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 59Fe from transferrin (TF) provided extracellularly (exogenous source) was compared with acquisition when MDM were loaded with 59Fe from TF prior to M. tuberculosis infection (endogenous sources). M. tuberculosis 59Fe 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 59Fe 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) 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 Fe3+ 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...
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 2-microglobulin and forms a complex with TFR. This alters TFR affinity for Fe-TF (30), the direction of which can alter resistance to infection with M. tuberculosis, the intracellular iron content of macrophages from patients with hemochromatosis, 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 H37Ra (ATCC 35801 and 27294, respectively). *M. tuberculosis* was cultivated (10 days) and harvested in RPMI 1640 containing 10 mM Heps, 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 megaelectrons for 18 h. The suspension was kept sterile at 4 °C, and washed three times in cold RPMI 1640. Cells were incubated with purified mouse anti-human TfR (mAb 25/26) and subsequent addition (24 h later) of [59Fe2]TF or with [59Fe2]TF alone (no M. tuberculosis). MDM and bacilli were processed as described above, and the amounts of 59Fe acquired by MDM and bacilli were determined using the γ counter.

Iron Uptake by Intraphagosomal M. tuberculosis: Endogenous Source—MDM monolayers were incubated with [59Fe2]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 59Fe was determined in the γ counter.

Iron Internalization into Macrophages—In order to determine whether the [59Fe2]TF delivered to MDM is internalized, MDM were exposed to [59Fe2]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, ferric citrate and nitritocitrin acid (NTA), to remove iron remaining on the cell surface. The bacterial suspension was washed through the 7H9 medium (consisted of a 5-min incubation at 37 °C) with 5 mM ascorbate in RPMI 1640 containing 1 mM ferric citrate, 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 to determine iron uptake by each group of treated cells.

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 [59Fe2]TF or with [59Fe2]TF alone (no M. tuberculosis). MDM and bacilli were processed as described above, and the amounts of 59Fe 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 × 106 MDM/well) were prepared in 48-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 then washed five times in PD. A drop of 20 μl acetic acid overlaid with 200 μl of blocking buffer (2.5% bovine serum albumin + 5% fetal calf serum in PD), placed on a nutator (Oxis Instruments, Iviland, PA) overnight at 4 °C, and washed three times in cold RPMI 1640. Cells were incubated with purified mouse anti-human TIR (mAb clone DF1513, IgG1, Ancell Corp., Bayport, MN, 1.25 μg/ml overnight at 4 °C in cold RPMI 1640). The supernatant was removed, 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 biotin-
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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. \(^{59}\text{Fe}\) 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 \(^{59}\text{Fe}\) were determined. Previous work has shown that M. tuberculosis remain intact throughout this procedure (39). We term this experimental paradigm exogenous iron acquisition.

As shown in Fig. 1, M. tuberculosis-associated iron increased with time of MDM incubation with \(^{59}\text{Fe}\) from 24 to 48 h. Significant iron acquisition was also seen with the M. tuberculosis strain H37Rv (Fig. 1). \(^{59}\text{Fe}\) uptake by M. tuberculosis is an active process requiring viable bacteria since irradiated M. tuberculosis phagocytosed by MDM showed no \(^{59}\text{Fe}\) association (Fig. 1). Our previous results showed that no \(^{59}\text{Fe}\) was detected associated with polystyrene microspheres coated with the M. tuberculosis cell wall lipoglycan, lipooarabinomannan (LAM), isolated from phagosomes (39). Thus, \(^{59}\text{Fe}\) 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 \(^{59}\text{Fe}\) TF was incubated with 5 mM ascorbic acid at pH 5 for 5 min, >98% of \(^{59}\text{Fe}\) initially bound to TF was released, as assessed by measuring retained \(^{59}\text{Fe}\) 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 \(^{59}\text{Fe}\) remained associated with M. tuberculosis (Fig. 1). 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.

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**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 [59Fe]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 [59Fe] (B) were determined. For comparison, exogenous iron acquisition is shown (paradigm as in Fig. 1). MDM and *M. tuberculosis* [59Fe] 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 [59Fe] was observed at 24 h between endogenous and exogenous sources of [59Fe]. Both MDM and *M. tuberculosis*-associated [59Fe] were significantly greater at 48 h with the exogenous [59Fe] source (p < 0.05).

**Iron Acquisition by Intraphagosomal Mycobacterium tuberculosis**—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 [59Fe]2TF 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 [59Fe]2TF was present during *M. tuberculosis* infection, only internal iron could serve as the [59Fe] source (termed endogenous sources). Culture supernatant showed negligible release of [59Fe]2 during the 24 h chase (data not shown), indicating that once internalized by the MDM, no significant amount of [59Fe] was returned to the extracellular environment, a process that could have confounded data interpretation.

*M. tuberculosis* [59Fe] uptake under this experimental condition was directly compared with the previous paradigm for iron acquisition from exogenous TF. Total MDM [59Fe] content and [59Fe] 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 [59Fe] 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. 2B) despite the fact that MDM-associated [59Fe] was constant (Fig. 2A). 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. 2B). 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 [59Fe]2TF at 37 °C for 24 h were treated with the reducing agent ascorbate, and the extracellular iron chelators, ferrozine and NTSA, to remove iron remaining on the cell surface. Results were compared with MDM incubated with [59Fe]2TF 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 [59Fe] detected following [59Fe]2TF exposure at 37 °C. Treated MDM retained 106.3 ± 4.7% of the pretreatment [59Fe] (p = 0.12, n = 3); whereas it removed 67.8 ± 5.2% (p < 0.02, n = 2) of the MDM-associated [59Fe] detected on the cells exposed to [59Fe]2TF at 4 °C. These data indicate that the MDM-associated [59Fe] 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...
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We found that M. tuberculosis-infected MDM exposed to \[\text{[}^{59}\text{Fe}\text{]}\text{TF}\] contained significantly less \(^{59}\text{Fe}\) 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 donors, we observed a marked decrease in the amount of \(^{59}\text{Fe}\) 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: 38 ± 3.8% and 17 ± 9.6% of control using the exogenous and endogenous iron sources, respectively (Fig. 4A). 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 \([^{59}\text{Fe}]\text{TF}\) (\(p < 0.001, n = 4\)).

The amount of total MDM-associated \(^{59}\text{Fe}\) at 24 h was not significantly decreased in the MDM from hemochromatosis patients relative to healthy donors (Fig. 4B), 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. \(^{59}\text{Fe}\) 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 \(^{59}\text{Fe}\) over time from \[^{59}\text{Fe}\text{-labeled TF}\] 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 \(^{59}\text{Fe}\) could not be removed by
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![Graph](image75x533 to 547x737)

**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 

|                | Exogenous | Endogenous |
|----------------|-----------|------------|
| Healthy donors |           |            |
| Hemochromatosis|           |            |

**A**

|                | Exogenous | Endogenous |
|----------------|-----------|------------|
| Healthy donors |           |            |
| Hemochromatosis|           |            |

**B**

|                | Exogenous | Endogenous |
|----------------|-----------|------------|
| Healthy donors |           |            |
| Hemochromatosis|           |            |

**M.tb-associated Fe (pmol)**

**MDM-associated Fe (nmol)**

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-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. tuberculo-

sis*-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 infection 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. 4B). 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
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Iron 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 59Fe 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 directly traffics 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 Mn2+ 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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