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Heme Catabolism by Heme Oxygenase-1 Confers Host Resistance to Mycobacterium Infection

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Heme oxygenases (HO) catalyze the rate-limiting step of heme degradation. The cytoprotective action of the inducible HO-1 isoform, encoded by the Hmox1 gene, is required for host protection against systemic infections. Here we report that upregulation of HO-1 expression in macrophages (Mφ) is strictly required for protection against mycobacterial infection in mice. HO-1-deficient (Hmox1−/−) mice are more susceptible to intravenous Mycobacterium avium infection, failing to mount a protective granulomatous response and developing higher pathogen loads, than infected wild-type (Hmox1+/+) controls. Furthermore, Hmox1−/− mice also develop higher pathogen loads and ultimately succumb when challenged with a low-dose aerosol infection with Mycobacterium tuberculosis. The protective effect of HO-1 acts independently of adaptive immunity, as revealed in M. avium-infected Hmox1−/− versus Hmox1+/+ SCID mice lacking mature B and T cells. In the absence of HO-1, heme accumulation acts as a cytotoxic pro-oxidant in infected Mφ, an effect mimicked by exogenous heme administration to M. avium-infected wild-type Mφ in vitro or to mice in vivo. In conclusion, HO-1 prevents the cytotoxic effect of heme in Mφ, contributing critically to host resistance to Mycobacterium infection.

Mycobacterial infections remain a major global burden on society. Mycobacterium tuberculosis, the causative agent of tuberculosis, infects as much as one-third of the world population and accounts for up to 2 million human deaths per year (1). The advent of the AIDS epidemic and the introduction of immunosuppressive therapies have dramatically increased the number of individuals at risk of infection not only with M. tuberculosis but also with other Mycobacterium species, which would otherwise not cause disease, such as Mycobacterium avium, an ubiquitous bacterium that acts as an opportunistic pathogen in immunocompromised individuals (2).

Given the tropism of mycobacteria toward macrophages (Mφ), host resistance to infection should operate within this specific cell compartment to confer protection against infection (3). In keeping with this notion, known factors in resistance to Mycobacterium infection include a series of mechanisms operating in Mφ, including those regulating lysosome fusion, intracellular iron availability, and production of antimicrobial peptides or reactive oxygen and nitrogen species (4, 5). Mφ antimicrobial capacity is also regulated by cytokines, including tumor necrosis factor (TNF) produced by Mφ and gamma interferon (IFN-γ) produced by natural killer (NK) and T cells (4, 6).

One of the hallmarks of Mycobacterium infection is the formation of large multilamellar structures composed of infected as well as noninfected Mφ, i.e., granulomas. These confine mycobacteria within the site of infection, restraining pathogen dissemination to other organs (7, 8). Granulomas also play a critical role in the recruitment of effector immune cells, including NK and T cells that are critical for host resistance to infection. However, at least (chronic) stages of Mycobacterium infection, granulomas can undergo central necrosis, promoting pathogen dissemination (9) and causing irreversible tissue damage in the lung, a hallmark of lethal forms of the disease. On the other hand, studies on the zebrafish embryo model of granuloma formation induced by Mycobacterium marinum suggest that the dynamics of granuloma formation actually favor the initial expansion and dissemination of mycobacterium infection (62).

Programmed cell death of infected Mφ can impact granuloma integrity and hence host resistance to Mycobacterium infection. While apoptosis promotes T cell cross-priming and as such restrains Mycobacterium growth, necrosis is used by mycobacteria to evade adaptive immunity and promote bacterial growth (10). This suggests that cytoprotective mechanisms that prevent Mφ necrosis, but not apoptosis, should favor granuloma integrity and as such limit Mycobacterium growth (11). We hypothesized that the cytoprotective enzyme heme oxygenase-1 (HO-1) (12, 13) might play such a role when expressed by infected Mφ.

Recognition of pathogen-associated molecular patterns by Mφ is associated with the induction of HO-1 expression, which catabolizes free heme into biliverdin, iron, and the gasotransmitter carbon monoxide (14). The cytoprotective action of HO-1 (reviewed in reference 15) confers host protection against systemic infections in mice, as demonstrated for malaria (16–18) or severe sepsis (19). The protective effect of HO-1 against systemic infections relies to a large extent on its ability to negate the cytotoxic effects of free heme, that is, heme released from hemoproteins such as hemoglobin or myoglobin (18, 19; reviewed in reference 15). In the context of severe malaria or sepsis, this protective effect acts without regard to pathogen load to promote host survival.
(16–19) and as such is said to confer disease tolerance (3). Here we demonstrate that the cytotoxic protective effect of HO-1, when excreted in Mδ, is essential to ensure granuloma integrity and to confer resistance, rather than tolerance, to mycobacterial infections.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 86/609/EEC and Portuguese rules (DL 129/92). The animal experimental protocols were approved by the competent national authority, Direcção Geral de Veterinária (DGV) (protocol permit numbers 520/000/000/2006 and 0420/000/000/2011). All animal experiments were planned in order to minimize mouse suffering.

Mice. We generated Hmox1<sup>+/−</sup> mice by mating Hmox1<sup>−/−</sup> mice, as previously described (20). Mice were bred at the pathogen-free facilities of the Instituto Gulbenkian de Ciência (IGC), housed at the Institute for Molecular and Cell Biology (IBMC) animal facility in high-efficiency particulate air (HEPA) filter-bearing cages, and fed sterilized food and water ad libitum. Genomic DNA was isolated from the tail, and the Hmox1 genotype was determined by PCR using the primers 5′-TCCTGACAGGTT CTTCTGAG-3′ with 5′-ACGAAGTGCACCATCTGT-3′ and 5′-GTT GACAGAAAGGCTAAG-3′ with 5′-CTGTAACCTCCATCCAC-3′.

Bacteria. Mycobacterium avium strain 2447, forming smooth transparent (Smt) colonies, was isolated from an AIDS patient and was a gift from F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium). Mycobacteria were grown to mid-log phase in Middlebrook 7H9 medium (Difco) containing 0.05% Tween 80 (Sigma) at 37°C. Bacteria were harvested by centrifugation, suspended in a small volume of saline containing 0.05% Tween 80, briefly sonicated to disrupt bacterial clumps, diluted, and stored in aliquots at −80°C until use. M. tuberculosis strain H37Rv, kindly provided by António Gil Castro (Life and Health Science Research Institute, Universidade do Minho, Portugal), was grown in Proskauer-Beck medium containing 0.05% Tween 80 to mid-log phase, harvested by centrifugation, and frozen at −80°C until use.

Mouse infection and quantification of bacterial loads in organs. Mice were infected with M. avium intravenously (i.v.), through a lateral tail vein, with 10<sup>6</sup> CFU. Control animals received the same volume of saline. Mice were infected with M. tuberculosis via the aerosol route, using an airborne infection apparatus (Glas-Col Inc., Terre Haute, IN), resulting in the implantation, on average, of 30 bacilli in the lung of each mouse. At different time points, mice were sacrificed, and organs were aseptically collected and homogenized in a 0.05% Tween 80 solution in distilled water. Serial dilutions were plated into Middlebrook 7H10 (M. avium) or 7H11 (M. tuberculosis) agar medium (Gibco), the plates were incubated at 37°C for 1 week or 3 weeks, respectively, and the number of colonies was counted.

Gene expression analysis. Tissue samples were collected and frozen at −80°C until use. Total RNA was extracted using the Micro-to-Midi total RNA purification system (Invitrogen) according to the manufacturer’s specifications. Two micrograms of total RNA was transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Fermentas), using an oligo(dT)18 primer. The primers used for amplification of cDNA were as follows: hprt1 (housekeeping), 5′-GTTGAATGAGCTGCAAGG-3′ (forward) and 5′-CCAGCAAGGCTTACCACTCAAC-3′ (reverse); hmox1, 5′-GACACGAAAGGCTAAG-3′ (forward) and 5′-GACTGTTTCGCTTAC-3′ (reverse). The primers were shown not to coamplify genomic DNA. All reactions were performed in a total reaction volume of 20 μl with iQ SYBR Green Supermix (Bio-Rad) and carried out in the iQ5 instrument (Bio-Rad). Baseline thresholds were calculated by the Bio-Rad iQ5 program, and the threshold cycles (C<sub>T</sub>) were used in the REST software (21), where C<sub>T</sub> values for target genes were normalized to expression levels of hprt1. Values are reported as fold difference relative to those for the control samples.

Western blot. Tissue samples were homogenized in radioimmuno-precipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM sodium chloride, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) containing protease inhibitors (Sigma). Fifty microliters of liver or 20 μg of spleen in electrophoresis sample buffer (50 mM Tris-HCl [pH 8.8], 2% SDS, 0.017% bromophenol blue, 10% glycerol, 2 mM EDTA, 100 mM dithiothreitol [DTT]) was subjected to electrophoresis in a 10% SDS-polyacrylamide gel and transferred to a 0.45-μm nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% fat-free milk and incubated with rabbit anti-HO-1 antibody (Ab) (Proteintech) or rabbit anti-β-actin (Abcam), followed by a horseradish peroxidase-conjugated sheep anti-rabbit IgG (The Binding Site). Immunoreactivity was visualized using an enhanced chemiluminescence (ECL) reagent (Pierce) according to the manufacturer’s instructions, and the signal was recorded on a ChemiDoc XRS system (Bio-Rad). Band densitometry was performed with the Quantity One program (Bio-Rad).

Histopathology. Samples of liver or lung were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H&E) and counterstained with methylgreen. Tissue samples were examined histologically by two independent observers, as well as by two independent pathologists.

Detection of cytokines in plasma and tissues. Blood was harvested into EDTA tubes from mice anesthetized with isoflurane (Abbott Laboratories) by retro-orbital bleeding. The plasma was collected after centrifugation and frozen at −80°C until use. At the time of sacrifice, portions of the organs were stored at −80°C. Later, these portions were homogenized and protein extracts were prepared according to Bio-Rad’s Bio-Plex recommended protocol. Cytokines were measured with the Bio-Plex multiplex cytokine assay system from Bio-Rad, according to the manufacturer’s instructions. Total protein content was determined in parallel. Data analysis was performed with Bio-Plex Manager software (Bio-Rad).

Detection of plasma hemoglobin and heme. Plasma hemoglobin was determined by spectroscopy at λ = 577 nm. Total plasma heme was measured using the 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase assay (BD Bioscience) at λ = 655 nm. Purified hemoglobin was used as a standard for hemoglobin and heme measurements.

Tissue iron measurements. Tissue samples were weighed and desiccated in a microwave oven (MDS-2000). The dried samples were weighed and mineralized by acid digestion and heating at 65°C for 20 h. The sample supernatant was collected, and iron was complexed to the bathophenentholin sulfonate chrome as described by Torrence and Bothwell (22). The nonheme iron concentration was measured spectrophotometrically at λ = 535 nm. Iron content in tissues was expressed as micrograms of nonheme iron per gram of dry tissue.

Protoporphyrins. Heme (Frontier Scientific Inc.) was dissolved in 0.2 M NaOH, the pH was adjusted to 7.4 with 0.1 M HCl, and the volume was completed with distilled water. The solution was then filter sterilized and stored protected from light at −80°C until use. Mice received 0.2 ml of the heme solution every other day by a lateral tail vein, starting 1 day before
the infection with M. avium. Control mice received an equal volume of vehicle.

**Cell culture.** Bone marrow-derived Mϕ (BMMϕ) were obtained by culturing bone marrow cells that were flushed from the femurs of the mice with Hanks’ balanced salt solution (HBSS) ( Gibco). The resulting cell suspension was centrifuged and the cells resuspended in Dulbecco’s modified essential medium (DMEM) ( Gibco) supplemented with 10 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS) ( Gibco), and 10% L929 cell-conditioned medium (LCCM) as a source of macrophage colony-stimulating factor (M-CSF). To remove fibroblasts, the cells were cultured overnight, at 37°C in a 7% CO2 atmosphere on cell culture dishes. The nonadherent cells were collected with warm HBSS, distributed in 24-well plates (5 x 10^5 cells/well) or 96-well plates (1 x 10^5 cells/well), and incubated at 37°C in a 7% CO2 atmosphere. After 3 days, 10% LCCM was added. On day 7, the medium was renewed. On day 10, when the cells were fully differentiated into Mϕ, they were infected with M. avium, with 10^8 CFU of M. avium (24-well plates) or 2 x 10^7 CFU (96-well plates) added to each well. Cells were incubated for 4 h at 37°C in a CO2 atmosphere and then washed with warm HBSS to remove noninternalized bacteria and reincubated in DMEM with 10% FBS and 10% LCCM. To quantify the mycobacteria, Mϕ from triplicate wells were lysed at different time points with 0.1% saponin (Sigma). Serial dilutions were plated into Middlebrook 7H10 agar medium, and the plates were incubated at 37°C for 1 week, when the colonies were counted. For cytotoxicity assays, BMMϕ were washed with HBSS and exposed to heme diluted in HBSS for 1 h. After the removal of heme, the cells were infected with M. avium as described above, and 24 h later, resazurin (Sigma) was added to the wells (125 μM) and incubated at 37°C for 3 h. The conversion of resazurin to the highly fluorescent resorufin by viable cells was evaluated by measuring the relative fluorescence units (RFU) in a SpectraMax GeminiXS instrument ( Molecular Devices).

**Statistical analysis.** Statistically significant differences between groups were determined using the unpaired Student t test. Significance is indicated as follows: *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

**RESULTS**

*M. avium* infection is associated with induction of HO-1 expression in Mϕ. Expression of HO-1 supports the viability of activated Mϕ (23, 24), a cell type that is critically involved in providing host resistance to mycobacterial infections (5). Therefore, we investigated whether *M. avium* infection modulates the expression of HO-1. We found that *M. avium* infection was associated with the induction of HO-1 in the livers of BALB/c mice, with the highest levels of expression at day 30 postinfection, i.e., 5.2 ± 2.7- and 9.4 ± 5.8-fold increases at the mRNA and protein levels versus noninfected controls, respectively (Fig. 1A). HO-1 expression in the liver was restricted to F4/80+ Mϕ, as assessed by immunofluorescence (Fig. 1B). Expression of HO-1 in the spleen was not significantly induced in response to *M. avium* infection (see Fig. S1 in the supplemental material). This was not unexpected, as the basal level of HO-1 expression in this organ is very high due to its function in erythrocyte recycling.

**HO-1 confers resistance to *M. avium* infection.** To assess whether host resistance to *M. avium* infection operates via a mechanism involving HO-1, we used a loss-of-function approach, comparing the outcomes of *M. avium* infection in wild-type (*Hmox1^+/+^*) versus heterozygous (*Hmox1^+/−^*) or *Hmox1*-deficient (*Hmox1^−/−^*) BALB/c mice (20). The bacterial load was about 100-fold higher in *Hmox1^−/−^* than in *Hmox1^+/+^* mice, as assessed in the liver, spleen, and lungs, at 60 days postinfection (Fig. 1D). This reveals that host resistance to *M. avium* relies on a mechanism involving the expression of HO-1, presumably in Mϕ. The bacterial load in heterozygous (*Hmox1^+/−^*) mice was similar to that in wild-type (*Hmox1^+/+^*) mice (Fig. 1D).

Given that host resistance to *Mycobacterium* infection relies on interleukin-12 (IL-12)-driven IFN-γ-production by CD4+ T helper (T_H) cells (25), we asked whether impaired resistance to *M. avium* infection in *Hmox1*-deficient mice is associated with inhi-
bition of this antimicrobial response. To test this hypothesis, we compared the outcomes of \(M. \text{avium}\) infection in \(Hmox1^{-/-}\) mice and \(Hmox1^{+/+}\) BALB/c \(^{scid}\) (SCID) mice lacking peripheral T and B cells. As expected (26), SCID mice were more permissive to \(M. \text{avium}\) growth than immunocompetent mice, as revealed by a 5- to 10-fold increase in bacterial load (Fig. 1D). The bacterial load was about 100-fold higher in \(Hmox1^{-/-}\) than in \(Hmox1^{+/+}\) SCID mice, revealing that the mechanism via which HO-1 contributes to host resistance to \(M. \text{avium}\) acts independently of adaptive immunity (Fig. 1D). This increase in bacterial growth in different organs of \(Hmox1^{-/-}\) mice was also observed at earlier infection time points, namely, at 15 and 30 days of infection (see Fig. S2A in the supplemental material).

Given that induction of HO-1 expression in response to \(M. \text{avium}\) infection is restricted to \(M. \text{avium}\)-deficient mice lack granulomas and develop tissue damage in response to \(M. \text{avium}\) infection. To gain further un-

The protective effect of HO-1 against \(M. \text{avium}\) infection acts independently of the regulation of key cytokines. Resistance to \(M. \text{avium}\) infection relies on the production of several proinflammatory cytokines (26, 29), while anti-inflammatory cytokines, e.g., IL-10, can increase host susceptibility (30). To assess whether enhanced susceptibility of \(Hmox1^{-/-}\) deficient mice to \(M. \text{avium}\) infection was associated with deregulated cytokine production, the concentrations of several cytokines functionally involved in host protection against \(M. \text{avium}\) infection were compared in the plasma, liver, and spleen of infected \(Hmox1^{-/-}\) versus \(Hmox1^{+/+}\) SCID mice. Expression of IFN-\(\gamma\), TNF, IL-17, and IL-10 was similar in \(Hmox1^{-/-}\) and \(Hmox1^{+/+}\) SCID mice, as assessed at 30 days postinfection (Fig. 2). Expression of IL-12p40 was higher in infected \(Hmox1^{-/-}\) mice than in wild-type controls (Fig. 2), which is at first inconsistent with the impaired resistance of \(Hmox1^{-/-}\) mice to \(M. \text{avium}\) infection (Fig. 2). Expression of IL-1\(\beta\) and IL-6 was also higher in the spleen (but not in the plasma or liver) of infected \(Hmox1^{-/-}\) versus \(Hmox1^{+/+}\) mice (Fig. 2). This indicates that the protective effect of HO-1 against \(M. \text{avium}\) infection acts via a mechanism that is probably not based on the regulation of cytokines involved in bacterial clearance, such as IL-12, TNF, IFN-\(\gamma\), or IL-10 (29), instead being associated with a downregulation of the proinflammatory cytokines IL-1\(\beta\) and IL-6.

Expression of several chemokines functionally involved in host protection against \(Mycobacterium\) infection (31) was also compared in \(Hmox1^{-/-}\) and \(Hmox1^{+/+}\) mice, 30 days after \(M. \text{avium}\) infection. The MCP-1/CCL2 concentration in plasma, liver, and spleen was higher in \(Hmox1^{-/-}\) than in \(Hmox1^{+/+}\) mice (Fig. 2), which is in keeping with a recent report suggesting that HO-1 controls MCP-1/CCL2 during \(Mycobacterium\) infection (32). This was also the case for CCL3, but only in the spleen (Fig. 2). Expression of CCL4 was similar in \(Hmox1^{-/-}\) and \(Hmox1^{+/+}\) mice (Fig. 2). Similar results were observed at 15 days of infection (see Fig. S2B in the supplemental material). \(Hmox1^{-/-}\)-deficient mice also showed higher hepatic expression of IL-12p40, Ccl2, Ccl5, and Icam-1 mRNAs than \(Hmox1^{+/+}\) mice (see Fig. S2C in the supplemental material).

FIG 2 \(Hmox1\)-deficient mice produce normal or elevated levels of inflammatory cytokines and chemokines in response to \(M. \text{avium}\) infection. Cytokine and chemokine concentrations in the plasma, liver, and spleens of SCID BALB/c \(Hmox1^{+/+}\) and \(Hmox1^{-/-}\) mice at 30 days after \(M. \text{avium}\) infection were assessed by a bead-based multiplex immune assay. Data are shown as mean ± standard deviation for a group of 3 mice per genotype. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
understanding of the mechanisms underlying the susceptibility of Hmox1-deficient mice to Mycobacterium infection, histological analysis of the livers of M. avium-infected mice was performed. As expected (33), intravenous M. avium inoculation induced the formation of granulomas in the livers of wild-type BALB/c mice. These multicellular structures are composed of a core of epithelioid Mφ surrounded by a lymphocytic cuff (Fig. 3A and B), with accumulation of collagen fibers (Fig. 3C). Granulomas were absent in Hmox1−/− mice (Fig. 3D to F). Although collagen fibers were detected in the liver, these were dispersed without apparent organization (Fig. 3F). Likewise, leukocyte infiltrates, composed mostly of granulocytes, were observed, but without granuloma-like organization (Fig. 3D and E). Several alterations in the overall architecture of the liver parenchyma were found in infected Hmox1-deficient versus wild-type mice. These include extensive vascular damage associated with smooth muscle cell proliferation (Fig. 3D), collagen deposition (Fig. 3F), and enlarged sinusoids and extensive cell infiltrations (Fig. 3E). Noninfected age-matched Hmox1-deficient mice did not show significant pathological alterations compared to wild-type controls (see Fig. S3A and E in the supplemental material). Similar pathological features, including the absence of granulomas, were also observed in M. avium-infected Hmox1−/− versus Hmox1+/+ SCID mice (see Fig. S3B to D and F to H in the supplemental material). This demonstrates that expression of HO-1 is required for granuloma formation and/or maintenance, independently of any effect on T or B lymphocytes.

When infected with M. avium, Hmox1−/− mice had twice as much iron accumulation in the liver as did wild-type mice (Fig. 4A). Moreover, Hmox1−/− mice accumulated cell-free hemoglobin and heme in plasma at levels that were 4-fold higher than those in wild-type mice (Fig. 4A). Given that heme and labile iron can act in a pro-oxidant manner during systemic infections (34), we asked whether infected Hmox1−/− developed oxidative stress, presumably leading to programmed cell death and tissue damage. We found that this is the case, as revealed by the higher accumulation of oxidized guanine in the livers of infected Hmox1−/− versus Hmox1+/+ mice, a hallmark of oxidative stress-induced nucleic acid damage (Fig. 4B).

Heme causes oxidative stress leading to programmed cell death in response to several agonists, including TNF (18). In the case of Mφ, heme promotes TNF-mediated necroptosis (24), a programmed form of necrosis (35). To investigate whether Mφ programmed cell death occurs during M. avium infection in vivo, we detected cell death by TUNEL assay in the livers of infected mice. We found that M. avium infection was associated with increased cell death in Hmox1−/− versus Hmox1+/+ mice (Fig. 5A and C). We did not find a corresponding increase in cleaved caspase-3-positive cells (Fig. 5B and C), which is consistent with the notion, although does not prove, that cells are dying by necroptosis (24, 35, 36). This increased cell death in the tissues of Hmox1−/− mice is observed as soon as 15 days after infection (see Fig. S2D in the supplemental material). We conclude that expression of HO-1, presumably in Mφ, acts in a cytoprotective manner to prevent programmed cell death during M. avium infection.

Heme induces infected Mφ to undergo programmed cell death and impair granuloma formation. Given the results presented above, we reasoned that heme might sensitize mycobacterium-infected Mφ to undergo programmed cell death (24), compromising granuloma formation and ultimately host resistance to infection. To test this hypothesis, we exposed bone marrow-derived Mφ to heme before M. avium infection in vitro. Heme-treated Mφ underwent programmed cell death, as assessed at 24 h after infection (Fig. 6A and B). This effect was dose dependent in that increasing the heme concentration progressively reduced Mφ...
viability (Fig. 6B). Concomitantly with heme-induced cell death, Mφ became more permissive to mycobacterial proliferation (Fig. 6C). Despite the increased growth of mycobacteria in heme-treated M/H9278, heme was equally efficient at inducing cell death in uninfected and infected M/H9278 (Fig. 6B), suggesting that necroptosis is induced not by higher pathogen loads but by the pro-oxidant effect of heme. These results further suggest that heme impairs the bactericidal activity of M/H9278, compromising host resistance to mycobacterial infection.

On the other hand, the in vivo administration of heme to M. avium-infected BALB/c mice inhibited granuloma formation (Fig. 6D and F). This was, however, not associated with the detection of cell death in the liver or the increase in bacterial load, as assessed at 15 days after infection (Fig. 6E). This suggests that heme suppresses granuloma formation at an early stage of infection, rather than disrupting preformed granulomas.

Host resistance to M. tuberculosis infection acts via a mechanism that requires the expression of HO-1. Unlike M. avium, Mycobacterium tuberculosis actively manipulates the death pathway of its host cells (10). Therefore, we asked whether expression of HO-1 is also required to confer host resistance to M. tuberculosis infection. It was previously reported that intravenous infection with high doses (10^7 and 10^8 CFU) of M. tuberculosis induces the expression of HO-1 in the lung (27, 28). Here, we used a model of infection with a low dose of M. tuberculosis (30 CFU) via the aerosol route, which more closely replicates the natural route of infection. After 60 days of infection, we detected the expression of HO-1 by immunohistochemistry in the lung. Akin to the case for M. avium, HO-1 was induced in granulomas, while it is absent from healthy tissue (Fig. 7A). In the absence of HO-1, mice developed higher bacterial loads in the lung.
and spleen than wild-type mice at 60 days postinfection (Fig. 7B). Furthermore, all Hmox1\textsuperscript{−/−} mice succumbed to M. tuberculosis infection, while wild-type (Hmox1\textsuperscript{+/+}) and heterozygous (Hmox1\textsuperscript{+/-}) mice all survived until day 240 postinfection, the last time point analyzed (Fig. 7C). The lung bacterial load at the time of death of Hmox1\textsuperscript{−/−} mice ranged from 5.1 to 8.6 log\textsubscript{10} CFU (Fig. 7C, values in brackets) compared to 3.4 to 4.3 log\textsubscript{10} CFU in Hmox1\textsuperscript{+/+} mice at the same time range after infection. When infected with M. tuberculosis, Hmox1\textsuperscript{−/−} mice also exhibited higher bacterial loads in other organs. At the time of death, the bacterial load of these mice ranged from 5.4 to 8.4 log\textsubscript{10} CFU in the spleen and from 5.2 to 8.5 log\textsubscript{10} CFU in the liver, while wild-type mice presented 4.1 to 4.3 log\textsubscript{10} CFU in the spleen and below the level of detection (below 100 bacteria) in the liver at the same time of infection. Histological analysis of the lungs of Hmox1\textsuperscript{−/−} mice at 60 days after M. tuberculosis infection showed rare lesions, composed predominantly of lymphocytes, surrounding a central Mφ core and corresponding to early-stage granulomas (Fig. 7D). Cell infiltrates were also observed in M. tuberculosis-infected Hmox1\textsuperscript{−/−} mice, but these were composed predominantly of Mφ and appeared to be less organized (Fig. 7D). Lung sections from Hmox1-deficient mice also showed high bacterial loads in this organ (Fig. 7E) and significantly increased levels of cell death, revealed by TUNEL staining (Fig. 7F). Overall, these observations reveal that expression of HO-1 also plays a critical role in controlling M. tuberculosis infection in the lung.

**DISCUSSION**

The present work shows that HO-1 plays a central role in the control of Mycobacterium infection. This heme-catabolizing enzyme is required to confer host resistance to Mycobacterium infec-
tion, acting via mechanisms that are independent of the classic IL-12–IFN-γ axis and the development of protective pathogen-specific T cells (Fig. 1D) (26, 29, 37). Instead, HO-1 is required to sustain MΦ viability during infection (Fig. 5 and 6) and for the formation and/or maintenance of protective granulomas (Fig. 3).

Mechanisms regulating MΦ viability can have a major impact on the outcome of *Mycobacterium* infections (10). Nonpathogenic mycobacterial species and attenuated strains of *M. tuberculosis* induce MΦ to undergo apoptosis, which promotes host resistance to infection (38–40). In contrast to this, virulent *M. tuberculosis* strains suppress MΦ apoptosis and instead trigger MΦ to undergo necrosis (41, 42), allowing mycobacteria to evade and spread. The recent finding that heme causes MΦ cell death with characteristics of programmed necrosis (24) is consistent with our observation that heme is cytotoxic to *M. avium*-infected MΦ (Fig. 6A and B), which underlies the increase in mycobacterial growth (Fig. 6C).

Furthermore, we show that this mechanism can occur in vivo, as observed during *Mycobacterium* infection in *Hmox1*-deficient mice (Fig. 3 and 7). Uninfected *Hmox1−/−* mice did not show increased programmed cell death (Fig. 5). It could be argued that the infection *per se* induces MΦ to undergo programmed cell death. However, in the absence of infection, heme can trigger MΦ programmed cell death (24) (Fig. 6).

The erythrocyte life span is reduced during anemia in chronic disease (43), a condition occurring during mycobacterial infections (44). Moreover, mice infected with *Mycobacterium leprae-murium* have increased erythropagocytosis (45), implying a higher hemoglobin turnover and supporting the hypothesis that MΦ are exposed to hemoglobin and therefore to heme during mycobacterial infection. In the case of HO-1 deficiency, heme is not adequately cleared, resulting in oxidative damage and programmed cell death. Heme-driven MΦ programmed cell death should occur predominantly in MΦ that recognize mycobacteria, based on the production of reactive oxygen species and TNF in response to *mycobacteria* (24). Presumably this explains why heme catabolism by HO-1 is required to mount a protective granulomatous response, a notion supported by two independent and complementary observations. First, granulomas are absent or significantly decreased in *Mycobacterium*-infected *Hmox1*-deficient mice, in contrast to wild-type controls, where these structures are observed (Fig. 3). Second, granuloma formation after *M. avium* infection in wild-type mice is drastically reduced by the administration of heme (Fig. 6). This suggests that the substrate of HO-1 activity, i.e., heme, impairs granuloma formation and/or maintenance, thus explaining why heme catabolism by HO-1 promotes granuloma formation and/or maintenance.

The molecular mechanisms regulating granuloma formation in response to *Mycobacterium* infection are not completely understood (46). It is clear, however, that expression of cytokines, e.g., TNF, IFN-γ, or IL-12, as well as adhesion molecules, e.g., ICAM-1, is required for the formation of protective granulomas (7, 9). The observation that the expression of these genes was not impaired in *M. avium*-infected *Hmox1*-deficient versus wild-type mice (Fig. 2) suggests that the mechanism via which HO-1 promotes the formation and/or maintenance of protective granulomas acts independently of a putative effect on the expression of these and probably other related inflammatory genes.

How the absence of granulomas in *Hmox1−/−* mice affects their resistance to the infection is not clear. The granuloma is thought to be protective to the host, by favoring a close contact between infected MΦ and lymphocytes, and the lack of granulomas is usually associated with increased bacterial proliferation (7). Hence, the increased susceptibility of *Hmox1−/−* mice could be attributed to their inability to form these lesions. However, heme administration to BALB/c mice blocks the assembly of the granuloma, as assessed at day 16 after *M. avium* infection, without affecting bacterial growth (Fig. 6), whereas *Hmox1−/−* mice already have significantly higher bacterial loads at this time point (see Fig. S1 in the supplemental material). One possible interpretation is that the lack of granulomas is not entirely responsible for the increased susceptibility of *Hmox1−/−* mice, at least during the initial phase of infection. Similar observations have been made using *Icam-1*-deficient mice, which present a deficient recruitment of MΦ to infected tissues. These mice do not form the granulomas after aerosol challenge with *M. tuberculosis* but are capable of mounting a Th1 cell-mediated response, with normal levels of IFN-γ and IL-12, which controls the infection until day 90 similarly to the case for wild-type mice (47). However, at 100 to 130 days after infection, ICAM-1-deficient mice undergo a surge in bacterial numbers, resulting in lung tissue damage and death (48), which suggests that although they are not necessary to control the infection initially, granulomas are necessary to control chronic infection. Our results show that *Hmox1−/−* mice have increased bacterial loads after 60 days of infection with *M. tuberculosis*, which are probably not attributable to a deficient granuloma formation. However, akin to *Icam-1−/−* mice, *Hmox1−/−* mice infected with *M. tuberculosis* undergo a surge in bacterial numbers before the death of the host (Fig. 7), corroborating the notion that the faulty assembly of granulomas may impair the control of infection at late stages.

In addition to acting as a cytotoxic agonist, heme is a putative source of labile iron that can promote microbial growth, thus limiting host resistance to infection (49). *Hmox1*-deficient mice accumulate heme in plasma and develop tissue iron overload following *M. avium* infection (Fig. 4A), which could contribute to the higher pathogen load observed in *Hmox1−/−* than in *Hmox1+/−* mice (Fig. 1D). However, iron overload *per se* is associated with most a 10-fold increase in pathogen load (50–53), but *Hmox1* deficiency and heme accumulation in plasma are associated with 100-fold increase in pathogen load, compared to that in wild-type mice (Fig. 1D). This suggests that HO-1 reduces the pathogen load by additional mechanisms, other than controlling iron overload, presumably through its cytotoxic effect exerted on infected MΦ. It should be noted that while exogenously administered heme inhibits granuloma formation, this is not enough to cause a significant increase in *M. avium* growth in vivo at early infection time points (Fig. 6D and E). Only in the absence of the protective action of HO-1 does heme cause massive MΦ death and concomitant uncontrolled bacterial growth.

Although the underlying mechanism via which HO-1 confers host protection against *Mycobacterium* infection is similar to the one described for other pathogens (16, 18, 19), i.e., cytoprotection against heme, HO-1 confers resistance rather than tolerance to *Mycobacterium* infection. The reason for this is most probably that HO-1 provides cytoprotection in a major cell compartment conferring resistance to *Mycobacterium* infection, i.e., MΦ. These findings should have implications for our understanding of the pathogenesis of diseases triggered by intramacrophagic pathogens that have evolved mechanisms to subvert the MΦ cell death for their advantage, such as *Listeria monocytogenes*, *Salmonella* spp., *Shigella*
spp., and *Yersinia pestis* (54–56). Akin to the case for *Mycobacterium*, HO-1 may determine the pathological outcome of these infections by virtue of its cytoprotective effect exerted on Mϕ.

There are most probably other mechanisms via which HO-1 controls host microbe interaction in the context of mycobacterial infection. Carbon monoxide produced via heme catabolism by HO-1 induces the expression of the dormancy regulon during *M. tuberculosis* infection *in vitro* (27, 28), suggesting that HO-1 activity may contribute to latency. Therefore, reduced carbon monoxide should prevent the induction of the dormancy regulon, contributing to the increased proliferation of mycobacteria. While this hypothesis remains to be tested, *M. tuberculosis* deficient in the induction of the dormancy regulon establishes *in vivo* infections in mice that are indistinguishable from those caused by the parental strain (57) or even present a growth defect (58), suggesting that a putative effect of CO on the dormancy regulon should have a limited impact on bacterial growth *in vivo*.

While we were preparing this article, Regev et al. (32) reported that *Hmox1*−/− mice infected through the intratracheal route with *M. avium* fail to develop organized granulomas in the lung. Those authors attributed this effect to an increased expression of CCL2/CCR2 in HO-1 deficient mice, which while consistent with our findings (Fig. 2; see Fig. S2 in the supplemental material) may be attributed to a higher bacterial load in *Hmox1*−/− than in *Hmox1*+/+ mice, thus inducing CCL2 expression (59). However, it is not clear whether there is a causal effect of increased CCL2 and lack of granulomas. While our present data confirm that HO-1 regulates MCP-1/CCL2 expression in response to *M. avium* infection (Fig. 2; see Fig. S2 in the supplemental material) (32), this should not have a major impact on pathogen clearance since MCP-1/CCL2-deficient mice are indistinguishable from wild-type mice in their ability to clear *Mycobacterium* infection (60). Instead we propose that it is the oxidative stress induced by the accumulation of heme in Mϕ that recognize mycobacteria that inhibits granuloma formation. This argues for the existence of an inverse relationship between the levels of reactive oxygen and nitrogen species and granuloma development, a notion is strongly supported by our previous observation that inducible nitric oxide synthase-deficient mice form larger and denser granulomas in response to *M. avium* infection than wild-type mice (33, 61). Of note, when we treated mice with heme, although they were unable to form granulomas in response to *M. avium* infection, the level of expression of CCL2 mRNA in the liver was not increased compared to that in control mice (data not shown).

In conclusion, we demonstrate that expression of HO-1 is strictly required to prevent Mϕ from undergoing programmed cell death induced by free heme, thus playing a key role in host resistance against mycobacterial infections. We propose that pharmacologic targeting of heme catabolism might be used therapeutically against *Mycobacterium*.

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**REFERENCES**

Heme Oxygenase-1 Protects against Mycobacteria


