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Intraphagosomal *Mycobacterium tuberculosis* Acquires Iron from Both Extracellular Transferrin and Intracellular Iron Pools

IMPACT OF INTERFERON-γ AND HEMOCHROMATOSIS*

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***Mycobacterium tuberculosis* multiplies within the macrophage phagosome and requires iron for growth. We examined the route(s) by which intracellular *M. tuberculosis* acquires iron. During intracellular growth of the virulent Erdman *M. tuberculosis* strain in human monocyte-derived macrophages (MDM), *M. tuberculosis* acquisition of ^{59}Fe from transferrin (TF) provided extracellularly (exogenous source) was compared with acquisition when MDM were loaded with ^{59}Fe from TF prior to *M. tuberculosis* infection (endogenous sources). *M. tuberculosis* ^{59}Fe acquisition required viable bacteria and was similar from exogenous and endogenous sources at 24 h and greater from exogenous iron at 48 h. Interferon-γ treatment of MDM reduced ^{59}Fe uptake from TF 51% and TF receptor expression by 34%. Despite this, intraphagosomal *M. tuberculosis* iron acquisition in IFN-γ-treated cells was decreased by only 30%. Macrophages from hereditary hemochromatosis patients have altered iron metabolism. Intracellular *M. tuberculosis* acquired markedly less iron in MDM from these individuals than in MDM from healthy donors, regardless of the iron source (exogenous and endogenous): 36 ± 3.8% and 17 ± 9.6% of control, respectively. Thus, intraphagosomal *M. tuberculosis* can acquire iron from both extracellular TF and endogenous macrophage sources. Acquisition of iron from macrophage cytoplasmic iron pools may be critical for the intracellular growth of *M. tuberculosis*. This acquisition is altered by IFN-γ treatment to a small extent, but is markedly reduced in macrophages from hemochromatosis patients.**

Iron is required by both the host and microbial pathogen for growth and metabolism, thus creating constant competition for available iron (1). Limiting the access of microorganisms to iron is an evolutionary strategy of host defense (1). This strategy involves the chelation of extracellular iron by host proteins, such as transferrin (TF)[1] and lactoferrin, and/or storage of iron intracellularly in ferritin (1). During infection, iron shifts from serum to reticuloendothelial system macrophages. This further restricts the availability of iron for extracellular pathogens (1).

Iron is transported intracellularly in macrophages and other cells through the binding of Fe-TF to a specific TF receptor (TFR) on the plasma membrane, with subsequent internalization of the complex via receptor-mediated endocytosis (2). The iron is released, in part, by reduction and acidification within the endosome, following which the iron is transported to the cytoplasm by the divalent metal transporter, DMT1 (also called Nramp2 and DCT-1) (3, 4). Once iron is internalized it enters the cytoplasmic labile iron pool, where it is chelated to small molecules such as citrate, ADP, ATP, and phosphate and then utilized to meet cellular metabolic needs (3, 5). There is a dynamic equilibrium between iron in the labile iron pool and iron stored in ferritin that is altered depending on states of iron sufficiency, deficiency, or overload (6).

Extracellular pathogens have evolved a variety of ways to compete for iron. Many produce siderophores, low molecular weight iron chelators that compete with and/or remove Fe^{3+} from host Fe-binding proteins (7, 8). Alternatively some bacteria bind and directly remove iron from TF or lactoferrin, without siderophores (7). Such strategies work well because the organism has direct access to host iron storage molecules.

However, not all pathogens reside in the extracellular environment. For example, *Mycobacterium tuberculosis* is an important human intracellular pathogen that survives phagocytosis and multiplies within unique phagosomes of macrophages (9–13). How *M. tuberculosis* gains access to adequate iron for growth while residing within the phagosome is unknown. The organism does produce siderophores (14), and their production appears to be important in growth of the organism within macrophages (15). However, the source(s) from which the iron is acquired and the site at which the siderophore encounters it remain unknown. *M. tuberculosis*-derived components can traffic into the macrophage cytoplasm and/or be secreted (12, 16, 17), but whether *M. tuberculosis* siderophores exit the phagosome is not known.

If *M. tuberculosis* siderophores do not leave the phagosome, iron could be brought to them. Extracellular TF is known to cycle to *M. tuberculosis*-containing phagosomes through

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[1] The abbreviations used are: TF, transferrin; IFN-γ, interferon-γ; MDM, monocyte-derived macrophages; MOI, multiplicity of infection; NTA, nitrilotriacetic acid; PD, phosphate buffer; TFR, TF receptor; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; DMT, divalent metal transporter.

plasma membrane TFR trafficking to early endosomes (18, 19) and it has been proposed that this provides *M. tuberculosis* with iron via phagosome-endosome fusion (18). However, evidence that iron accompanies TF to the phagosome has not been obtained. Interferon-γ, a cytokine linked to host defense against *M. tuberculosis* (20), has been reported to decrease the ability of macrophages to acquire iron from TF (21–26). Thus, a portion of the antimicrobial activity of interferon-γ has been attributed to decreasing iron availability to intracellular pathogens (25, 27). However, direct assessment of the effect of interferon-γ on iron acquisition by an intracellular microbe has not been studied.

Another situation in which macrophage iron acquisition from TF is altered is in patients with hereditary hemochromatosis. Most cases of hereditary hemochromatosis result from mutations in a membrane protein termed HFE (28, 29). HFE binds to cell surface β_2-microglobulin and forms a complex with TFR. This alters TFR affinity for Fe-TF (30), the direction of which appears to depend upon whether or not β_2-microglobulin is also present (31, 32). HFE mutations associated with hemochromatosis result in reduced HFE cell surface expression. Paradoxically, in hereditary hemochromatosis the intracellular iron content of macrophages is reported to be unusually low (33–35), in part related to acceleration of macrophage iron export (33, 35, 36). Given the reported decrease in iron content of macrophages from patients with hemochromatosis, we postulated that such cells might be a poor source of iron for intraphagosomal *M. tuberculosis,* and their use would provide additional insight into the iron trafficking to *M. tuberculosis*.

Herein, we report studies that were undertaken to clarify the route and mechanism of iron acquisition by virulent *M. tuberculosis* residing within the phagosome of human macrophages. We also provide data on the effect of interferon-γ and for the first time report on the influence of mutations in HFE on these events, which prompt speculation that the hereditary hemochromatosis phenotype could be associated with increased resistance to infection with *M. tuberculosis*.

MATERIALS AND METHODS

M. tuberculosis—All experiments were carried out using the virulent *M. tuberculosis* strains Erdman and H37Rv (ATCC 35801 and 27294, respectively). *M. tuberculosis* was cultivated (10 days) and harvested in RPMI 1640 containing 10 mM Hepes, to form predominantly single-cell suspensions (37). The bacterial suspension was used within an hour of preparation in all experiments.

For experiments employing non-viable *M. tuberculosis*, Erdman strain *M. tuberculosis* was suspended in 7H9 medium and irradiated with 2.5 megarads for 18 h. The suspension was kept sterile at 4 °C. Single-cell suspensions of bacilli were prepared by adding 250 μl of the *M. tuberculosis* suspension to 750 μl of RPMI 1640 containing 10 mM Hepes.

Macrophage Culture—Peripheral blood mononuclear cells were obtained from healthy adult volunteers who were purified protein derivative negative and without a history of mycobacterial infection (38) or from hemochromatosis patients (approved Human Subjects Protocol, University of Iowa Institutional Review Board). Mononuclear cells were cultured for 5 days in teflon wells (Savillex, Minnetonka, MN) in RPMI 1640 supplemented with 20% autologous serum. The resultant monocyte-derived macrophage (MDM) fraction was isolated by adherence to tissue culture wells for 2 h with 10% autologous serum and washed. MDM monolayers (1–2 × 10^6 MDM/well) were prepared in 4-well tissue culture plates (ICN Biomedicals, Aurora, OH) or 1 × 10^5 MDM/well in 96-well culture plates (Falcon, Lincoln Park, NJ). They were incubated for 7 days at 37 °C in RPMI 1640 (Invitrogen) supplemented with 20% autologous serum, as previously reported (38, 39).

Iron Uptake by Intraphagosomal M. tuberculosis: Exogenous Source—M. tuberculosis was added to 12-day-old MDM monolayers as previously reported (39). Briefly, the monolayers (duplicate wells) were washed three times in RPMI 1640 and incubated with single suspensions of *M. tuberculosis* at a bacteria:MDM ratio of 5 (multiplicity of infection, MOI = 5) for 2 h in RPMI 1640 containing 10 mM Hepes and 1 mg/ml of human serum albumin. Monolayers were washed in RPMI 1640 and repleted with RPMI 1640 supplemented with 1% autologous serum. After 24 h, 10 μM [$^{59}Fe_2$]TF was added to the monolayers and incubated for another 24 h. Intracellular bacilli were harvested as described (39). Briefly, MDM were lysed in 0.1% SDS in the presence of 1000 units/ml DNase (Invitrogen) and EDTA-free protease inhibitor mixture tablet (Roche Molecular Biochemicals) prepared as specified by the manufacturer to release the bacilli. Duplicate (30-μl) aliquots of the cell lysate were withdrawn into γ counter tubes for assessment of total iron in the MDM lysate. The released bacilli were centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was removed, and the bacterial pellet was washed three times with 0.01% SDS in RPMI 1640. The bacilli were finally resuspended in 500 μl of 0.01% SDS in RPMI 1640 and filtered through a 0.22 μm (pore size) Spin-X centrifuge tube filter (Costar, Corning, NY). The filter containing the trapped bacilli was cut and placed into a γ counter tube. The amounts of MDM and bacilli-associated ^{59}Fe were assessed using a γ counter (Packard, Meriden, CT).

In certain experiments to ascertain whether acquisition of iron by *M. tuberculosis* within MDM phagosomes requires viable bacteria, live or γ-irradiated *M. tuberculosis* was incubated with MDM monolayers for 2 h and washed. The infected monolayers were repleted with RPMI 1640 containing 1% autologous serum and incubated for 24 h. [^{59}Fe]TF (10 μM) was added, and bacilli were harvested from lysed MDM 24 h later as above. Bacteria-associated ^{59}Fe was determined in the γ counter.

Iron Uptake by Intraphagosomal M. tuberculosis: Endogenous Source—MDM monolayers were incubated with [$^{59}Fe_2$]TF for 24 h and washed. After a 24-h chase period, the monolayer was incubated with *M. tuberculosis* at an MOI of 5 for 2 h and washed. Intracellular *M. tuberculosis* bacilli were harvested from macrophages as above at various time periods, and bacterial-associated ^{59}Fe was determined in the γ counter.

Iron Internalization into Macrophages—In order to determine whether the [$^{59}Fe_2$]TF delivered to MDM is internalized, MDM were exposed to [$^{59}Fe_2$]TF at 37 °C for 24 h or 4 °C for 1 h and then washed with the reducing agent, ascorbate, and the extracellular iron chelators, ferrozine and nitrilotriacetic acid (NTA), to remove iron remaining on the membrane surface. The cells were washed three times (each wash consisted of a 5-min incubation at 37 °C) with 5 mM ascorbate in RPMI 1640 containing 1 mM ferrozine, pH 5, followed by three washes with 1 mM NTA, pH 7. The control group was washed with RPMI 1640 alone. The cells were then placed in phosphate-buffered saline, cooled on ice for 30 min, and released by scraping. The cell suspension was transferred into a test tube and centrifuged at 400 × *g* for 10 min at 4 °C. The supernatant was removed, the cell pellet resuspended in phosphate-buffered saline, and an aliquot was withdrawn for cell count using a hemocytometer. The cell suspension was again pelleted and after removing the supernatant, the bottom of the tube containing the cell pellet was cut into a γ counter tube, and cell-associated iron was determined. Iron uptake by each group of treated cells (37 °C for 24 h or 4 °C for 1 h) was compared with uptake from its control.

Analysis of Iron Acquisition by IFN-γ-treated MDM and Intraphagosomal M. tuberculosis Harvested from These Cells—12-day-old MDM monolayers were treated with 1,000 or 10,000 units/ml of IFN-γ (or medium only for control) for 5 days prior to the addition of *M. tuberculosis* and subsequent addition (24 h later) of [$^{59}Fe_2$]TF or with [$^{59}Fe_2$]TF alone (no *M. tuberculosis*). MDM and bacilli were processed as described above, and the amounts of ^{59}Fe acquired by MDM and bacilli were determined using the γ counter.

Determination of Transferrin Receptor Expression on MDM by ELISA—12-day-old MDM monolayers (1 × 10^5 MDM/well) were prepared in 96-well tissue culture plates (triplicate wells). Some of the wells were then treated with IFN-γ (1,000 or 10,000 units/ml) or medium (control) for 5 days. The monolayers were washed three times with RPMI 1640, *in situ* fixed with 1% paraformaldehyde in phosphate buffer (PD) for 5 min. The fixative was aspirated off, and the wells were fixed further with 1% paraformaldehyde at room temperature for 10 min. The monolayers were washed four times in PD. The monolayers were then overlaid with 200 μl of blocking buffer (2.5% bovine serum albumin + 5% fetal calf serum in PD), placed on a nutator (Oxis Instruments, Ivyland, PA) overnight at 4 °C, and washed three times in cold RPMI 1640. Cells were incubated with purified mouse anti-human TfR (mAb clone DF1513, IgG1, Ancell Corp., Bayport, MN, 1.25 μg/ml) overnight at 4 °C in medium containing 2.5% bovine serum albumin in PD, and washed three times in PD. A group of cells was incubated with a subtypic control mAb (mouse IgG1, PharMingen, San Diego, CA) or buffer alone (2 control groups). Monolayers were washed three times in cold PD and then incubated for 2 h at room temperature with biotiny-

lated goat anti-mouse IgG1 (λ1 chain specific, 83.3 ng/ml Southern Biotech, Birmingham, AL) in medium containing 2.5% bovine serum albumin in PD, and washed three times in PD. The monolayers were incubated with streptavidin-horseradish peroxidase conjugate (6,000-fold dilution in 2.5% bovine serum albumin in PD) for 3 h at room temperature on a nutator and washed three times in PD. Reaction color was developed for 15 min with addition of the Bio-Rad horseradish peroxidase reagent. The reaction was stopped with addition of 1% oxalic acid and absorbance read at 405 nm on a microplate ELISA reader (BioRad). In all experiments, absorbance from cells treated with the subtypic control primary mAb was equal to those treated with buffer alone. This value was <0.2 and was subtracted from the absorbance values obtained for cells treated with anti-human TfR mAb.

Measurement of Ferritin Content of MDM—MDM monolayers were washed three times with warm RPMI 1640 and repleted with RPMI containing 1% autologous serum. IFN-γ (1,000 units/ml) was added for 5 days. ^{59}Fe (10 μM as transferrin) or medium (control) was added for 24 h. Cell monolayers were washed three times, overlaid with phosphate-buffered saline, cooled on ice for 30 min, and released by scraping with rubber policeman. Cells were washed two times in phosphate-buffered saline and finally resuspended in 1 ml of buffer containing EDTA-free protease inhibitor mixture tablet. The cells were cavitated under 350 psi N_2, and the cell lysates were sequentially centrifuged at 500 (pellet, intact cells) and 11,000 × *g* (pellet, membranes) for 15 min at 4 °C. The 11,000 × *g* pellet was solubilized in the same buffer containing 0.1% Triton x-100 and sonicated with four short pulses. All fractions (supernatants and pellets) were stored at −80 °C until analyzed. Both the pellets and supernatants were assayed for ferritin using the turbidimetric assay method (Roche Molecular Biochemicals) (40). Samples of cell lysates (100–250 μl) were added to 125 μl of 0.18 M Tris buffer containing 100 mM NaCl (R1) and 125 μl of anti-human ferritin-coated 0.1% latex beads (R2). Saline was added to a final volume of 500 μl. The tubes were incubated overnight at 4 °C on a shaker. The suspension was centrifuged at 1,000 × *g* for 10 min at 4 °C, and the supernatants were removed. The pellets were finally resuspended in 500 μl of saline and the absorbance of the supernatants, and the pellet suspensions were read at 700 nm on a spectrophotometer (Beckman, Fullerton, CA). In some experiments to determine total cell ferritin, cells were lysed in 0.1% Triton X-100 containing the protease inhibitors, and the lysate was analyzed.

The quantity of cellular ferritin was assessed from the absorbance using standard ferritin concentrations (Roche Molecular Biochemicals). A plot of absorbance at 700 nm against ferritin concentrations exhibited a negative slope as a result of the increased rate of settling of antibody-coated latex beads in supernatants in response to increasing concentrations of ferritin. A similar plot against resuspended pellets exhibited a positive slope. A double inverse plot of the curve gave a straight line. The concentrations of the unknowns using both supernatants and resuspended pellets gave similar values.

Measurement of Iron Incorporation into Ferritin within MDM—To determine the amount of radioactive iron incorporated into ferritin, lysates of cells pre-exposed to ^{59}Fe, were incubated with the latex beads to which anti-ferritin antibody had been linked as described above. The turbid suspension was centrifuged for 10 min (1,500 × *g*, 4 °C). The supernatant was removed. The bottom of the tube containing the pellet was cut into a 5-ml γ tube and counted on a γ counter. The amount of iron in the pellet was compared with the total iron incorporated into the cell, the membrane, and the supernatant.

Statistical Analyses—Results obtained under different experimental conditions were compared by Student's paired *t* test when independent variables were being assessed or by analysis of variance (ANOVA) when analyses of trends were being determined. For both types of analyses results were considered significant at $p \leq 0.05$. Since absolute results vary from MDM donor to donor, each experiment was interpreted relative to its own control group(s).

RESULTS

Intraphagosomal M. tuberculosis Acquire Iron from Exogenous Transferrin—Although it was previously shown that exogenous TF traffics to *M. tuberculosis*-containing macrophage phagosomes (18, 19), the actual delivery of iron to *M. tuberculosis* during the process was not tested. To address this critical point, we utilized an assay that allows for detection of iron acquisition by intraphagosomal *M. tuberculosis* (39). Virulent Erdman *M. tuberculosis* were added to MDM monolayers for 2 h to allow for phagocytosis, washed, and incubated an additional 24 h. [^{59}Fe$_2$]TF was then added to the *M. tuberculosis*-infected monolayers and at defined time points, the monolayers were lysed, and both total MDM- and *M. tuberculosis*-associated ^{59}Fe were determined. Previous work has shown that *M. tuberculosis* remain intact throughout this procedure (39). We term this experimental paradigm exogenous iron acquisition.

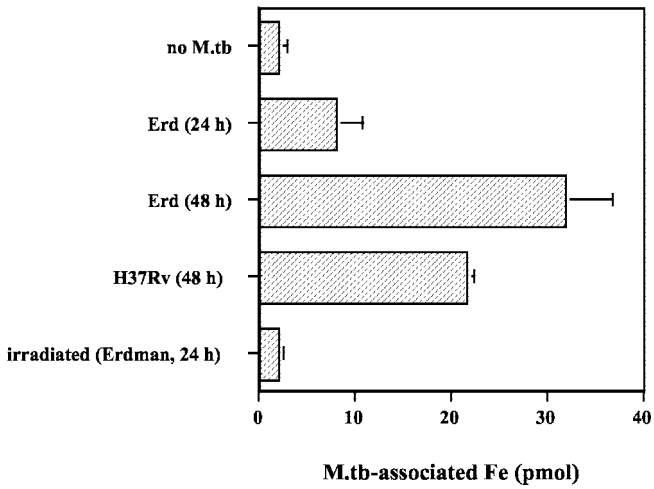

FIG. 1. **Iron acquisition from TF (exogenous source) by live intraphagosmal *M. tuberculosis*.** MDM monolayers infected with no *M. tuberculosis* (*no M. tb.*), viable *M. tuberculosis* Erdman strain, or irradiated *M. tuberculosis* for 24 h were then incubated with 10 μM [^{59}Fe$_2$]TF for the indicated time periods, following which the monolayers were lysed with SDS and released *M. tuberculosis* washed and trapped on a 0.22-μm filter. Shown is the mean ± S.E. of *M. tuberculosis* (*M. tb.*)-associated ^{59}Fe ($n = 7$ for live Erdman 24 h, $n = 7$ for live Erdman 48 h, $n = 2$ for live H37Rv, and $n = 2$ for irradiated Erdman). *M. tuberculosis*-associated ^{59}Fe at 48 h with the *M. tuberculosis* Erdman strain is significantly increased over the value at 24 h, and *M. tuberculosis*-associated ^{59}Fe is significantly reduced for irradiated *M. tuberculosis* compared with live *M. tuberculosis* ($p < 0.005$).

As shown in Fig. 1, *M. tuberculosis*-associated iron increased with time of MDM incubation with [^{59}Fe$_2$]TF from 24 to 48 h. Significant iron acquisition was also seen with the *M. tuberculosis* strain H37Rv (Fig. 1). ^{59}Fe uptake by *M. tuberculosis* is an active process requiring viable bacteria since irradiated *M. tuberculosis* phagocytosed by MDM showed no ^{59}Fe association (Fig. 1). Our previous results showed that no ^{59}Fe was detected associated with polystyrene microspheres coated with the *M. tuberculosis* cell wall lipoglycan, lipoarabinomannan (LAM), isolated from phagosomes (39). Thus, ^{59}Fe acquisition is seen only with viable intraphagosomal bacteria.

Given the previous work demonstrating that TF is delivered to the *M. tuberculosis* phagosome (18), we next sought to distinguish between iron acquisition from TF by the microbe and attachment of the Fe-TF complex to the *M. tuberculosis* surface within the phagosome. To do so, we took advantage of the fact that iron is readily released from TF at pH < 5.0, particularly if a reducing agent such as ascorbate is present (41). Consistent with this, when a solution of [^{59}Fe$_2$]TF was incubated with 5 mM ascorbic acid at pH ≤ 5 for 5 min, >98% of ^{59}Fe initially bound to TF was released, as assessed by measuring retained ^{59}Fe after centrifugation of the solution through a 30-kDa cutoff Centriprep filter (42). In contrast, when *M. tuberculosis* recovered from the phagosome was subjected to ascorbic acid washes under the same conditions, nearly all (88.1 ± 8.3%, $n = 6$) of the ^{59}Fe remained *M. tuberculosis*-associated. Thus, these data suggest that iron associated with intraphagosomal *M. tuberculosis* does not reflect simple attachment of Fe-TF to the bacterial surface as assessed by acid washes, but is indicative of microbial removal of iron from TF.

Intraphagosomal M. tuberculosis Acquire Iron from Endoge-

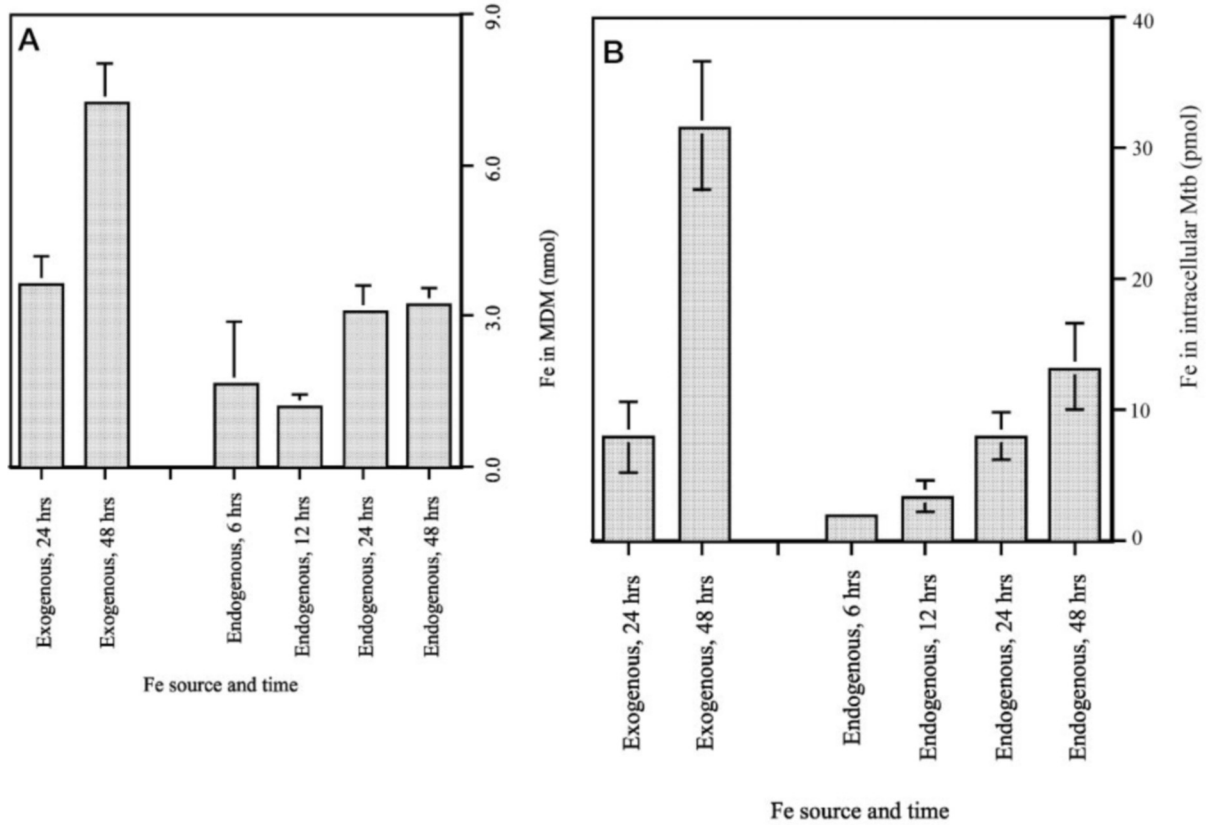

FIG. 2. **Intraphagosomal *M. tuberculosis* acquires iron from both endogenous iron pool(s) within the macrophage and the exogenous source (Fe-TF).** For the endogenous paradigm, MDM were incubated with 10 μM [^{59}Fe]TF for 24 h, washed, "chased" for 24 h, and then infected with Erdman *M. tuberculosis*. At 6, 12, 24, and 48 h the MDM monolayer was lysed, released bacilli were harvested, and both MDM (*A*) and *M. tuberculosis* (*M. tb.*)-associated ^{59}Fe (*B*) were determined. For comparison, exogenous iron acquisition is shown (paradigm as in Fig. 1). MDM and *M. tuberculosis* ^{59}Fe were quantitated at 24 and 48 h from the exogenous iron source. Results are expressed as mean ± S.E. (n = 3–7). No significant difference ($p > 0.05$) in MDM or *M. tuberculosis*-associated ^{59}Fe was observed at 24 h between endogenous and exogenous sources of ^{59}Fe. Both MDM and *M. tuberculosis*-associated ^{59}Fe were significantly greater at 48 h with the exogenous ^{59}Fe source ($p < 0.05$).

nous Macrophage Sources—The above data show that intraphagosomal *M. tuberculosis* can acquire iron from extracellular TF (exogenous source). However, it is not known whether *M. tuberculosis* can acquire iron from intracellular sources such as the labile iron pool or ferritin. To study *M. tuberculosis* iron acquisition from intracellular pools, we changed our experimental paradigm as follows.

MDM were incubated with [^{59}Fe]$_2$TF for 24 h at 37 °C, followed by extensive washing and an additional incubation for 24 h (chase period) prior to adding *M. tuberculosis*. Since no extracellular [^{59}Fe]TF was present during *M. tuberculosis* infection, only internal iron could serve as the ^{59}Fe source (termed endogenous sources). Culture supernatant showed negligible release of ^{59}Fe during the 24 h chase (data not shown), indicating that once internalized by the MDM, no significant amount of ^{59}Fe was returned to the extracellular environment, a process that could have confounded data interpretation.

M. tuberculosis ^{59}Fe uptake under this experimental condition was directly compared with the previous paradigm for iron acquisition from exogenous TF. Total MDM ^{59}Fe content and ^{59}Fe acquisition by intraphagosomal *M. tuberculosis* were equivalent from endogenous and exogenous sources at 24 h (Fig. 2, *A* and *B*). In the next 24 h of *M. tuberculosis* infection, there was only a modest and variable (66 ± 44.7%) increase in ^{59}Fe acquisition by *M. tuberculosis* above that at 24 h from the endogenous sources, which did not reach statistical significance (n = 5, $p > 0.05$, Fig. 2*B*) despite the fact that MDM-associated ^{59}Fe was constant (Fig. 2*A*). In contrast, there was a marked (4.0 ± 0.3-fold) increase in iron acquisition from exogenous TF for the time period of 24–48 h (n = 2–5, $p < 0.002$, Fig. 2*B*). Thus, these data provide evidence that intraphagosomal *M. tuberculosis* can acquire iron from both exogenous TF and endogenous cytoplasmic pools, although the amount of iron acquired from the two sources differs over time. *M. tuberculosis* can continue to readily acquire iron from exogenous Fe-TF.

In the iron acquisition protocol utilized (endogenous sources), it was possible for the iron that associated with macrophages over the 24-h chase period prior to adding *M. tuberculosis*, to remain attached to TF on the cell surface rather than to be internalized into endogenous pools. In order to confirm that the iron delivered was internalized, MDM exposed to [^{59}Fe$_2$]TF at 37 °C for 24 h were treated with the reducing agent ascorbate, and the extracellular iron chelators, ferrozine and NTA, to remove iron remaining on the cell surface. Results were compared with MDM incubated with [^{59}Fe$_2$]TF at 4 °C for 1 h, to allow for Fe-TF attachment but not internalization. Treatment with the reducing agent and iron chelators failed to decrease the MDM-associated ^{59}Fe detected following [^{59}Fe$_2$]TF exposure at 37 °C. Treated MDM retained 106.3 ± 4.7% of the pretreatment ^{59}Fe (p = 0.12, n = 3); whereas it removed 67.8 ± 5.2% ($p < 0.02$, n = 2) of the MDM-associated ^{59}Fe detected on the cells exposed to ^{59}Fe$_2$TF at 4 °C. These data indicate that the MDM-associated ^{59}Fe following a 24-h incubation at 37 °C represented internalized Fe, rather than iron bound to TF and retained on the cell surface.

Effect of IFN-γ on Iron Acquisition by MDM and Intracellular M. tuberculosis—Interferon-γ plays a major role in host defense against *M. tuberculosis* (20), as well as other intracellular pathogens (43). Part of the antimicrobial mechanism of IFN-γ has been linked to its ability to decrease the availability

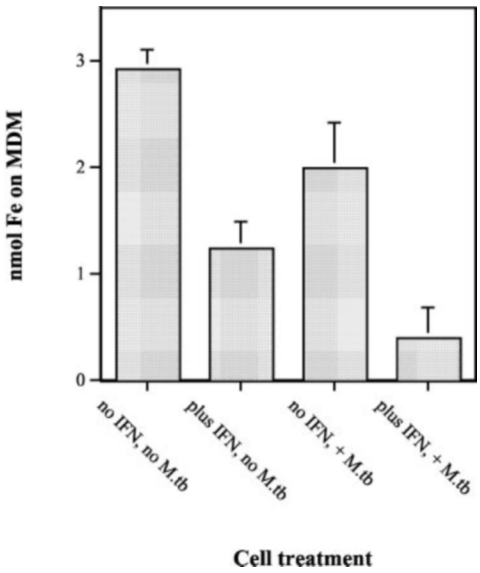

FIG. 3. **IFN-γ treatment reduces exogenous iron acquisition by uninfected and *M. tuberculosis*-infected macrophages.** MDM were treated with or without IFN-γ (1,000 units/ml) for 5 days, incubated with *M. tuberculosis* (+ *M.tb*) for 48 h or no *M. tuberculosis* (*no M. tb.*), and then incubated for an additional 24 h with [^{59}Fe]TF. MDM-associated ^{59}Fe was then determined. Shown is mean ± S.E. (n = 2–4) MDM-associated ^{59}Fe. IFN-γ treatment was continued throughout the experiment. There was a significant decrease in MDM-associated ^{59}Fe with IFN-γ treatment when compared with the untreated *M. tuberculosis* or uninfected control ($p < 0.05$). *M. tuberculosis* infection in the absence of IFN-γ treatment also resulted in a significant decrease in MDM-associated ^{59}Fe ($p < 0.05$).

of macrophage iron for use by the pathogen (25, 26). IFN-γ decreases macrophage iron content, ferritin, and TFR expression (44, 44).

Therefore, we sought to determine whether IFN-γ treatment of MDM limits intraphagosomal *M. tuberculosis* acquisition of iron from TF. We found that treatment of MDM with IFN-γ (1,000 units/ml or 10,000 units/ml) for 5 days significantly decreased MDM iron acquisition from TF (50.6 ± 13.7% of control, $n = 4$, $p < 0.05$, Fig. 3). This decreased further if the MDM were also infected with *M. tuberculosis* (Fig. 3). Despite the IFN-γ effect on MDM-associated iron, iron uptake by intraphagosomal *M. tuberculosis* decreased to a lesser extent, remaining 69.9 ± 0.5% of that observed in non IFN-γ-treated control MDM ($p < 0.05$, $n = 2$). Thus, despite IFN-γ's ability to limit iron acquisition by macrophages, *M. tuberculosis* continues to have access to iron. This observation may explain in part the lack of effectiveness of IFN-γ in limiting *M. tuberculosis* growth *in vitro* within human MDM that we (39) and others (45) have previously observed.

Since IFN-γ treatment significantly reduced iron acquisition by MDM, we explored whether IFN-γ reduced TFR expression and/or ferritin content as reported (44, 46). We found that IFN-γ treatment of MDM for 5 days (1,000 units/ml) decreased MDM TFR expression by 34.3 ± 5.1% ($n = 5$, $p < 0.005$) and cellular ferritin by 27.9 ± 3.6% ($p < 0.05$, $n = 3$). The proportion of new iron taken up from exogenous TF that became associated with ferritin was minimally altered from control by IFN-γ treatment (10.3 ± 2.2% *versus* 9.0 ± 0.4%, respectively $n = 2$, $p < 0.05$).

M. tuberculosis Infection Decreases Iron Acquisition from TF by Macrophages—In our experiments reported above with IFN-γ, we observed that iron acquisition by MDM was most significantly reduced in *M. tuberculosis*-infected cells (Fig. 3). This raised the possibility that *M. tuberculosis* infection itself can down-regulate iron acquisition by macrophages. Interestingly, we found that *M. tuberculosis*-infected MDM exposed to [$^{59}Fe_2$]TF contained significantly less ^{59}Fe than uninfected control MDM (2.0 ± 0.4 nmol for infected *versus* 2.8 ± 0.2 nmol for uninfected, $p < 0.05$, Fig. 3). In contrast, MDM that ingested irradiated *M. tuberculosis* showed no significant difference in iron acquisition from control MDM (data not shown). These results indicate that live *M. tuberculosis* alters one or more aspects of MDM metabolism that are involved in acquisition and/or storage of iron from TF. Under these experimental conditions we find no significant differences between the number of control and *M. tuberculosis*-infected MDM recovered (data not shown), indicating that the results do not reflect a simple loss of MDM with *M. tuberculosis* infection.

Iron Acquisition by M. tuberculosis within Macrophages from Hemochromatosis Patients—In contrast to hepatocytes and most other cell types, monocytes and macrophages from patients with hereditary hemochromatosis have low intracellular iron (33–36). If *M. tuberculosis* acquires iron from internal macrophage pools during its intraphagosomal residence, *M. tuberculosis* iron uptake in these cells should be lower than that seen in MDM from healthy donors. To explore this possibility, we studied MDM from patients with genetically confirmed hereditary hemochromatosis. Compared with cells from normal controls we observed a marked decrease in the amount of ^{59}Fe acquired by intraphagosomal *M. tuberculosis* residing within MDM from hemochromatosis patients, regardless of whether iron acquisition from exogenous TF or endogenous MDM iron stores was examined: 36 ± 3.8% and 17 ± 9.6% of control using the exogenous and endogenous iron sources, respectively (Fig. 4*A*). Pretreatment of MDM from hemochromatosis patients with the IFN-γ for 5 days prior to *M. tuberculosis* infection resulted in a 58 ± 7% further decrease in *M. tuberculosis* iron content after a 24 incubation with exogenous [^{59}Fe]TF ($p \leq 0.001$, $n = 4$).

The amount of total MDM-associated ^{59}Fe at 24 h was not significantly decreased in the MDM from hemochromatosis patients relative to healthy donors (Fig. 4*B*), although a trend in that direction was noted. IFN-γ treatment (1,000 units for 5 days) decreased iron acquisition from exogenous TF by MDM from patients with hemochromatosis to a similar degree as that observed with MDM from healthy donors. ^{59}Fe acquisition from exogenous TF by IFN-γ MDM from hemochromatosis patients was 52 ± 11% of untreated control ($p < 0.01$, $n = 4$) compared with 50.6 ± 13.7% of control for MDM ($p < 0.05$, $n = 4$) from healthy donors, as noted earlier in Fig. 3. Under the same IFN-γ treatment conditions, TFR expression fell to a greater extent (63 ± 3%) in the MDM from hemochromatosis patients compared with MDM from healthy donors 34.3 ± 5.1% ($p <$ 0.5).

DISCUSSION

While extracellular pathogens have direct access to host extracellular iron chelates, bacteria that replicate within macrophage phagosomes, such as *M. tuberculosis*, face a greater challenge to obtain adequate iron to meet their metabolic needs. Extending our previous findings (39), we found that viable but not irradiated *M. tuberculosis* residing within the phagosomes of infected human MDM acquired ^{59}Fe over time from ^{59}Fe-labeled TF added to the culture medium (exogenous source). This finding is not surprising given that only live bacteria would be expected to synthesize the exochelins that facilitate iron uptake by *M. tuberculosis* (47). It is also possible that this result reflects differences in intracellular trafficking of dead *versus* live *M. tuberculosis* in which live organisms are in an endosomal compartment whereas non-viable ones are in phagolysosomes.

M. tuberculosis-associated ^{59}Fe could not be removed by

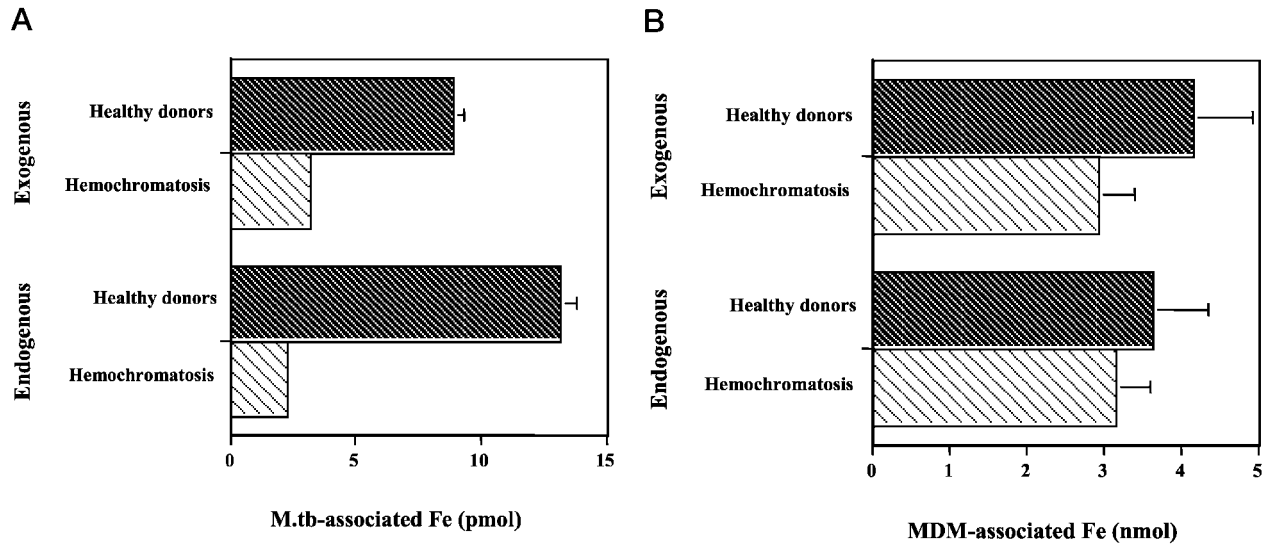

FIG. 4. **Iron uptake by intraphagosomal *M. tuberculosis* within MDM from healthy donors and patients with hemochromatosis.** MDM from patients with hemochromatosis (*light cross-hatch*) or healthy donors (*dark cross-hatch*) were infected with *M. tuberculosis* and provided ^{59}Fe from exogenous and endogenous sources. After 24 h, the monolayers were harvested and *M. tuberculosis* (*M. tb.*)- associated (*A*) and MDM (*B*) ^{59}Fe were determined. Results are shown as mean ± S.E. of *M. tuberculosis*- and of MDM-associated ^{59}Fe (n = 3–4). p values for comparisons between *M. tuberculosis*-associated ^{59}Fe for organisms growing in MDM derived from hemochromatosis patients *versus* healthy controls using endogenous or exogenous TF were <0.02 and <0.05, respectively. Total MDM iron was not significantly different in hemochromatosis patients *versus* healthy controls.

treating the bacteria with an acid wash protocol that removes iron from TF, indicating that the ^{59}Fe detected does not result from the adherence of ^{59}Fe-TF to the bacterial surface and is thus consistent with internalization of that iron by the bacteria. However, we cannot eliminate the possibility that the iron is located within the *M. tuberculosis* cell wall and as such is resistant to removal by our protocol. Nevertheless, our data confirm that *M. tuberculosis* residing within MDM phagosomes can acquire iron from exogenous TF via a process that requires viable *M. tuberculosis*.

Based on the work of others (18), our initial expectation was that iron acquisition from TF by *M. tuberculosis* occurs through delivery of Fe-TF to the phagosome via receptor-mediated endocytosis and fusion of the early endosome with the *M. tuberculosis*-containing phagosome. However, we find that intraphagosomal *M. tuberculosis* readily acquire ^{59}Fe from MDM preloaded with ^{59}Fe from TF prior to their infection with *M. tuberculosis*. Thus, *M. tuberculosis* are able to acquire iron from an endogenous MDM site(s) and do not require the iron to be present extracellularly. To our knowledge, this is the first direct evidence that *M. tuberculosis* can acquire iron from an MDM-associated pool.

Our data raise questions about the paradigm of direct transfer of iron bound to TF as the primary means for iron trafficking from extracellular TF to intraphagosomal *M. tuberculosis*. If that paradigm is correct, then the amount of ^{59}Fe acquired from extracellular TF should have greatly exceeded that from the endogenous MDM ^{59}Fe pool at 24 h. But, this was not the case. The magnitude of iron taken up by *M. tuberculosis* at 24 h from the ^{59}Fe-preloaded MDM was essentially identical to that which occurred when the ^{59}Fe was presented as extracellular [$^{59}Fe_2$]TF. This could not be explained on the basis of differences in the amount of iron that associated with the MDM, as this was essentially identical under the two conditions. Our data raise the possibility that a portion of the iron uptake from extracellular TF by *M. tuberculosis* may instead involve initial iron transfer from endocytosed TF, presumably via DMT-1, to the MDM cytoplasm or another internal site, where *M. tuberculosis* then gain access to it.

We also observed a relative lack of increase in *M. tuberculosis*-associated iron between 24 and 48 h following infection of ^{59}Fe-preloaded MDM, despite a stable amount of endogenous ^{59}Fe in the MDM. This contrasted with a major increase in *M. tuberculosis*-associated ^{59}Fe over the same time period with the continuous presence of an exogenous iron source. Over time, iron taken up by the MDM may become less accessible to *M. tuberculosis* as it moves from its initial intracellular locale (*e.g.* labile iron pool) to other ones (*e.g.* ferritin).

Additional data emphasize the potential differential access of intracellular *M. tuberculosis* to various intracellular macrophage iron stores. MDM from individuals with hemochromatosis exhibited similar amounts of MDM-associated ^{59}Fe relative to MDM from healthy donors following incubation with [^{59}Fe]TF. However, *M. tuberculosis* had a much more difficult time accessing ^{59}Fe from the hemochromatosis cells. Low macrophage intracellular iron availability in the setting of genetic mutations of HFE leading to hemochromatosis could limit *M. tuberculosis* growth by decreasing its access to iron. We speculate that resistance to *M. tuberculosis* infection could remotely have been a positive selection factor for retention of HFE mutations in the gene pool. If such mutations increased resistance to *M. tuberculosis* infection, they would increase the likelihood of survival to a reproductive age in locations of high tuberculosis prevalence. The negative effects of the HFE$^-$ phenotype are not manifested until later in life. Thus, they should not provide a negative evolutionary selective pressure. To our knowledge this possibility has not been studied in either a population-based fashion or in laboratory studies. Studies on the effect of the hemochromatosis phenotype on the growth of intracellular pathogens such as *M. tuberculosis* are currently ongoing.

In contrast to most other cell types, monocytes and MDM from individuals with hereditary hemochromatosis have low intracellular iron (33, 34). We observed a trend toward lower net iron uptake by hemochromatosis compared with control MDM following exposure to Fe-TF, but this did not reach statistical significance (Fig. 4*B*). Not all studies have found impaired iron acquisition from TF by mononuclear phagocytes from individuals with hemochromatosis, perhaps related to the extent to which the individuals have been phlebotomized prior

to study (48–50).

IFN-γ plays a key role in host defense against *M. tuberculosis*, as it helps convert macrophages from a quiescent to activated state (20). At least some of the antimicrobial effects of IFN-γ have been attributed to alterations of macrophage iron stores. We confirmed previous data that IFN-γ decreases macrophage ferritin, TFR expression, and MDM iron acquisition from TF (21–23, 44, 46, 51).

Even though IFN-γ lowered the total iron content of MDM, the ability of *M. tuberculosis* to acquire intracellular iron from IFN-γ-treated macrophages was only impaired to a small extent. This suggests that IFN-γ does not effectively alter the amount of iron in the intracellular macrophage pools that can be accessed by intraphagosomal *M. tuberculosis* and may explain in part why IFN-γ does not slow the growth of *M. tuberculosis* in human MDM (39, 45). These data, in conjunction with observations using macrophages from patients with hemochromatosis, reveal that the total iron content of the macrophage may not be the key determinant in the ability of intraphagosomal *M. tuberculosis* to acquire iron. Rather it is the iron content of as yet to be determined intracellular sites in the macrophage that are critical to these dynamics.

In addition to macrophage factors impacting *M. tuberculosis* iron metabolism, *M. tuberculosis* infection may also modulate macrophage iron acquisition. MDM containing live (but not dead) *M. tuberculosis* acquired significantly less ^{59}Fe from TF than uninfected MDM. Thus, live *M. tuberculosis* may alter one or more aspects of MDM metabolism involved in acquisition and/or storage of iron from TF. Consistent with this, MAC infection of murine peritoneal macrophages has been reported to decrease macrophage TFR and ferritin mRNA levels (44). This is not surprising given that *M. tuberculosis* infection alters expression of a number of MDM genes (52, 53). These data further underscore the complex nature of macrophage intracellular iron pools and their relationship to mycobacterial and macrophage iron metabolism.

Although we have shown that *M. tuberculosis* can acquire iron from both endogenous MDM iron pools and from iron bound to extracellular TF, the exact means whereby iron crosses the phagosome membrane to reach *M. tuberculosis* remains unclear. Our data do not exclude the possibility that a portion of the iron traffics directly to the phagosome bound to TF via receptor-mediated endocytosis and early endosome fusion, as previously proposed (18). Previous work has shown that *M. tuberculosis* cannot directly remove iron from TF (47). Thus, iron would have to be released from the TF in order to be acquired by *M. tuberculosis*. This could occur through the action of its exochelins (47) or as a consequence of the lowered pH of the phagosome.

However, this does not explain how *M. tuberculosis* accesses endogenous MDM iron. Siderophore production appears to be very important to *M. tuberculosis* growth within macrophages. A genetically modified *M. tuberculosis* strain unable to synthesize siderophores grew very poorly within the human macrophage THP-1 cell line (15). However, it could grow in Fe-rich culture media (15), suggesting that siderophore production may not be required outside of the macrophage. Perhaps *M. tuberculosis* siderophores escape the phagosome, gain access to MDM-associated iron, and transport it back to the intraphagosomal organism. *M. tuberculosis*-derived components can traffic into the macrophage cytoplasm and/or be secreted (12, 16, 17). However, siderophore trafficking in the macrophage has not been explored.

If *M. tuberculosis* siderophores do not leave the phagosome, iron must be brought to the bacterium. Iron could move to *M. tuberculosis* from the cytoplasm by a phagosome-associated iron transporter. DMT-1 (Nramp2) moves iron out of the endosome, not into it (4). Nramp1 is an integral membrane protein expressed in macrophage late endosomes and lysosomes that are related to DMT-1 (54). Nramp1 has been linked to resistance to infection with BCG and other intracellular pathogens in mice (55, 60), but not virulent *M. tuberculosis* (56–58). Intriguing data suggest that Nramp1 moves iron into MAC-containing phagosomes of a murine macrophage cell line (59,61). However, studies by Gros and co-workers (62) show that Nramp1 functions primarily as a H^+ and Mn^{2+} transporter, leading to the acidification of the phagosome. Thus, the role of human Nramp1 in iron acquisition by intraphagosomal *M. tuberculosis*, remains unclear at this time.

We have made novel observations that virulent *M. tuberculosis* residing within human MDM phagosomes can acquire iron from both extracellular TF and endogenous MDM iron stores. Several potential routes exist for that iron to reach intraphagosomal *M. tuberculosis*. Our data raise the possibility that *M. tuberculosis* iron acquisition from extracellular TF may in part involve initial removal and transfer of iron from TF to the MDM cytoplasm (or another internal site) rather than entirely by direct transport to the phagosome bound to TF. Access to internal macrophage iron pools may be critical for the replication of intraphagosomal *M. tuberculosis* and therefore to *M. tuberculosis* pathogenesis. Conditions such as hemochromatosis, which alter the normal status of these pools, may impact on *M. tuberculosis* iron acquisition. Additional work to define the intracellular iron pools accessible to *M. tuberculosis*, the mechanism of delivery of cytoplasmic iron to the phagosome, and the applicability of these findings to other biologically relevant extracellular iron chelates (*e.g.* lactoferrin) and other intracellular pathogens are areas of particular interest and importance.

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