Correction of the Iron Overload Defect in β-2-Microglobulin Knockout Mice by Lactoferrin Abolishes Their Increased Susceptibility to Tuberculosis

Ulrich E. Schaible,[1] Helen L. Collins,[1] Friedrich Priem,[2]
and Stefan H.E. Kaufmann[1]

[1]*Max-Planck-Institute for Infection Biology, Schumannstr. 21-22, and* [2]*Institut für Laboratoriumsmedizin und Pathochemie, Charité, Humboldt-University, Schumannstr. 20-21, D-10117 Berlin, Germany*

Abstract

As a resident of early endosomal phagosomes, *Mycobacterium tuberculosis* is connected to the iron uptake system of the host macrophage. β-2-microglobulin (β2m) knockout (KO) mice are more susceptible to tuberculosis than wild-type mice, which is generally taken as a proof for the role of major histocompatibility complex class I (MHC-I)–restricted CD8 T cells in protection against *M. tuberculosis*. However, β2m associates with a number of MHC-I–like proteins, including HFE. This protein regulates transferrin receptor mediated iron uptake and mutations in its gene cause hereditary iron overload (hemochromatosis). Accordingly, β2m-deficient mice suffer from tissue iron overload. Here, we show that modulating the extracellular iron pool in β2m–KO mice by lactoferrin treatment significantly reduces the burden of *M. tuberculosis* to numbers comparable to those observed in MHC class I–KO mice. In parallel, the generation of nitric oxide impaired in β2m–KO mice was rescued. Conversely, iron overload in the immunocompetent host exacerbated disease. Consistent with this, iron deprivation in infected resting macrophages was detrimental for intracellular mycobacteria. Our data establish: (a) defective iron metabolism explains the increased susceptibility of β2m-KO mice over MHC-I–KO mice, and (b) iron overload represents an exacerbating cofactor for tuberculosis.

Key words: mycobacteria • MHC • innate immunity • macrophages • endosomes

Introduction

Mycobacterium tuberculosis is a facultative intracellular bacterium, which resides in macrophages in early endosomal compartments. Characterization of the mycobacterial phagosome has established that this compartment has access to transferrin (Tf) and the transferrin receptor (TfR) and thus to the iron (Fe) transport pathway of the host cell (1). Access to the host iron source is crucial for mycobacterial survival, as disconnecting their phagosomes from the Tf-pathway by IFN-γ activation inhibits mycobacterial growth (2). The notion that mycobacteria require Fe for intracellular growth is also supported by their expression of soluble high affinity Fe binding molecules, exochelins, which capture Fe in the environment for its delivery to cell wall associated siderophores (3).

The contribution of MHC-I–restricted CD8 T cells in protection against tuberculosis has been deduced from experiments showing that β-2-microglobulin (β2m)–KO mice are highly susceptible to *M. tuberculosis* when compared with C57BL/6 (B6) mice (4). Surface expression of MHC-I molecules requires β2m, and consequently these mice lack MHC-I–restricted CD8 T cells. However, MHC-I KO mice (K^bD^b-KO) are far less susceptible to tuberculosis than β2m–KO mice (5). Therefore, factors other than the absence CD8 T cells must be involved in the higher susceptibility of β2m–KO mice. In addition to molecules involved in antigen presentation, β2m noncovalently associates with the MHC I homologue HFE, which regulates Tf/TfR uptake and Fe release from Tf inside recycling endosomes. In patients with hereditary hemochromatosis HFE is nonfunctional as a result of several point mutations which results in tissue iron overload (6, 7). Here we show that *M. tuberculosis* infection is exac-

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U.E. Schaible and H.L. Collins contributed equally to this work.

H.L. Collins' present address is Division of Life Sciences, Kings College London, Franklin Wilkins Building, 150 Stamford St., London SE1 9NN, UK.

Address correspondence to U.E. Schaible, Max-Planck-Institute for Infection Biology, Schumannstr. 21-22, D-10117 Berlin, Germany. Phone: 49-30-28460-520; Fax: 49-30-28460-503; E-mail: schaible@mpiib-berlin.mpg.de

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erbated under Fe overload. Moreover, this defect can be corrected by the administration of lactoferrin, which results in the amelioration of bacterial burden.

Materials and Methods

Bacteria

M. tuberculosis (Erdman) and *M. bovis* BCG were grown in Middlebrook medium (7H9; Difco) and harvested at a density of ~2–4 × 10^8/ml. For in vitro growth measurements, 5 ml 7H9 containing either 1 mg/ml Fe citrate (Fe^{3+}Ci; Sigma-Aldrich), 1 mg/ml bovine lactoferrin (Sigma-Aldrich/ICN Biomedicals), 0.5 mg/ml deferroxamine (ICN Biomedicals), or deferroxamine/Fe^{3+}Ci together, were inoculated with 10^7 *M. tuberculosis*. OD_{600} or CFUs were determined as indicated.

Mice, Treatment, and Infection

Wild-type B6, β2m-KO, and MHC-I KO mice (provided by Dr. Lemonnier, Institute Pasteur, Paris, France; reference 8) were bred under SPF conditions at the central animal facilities of the Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (Berlin, Germany). The KO mice were backcrossed for at least 10 generations on the genetic background of B6 mice. Mice were infected with 3–5 or 15–200 *M. tuberculosis*/lung by aerosol using an aerosol chamber (Glas-Col). Inocula were confirmed at day 1 after infection by plating the complete lung onto Middlebrook 7H11/ampicillin plates. CFU in lung, spleen, and liver were determined at the time points indicated by mechanical disruption of the organs in water/1% albumin/0.5% Tween 80 (WTA), and plating serial dilutions onto Middlebrook 7H11/ampicillin agar. Mice were treated twice a week intranasally with 1 mg/mouse bovine lactoferrin (iron saturability, 72%; Sigma-Aldrich), recombinant human lactoferrin (iron saturability of 88.5%; provided by Dr. Pauline Ward, Baylor College, and Dr. Karel Petrak, Agennix Inc., Houston, TX) or intraperitoneally with deferroxamine in PBS or with PBS alone. To overload mice with iron, animals were given 25 mg/ml Fe^{3+}Ci in the drinking water for the duration of the experiment.

Nitric Oxide Measurement

Nitric oxide was determined in sera from infected mice as NO_2 upon reduction of NO_3 using the Griess-reaction as described previously (9).

IFN-γ ELISA

Sera from infected mice were diluted in 96-well plates precoated with anti–IFN-γ mAb R46A2 and blocked with 1% BSA. After several washings, plates were incubated with the biotinylated mAb XMG1.2 followed by peroxidase-coupled streptavidin and substrate. Recombinant murine IFN-γ (R&D Systems) was applied as standard. The OD was measured at 560 nm. The limit of detection was 1.5 U/ml.

Intramacrophage Killing Assay

Bone marrow cells from B6 or β2m–KO mice were harvested and differentiated to macrophages in L-cell supernatant (20%) supplemented medium as described (9). Cells were infected with *M. tuberculosis* at a MOI of 10:1 for 2 h in the presence of 5% heat-inactivated horse serum and further cultured with or without 0.5 mg/ml lactoferrin or 0.1 mg/ml rat anti-TfR antibody (Tib219; American Type Culture Collection) for 3 d. Cells were lysed in 0.5% Triton X-100 in PBS at the time points indicated and plated onto Middlebrook 7H11 plates.

Analysis of Iron Content

Tissue. Organs were weighed, lyophilized, and digested in nitric acid and H_2O_2. After evaporation iron was measured in an Atomic Absorption Spectrometer (AAS) "Unicam 939 SOLAR" (Thermo) using an acetylene/air flame at 248.3 nm. The calibration range was linear up to 40 μmol/l iron.

Serum. Serum iron was measured with the colorimetric FerroZine assay and the Hitachi 747E analyser (Roche Diagnostics).

Histology

Snap frozen tissues from wild-type or β2m–KO mice were cryosectioned and stained for Fe^{3+} using Prussian blue (Merck) according to the manufacturer's protocol.

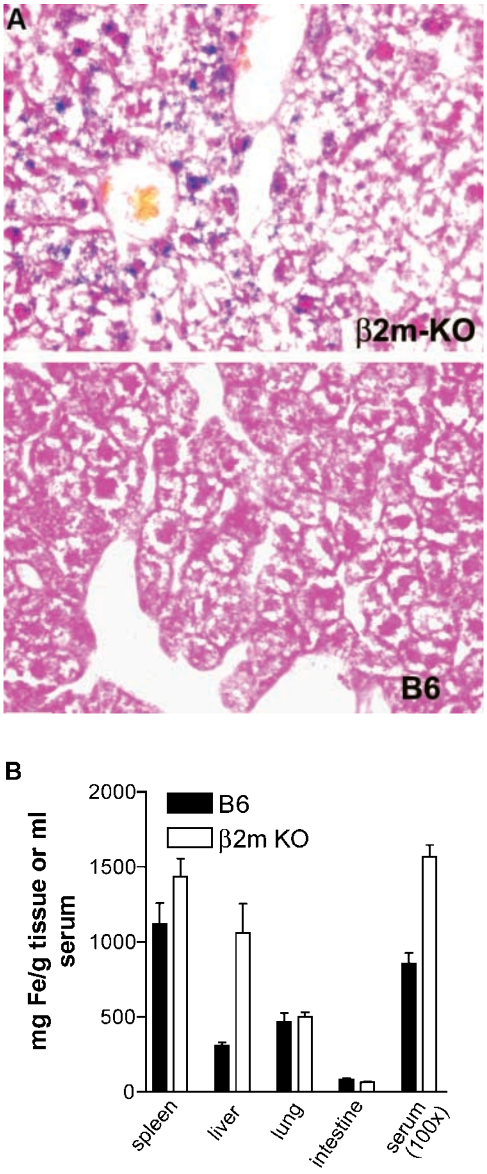
Flow Cytometry

Bone marrow–derived macrophages were treated with 1,000 U/ml IFN-γ, mycobacteria, or lactoferrin for 48 h. Cells were harvested by placing them in cold PBS, blocked for 30 min in PBS plus 5% goat serum, and incubated with Cy-5–labeled rat anti–murine TFR mAb Tib219 (American Type Culture Collection) for 1 h. Flow cytometry was performed using FACScan™ (Becton Dickinson) and analyzed using CELLQuest™ software.

Results

To evaluate the iron status of β2m–KO mice, the total Fe content of the organs from β2m–KO mice and B6 controls was compared by Prussian blue staining and chemical analysis. Consistent with previous findings, β2m–KO mice revealed Fe overload, notably in liver and spleen (10–12; Fig. 1, A and B). To determine whether iron overload contributes to increased susceptibility to tuberculosis, Fe^{3+}Ci was administered to B6 mice via the drinking water. When compared with untreated animals, this diet led to enhanced Fe values in liver, spleen, and lung (Fig. 1 C). These mice were infected by aerosol with a low dose of *M. tuberculosis*. At 15 d after infection, the mycobacterial burdens in the lungs were approximately 10-fold higher in Fe-overloaded as compared with nontreated mice (Fig. 1 D). Hence, experimental Fe overload exacerbated tuberculosis in immunocompetent mice, consistent with one other study (13) and reflecting the situation in β2m–KO animals. In line with these in vivo observations, the addition of excess Fe to complete mycobacterial medium enhanced bacterial growth. Although not statistically significant, the increase in growth rate in the presence of excess iron was observed in all experiments (Fig. 2, A and B). Furthermore, chelation of free Fe by deferroxamine inhibited growth of *M. tuberculosis*, which was rescued by adding a surplus amount of Fe (Fig. 2 B).

We reasoned that restriction of iron supply could prevent growth of *M. tuberculosis* in vivo, therefore extracellular Fe was depleted by the intranasal administration of lactoferrin to B6 and β2m–KO mice prior, and subsequent, to low dose aerosol infection with *M. tuberculosis*. Lactoferrin treatment did not significantly alter mycobac-



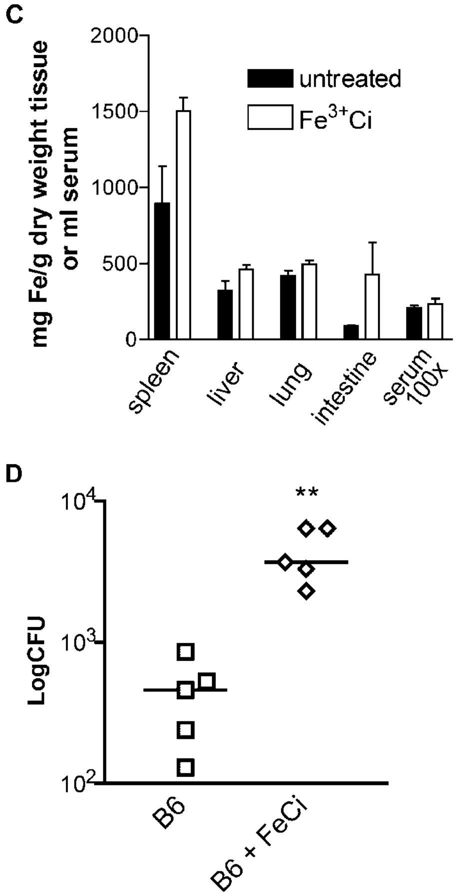


Figure 1. Iron overload in β2m KO mice and susceptibility to *M. tuberculosis* of experimentally iron overloaded mice. (A) Iron-specific staining by Prussian blue of liver sections revealed iron overload in β2m–KO as compared with B6 mice (representative specimens from one out of five mice per group). (B) Iron overload in β2m–KO mice was verified by measuring iron content in organs of each mouse strain (data from five mice ± SD out of three experiments). (C) Elevated iron concentrations in organs from mice treated with $Fe^{3+}Ci$ (values from five mice ± SD, one out of three experiments). (D) Experimental iron overload increased growth of *M. tuberculosis* in B6 mice. Mice ($n = 5$) were infected with *M. tuberculosis* by aerosol and treated with Fe via the drinking water. Bacterial load was measured in the lungs at day 15 (**$P < 0.005$ as determined by the non paired Mann-Whitney test; one representative experiment out of three).

terial burdens in B6 mice. In marked contrast, bacterial loads were 100-fold lower in lactoferrin treated β2m–KO animals as compared with untreated β2m–KO mice (Fig. 3, A and B). The numbers of *M. tuberculosis* in the organs of lactoferrin-treated β2m–KO mice were comparable to those seen in organs of nontreated MHC-I–KO mice. At this early time point, untreated β2m–KO mice had at least fivefold higher bacterial numbers in the respective organs than B6 and MHC-I–KO mice (Fig. 3 B). Thus, treatment with lactoferrin ameliorated *M. tuberculosis* infection in β2m–KO mice reducing bacterial loads to those seen in B6 and MHC-I–KO mice. Similarly, depletion of Fe by deferroxamine decreased mycobacterial numbers in β2m–KO and B6 mice (unpublished data). This is in contrast to a recent study on murine salmonellosis, which revealed strong exacerbation of infection by deferroxamine treatment through inhibition of the respiratory burst in host cells (14).

As correlates of the protective host response, nitric oxide (·NO) and IFN-γ were measured in sera from β2m–KO mice infected with *M. tuberculosis*. IFN-γ levels were comparable in both treated and untreated mice (unpublished data). However, ·NO was undetectable in sera from β2m KO mice infected with *M. tuberculosis*, whereas B6 mice produced low but detectable levels (Fig. 3 C). In contrast, lactoferrin increased ·NO production in *M. tuberculosis*–infected β2m–KO mice resulting in even higher serum levels in comparison with treated or untreated B6 mice. We conclude that lactoferrin not only sequesters extracellular Fe, but also promotes ·NO production. Lactoferrin has been previously shown to contain a microbicidal peptide (15–17). In contrast to the complete protein, however, intranasal treatment with this peptide (FKCRRWQWRM) after aerosol infection with *M. tuberculosis* did not reduce growth of *M. tuberculosis* in β2m–KO mice (data not depicted). This suggests that the Fe binding property of lactoferrin is

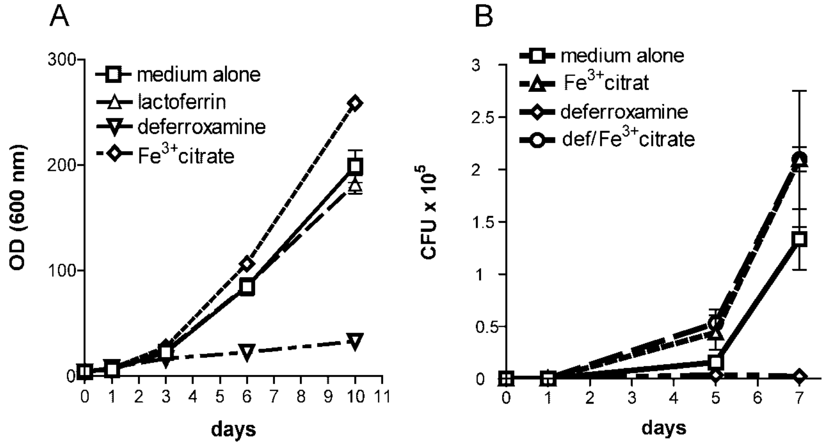


Figure 2. Depletion of iron inhibits and excess iron enhances growth of *M. tuberculosis* in broth culture. Mycobacterial growth was monitored by OD_{600} (A) and CFU (B) in the presence or absence of Fe^{3+}Ci, deferroxamine (def), a mixture of both or lactoferrin. Results indicate mean ± SD of three individual cultures. Shown are representative independent experiments out of three for each set up.

responsible for the inhibition of mycobacterial growth in the β2m–KO mouse.

Macrophages are the primary host cells for *M. tuberculosis* and Fe supply is provided by the Tf/TfR/HFE uptake system in these cells (18). Macrophages from B6 and β2m–KO mice were infected in the presence or absence of lactoferrin and viable intracellular *M. tuberculosis* organisms were enumerated at days 1, 2, and 3. In untreated cultures, numbers of mycobacteria increased 50- to 100-fold by day 3, whereas lactoferrin reduced and finally terminated growth of *M. tuberculosis* between days 2 and 3 after infection (Fig. 4). Hence, depletion of extracellular Fe by lactoferrin is detrimental for *M. tuberculosis* growth in macrophages in vitro, even in the absence of activation by IFN-γ. Similar to lactoferrin, treatment with an anti-TfR monoclonal antibody also restricted growth of *M. tuberculosis* in macrophages (Fig. 4). As lactoferrin has no effect on mycobacterial growth in broth culture (Fig. 2 A), these results strongly suggest that interference with Fe transport into infected macrophages leads to inhibition of mycobacterial growth.

The TfR associates with HFE to facilitate correct Tf/Fe import into the cell, a process disrupted in cells carrying mutated HFE or lacking β2m. To study the influence of lactoferrin on this system, TfR expression on B6 and β2m–KO macrophages was measured after treatment with IFN-γ and/or mycobacteria. Surface expression of TfR was reduced in β2m–KO macrophages. The small constitutive surface expression of the TfR on β2m–KO macrophages remained unaltered after treatment with IFN-γ or mycobacteria (Fig. 5). However, in B6 macrophages, IFN-γ de-

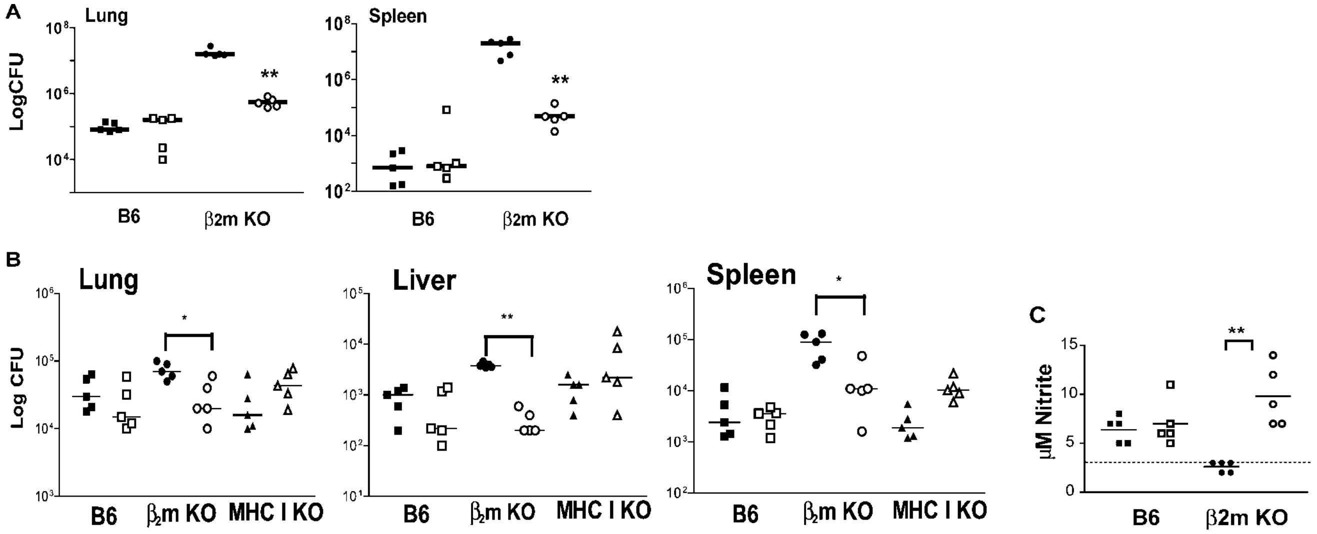


Figure 3. Lactoferrin decreased growth of *M. tuberculosis* in β2m KO but not in MHC class I KO mice. (A) Lactoferrin treatment reduced the growth of *M. tuberculosis* in β2m–KO but not B6 mice. Mice were infected with 159 bacteria/lung by aerosol and bacterial load was measured at 22 d after infection (untreated, closed symbols; lactoferrin treatment, open symbols). (B) Lactoferrin treatment decreased growth of *M. tuberculosis* in β2m–KO but less so in B6 or MHC class I KO mice. Mice were infected with 155 bacteria/lung by aerosol and bacterial load was measured at 27 d after infection (untreated, closed symbols; lactoferrin treatment, open symbols). Statistics: *$P < 0.01$, **$P < 0.005$ as determined by the non paired Mann-Whitney test (data from one representative experiment out of three). (C) Serum levels of NO measured as NO_2 in mice at 27 d after infection by aerosol with five *M. tuberculosis*/lung (untreated, closed symbols; lactoferrin treated, open symbols).

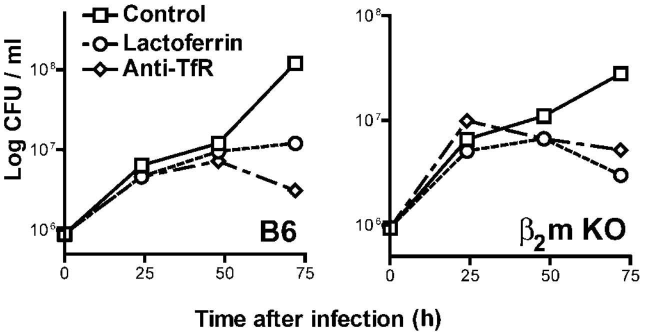


Figure 4. Lactoferrin decreased growth of *M. tuberculosis* in macrophages from both B6 and β2m-KO mice. Bone marrow–derived macrophages were infected with *M. tuberculosis* at a MOI of 2:1. 2 h after infection the cells were washed and further cultured for 4 d in the absence or presence of lactoferrin or an anti-TfR antibody. Mycobacterial growth was measured by CFU at the indicated time points (data ± SD from one representative experiment out of four).

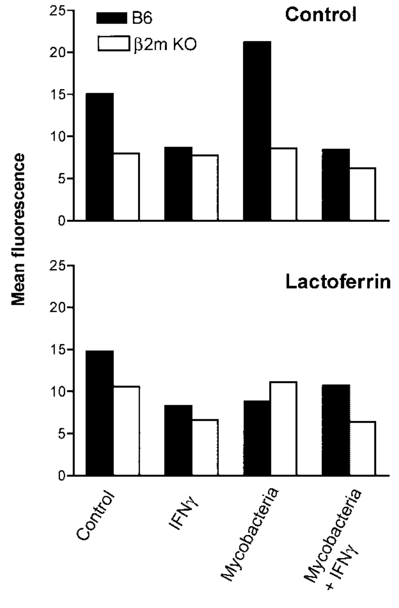


Figure 5. Reduced TfR expression on macrophages from β2m–KO mice. Bone marrow–derived macrophages from B6 and β2m KO mice were treated with IFN-γ, lactoferrin, and/or mycobacteria (10:1) for 48 h and analyzed by flow cytometry using the rat anti–murine TfR Tib-219 (data ± SD from one representative experiment out of three).

creased and mycobacterial infection up-regulated surface expression of TfR. Interestingly, mycobacteria-induced TfR up-regulation was down-modulated by lactoferrin (Fig. 5), probably as a counter mechanism to reduce Fe uptake by infected cells, similar to observations in mycobacteria-infected macrophages upon IFN-γ activation (19). In addition to depleting Fe from the extracellular environment, lactoferrin-mediated TfR down-regulation limits Fe availability to intracellular mycobacteria adding a new antibacterial property to lactoferrin.

Discussion

Since the early days of tuberculosis, a correlation between host iron status and exacerbation of the disease has been recognized, but widely forgotten (20, 21). On the one hand, host cells require Fe as a cofactor for mycobactericidal effector mechanisms, while on the other hand the pathogen must gain access to Fe to ensure its intracellular survival. Therefore, host and mycobacteria compete for this critical element. Here we show that when this balance is tipped in favor of the pathogen, under conditions of hereditary or experimental Fe overload, *M. tuberculosis* flourishes in vivo. Our findings provide strong evidence, that iron is a crucial growth factor for *M. tuberculosis*, and explain why exploitation of the iron-rich early endosomal compartment of host macrophages by mycobacteria represents an important survival strategy for these pathogens. Intraphagosomal mycobacteria take up Fe from exogenous transferrin (reference 1, and D.G. Russell, Cornell University, Ithaca, NY, personal communication), and mycobacterial phagosomes concentrate Fe as demonstrated by electron energy loss spectroscopy (unpublished data). Mycobacteria require iron as obligate cofactor for at least 40 different enzymes encoded by the *M. tuberculosis* genome (21). However, in the intracellular environment, mycobacteria are faced with limited access to this nutritive element. In response to this predicament, *M. tuberculosis* expresses a number of high affinity, iron-binding siderophores such as mycobactins to compete for iron with host cell iron capture systems (3, 21). The imminent need for iron by pathogenic mycobacteria is documented by *M. tuberculosis* mutants deficient for siderophores, which are attenuated for growth in macrophages (22). Mycobacterial genes involved in iron acquisition and storage such as those involved in biosynthesis of siderophores and iron storage proteins are regulated by the iron-dependent-repressor (IdeR), a transcription suppressor with high homology to the corynebacterial diphtheria-toxin-repressor (DtxR; references 23 and 24). *M. tuberculosis* mutants expressing a constitutively active DtxR are attenuated in mice (24). The fact that a number of IdeR controlled genes are strongly up-regulated upon iron starvation as well as inside macrophages further indicates the importance of iron for the intracellular survival of mycobacteria (23). Furthermore, treatment of infected macrophages with Tf saturated with gallium, which substitutes for Fe without undergoing redox recycling, inhibits the intracellular growth of *M. tuberculosis* in vitro (25).

Hereditary iron overload in β2m–KO mice meets the demand for Fe of intracellular *M. tuberculosis* thereby supporting their replication and, as a consequence, exacerbating tuberculosis. However, decreasing the extracellular Fe pool by treatment with lactoferrin limited available iron for transport into the host cell, and reduced the growth of *M. tuberculosis* to levels seen in normal mice. Although a higher risk of developing tuberculosis has not been described in hemochromatosis patients to date, epidemiological studies in human populations show a correlation between dietary

Fe overload and susceptibility to, and prevalence of, tuberculosis in subsaharan Africa (26).

The finding that NO production in infected β2m–KO mice is impaired but can be rescued by lactoferrin indicates that iron overload can hamper NO synthesis by a so far unknown mechanism. Similar to hemochromatosis patients, experimentally iron overloaded mice suffer from constitutive oxygen-free radical generation (27). Preliminary experiments show that constitutive generation of reactive oxygen intermediates (ROIs) in β2m–KO peripheral blood leukocytes can be reduced by lactoferrin suggesting a negative feedback mechanism by which ROI can control NO production (unpublished data).

Apart from classical MHC-I molecules and HFE, β2m also associates noncovalently with a number of nonclassical MHC-Ib and other MHC-I–like molecules such as Qa, H2-M3, and CD1. Therefore, a lack of these molecules could also contribute to the higher susceptibility of β2m–KO mice as compared with MHC-I–KO mice. However, mice lacking CD1d or CD8 T cells including H2-M3–specific CD8 T cells are less susceptible to *M. tuberculosis* than β2m–KO mice (28–30). This further supports the idea that defective surface expression of HFE causes an imbalance in Fe transport which forms the basis for unrestrained growth of *M. tuberculosis* in β2m–KO mice as compared with B6 or MHC-I–KO mice. Our data provide further insights into the multifaceted biology of tuberculosis and provide guidelines for rational development of novel treatment regimes for this threatening disease, especially in areas of the world where dietary Fe overload and tuberculosis are concurrent problems.

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