mtb* Dos regulon. HO-1^{-/-} and HO-1^{+/+} BMM were independently infected with *Mtb* and RNA isolated from intracellular bacilli. Q-PCR was used to analyze the expression of *dosR*, *hspX*, and *fdxA* (an established "fingerprint" of the Dos regulon). Results are expressed as mean \pm S.D. ($n = 3$ in triplicate).

response to *Mtb* infection (Fig. 3*A*), we sought to examine the contribution of the NO signaling pathway in modulating HO-1 induction. We infected RAW 264.7 macrophage cells with *Mtb* and exploited Q-PCR to examine HO-1 expression 24 h post-infection. We found that *Mtb* significantly up-regulates HO-1 expression (Fig. 4*A*) compared with uninfected RAW 264.7 cells and the induction of HO-1 mRNA was directly associated with increased HO-1 protein (Fig. 4*B*) after infection with *Mtb*. Note that HO-1 can exist in a 32-kDa wild type and a cleaved 28-kDa form in immunoblot blots (33). Furthermore, time course experiments demonstrated that HO-1 expression was induced within 2 h of *Mtb* infection and was maintained for at least 24 h (data not shown). To specifically examine the potential link between HO-1 and the NO signaling pathway, we also infected the RAW 264.7 γ -NO(-) cell line, which is defective in the IFN- γ -mediated induction of iNOS (34). Our Q-PCR analysis showed that HO-1 expression following *Mtb* infection (Fig. 4*A*) is significantly increased in the RAW 264.7 γ -NO(-) cell line at levels comparable with that of the parental cell line (Fig. 4*A*). Corroborating these data is the Western blot analysis showing increased HO-1 protein levels in *Mtb*-infected RAW 264.7 γ -NO(-) cells (Fig. 4*B*). We also utilized the highly specific NOS inhibitor *N*-(3-(amino-methyl) benzyl) acetamidine (1400W) and found that HO-1 mRNA and protein levels were significantly increased (Fig. 4, *A* and *B*) in the presence of the inhibitor during *Mtb* infection. Finally, we infected iNOS2^{-/-} BMM with *Mtb* and again observed a significant increase in HO-1 expression upon *Mtb* exposure (Fig. 4*A*).

HO Enzymatic Activity Is Increased upon Infection of BMM with *Mtb*—Having observed that HO-1 mRNA and protein levels increase in macrophages upon infection with *Mtb*

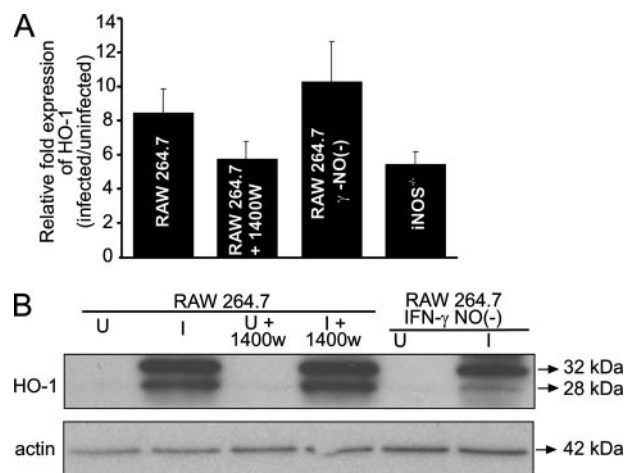


FIGURE 4. Induction of HO-1 in response to *Mtb* infection is independent of the NO signaling pathway. *A*, increase in HO-1 mRNA in response to *Mtb* infection. RAW cells and BMM from iNOS2^{-/-} mice were infected with *Mtb*, and HO-1 expression was analyzed using Q-PCR. Results are expressed as mean \pm S.D. ($n = 3$ in triplicate). *B*, increase in HO-1 protein levels in response to *Mtb* infection. HO-1 protein is increased in RAW cells 24 h post infection and in the presence and absence of 1400W. Note that HO-1 can migrate as two bands (see "Results" for details).

(Figs. 3*A* and 4, *A* and *B*), we now sought to examine (i) whether increased HO-1 expression reflects an increase in HO enzymatic activity leading to increased CO levels, and (ii) the role of the NO pathway in modulating HO enzymatic activity upon infection. RAW 264.7 cells were infected with *Mtb* and used in the HO enzymatic activity assay. In this widely used assay, HO stoichiometrically releases CO, molecular iron, and biliverdin. The latter is then converted to bilirubin by biliverdin reductase and is therefore an exact indicator of the amount of released CO. Subsequently, it was observed that macrophages infected with *Mtb* displayed ~8-fold higher HO enzymatic activity compared with uninfected control cells (Fig. 5*A*). Importantly, identical experiments were conducted with RAW 264.7 γ -NO(-) and RAW 264.7 cells treated with 1400W. Again, the results demonstrate that HO activity was not affected by a defective IFN- γ signaling pathway or a specific iNOS inhibitor (Fig. 5, *B* and *C*). Collectively, these results suggest that the induction of HO-1, and therefore CO, upon *Mtb* infection is independent of IFN- γ -mediated NO production and point toward an alternative pathway by which *Mtb* induces HO-1.

HO-1 Is Induced in Lesions of *Mtb*-infected Mice—Using a systematic series of *in vitro* culture and macrophage infection experiments described above, we made the observation that *Mtb* specifically senses and responds to physiological levels of CO to induce the Dos regulon. To determine whether CO was produced at the site of infection, as is the case for NO (3), we assessed whether HO-1 mRNA is up-regulated in mouse lungs following *Mtb* challenge. Upon infection with *Mtb*, HO-1 expression was significantly increased (19.5-fold) (Fig. 6*A*). Staining of lung lesions with HO-1-specific antibodies demonstrated that HO-1-expressing cells were associated with TB lesions. Also, a high proportion of HO-1-positive cells were found within extensive inflammatory lesions containing necrotic areas in the lungs of the mice

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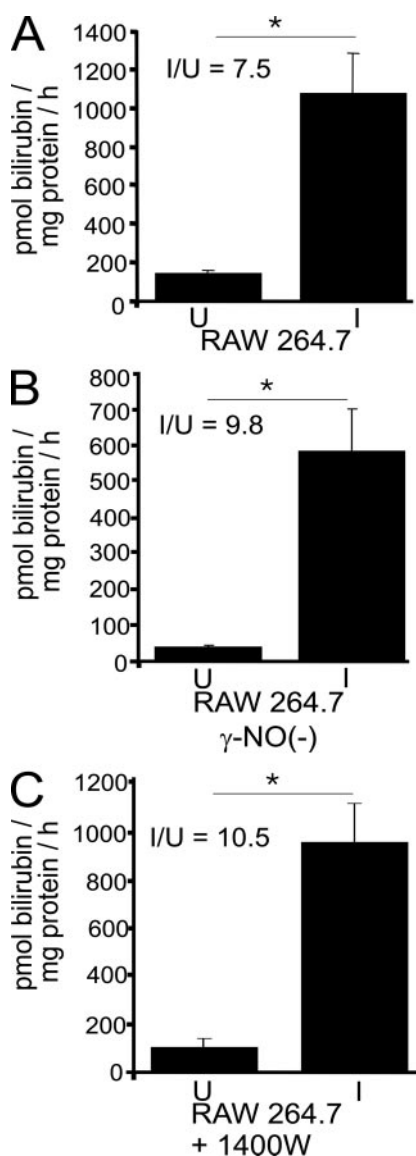


FIGURE 5. HO enzymatic activity is increased in response to *Mtb* infection. Biliverdin is a precise indicator of the amount of released CO (see "Results" for details). HO enzymatic activity was measured in RAW 264.7 (A), RAW 264.7 γ -NO(-) (B), and RAW 264.7 (C) cells with 1400W 24 h post-infection with *Mtb*. U, uninfected; I, infected. Results are expressed as mean \pm S.E.*, $p < 0.001$ ($n = 3-6$ /gp).

(Fig. 6B, I and II). The staining was localized predominantly in alveolar epithelial cells and infiltrating macrophages (Fig. 6B, I, inset).

In sum, we have shown that increased levels of HO-1 mRNA, protein, and therefore CO are produced in the lungs of *Mtb*-infected mice. The data suggest both diverging and complementary roles for NO and CO in *Mtb* pathogenesis and identify CO as a signaling ligand for DosS and DosT to modulate the *Mtb* Dos regulon.

DISCUSSION

The *Mtb* DosR/S/T two-component system responds to at least two physiologically relevant dormancy signals, O_2 (6) and NO (7, 8). In this study, we discovered for the first time that CO is a third physiologically relevant signal capable of inducing the complete 48-member *Mtb* Dos regulon. We have also shown

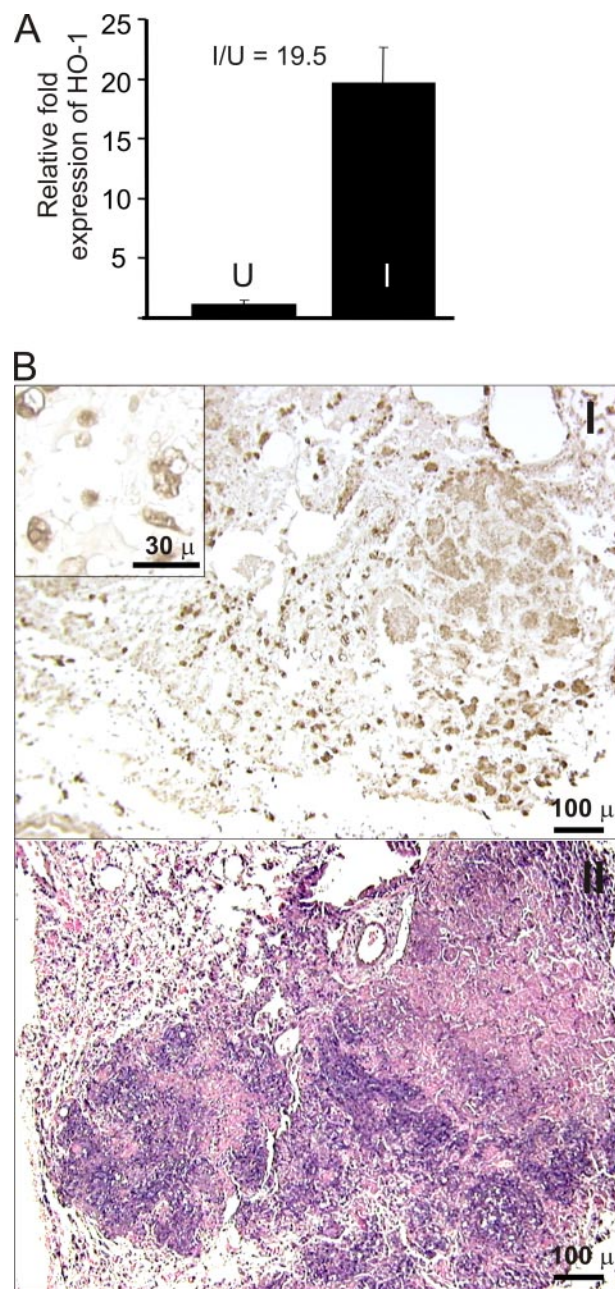


FIGURE 6. HO-1 is significantly induced in the lungs of *Mtb*-infected mice. A, relative HO-1 mRNA abundance measured in the lungs of uninfected (U) or *Mtb*-infected (I) (4 weeks post infection) C3HeB/FeJ mice. Results are expressed as mean \pm S.D. ($n = 4$ in triplicate). B, immunohistochemistry of *Mtb*-infected lungs (4 weeks post infection). Expression of HO-1 protein within TB lung lesions was demonstrated using HO-1-specific antibodies. Panel I, staining with HO-1-specific antibodies; panel II, hematoxylin and eosin staining. TB lung lesions of C3HeB/FeJ mice (panels I and II) contained numerous HO-1-positive cells (brown color in panel I and inset) within cell wall surrounding areas of necrosis.

that CO is primarily sensed through DosS. Importantly, we have demonstrated that the levels of CO produced within macrophages via HO-1 were sensed by *Mtb* and resulted in the induction of key members of the Dos regulon. We further demonstrated that HO-1 expression and protein levels, as well as HO enzymatic activity in macrophages, are significantly increased upon *Mtb* infection and are independent of the NO signaling pathway. Lastly, we have demonstrated that HO-1 is

significantly increased in the lungs of *Mtb*-infected mice. These findings demonstrate an important and previously unknown function for HO-1 and, thus, CO. Notably, the ability of three diatomic gases, CO, NO, and O₂, to induce an identical set of *Mtb* genes is an unparalleled finding and presents a useful paradigm for redox signal transduction in prokaryotic cells.

Recently, our laboratory (14) as well as others (15–17) has shown that DosS and DosT are GAF-containing heme proteins. We proposed a sense-and-lock model that suggests that the ligation state of *Mtb* DosS and DosT can be “locked” by gradients of NO and CO throughout progression of disease (14). In a recent study demonstrating a protective role for CO in experimental cerebral malaria (21), it was proposed that CO “locks” cell-free hemoglobin in the Fe(II) state, thereby preventing oxidation to the unstable met Fe(III) state, which can react with reactive oxygen species to disrupt the blood brain barrier (21). The overlap between this model and the sense-and-lock model for the *Mtb* Dos regulon (14) illustrates one of the most important properties of CO: the ability to react only with Fe(II) and not Fe(III). NO, on the other hand, can react with both Fe(II) and Fe(III) species.

In this study, we have utilized microarray technology and Q-PCR and conclusively demonstrated that similar to hypoxia and NO (6–8), low concentrations of exogenously provided CO induce the complete Dos regulon within minutes of CO exposure. Earlier studies have suggested that *M. bovis* BCG, *M. gordonae*, *M. smegmatis*, and *M. tuberculosis* H37Ra oxidize high concentrations of CO (35) via CO dehydrogenase (Rv0373c/Rv0375c). However, earlier microarray studies as well as this study have shown that neither hypoxia (6), NO (7, 8), nor CO (Fig. 1) differentially regulates expression of these genes.

Importantly, our findings demonstrating that CO is sensed primarily through DosS, with DosT playing a less prominent role, agree with recent kinetic data reporting that DosS ($K_d = 36$ nM) has a higher affinity for CO than DosT ($K_d = 940$ nM) (17). Nonetheless, this should be viewed with caution, because an earlier study has shown that DosS and DosT contributed essentially equally toward regulating *fdxA* (9) in response to hypoxia despite differences in binding of O₂ to DosS ($K_d = 3$ μM) or DosT ($K_d = 26$ μM) (9). Also, because *dosT* expression is not responsive to either O₂, NO, or CO (or any other condition according to our knowledge) and appears to be constitutively expressed, DosS or DosT protein levels may also influence regulation of the Dos regulon. Thus, the biological significance of the highly regulated nature of *dosS*, as opposed to constitutively expressed *dosT*, is an important, albeit unexplained, finding.

Because HO generates equimolar amounts of CO, Fe(II), and biliverdin using heme as a substrate *in vivo*, it was important to demonstrate that not only exogenously provided CO but also physiological levels of CO are capable of inducing the *Mtb* Dos regulon upon infection. Using mouse HO-1^{+/+} and HO-1^{-/-} BMM cells, we demonstrated that CO produced within macrophages significantly induces expression of the *Mtb* Dos regulon. Because HO generates not only CO, but also Fe(II) and biliverdin, it could be argued that these two components (or even heme) rather than CO induce the Dos regulon. However, neither heme (see supplemental text note 2) nor iron (36) was

shown to induce the Dos regulon, and there is no evidence to suggest that biliverdin is capable either. Rather, data from several independent studies (14, 15, 17) as well as our *in vitro* cell-based studies (Fig. 1) provide convincing evidence that DosS and DosT are exclusively sensing CO and not the other heme breakdown products.

HO-1 is the most important endogenous source of CO and is highly induced upon oxidative stress (37–39), whereas HO-2 is the low level, constitutive HO that contributes minimally to overall CO levels (40). Heme-independent CO arises primarily from photo-oxidation, peroxidation of lipids, and xenobiotic compounds and represents a fraction of total endogenous CO production (41).

Because continuous production of IFN-γ and tumor necrosis factor α, which synergistically regulate iNOS expression, were previously shown to be essential to prevent reactivation of persistent *Mtb* in infected mice (5), we examined the contribution of the NO signaling pathway in modulating HO-1 pathway. We independently infected three different cell types, two of which had defects in the IFN-γ/NO response pathway, and demonstrated that the induction of HO-1 in response to *Mtb* infection occurs via an unknown mechanism that is independent of NO, and possibly IFN-γ, signaling. Notably, we documented a significant increase of HO-1 in the lungs of *Mtb*-infected mice. This is not unusual, as HO-1 is rapidly induced in response to oxidative stress and inflammatory reactions, which are the major underlying causes of many diseases. However, a surprising finding was that this response appears to be independent of the NO pathway.

The combined production of NO and CO at the site of infection now provides insight into how gradients of NO and CO (and O₂) may shape disease progression. For example, “optimal” levels of O₂, NO, and CO could maintain *Mtb* in a metabolically quiescent state. On the other hand, because of the strong anti-apoptotic and anti-inflammatory properties of CO (18, 19, 42), excessive amounts of CO and also NO (5) could cause destructive immunopathology. Regardless, these findings provide mechanistic insight into how the presence of multiple gases (Fig. 7 and supplemental note 3) generates a “double-edged sword” that can either be beneficial or detrimental. Furthermore, differences in the on- and off-rates of the gases for DosS and DosT (17) and differential host and bacillary responses are evidence of a dynamic mechanism that *Mtb* has evolved to modulate disease during discrete disease states. *Mtb* may also exploit other strategies to sense O₂ or NO. For example, *Mtb* WhiB3 was shown to bind NO and react with O₂ via its 4Fe-4S cluster, which might initiate a metabolic switchover to a preferred *in vivo* carbon source, fatty acids (43). Lastly, the recent link between TB and CO air pollution (44), as well as the role of HO-1 in suppressing human immunodeficiency virus replication (45), suggests that the findings in this study may have important socioeconomic and environmental health implications.

In sum, although the exact role of HO-1 and CO in *Mtb* pathogenesis remains to be established, the findings in this study significantly advance our understanding of the *Mtb* Dos dormancy response, conventionally viewed as only being induced by two *in vivo* relevant signals, hypoxia and NO, and

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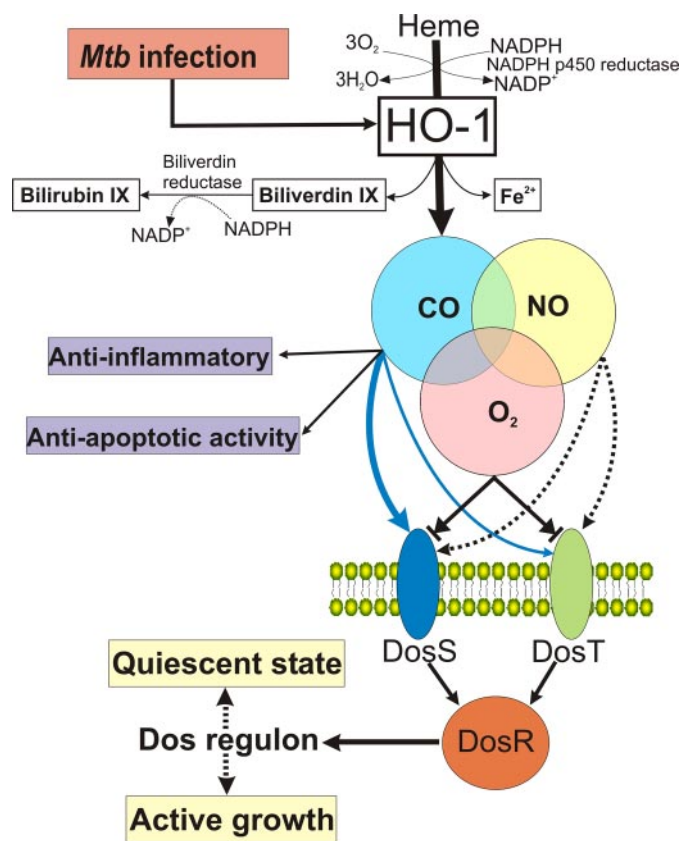


FIGURE 7. Hypothetical model depicting a role for CO in *Mtb* pathogenesis. Because numerous studies reported striking differences in local O₂, NO, and CO concentrations in organs, tissues, and single cells (see supplemental note 3), the model predicts that gradients of O₂, NO, or CO are sensed in conjunction by DosS and DosT, rather than independently. DosS is the preferred sensor of CO (thick blue arrow), whereas DosT is less capable (thin blue arrow) of inducing the Dos regulon. This contrasts with O₂, which inhibits expression of the Dos regulon (blocked arrow), albeit not during hypoxia. The model predicts that a combination of microenvironmental O₂, NO, or CO (overlapping circles) is crucial in modulating the state of the disease. Although modeling the above events in the context of chronic TB is technically difficult, the well established role of HO-1 in modulating apoptosis/necrosis, its anti-inflammatory properties (specifically the effect on tumor necrosis factor α), and the release of Fe(II) have significant implications for understanding the mechanism of *Mtb* persistence.

therefore represent a hitherto unexplored area of TB research. We have shown that CO generates expression profiles identical to that of *Mtb* cells exposed to hypoxia or NO and that DosS is the preferred sensor of CO. We have also shown that physiological levels of CO specifically induce the *Mtb* Dos regulon. Furthermore, we have demonstrated that HO-1 expression, protein, and enzymatic activity significantly increase in response to *Mtb* infection and that these events are independent of the NO signaling pathway. Lastly, we have demonstrated that HO-1 is produced at the site of infection, the lungs of *Mtb*-infected mice. These findings have broad implications for understanding the mechanistic basis for *Mtb* persistence.

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