Macrophage migration inhibitory factor (MIF) is a critical mediator of the innate immune response to *Mycobacterium tuberculosis*

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Macrophage migration inhibitory factor (MIF), an innate cytokine encoded in a functionally polymorphic genetic locus, contributes to detrimental inflammation but may be crucial for controlling infection. We explored the role of variant *MIF* alleles in tuberculosis. In a Ugandan cohort, genetic low expressers of MIF were 2.4-times more frequently identified among patients with *Mycobacterium tuberculosis* (TB) bacteremia than those without. We also found mycobacteria-stimulated transcription of *MIF* and serum MIF levels to be correlated with *MIF* genotype in human macrophages and in a separate cohort of US TB patients, respectively. To determine mechanisms for MIF's protective role, we studied both aerosolized and i.v. models of mycobacterial infection and observed MIF-deficient mice to succumb more quickly with higher organism burden, increased lung pathology, and decreased innate cytokine production (TNF-α, IL-12, IL-10). MIF-deficient animals showed increased pulmonary neutrophil accumulation but preserved adaptive immune response. MIF-deficient macrophages demonstrated decreased cytokine and reactive oxygen production and impaired mycobacterial killing. Transcriptional investigation of MIF-deficient macrophages revealed reduced expression of the pattern recognition receptor dectin-1; restoration of dectin-1 expression recovered innate cytokine production and mycobacterial killing. Our data place MIF in a crucial upstream position in the innate immune response to mycobacteria and suggest that commonly occurring low expression *MIF* alleles confer an increased risk of TB disease in some populations.

Although one third of the world's population is infected with *Mycobacterium tuberculosis* (TB), only 5–10% of those infected go on to give rise to the 8.6 million annual incident TB cases and 1.5 million deaths (1). HIV infection increases the risk of TB reactivation 20-fold, and coinfected individuals contribute to 13% of worldwide TB cases but almost 30% of TB-related deaths, further highlighting the importance of host immunity in determining TB outcome (2, 3). The convergence of these epidemics is particularly prominent on the African continent, which has the highest rates of TB cases and deaths per capita, 80% of which occur in people living with HIV (1).

A genetic component to immune control of TB leading to differences in disease susceptibility and outcome has long been recognized (4, 5). Although exquisite mycobacterial susceptibility has been identified in children who have defects in the IFN-γ/IL-12/IL-23 axis, TB genetics in adults has been more difficult to define (6). A number of the candidate genes that have emerged are postulated to affect macrophage handling of mycobacteria. For example, multiple polymorphisms in *SLC11A1*, involved in phagosome maturation and vesicle trafficking, have been associated with differing degrees of TB risk in different populations (7). Additionally, polymorphisms in the genes for the pattern recognition receptors (PRRs) DC-SIGN (Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), TLR (Toll-like receptor) 1/2/8/9, and NOD2 (Nucleotide-binding oligomerization domain-containing protein 2) have emerged in TB as well as leprosy susceptibility (6).

Challenges in establishing candidate genes for TB susceptibility and severity have come from incomplete functionality or mechanistic data for some polymorphisms as well as nonreproducibility among different populations. The different frequencies with which some candidate polymorphisms occur in populations from different ethnicities as well as heterogeneity of clinical phenotyping within cohorts contribute to this challenge (8). Studying cohorts at the extremes of TB diseases (i.e., bacteremia, meningitis) has been suggested as a method for uncovering novel TB susceptibility genes (6). In a study of TB meningitis, this approach has uncovered polymorphisms in the *LTA4H* promoter that highlight the dual role of the inflammatory response in TB—facilitating mycobacterial control vs. causing inflammatory pathology (9). Additionally, although HIV infection a major risk factor for TB, there is increasing evidence that host genetic factors also influence TB among coinfected patients (10).

In this context, we explored the role of macrophage migration inhibitory factor (MIF), an important mediator of innate immunity and macrophage responses, in TB. MIF was first described in the context of TB disease (11, 12). Encoded on human chromosome 22q11, MIF is released by macrophages, lympho-

Significance

Failure of the host immune system to control infection with *Mycobacterium tuberculosis* is a major determinant of tuberculosis (TB) disease. In this work, we examined the role of macrophage migration inhibitory factor (MIF), a cytokine that is encoded in a functionally polymorphic locus in humans, in TB. We found genetic low expressers of MIF to be enriched in a population of patients with HIV and disseminated TB. From our work in cellular and mouse models, we propose a key mechanism by which MIF regulates bacterial recognition as the first step in triggering inflammatory pathways to enable mycobacterial control.

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Data deposition: The microarray data have been deposited in the International MIF Consortium at http://www.biochemmcb.rwth-aachen.de/mif_consortium_public/index.php.

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cytes, and pulmonary epithelial cells in response to microbial stimuli (13–15). Several of MIF's downstream functions support its proinflammatory actions, including overriding glucocorticoid-mediated immune suppression; inducing sustained activation of the ERK 1/2 MAP kinase pathway; inhibiting activation-induced, p53-dependent apoptosis; and up-regulating TLR4 expression on macrophages (16–19). MIF knockout ($Mif^{-/-}$) mice are protected from pathogenic inflammation in some models (20) but are susceptible to infection in others (21–23). Thus, whether MIF mediates protection from infection or potentiates damaging host response depends on the nature of the pathogen as well as the type of immunity induced.

Two *MIF* promoter polymorphisms occur commonly across different populations. The number of CATT tetranucleotide repeats at -794 is associated with differential *MIF* expression: the -794 $CATT_5$ variant is a low-expression allele, and the -794 $CATT_{6,7,8}$ variants are higher-expression alleles. An SNP at -173 (G/C) of the *MIF* promoter also has been found in many studies to be in linkage disequilibrium with the high-expression, -794 $CATT_7$ *MIF* promoter allele and to correlate with disease susceptibility or severity (24). Globally, the $CATT_5$ allele is more frequently identified among African Americans and Africans compared with their Caucasian American or Western European counterparts (25).

Differences in the distribution of high- and low-expression *MIF* alleles relating to differing phenotypes in clinical disease have been described in a variety of inflammatory conditions, including rheumatoid arthritis, scleroderma, lupus, and asthma (26–28). In infectious diseases, there are reported associations between high-expression *MIF* alleles and improved outcomes in outpatient pneumonia and meningococcal meningitis, but more severe malaria (29–31). Analogous to the role of MIF defined in mouse models, it seems that the influence of MIF on the severity of infectious diseases (exacerbation or protection) depends on the particular pathogen or disease pathology.

Experimental studies suggest that MIF is necessary for development of PPD (Purified protein derivative)-elicited delayed type hypersensitivity lesions, that it contributes to macrophage control of *M. tuberculosis* infection in vitro, and that partially backcrossed $Mif^{-/-}$ mice are more susceptible to mycobacterial lethality (32–34). To date, however, there has been no systematic evaluation of the mechanism by which MIF may play a role in the tuberculous immune response. Initial studies of the role of variant *MIF* alleles in TB have yielded disparate results. Although investigations of the -794 CATT and -173 SNP have suggested associations between the -794 $CATT_{7,8}$ alleles and TB in a Chinese Han population, and the -173 C allele and TB in Columbian and Moroccan populations, an investigation of MIF levels in HIV/TB-coinfected patients in Tanzania found that patients with low circulating MIF levels have higher mortality (35–38). Notably, *MIF* genotype investigations have not been undertaken in African HIV/TB-coinfected patients despite the critical role of immune control and the enormity of disease burden in this population.

We report herein an examination of the role of MIF and its variant alleles in human mycobacterial infection and in experimental models. We found the low-expresser *MIF* genotype, -794 $CATT_{5/5}$, to be enriched in an HIV-positive population with disseminated TB. Mycobacteria-stimulated transcription in human monocytes was found to be strictly dependent on the number of CATT repeats in the *MIF* promoter, and MIF serum levels to correlate with *MIF* genotype in TB infected subjects. We found MIF to be crucial to the control of *M. tuberculosis* and *Mycobacterium bovis* disease in mouse models, with innate immune deficits leading to increased neutrophil-mediated tissue damage in genetically MIF-deficient animals. Finally, we uncovered a unique MIF-dependent mechanism for exacerbated mycobacterial disease in which reduced macrophage expression of the innate recognition receptor dectin-1 decreased mycobacteriacidal cytokine and reactive oxygen species (ROS) production, impairing macrophage mycobacterial control.

Results

Increased Frequency of the Low-Expression *MIF* Genotype $CATT_{5/5}$ Is Demonstrated in Patients with *M. tuberculosis* Bacteremia. The prevalence of the *MIF*-794 $CATT_{5–8}$ microsatellite length polymorphism was investigated in a cohort of patients who presented to two hospitals in Uganda with severe sepsis, for whom *M. tuberculosis* was isolated from blood cultures in 21% of the group (81 of 394) (39). Clinical characteristics and CATT genotyping results were compared in the patients with *M. tuberculosis* bacteremia vs. those without (Table 1). As expected, a greater proportion of mycobacteremic subjects were HIV positive, had lower CD4 counts, and were less likely to have initiated HAART (Highly active antiretroviral therapy) therapy. Mycobacteremic subjects also were more likely to be genotypic low expressers of *MIF* [-794 $CATT_{5/5}$, 33% vs. 18%, odds ratio (OR) 2.2, $P = 0.009$]. This association remained significant in multivariate analysis adjusting for age, sex, CD4 count, and use of HAART (OR 2.4; Table 2).

The -794 CATT Length *MIF* Polymorphism Is Functional upon Mycobacterial Stimulation, and Serum MIF Levels Correlate with Patient CATT Genotype in TB Disease. To assess the functionality of the *MIF* -794 CATT microsattelite repeat in response to mycobacteria, we measured the transcriptional activity of human monocytes transfected with -794 $CATT_{5–8}$ *MIF* promoter luciferase fusion plasmids at baseline and upon stimulation with heat-killed and γ-irradiated *M. bovis* and *M. tuberculosis*, respectively. Both basal and stimulated transcriptional activity of the *MIF* promoter increased proportionally as a function of CATT length, with $CATT_5$ showing the lowest and $CATT_8$ the highest gene transcription (Fig. 1 *A* and *B*).

MIF has been reported to be elevated in both serum and bronchoalveolar lavage fluid in patients with pulmonary TB (35, 40). *MIF* genotype and serum levels were investigated in 133 HIV-negative patients whose sera were obtained from the US Centers for Disease Control and Prevention (CDC) Tuberculosis Trials Consortium (TBTC) Study 22 (41). The mean serum MIF was elevated threefold in the TB patients, before the initiation of drug therapy, compared with disease-free controls (18.6 ng/mL vs. 5.4 ng/mL; Fig. 1*C*). In the TB cohort, patients carrying at least one high-*MIF* expresser allele, -794 $CATT_7$, also had higher serum MIF levels than those with low-*MIF* expresser alleles, -794 $CATT_{5,6}$, alone (25.4 ng/mL vs. 16.8 ng/mL; Fig. 1*D*). We did not find any correlation between MIF genotype and serum MIF levels in disease-free controls, in agreement with prior reports (27).

MIF Is Expressed and Released from Macrophages in Response to Tuberculous Stimuli. To determine the kinetics of MIF release from macrophages, human THP-1 macrophages were stimulated with γ-irradiated *M. tuberculosis*, and the conditioned media was sampled. MIF was released quickly after stimulation and plateaued after 1 h (Fig. 2*A*). Transcriptional up-regulation of MIF was not detected at

Table 1. Patients with the *MIF* low-expression genotype, $CATT_{5/5}$, are enriched among patients with disseminated *M. tuberculosis* infection

Variable	Case $n = 83$	Control $n = 311$	P value
Age, y	34.4 ± 8.8	34.2 ± 10.2	
Female	38 (45.8)	161 (51.9)	
HIV positive	83 (100)	256 (82.9)	<0.001
CD4 count (cells/μL)	36.1 ± 48.4	185.9 ± 253.7	<0.001
Receiving HAART	7 (8.4)	73 (23.6)	0.002
Mortality at 30 d	41 (52.6)	75 (26.6)	<0.001
MIF low-expresser, $CATT_{5/5}$	27 (32.5)	56 (18.0)	0.009

The *MIF*-794 $CATT_{5–8}$ microsatellite length polymorphism (*rs5844572*) was genotyped in a Ugandan population with sepsis, with (case) and without (control) disseminated TB, using DNA isolated from PBMCs. Groups were compared by Student t test or χ^2, and corrected P values were calculated as appropriate. Values are number (percentage) or mean ± SD.

Table 2. Relationship between low-expresser *MIF* genotype and risk for disseminated TB is preserved in multivariate analysis

Characteristic	Adjusted odds ratio (95% confidence interval)
MIF low-expresser, $CATT_{5/5}$	2.421 (1.300–4.524)*
Age	1.014 (0.985–1.045)
Female	0.683 (0.390–1.196)
Higher CD4 count	0.986 (0.981–0.991)*
Use of HAART	0.212 (0.089–0.508)*

Adjustment was performed for age, sex, higher CD4 count, and use of HAART. Higher CD4 count (per unit increase) and use of HAART were independently protective against disseminated TB. *$P \leq 0.05$.

2 h after stimulation but occurred thereafter, at 4 and 6 h (Fig. 2*B*). This temporal pattern of MIF production from macrophages—early release of protein followed by transcriptional up-regulation—reflects initial MIF release from preformed intracellular pools (14, 42). Similar results also were observed upon stimulation of human THP-1 macrophages with PPD, and *M. tuberculosis* ligands—lipomannan and phosphatidylinositol mannosides-6.

MIF is known to act in an autocrine/paracrine fashion to promote downstream cytokine production (16, 17, 19). The effect of MIF on macrophage cytokine production was evaluated in the setting of mycobacterial stimulation by incubating THP-1 macrophages with a neutralizing anti-MIF monoclonal antibody before stimulation with irradiated *M. tuberculosis*. Analysis of the conditioned media showed significantly lower concentrations of TNF-α (Fig. 2*C*). MIF neutralization also led to decreased IL-1β (1.41 ng/mL vs. 2.01 ng/mL, $P = 0.01$) and IL-6 (0.41 ng/mL vs. 0.50 ng/mL, $P = 0.04$) production from *M. tuberculosis* ligand-stimulated macrophages. When primary macrophages prepared from differentiated human peripheral blood mononuclear cells (PBMCs) were subject to antibody neutralization of MIF before infection with *M. bovis*, significantly less TNF-α release also was observed (Fig. 2*D*).

***Mif*$^{-/-}$ Mice Succumb More Rapidly to Aerosolized *M. tuberculosis* Infection, with Increased Pulmonary Pathology and Decreased Innate Cytokine Production.** To better understand the genetic association between variant *MIF* alleles and TB disease, we studied a mouse model of virulent mycobacterial infection. WT and *Mif*$^{-/-}$ mice were infected with 200 cfu of *M. tuberculosis* clinical isolate HN878 (W. Beijing Family) by aerosol exposure. *Mif*$^{-/-}$ animals succumbed more rapidly to TB disease than their WT counterparts (Fig. 3*A*). Lung bacterial loads, which were similar at 1 mo after infection, were threefold higher in the *Mif*$^{-/-}$ animals compared with WT later in the course of disease (Fig. S1). Increased bacterial burden also was reflected in the histology of the formalin fixed lungs, in which a greater number of aggregates of acid-fast bacilli were observed in the *Mif*$^{-/-}$ animals (Fig. 3*B*).

Although the survival curves of the WT and *Mif*$^{-/-}$ animals in the setting of *M. tuberculosis* infection did not diverge until 4 mo after infection, histopathologic differences in the lungs were more immediately apparent (Fig. 3*C*). At 1 mo after infection, WT mice developed small granulomatous lesions occupying ≤20% of the lung parenchyma, as assessed by histologic examination. In contrast, *Mif*$^{-/-}$ mice developed larger, more diffuse lesions that involved 50–60% of parenchyma. By 3 mo, WT mice had more organized, coalescent granulomas, whereas *Mif*$^{-/-}$ animals had diffuse mononuclear cell infiltration of the lung parenchyma.

To uncover possible mechanisms by which MIF contributes to the killing of intracellular pathogens or control of pathogen dissemination, we investigated serum and lung lysates after *M. tuberculosis* infection for cytokine and chemokine production by multiplex array. We noted a pattern of decreased levels of macrophage innate cytokines (IL-6, TNF-α, IL-10, and IL-12), increased IFN-γ, and increased neutrophil chemoattractants [G-CSF, MIP-2 (Macrophage inflammatory protein 2)] in the *Mif*$^{-/-}$ animals (Fig. 3*D*). This innate cytokine defect in the *Mif*$^{-/-}$ animals also was apparent in the serum (Fig. S1). Greater neutrophil accumulation in the *Mif*$^{-/-}$ lungs over the course of infection was suggested by increased myeloperoxidase (MPO) activity in the lung lysates of these animals (Fig. 3*E*). Pulmonary MIF production was induced by *M. tuberculosis* infection and detectable both by ELISA (Fig. 3*F*) and immunohistochemical staining (Fig. S2). MIF was detected within the granuloma structures, but the density of the inflammatory infiltrate as well as the limitations of staining precluded determination of its precise cellular source.

***Mif*$^{-/-}$ Mice Have Higher Lung Bacterial Burdens After i.v. *M. bovis* Infection, with Increased Neutrophil Accumulation and Decreased Innate Cytokine Production, but a Preserved Adaptive Immune Response.** We also studied a murine model of i.v. infection with *M. bovis*, bacillus Calmette–Guérin, which is amenable to experimental manipulation without the requirement for strict biosafety level 3 (BSL3) isolation. Although WT or *Mif*$^{-/-}$ mice infected with *M. bovis* did not demonstrate overt signs of illness for many weeks, earlier mortality in the *Mif*$^{-/-}$ animals was eventually observed (Fig. S3). The inability of the *Mif*$^{-/-}$ animals to control mycobacterial infection nevertheless was evident in *M. bovis* infection, with infected mice demonstrating 10-fold higher lung bacterial burdens after 4 mo (Fig. 4*A*). Lung histology at 6 mo showed greater inflammatory pathology in the *Mif*$^{-/-}$ mice, marked by the prominence and persistence of neutrophilic infiltrates (Fig. 4*B*). Neutrophilia in the lungs and spleens of *M. bovis*-infected animals was quantified using flow cytometry to identify the $Gr1^{hi}$ population in single-cell suspensions of the organs. *Mif*$^{-/-}$ animals demonstrated a greater number of neutrophils in

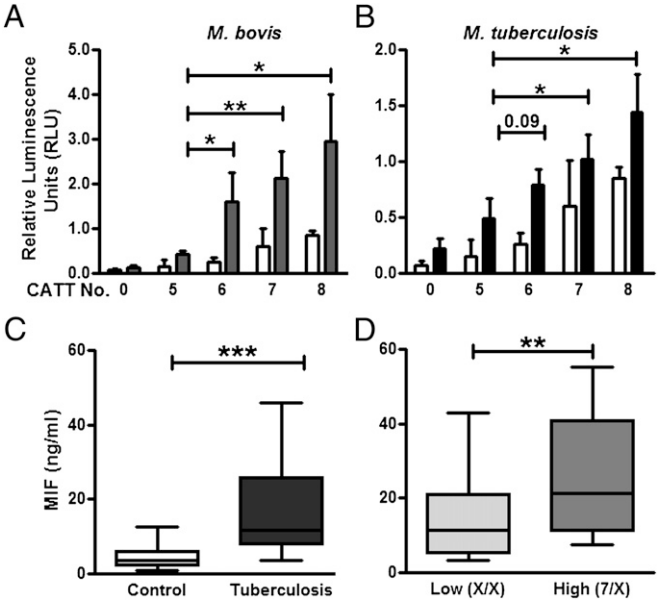

Fig. 1. Human monocytes demonstrate -794 CATT length-dependent *MIF* transcription upon mycobacterial stimulation, and serum MIF levels correlate with CATT genotype in TB disease. (*A* and *B*) Human THP-1 monocytes transfected with *MIF* promoter/luciferase reporter fusion plasmids bearing 0, 5, 6, 7, and 8 CATT repeats showed a CATT–dependent enhancement of transcription upon stimulation with heat-killed *M. bovis* (*A*) or irradiated *M. tuberculosis* (*B*). Serum MIF levels were evaluated in a cohort of patients with pulmonary TB ($n = 133$, TBTC) vs. in a cohort of disease-free controls ($n = 143$). (*C*) TB patients had higher circulating MIF than controls. (*D*) Patients with TB who have the high expression *MIF* genotype ($CATT_{7/X}$, $n = 36$) had higher circulating levels of the cytokine compared with their lower MIF expresser counterparts ($CATT_{X/X}$, X = the low expresser $CATT_5$ or $CATT_6$ alleles, $n = 97$). Mean ± SEM, and box and whisker plots with 10th and 90th percentiles reported. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

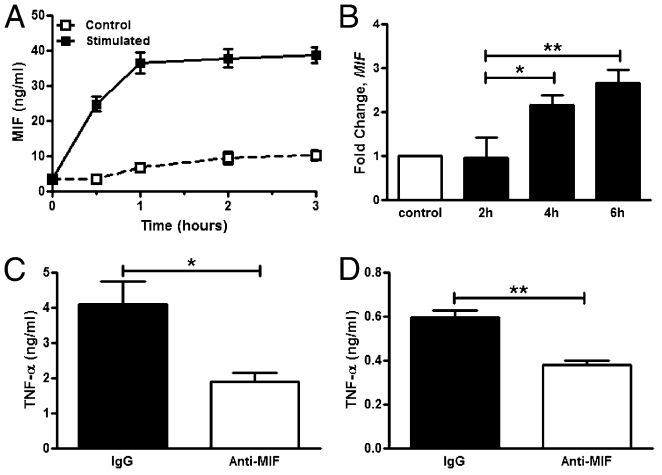

Fig. 2. MIF is released from human macrophages upon mycobacterial stimulation, and anti-MIF reduces TNF-α production. Human THP-1 monocytes were differentiated and stimulated with irradiated *M. tuberculosis*. (*A* and *B*) MIF release, measured by ELISA of conditioned media, was observed (*A*), and subsequently *MIF* transcription, measured by RT-PCR, was up-regulated (*B*). (*C* and *D*) Antibody neutralization of MIF (IgG1, clone IIID.9) reduced production of downstream TNF-α in THP-1 (*C*) as well as primary human macrophages (*D*) compared with IgG control treatment. Mean ± SEM reported. $*P \leq 0.05$, $**P \leq 0.01$.

both lungs and spleens (Fig. 4*C*). Interestingly, this difference was observed at 1 mo, when organ bacterial burdens were similar, and persisted over the 6-mo course of the experiment.

Cell activation and cytokine production responses in infected hosts were additionally investigated by restimulation of splenocytes with *M. bovis* ex vivo. Multiplex profiling of the conditioned media showed an MIF-related defect in the production of TNF-α and IL-10, augmented production of T-cell cytokines (IFN-γ, IL-17), and a threefold increase in the production of the neutrophil attractant G-CSF (Fig. 4*D*). Although macrophage numbers (F4/80$^+$CD11b$^+$) were identical in WT and *Mif*$^{-/-}$ animals, macrophage function, measured by intracellular TNF-α content, was impaired in both lung and spleen (Fig. 4*E*). The pulmonary and splenic content of CD4 and CD8 T cells was preserved in the *Mif*$^{-/-}$ animals. However, the ability of the *Mif*$^{-/-}$ CD4 T cells to produce IFN-γ upon restimulation was increased compared with WT cells, particularly in T cells obtained from the lungs (Fig. 4*F*).

Neutrophils play an important role in the outcome of infection by contributing both to enhanced pathogen clearance as well as to increased inflammatory tissue damage (43). To investigate more closely the dynamics of MIF-dependent neutrophil accumulation, we modeled the innate response to mycobacteria by injection of *M. bovis* into the peritoneums of WT and *Mif*$^{-/-}$ mice. Peritoneal lavage cells were collected at 8 h and neutrophils enumerated by flow cytometry. As in lungs, an enhancement in neutrophil accumulation was observed in *Mif*$^{-/-}$ animals compared with WT; this was reflected both by the proportion and the absolute number of neutrophils (Figs. 5 *A* and *B*). No differences between macrophages and T-cell numbers in WT and *Mif*$^{-/-}$ lavage were evident. Among mediators of neutrophil recruitment and survival, we found reduced expression of IL-10 in *Mif*$^{-/-}$ vs. WT lavage fluid (Fig. 5*C*), which was similar to the reduction in IL-10 observed in the *M. tuberculosis* and *M. bovis* pulmonary infection models. No differences in G-CSF or MIP-2 levels were detected, and there was no difference in the chemotaxis responses of *Mif*$^{-/-}$ vs. WT neutrophils in vitro (Fig. 5*D*). IL-10 mediates the efferocytosis of neutrophils (43), and imunoneutralization of IL-10 in WT mice quantitatively recapitulated the effect of MIF deficiency on *M. bovis*-induced neutrophil accumulation (Fig. 5 *E* and *F*).

These data collectively support an MIF-dependent defect in innate immunity that leads to increased lethality from mycobacterial infection. The enhanced IFN-γ response of CD4 T cells in *Mif*$^{-/-}$ mice may be in compensation to macrophage failure or to increased antigen exposure because of an accruing mycobacterial burden.

***Mif*$^{-/-}$ Macrophages Have Impaired Mycobacterial Control Resulting from Reduced Cytokine and ROS Production.** We next infected bone marrow-derived macrophages (BMDMs) from WT and *Mif*$^{-/-}$ mice with *M. bovis* and enumerated intracellular mycobacterial content by fluorescent detection. Macrophage mycobacterial uptake was identical in WT and *Mif*$^{-/-}$ cells, but there were decreased mycobacterial numbers at 3 d in infected WT macrophages, and no reduction was detected in *Mif*$^{-/-}$ macrophages (Fig. 6 *A* and *B*). These results were confirmed, accounting for bacterial viability, by plating the infected macrophage lysates at the indicated times and enumerating bacterial cfus. At 3 d after infection, WT macrophages showed a 1.4 log decrease in intracellular mycobacterial content relative to *Mif*$^{-/-}$ macrophages (Fig. 6*C*). Given the antiapoptotic effects of MIF, cell viability in these cultures was assessed by enumeration and by supernatant lactate dehydrogenase measurement. Both were equivalent in WT and *Mif*$^{-/-}$ macrophages throughout the course of the study.

Immunoneutralization of MIF, which has been reported to reduce *M. tuberculosis* killing by cultured human macrophages (34), also reproduced the mycobactericidal defect of genetic MIF deficiency (Fig. 6*D*), indicating that MIF's action on *M. bovis*-infected macrophage is mediated through an extracellular, autocrine/paracrine pathway.

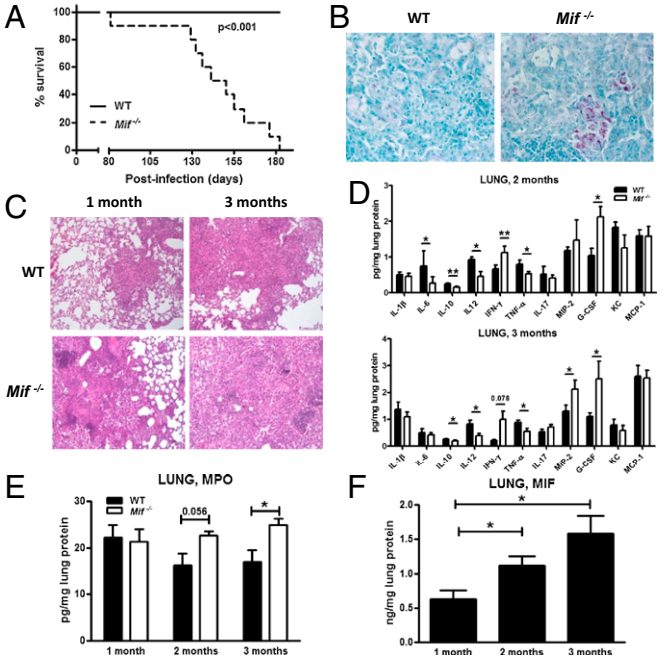

Fig. 3. Tuberculous immune response is impaired in the setting of MIF deficiency. Mice were infected with 200 cfu of *M. tuberculosis* HN878 via aerosol route. *Mif*$^{-/-}$ mice succumbed to infection with *M. tuberculosis* more rapidly than their WT counterparts (*A*) and had increased accumulation of acid-fast bacilli-positive bacteria within the lungs (*B*), exacerbated lung pathology H&E sections (*C*), and defective production of innate cytokines (IL-6, IL-12, and TNF-α) (*D*). The expression of IFN-γ and cytokines promoting neutrophil migration (G-CSF, MIP-2) as well as markers of neutrophil presence in the lung (MPO) were increased in *Mif*$^{-/-}$ mice (*D* and *E*). Lung MIF content was increased over the course of infection (*F*). $n = 10$ mice in the survival groups, $n \geq 4$ per group otherwise. Experiments repeated at least twice, and mean ± SEM reported. $*P \leq 0.05$, $**P \leq 0.01$. (Magnification: *B*, 40×; *C*, 10×.)

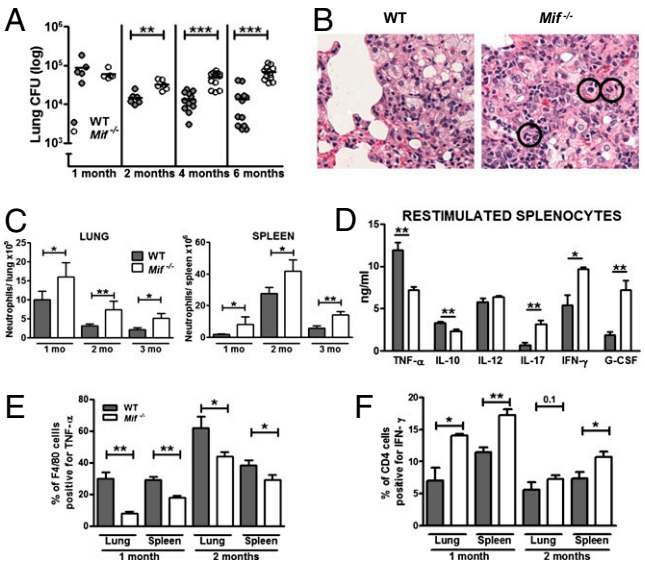

Fig. 4. *Mif*$^{-/-}$ animals have increased bacterial load, greater neutrophil accumulation, and impaired innate cytokine production during *M. bovis* infection. Animals were infected with 1×10^7 *M. bovis* via tail vein injection. (*A*) *Mif*$^{-/-}$ animals had higher lung bacterial loads at 2, 4, and 6 mo after infection compared with WT. (*B*) More severe lung pathology was observed in the *Mif*$^{-/-}$ animals, marked by increased neutrophil accumulation, H&E sections, neutrophils indicated in black circles. (*C*) Flow cytometric analysis of single-cell suspensions of lungs and spleens confirmed increased neutrophilia in the *Mif*$^{-/-}$ mice. (*D*) When 10^6 splenocytes from infected animals were plated and restimulated ex vivo with *M. bovis*, decreased TNF-α and IL-10 were noted in the culture supernatants along with increased IL-17, IFN-γ, and G-CSF. (*E* and *F*) Decreased macrophage production of TNF-α and increased CD4 cell production of IFN-γ were confirmed by flow cytometry. $n \geq 4$ animals per group, experiments repeated at least twice. Mean ± SEM reported. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. (Magnification: *B*, 40×.)

In terms of direct effectors of mycobacterial killing, we found impaired ROS generation but preserved nitric oxide (NO) production in *M. bovis*-infected *Mif*$^{-/-}$ macrophages (Fig. 7 *A* and *B*). The production of TNF-α and IL-10 from *M. bovis*-infected *Mif*$^{-/-}$ BMDMs also was impaired (Fig. 7 *C* and *D*), suggesting that the macrophage is responsible for the reduced levels of these cytokines observed in vivo. A similar 50% reduction in TNF-α and IL-10 production was observed in *M. tuberculosis*-infected *Mif*$^{-/-}$ BMDMs (Fig. 7 *C* and *D*). Although there is evidence that IL-10 may promote mycobacterial infection in certain settings (44, 45), the observations herein of increased mycobacterial burden, increased neutrophil numbers, and inflammatory lung damage, with decreased IL-10 production, suggest an intrinsic failure of *Mif*$^{-/-}$ macrophages to down-regulate a pathogenic immune response.

Dectin-1 Up-Regulation and Downstream Spleen Tyrosine Kinase Phosphorylation Are Impaired in *Mif*$^{-/-}$ Macrophages. Transcriptional microarray analysis of *M. bovis*-infected WT and *Mif*$^{-/-}$ BMDMs was performed to identify potential downstream mediators that may account for the MIF-dependent defects in the macrophage response to mycobacteria. Principal component analysis mapping demonstrated grouping of the transcription profiles by genotype and infection status (Fig. S4). A list of differentially regulated genes in infected *Mif*$^{-/-}$ vs. WT macrophages was generated, and a selection of the most significant genes is shown in Fig. S4. This list identified a number of genes, including the CD74 cell surface receptor for MIF, which may be down-regulated in the absence of MIF stimulation (46). Interestingly, the most significant, *Mif*-dependent reduction in mRNA expression ($P = 0.006$) was observed for *dectin-1*, which encodes a cell surface receptor for fungal β-1,3-glucans and for mycobacterial ligand(s) (47, 48).

We confirmed impaired up-regulation of dectin-1 in *M. bovis*- and *M. tuberculosis*-infected *Mif*$^{-/-}$ macrophages in independent experiments (Fig. 8 *A* and *B*). The magnitude of dectin-1 up-regulation in *M. tuberculosis*-infected WT macrophages also was consistent with previous observations in vivo (49). The proinflammatory effects of dectin-1 are mediated by phosphorylation of spleen tyrosine kinase (Syk) (50). Activation of this signal transduction pathway, which proceeds downstream through the adaptor molecule CARD9 (Caspase recruitment domain-containing protein 9), resulting in cytokine and ROS production, has been confirmed to occur during mycobacterial infection (51, 52). The phosphorylation of Syk was deficient in *Mif*$^{-/-}$ BMDMs in response to *M. bovis* infection, further implicating a functional impairment of dectin-1 signaling in the setting of MIF deficiency (Fig. 8*C*). Finally, we investigated the relationship between MIF and dectin-1 in human monocytes. *DECTIN-1* transcription was up-regulated in differentiated PBMCs upon *M. bovis* infection, and immunoneutralization of MIF-diminished *M. bovis*-induced up-regulation of *DECTIN-1* by twofold (Fig. 8*D*).

Dectin-1 Deficiency Is Associated with Defects in Cytokine and ROS Production, Which Can Be Restored by Overexpression of Dectin-1 in *Mif*$^{-/-}$ Macrophages. The deficiency in the downstream production of TNF-α, IL-10, and ROS observed in *M. bovis*-infected *Mif*$^{-/-}$ macrophages was mimicked by dectin-1 deficiency, although the magnitude of the defect for ROS was more pronounced in *Dectin-1*$^{-/-}$ than in *Mif*$^{-/-}$ cells (Fig. 9 *A*–*C*). The impact of these defects on the ability of the macrophages to control intracellular mycobacterial infection was evident in both *Mif*$^{-/-}$ and *Dectin-1*$^{-/-}$ cells (Fig. 9*D*). Inhibition of cellular dectin-1 function has been suggested to influence cytokine and ROS production in macrophages (53). We overexpressed dectin-1

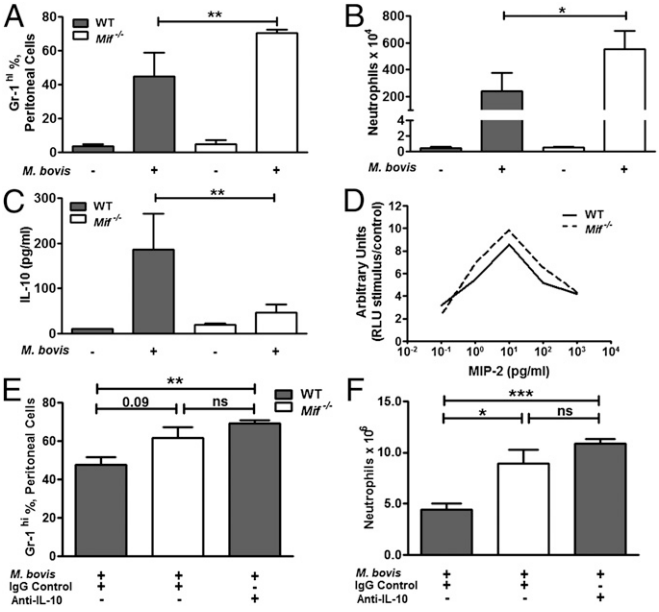

Fig. 5. Neutrophil accumulation and decreased levels of IL-10 are demonstrated in *Mif*$^{-/-}$ mice upon peritoneal *M. bovis* injection. Peritoneal exudate was collected by lavage from mice injected i.p. with 1×10^7 cfu *M. bovis* for 8 h. (*A*) Proportion of neutrophils in the peritoneal exudate was increased in the *Mif*$^{-/-}$ compared with WT. (*B*) Absolute neutrophil numbers were also increased in the *Mif*$^{-/-}$ compared with WT. (*C*) IL-10 was found to be decreased in the lavage fluid in *Mif*$^{-/-}$ compared with WT. (*D*) Bone marrow neutrophils, isolated from WT and *Mif*$^{-/-}$ mice by density gradient separation, were found to have identical migration in response to MIP-2 stimulus. (*E* and *F*) Antibody neutralization of IL-10 before *M. bovis* injection resulted in increased peritoneal exudate neutrophils. $n = 5$ animals per group, experiments repeated at least twice. Mean ± SEM reported. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

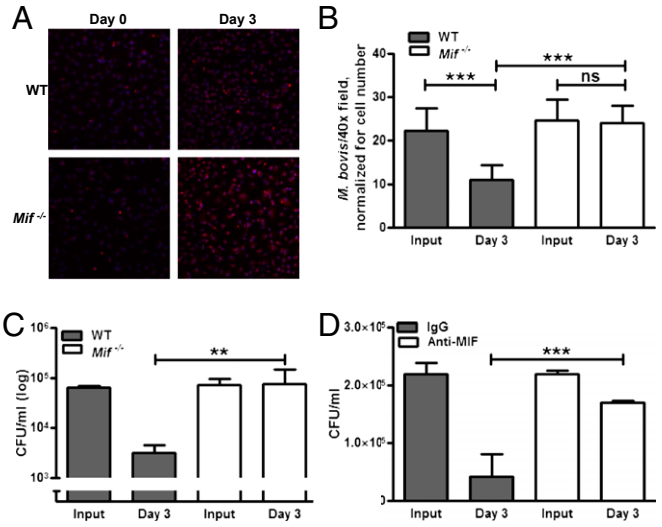

Fig. 6. MIF-deficient macrophages have a defect in their ability to control mycobacterial infection. BMDMs from WT and *Mif*$^{-/-}$ mice were infected with 10:1 MOI *M. bovis*. (*A* and *B*) *Mif*$^{-/-}$ macrophages demonstrated no reduction in intracellular bacteria at 3 d after infection compared with a significant reduction noted in the WT (detected by immunofluorescent antibody staining; in *A*, red, *M. bovis*; blue, DAPI; quantified in *B*). Subsequently, colony counts (cfus) in infected macrophage lysates were enumerated at 3 d after infection. (*C*) Although bacterial input (assessed 3 h after invasion) was identical, WT macrophages demonstrated a reduction in intracellular bacteria at 3 d, whereas no reduction was observed in *Mif* $^{-/-}$ cells. (*D*) Similar findings were observed with monoclonal antibody neutralization of MIF (clone IIID.9). Mean ± SEM reported. $*P \leq 0.05$ $**P \leq 0.01$ $***P \leq 0.001$. (Magnification: *A*, 40×.)

in *Mif*$^{-/-}$ BMDMs using a dectin-1–containing pCMV vector and confirmed a resulting 40% increase in *dectin-1* mRNA levels by RT-PCR. Overexpressing dectin-1 in *Mif*$^{-/-}$ BMDMs restored TNF-α transcription to WT levels upon *M. bovis* infection (Fig. 9*E*). Additionally, the mycobacterial killing capacity of the *dectin-1*–transfected *Mif*$^{-/-}$ macrophages was increased compared with the vector control transfected *Mif*$^{-/-}$ cells (Fig. 9*F*).

Discussion

The outcome of *M. tuberculosis* infection varies from rapid asymptomatic clearance through latent infection to different manifestations of clinical disease and lethality. Understanding the immune mechanisms responsible for these disparate outcomes is crucial to developing improved preventive and therapeutic strategies. We systematically examined the role of the innate cytokine MIF, which is encoded in a functionally polymorphic locus, in human TB disease, murine models of *M. tuberculosis* and *M. bovis* infection, and macrophage responses to mycobacterial challenge. Several findings are reported: (*i*) human *MIF* genetics influence mycobacteria-stimulated MIF production and may serve as a risk for disseminated TB disease; (*ii*) MIF deficiency renders mice more susceptible to mycobacterial pathology as a result of an inadequate innate immune response; and (*iii*) MIF-related defects in macrophage mycobacterial clearance are mediated in part by reduced expression of the microbial PRR dectin-1.

Macrophage control of mycobacterial infection is at the center of our model for MIF-related immune defects in TB disease. Failure of macrophage control is highlighted in the murine infection models, with staining of prominent mycobacterial aggregates in the lungs of *M. tuberculosis*-infected, *Mif*$^{-/-}$ mice, as well as 10-fold higher cfus of both *M. tuberculosis* and *M. bovis* isolated from these animals. On the macrophage level, genetic deficiency as well as antibody neutralization of MIF leads to failure of mycobacterial control in the setting of preserved bacterial uptake. Similarly, in our patient cohort of immunosuppressed (CD4 <50) Ugandan patients with disseminated TB, we find that genetic low expressers of MIF ($CATT_{5/5}$) are enriched 2.4-fold over TB culture-negative controls. Although other studies have examined MIF genetics in TB, we examined Sub-Saharan African, HIV-coinfected patients, a population that is critical to the global burden of disease. Additionally, we demonstrate that increasing CATT repeat length in the *MIF* promoter is associated with greater mycobacteria-stimulated *MIF* transcription in human monocytes and that pulmonary TB patients with the high-expresser, *MIF* $CATT_{7/X}$ genotype have higher circulating MIF levels than their low-expresser counterparts. The contribution of a genetic polymorphism to the risk for a common disease such as TB depends in part on the frequency at which the susceptibility allele occurs in the population (8). The proposed *MIF* TB susceptibility allele (-794 $CATT_5$) is found commonly in Caucasians (~45%) and is more prevalent in African Americans and in Africans (60–80%), indicating that *MIF* genotype may make a clinically meaningful contribution to TB disease risk (5, 25).

Although our human genetic data differ from previously published findings in cohorts with TB, this may be attributed to key disease as well as population differences among the groups. Consistent with the published geographic stratification of *MIF* polymorphisms, our cohort's CATT distribution was distinct from the other populations in which *MIF* genotypes have been studied. Disseminated *M. tuberculosis* is well described in populations with high HIV seroprevalence and depressed CD4 T-cell counts and is associated with a high risk of mortality (54, 55). Although the importance of CD4 T-cell function in TB immunity is well established, the contribution of innate immunity to TB outcome also has been described in this group (56, 57). Accordingly, it may be the case that in our Ugandan population, impaired adaptive responses in the setting of HIV infection amplifies the immunologic impact of *MIF* genotype on macrophage TB control. Our mouse model data highlight the importance of MIF to the innate immune response to TB, even in the face of a largely preserved adaptive immune response. The role of *MIF* genotype in modulating the balance between immune control and inflammatory pathology in HIV-negative patients also may be distinct from that in HIV-positive patients.

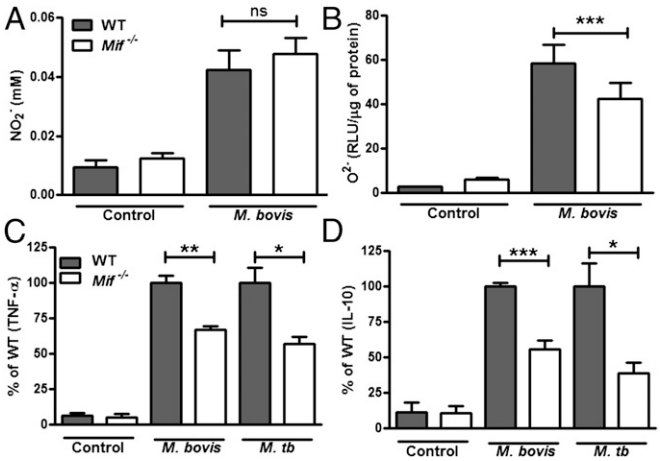

Fig. 7. MIF-deficient macrophages have reduced ROS and cytokine production. BMDMs from WT and *Mif*$^{-/-}$ were infected with *M. bovis* and the conditioned media collected for NO (NO_2^-) quantification, ROS (O_2^-) determination, and cytokine analysis. (*A* and *B*) Although *Mif*$^{-/-}$ BMDMs were not impaired in their ability to produce NO (*A*), they generated reduced amounts of ROS in response to *M. bovis* infection (*B*). (*C* and *D*) Additionally, the *Mif*$^{-/-}$ BMDMs demonstrated impaired TNF-α and IL-10 production after infection with *M. bovis* or *M. tuberculosis*. Mean ± SEM reported. $*P \leq 0.05$ $**P \leq 0.01$, $***P \leq 0.001$.

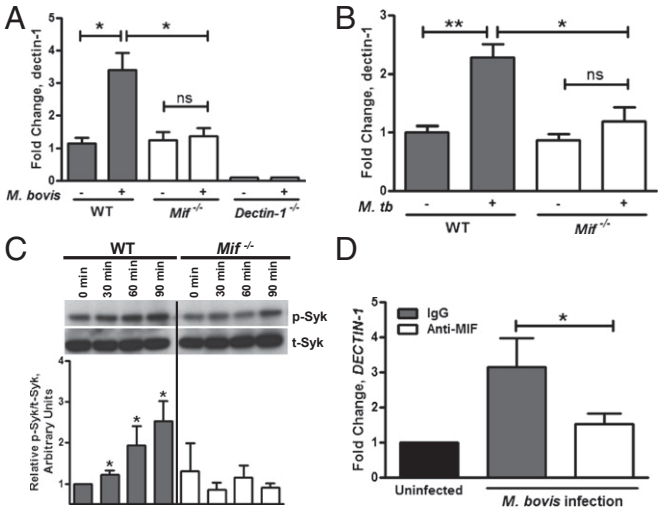

Fig. 8. Dectin-1 expression is MIF-dependent in both mouse and human macrophages, and dectin-1 signaling is reduced in *Mif*$^{-/-}$ cells. (*A* and *B*) RT-PCR using RNA from *M. bovis* and *M. tuberculosis* infected BMDMs (10:1 MOI) demonstrated decreased up-regulation of dectin-1 in *Mif*$^{-/-}$ macrophages compared with WT. (*C*) BMDMs infected with 100:1 MOI *M. bovis* showed decreased Syk phorphorylation by Western Blot (calculated as relative p-Syk/t-Syk) in *Mif*$^{-/-}$ macrophages compared with WT. (*D*) In primary human macrophages, treatment with anti-MIF antibody decreased the transcriptional up-regulation of *DECTIN-1* after *M. bovis* infection. Representative blots are shown and densitometry is performed on blots from three independent experiments. Mean ± SEM reported. $*P \leq 0.05$, $**P \leq 0.01$.

A defect in the innate immune response attributable to MIF expression was apparent in both the mouse and macrophage studies, and consistent findings were observed between virulent *M. tuberculosis* and more indolent *M. bovis* infection. In two well-characterized models of mycobacterial infection—an aerosolized (*M. tuberculosis*) and an i.v. (*M. bovis*) infection model—*Mif*$^{-/-}$ mice succumbed more quickly and with a higher organism burden, increased lung pathology, and reduced innate cytokine production. MIF-deficient mice also showed increased pulmonary neutrophil accumulation but a preserved and possibly augmented or compensatory adaptive immune response. The increased IFN-γ production of CD4 T cells in *Mif*$^{-/-}$ mice may not sufficiently compensate for the macrophage defect, leading to significantly reduced survival in both *M. tuberculosis* and *M. bovis* infection. We attributed the increase in pulmonary disease in *Mif*$^{-/-}$ mice both to the reduced inflammatory and mycobactericidal capacity of the macrophages, as well as their decreased expression of IL-10, which may down-regulate tissue-damaging neutrophilia by promoting efferocytosis (43). This latter conclusion was supported by recapitulating MIF- and IL-10–dependent neutrophil responses in a model of i.p. *M. bovis* infection. In vitro, MIF-deficient macrophages also demonstrated decreased TNF-α, IL-10, and ROS production and impaired mycobacterial killing. Although NO is known to be an effector of mycobacterial killing (58), we and others have found the NO pathway to be preserved in the *Mif*$^{-/-}$ macrophages (59).

By whole-genome transcriptional profiling, reduction in the expression of the mycobacterial PRR dectin-1 was identified in *Mif*$^{-/-}$ macrophages. MIF has been reported previously to up-regulate the expression of TLR4, which activates signal transduction in response to LPS by a pathway that requires the transcription factor PU.1 (16). Both *dectin-1* and *Tlr4* share functional promoter PU.1 binding sites, which suggests a common MIF-dependent mechanism for the inflammatory regulation of these two PRRs (16, 60). Our whole-genome array analysis did not reveal changes in the expression of different TLRs in response to mycobacterial infection, although a down-regulation of additional PU.1 associated genes, such as *Spib*, was detectable.

Although a dominant role for dectin-1 in mycobacterial control was not evident in one published mouse model (61), genetic deficiency in the dectin-1 adaptor molecule CARD9 results in diminished host survival, increased bacterial burden, neutrophilia, and more severe lung pathology despite a preserved adaptive response (62). Each of these features phenocopies our observations for mycobacteria-infected *Mif*$^{-/-}$ mice. The connection between MIF and dectin-1 in macrophages is evidenced in our work by the downstream similarities between the two defects, as well as the impact of MIF on *DECTIN-1* up-regulation in human macrophages. Moreover, dectin-1 overexpression rescues the MIF-dependent cytokine production and mycobacterial killing. Redundant roles for the C-type lectin receptors, as well as TLRs, have been suggested in other mouse models of mycobacterial infection (63–65). The MIF-related reduction in dectin-1 expression may be less likely to be compensated by an alternate receptor on the organism level. Taken together, our work suggests that MIF's role in mycobacterial control is mediated, at least in part, by dectin-1.

Although neutrophils are considered to be beneficial in early mycobacterial infection, they accumulate in situations of high pathogen load or immunological dysfunction and contribute to pulmonary pathology in established TB (66, 67). The persistence of a prominent neutrophilic infiltrate in both *M. tuberculosis*- and *M. bovis*-infected *Mif*$^{-/-}$ mice may be attributed to increased bacterial burden, innate immune failure, or a combination of both. There is experimental evidence that dectin-1 regulates IL-10 production (68, 69), and an interesting congruency between *Mif*$^{-/-}$ and

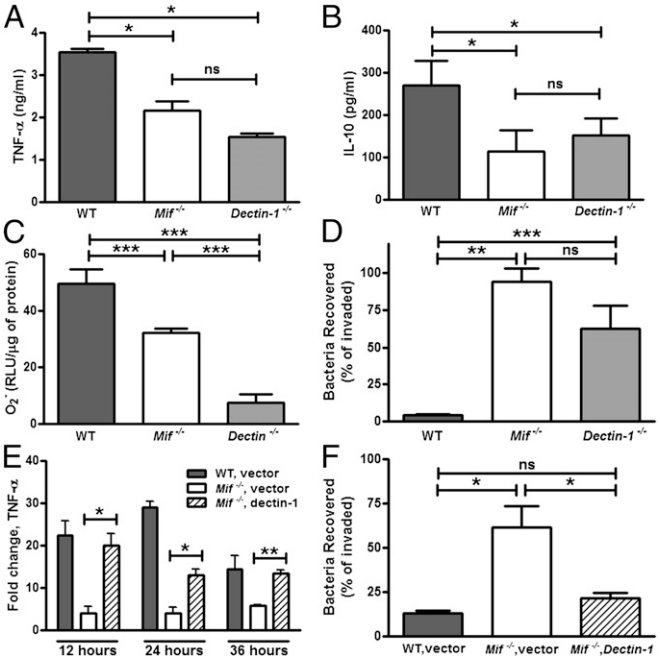

Fig. 9. *Mif*$^{-/-}$ and *Dectin-1*$^{-/-}$ macrophages have similar defects in cytokine and ROS production as well as mycobacterial control. TNF-α expression and mycobacterial killing can be recovered by *dectin-1* overexpression in *Mif*$^{-/-}$ cells. (*A* and *B*) *Dectin-1*$^{-/-}$ and *Mif*$^{-/-}$ BMDMs both had impaired TNF-α and IL-10 production induced by *M. bovis*. (*C*) ROS (O_2^-) in response to depleted zymosan was reduced in *Mif*$^{-/-}$ and absent in *Dectin-1*$^{-/-}$ BMDMs. (*D*) Intracellular control of mycobacterial infection, displayed by cfu recovered at day 3 after infection/cfu after invasion (day 0), was impaired in both *Mif*$^{-/-}$ and *Dectin-1*$^{-/-}$ BMDMs. (*E* and *F*) Overexpression of dectin-1 in *Mif*$^{-/-}$ BMDMs by transfection of a dectin-1 expression vector restored TNF-α transcription and mycobacterial killing after *M. bovis* infection. Mean ± SEM reported. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Card9$^{-/-}$ mice is reduced IL-10 expression. It also is notable that *Mif*$^{-/-}$ mice and sepsis patients with low-expresser *MIF* alleles have reduced IL-10 production (21, 30). These observations indicating a role for MIF in regulating IL-10 expression and neutrophil accumulation may explain variation in the progression of mycobacterial disease and provide an example of how a single genetic determinant may influence the balance between antimicrobial mechanisms and tissue-damaging inflammatory responses.

Both MIF and dectin-1 are expressed by immune cells at key portals of pathogen invasion (70, 71). MIF is expressed within the mycobacterial granuloma, and we identified defects in TNF-α and ROS production as well as mycobacterial control that are shared by MIF and dectin-1–deficient macrophages. MIF has been shown to up-regulate inflammatory cytokine production by mechanisms that include sustaining MAPK phosphorylation and inhibiting the synthesis of an inhibitor of NF-κB (18, 69, 72, 73). MIF-deficient macrophages also have impaired dectin-1 expression as well as decreased dectin-1 signal transduction, as evidenced by reduced stimulation-induced phosphorylation of Syk.

Many PRRs contribute to mycobacterial recognition, including DC-SIGN, TLRs 1, 2, 4, 8, and 9, NOD2, Mincle, dectin-1, and MARCO (macrophage receptor with a collagenous structure), and a number of human genetic polymorphisms in PRRs have been associated with TB susceptibility (6, 8, 74, 75). The *MIF* promoter CATT repeat length is related to serum MIF levels in TB patients and to mycobacteria-stimulated *MIF* expression in human macrophages. Expression of dectin-1 in TB-infected patients of different *MIF* genotype has not yet been undertaken. Genetic *DECTIN-1* defects have been shown to mediate susceptibility to fungal infections but have not yet been examined in TB (76). It will be important to determine whether impairment in dectin-1–mediated recognition, downstream signaling, and effector responses are recapitulated in macrophages from *MIF* low-expressers to understand whether decreased mycobactericidal capacity in these subjects may be a precursor to TB disease risk. Additionally, further examination of the mechanism for MIF control of dectin-1 expression in human macrophages and dendritic cells, as well as the possibility of augmenting MIF function, potentially by pharmacologic means (77), in subjects genetically predisposed to low *MIF* expression should be pursued.

Methods

Patient Cohorts and Genotyping. DNA isolated from PBMC pellets was available from 404 patients in a severe sepsis cohort recruited from Mulago and Masaka Hospitals in the Central Region of Uganda from July 2006 to May 2009. Full study details are described elsewhere, but patients with suspected infection, meeting criteria for severe sepsis, with portable whole-blood lactate concentration >2.5 mmol/L or Karnofsky performance scale score ≤40 were included (39, 78). All patients were investigated with bacterial and mycobacterial blood cultures. The study database was queried for patient demographics, HIV status, and CD4 count/use of HAART, where applicable, as well as results of blood cultures. All patients provided informed consent and the studies were approved by the institutional review boards (IRB) at the collecting institutions. Transfer of the samples was approved by the providing institution and the Yale human investigation committee (HIC).

Analysis of the *MIF* promoter polymorphism, -794 $CATT_{5–8}$ microsatellite repeat [*rs5844572*], was carried out by PCR using a forward primer (5′-TGCAGGAACCAATACCCATAGG-3′) and a fluorescence-labeled reverse primer (5′-AATGGTAAACTCGGGGGAC-3′). Automated capillary electrophoresis on a DNA sequencer was performed on the PCR products, and the CATT alleles were identified using Genotyper version 3.7 software (Applied Biosystems) (26). Of note, the -794 $CATT_{5–8}$ microsatellite is not represented in the high-density genotyping chips that have been used previously in genome-wide association studies.

Differences in relevant population characteristics in the Uganda cohort were analyzed using the Student *t* test. The proportion of *MIF* genotypic low expressers in the cases and controls were compared by χ^2 analysis. Multivariate odds ratios were calculated using logistic regression, controlling for age, sex, race, HIV status, CD4 count, and HAART status.

Serum samples from 133 patients with pulmonary TB were obtained from the TBTC sample repository at the CDC. The samples originated from patients enrolled at various sites across the United States in TBTC Study 22 (41). Approval to use the samples was granted by the TBTC Core Science Group. Clinical and demographic data were queried from the TBTC database. Control sera were obtained from 143 disease-free subjects with similar demographics enrolled in the Genetic and Inflammatory Markers of Sepsis Study (30). Serum MIF levels were measured by sandwich ELISA using specific antibodies (27). Genomic DNA was isolated from serum samples using the easy-DNA kit (Invitrogen), and -794 CATT genotyping was performed as indicated above. Analysis in the CDC-TBTC cohort was performed using the Student *t* test.

Human Macrophage Experiments. For the *MIF* promoter activity experiments, a dual-Luciferase reporter assay system was used (Promega). One million human THP-1 monocytes (ATCC) were cultured in RPMI 1640 supplemented with FBS and were cotransfected with 1 μg each of the respective CATT repeat construct (0-8) and a β-actin construct using the cell-line Amaxa nucleofactor platform (Lonza). The cells were incubated at 37 °C for 24 h, stimulated with either γ-irradiated *M. tuberculosis* (a generous gift from BEI Resources, Manassas, VA) or heat-killed *M. bovis* (5 μL of a 50 mg/mL solution) and incubated for an additional 8 h. The cells then were harvested, lysed, and luminescence was read at 470 and 560 mm using the Synergy Mx Monochromator-Based Microplate Reader (BioTek). Baseline and stimulated *MIF* promoter activity was determined relative to the β-actin activity in the respective samples.

For stimulation experiments, THP-1 monocytes were differentiated into macrophages by adding 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) to the culture medium for 16 h. Human PBMCs were isolated by Ficoll-Hypaque gradient centrifugation. The cells were resuspended in RPMI 1640 supplemented with human AB serum (Cambrex) and plated. After 2 h of culture, the adherent cells were washed extensively with PBS and cultured for 1 wk with the human serum-supplemented media to allow differentiation into monocyte-derived macrophages. Macrophages were either stimulated with mycobacterial glycolipids (PIM-6, lipomannan, also obtained from BEI Resources) or infected with *M. bovis*, bacillus Calmette–Guérin Pasteur. *M. bovis* was grown to log phase in Middlebrook 7H9 media (Difco) supplemented with oleic albumin dextrose catalase (Worthington Biochemicals), and single cell preparations were prepared for infection by sonicating bacteria and dispersing in 0.05% Tween 80 (Sigma) solution. Infection experiments were performed at multiplicity of infection (MOI) 10:1. Bacteria were quantified by plating of serial dilutions on 7H10 plates, and counting colonies after 3 weeks incubation at 37 °C.

MIF neutralization was performed using 100 μg/mL of monoclonal mouse anti-MIF (IgG1, clone IIID.9) added to the culture media 24 h before and maintained over the course of the experiment. Cytokine ELISAs were performed using commercially available kits (eBioscience).

RNA was isolated using the RNeasy kit (Qiagen). Reverse transcription was performed using QuantiTect kit (Qiagen) according to the manufacturer's instructions. For quantitative PCR, the following primers were used: Hu *MIF* forward, 5′-CGGACAGGGTCTACATCAA-3′ and Hu *MIF* reverse, 5′-CTTAGGCGAA-GGTGGAGTT-3′ and Hu *β-actin* forward, 5′-GGATGCAGAAGGAGATCACTG-3′ and Hu *β-actin* reverse: 5′-CGATCCACACGGAGTACTTG-3′.

Animal Experiments. Female, 8- to 10-wk-old C57BL/6 mice (WT) were purchased from Jackson Laboratories. All animal experiments were approved by IRBs at Yale and Public Health Research Institute Center. *Mif*$^{-/-}$ mice in the C57BL/6 background (N10) were bred by homozygous mating in a pathogen-free facility and were used with age and sex matching to the WT. *M. tuberculosis* infection was performed with the clinical strain, HN878, grown to log phase in Middlebrook 7H9 broth, 0.2% glycerol, and 0.05% Tween 80. Mice were infected with a low dose (~10^2 cfu) of *M. tuberculosis* using an aerosol exposure system (CH Technology) in a BSL3 facility as described previously (79). Alternately, animals were infected with ~1×10^7 cfu *M. bovis*, injected via tail vein injection in 200 μL of PBS. *M. bovis* infected mice were housed in BSL2 plus pathogen-free conditions.

Animals were either observed for survival (10 mice per experiment) or killed at the indicated time points (at least 4 mice per group, per experiment). Killing was performed by CO_2 asphyxiation. Blood was removed by cardiac puncture and serum prepared. Lungs and spleen were moved by dissection. Burden of viable mycobacteria was evaluated after infection and during the disease course by homogenizing the respective organ and spreading on Middelbrook plates. Segments of lung tissue were fixed in 10% buffered formalin (Sigma-Aldrich) and paraffin embedded. Lysates were prepared by snap-freezing a lobe of the lungs, mechanically disrupting the tissue, and addition of lysis buffer with protease inhibitor mixture (Sigma-Aldrich, Santa Cruz Biotechnology) before passing through a 0.2-μm filter (to allow removal from BSL3). Serum and lung lysates were evaluated by specific ELISA

(for MIF and MPO; Cell Signaling) and Luminex cytokine magnetic bead array (Bio-Rad, for all others described).

Histologic sections were stained with H&E or Ziehl-Neelsen (ZN) acid-fast stain for evaluation of pathology and mycobacterial load, respectively, as previously described (80). Briefly, photographs of the H&E- and ZN-stained sections were taken and analyzed using Nikon FX-35DX. For morphometric analysis of granulomas, H&E-stained lung sections were scanned with a PathScan Enabler IV scanner (Meyer Instruments). SigmaScan Pro-5 software was used to count the number and size of lesions. The extent of lung involvement was calculated using the average size of granulomas, the number of lesions per cm^2 of tissue, and the percentage of the lung sections occupied by granulomas. Morphometric analysis was carried out by an independent pathologist blinded to the source of tissues.

Lungs and spleen from the *M. bovis*-infected animals were used for flow cytometry experiments. Single-cell suspensions from lung tissue were prepared by incubating minced tissue with collagenase IV (1 mg/mL; Worthington Biochemicals) and DNase I (25–50 U/mL; Sigma-Aldrich) for 1 h at 37 °C. The digested lung was further disrupted by passing through a cell strainer (BD Bioscience). Splenocytes were obtained by passing spleens through a cell strainer, lysing red cells, and washing with RPMI/FBS. Cells were counted and viability determined by Trypan Blue exclusion. For cytokine production experiments, cell suspensions (2×10^5cells/100 μL) were incubated in 96-well U-bottom plates (Corning) with *M. bovis* for 24 h. Where appropriate, Brefeldin A (1.0 μg/mL; BD Biosciences) was added to the wells for 4 h before harvest.

For the peritonitis model, *M. bovis* (1×10^7 in 200 μL of sterile PBS), prepared as described above, was injected i.p. into mice of the designated genotype. Sterile PBS alone was used as control. In the IL-10 neutralization experiments, mice were injected with 200 μg of anti-IL-10 mAb (clone JES052A5; eBioscience) or Rat IgG1 Isotype Control (eBioscience), 16 h before *M. bovis* injection.

The following antibodies were used for surface staining: F4/80-APC, CD11b-PerCP, Gr-1-PE, CD8-APC-Cy7, CD4-Pacific Blue, CD62L-PE-Cy7, CD44-PerCP, and CD69-FITC (eBioscience). For intracellular staining, IFN-γ-PE-Cy7 and TNF-α-PE (eBioscience) were used along with the Cytofix/Cytoperm kit from BD Biosciences, according to the manufacturer's instructions. All samples were fixed in 4% paraformaldehyde before analysis. Data were acquired using the LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Bone Marrow Macrophage Experiments. BMDMs were prepared by differentiating cells flushed from the femur/tibias of C57BL/6 mice of the appropriate genotype (WT, *Mif*$^{-/-}$, and *Dectin-1*$^{-/-}$; the latter were obtained under MTA (material transfer agreement) from the University of Aberdeen, Scotland) in the presence of supernatant from L929 cells for 1 wk. For intracellular infection experiments, 2×10^5 BMDMs were plated in 12-well tissue culture plates or chamber slides (Corning) and allowed to adhere overnight. Cells were infected with *M. bovis*, MOI 10:1, and allowed to invade for 3 h. Thereafter, cells were washed thoroughly with PBS and fresh media added. For immunofluorescence, cells were fixed and permeabilized before staining with rabbit polyclonal anti-*M. tuberculosis* complex antibody (Biodesign International) and goat anti-rabbit-Alexa 594 (Invitrogen), as well as DAPI, at day 0 and day 3 after infection. Cells were visualized using a Nikon Perfect-Focus epifluorescent microscope, and intracellular mycobacteria were quantified by counting at least 30 high-power fields per condition. For cfu experiments, cells lysates were collected at days 0 (after invasion, bacterial input), 3, and 7 using 0.5% Triton X, and serial dilutions of lysates plated on Middlebrook agar to enumerate colony numbers. Antibody neutralization of MIF was performed for 24 h before *M. Bovis* infection, and 100 μg/mL of anti-MIF IgG1, clone IIID.9 was maintained in the culture supernatant throughout the infection course. Intracellular mycobacteria were enumerated by cfu assay.

The ability of WT and *Mif*$^{-/-}$ and *Dectin-1*$^{-/-}$ macrophages to produce cytokines (TNF-α, IL-10), NO, and ROS in response to *M. bovis* or *M. tuberculosis* was assessed as follows. Culture supernatants of 1×10^6 BMDMs plated in 12-well tissue culture dishes were collected 4 h after infection and evaluated for TNF-α and IL-10 by specific ELISA (eBioscience). NO was measured in the supernatants as NO_2- after reduction of NO_3- using the Griess reaction. ROS production from the macrophages was assessed using the Diogenes Reactive Oxygen Assay Kit (National Diagnostics). Briefly, 2×10^5 macrophages of the appropriate genotype were plated in 96-well opaque plates (Corning). Cells were either infected with *M. bovis* or stimulated with depleted zymosan (Sigma-Aldrich) for the indicated duration. Chemiluminescence after incubation with the Diogenes reagent was measured and normalized for the protein concentration in the respective well (determined by the Bradford Assay, BioRad, of cell lysates).

Dectin-1 Studies. RNA was isolated from murine BMDMs of the appropriate genotype after infection with 10:1 MOI of *M. bovis* and reverse transcribed as described previously. *M. bovis* or *M. tuberculosis* infection-dependent transcription of *DECTIN-1* in human macrophages, with and without antibody neutralization of MIF, was determined using RT-PCR. Primer sets for murine and human dectin-1 as well as murine TNF-α were purchased from Qiagen. Phosphorylation of Syk after *M. bovis* infection in WT and *Mif*$^{-/-}$ BMDMs was determined by Western blot using specific antibodies. Cells were lysed at the indicated time points using lysis buffer (Invitrogen) supplemented with protease inhibitor (Roche) and phosphatase inihibitor mixtures (Sigma-Aldrich). Lysates were run on a 4–12% Bis-Tris NuPage gel (Invitrogen) and transferred onto polyvinylidene difluoride membrane. Phosphorylation of Syk was determined using a specific antibody and normalized for total Syk in the respective lysates (Cell Signaling). Densitometry was performed using ImageJ.

Murine *dectin-1* was cloned into a pCMV vector. The pCMV-dectin-1 and the pCMV vector alone (control) were transfected into equal numbers of WT and *Mif*$^{-/-}$ BMDMs via the Amaxa nucleofactor platform (Lonza). Transfected cells were plated into 12-well tissue culture dishes. On the following day, cells were infected with *M. bovis* as previously described. RNA was isolated at the indicated time points and RT-PCR performed with murine TNF-α and dectin-1 primers (Qiagen). Alternately, cfu assay was performed after invasion and at 3 d to determine mycobacterial killing capacity, which was depicted relative to the respective WT vector control.

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