Iron Loading Exaggerates the Inflammatory Response to the Toll-like Receptor 4 Ligand Lipopolysaccharide by Altering Mitochondrial Homeostasis

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ABSTRACT

Background: Perioperative and critically ill patients are often exposed to iron (in the form of parenteral-iron administration or blood transfusion) and inflammatory stimuli, but the effects of iron loading on the inflammatory response are unclear. Recent data suggest that mitochondrial reactive oxygen species have an important role in the innate immune response and that increased mitochondrial reactive oxygen species production is a result of dysfunctional mitochondria. We tested the hypothesis that increased intracellular iron potentiates lipopolysaccharide-induced inflammation by increasing mitochondrial reactive oxygen species levels.

Methods: Murine macrophage cells were incubated with iron and then stimulated with lipopolysaccharide. C57BL/6 wild-type mice were intraperitoneally injected with iron and then with lipopolysaccharide. Markers of inflammation and mitochondrial superoxide production were examined. Mitochondrial homeostasis (the balance between mitochondrial biogenesis and destruction) was assessed, as were mitochondrial mass and the proportion of nonfunctional to total mitochondria.

Results: Iron loading of mice and cells potentiated the inflammatory response to lipopolysaccharide. Iron loading increased mitochondrial superoxide production. Treatment with MitoTEMPO, a mitochondria-specific antioxidant, blunted the proinflammatory effects of iron loading. Iron loading increased mitochondrial mass in cells treated with lipopolysaccharide and increased the proportion of nonfunctional mitochondria. Iron loading also altered mitochondrial homeostasis to favor increased production of mitochondria.

Conclusions: Acute iron loading potentiates the inflammatory response to lipopolysaccharide, at least in part by disrupting mitochondrial homeostasis and increasing the production of mitochondrial superoxide. Improved understanding of iron homeostasis in the context of acute inflammation may yield innovative therapeutic approaches in perioperative and critically ill patients. **(Anesthesiology 2017; 127:121-35)**

IRON is an essential trace element.[1] Increased appreciation of the adverse effects of anemia in the perioperative period together with awareness of the risks of blood transfusion have led to the preoperative use of intravenous iron preparations as part of patient blood management protocols.[2–4] Critically ill patients in intensive care units are also exposed to acute iron loading (defined here as an increase in intracellular iron concentration in response to iron administration) in the form of blood transfusions, as well as oral and parenteral iron treatment.[5,6] Although patients are often exposed to both iron loading and inflammatory stimuli such as major surgery or critical illness, the effects of iron loading on the inflammatory response are incompletely understood.

Because of the ability of iron to generate reactive oxygen species (ROS) by the Fenton reaction,[7] iron administration might be expected to potentiate the response to subsequent inflammatory stimuli. Studies supporting a proinflammatory role for iron include those by Zager *et al.*,[8] who showed that

What We Already Know about This Topic

- Inflammation may play a role in critical illness
- Iron can increase the formation of reactive oxygen species, potentially affecting inflammation
- Critically ill patients may be exposed to iron through transfusion

What This Article Tells Us That Is New

- In rodent and cellular models, iron loading potentiated inflammation caused by lipopolysaccharide
- Iron loading in this model increased the production of mitochondrial superoxide and disrupted mitochondrial homeostasis

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iron administration worsened *Escherichia coli*–induced sepsis, and Wang *et al.*,[9] who reported that the iron chelator deferoxamine blunted lipopolysaccharide-induced inflammation. However, Pagani *et al.*[10] found that iron-deficient mice had a more robust inflammatory response to lipopolysaccharide than iron-replete mice, and De Domenico *et al.*[11] demonstrated that iron administration diminished the inflammatory response to lipopolysaccharide. The reason for the lack of agreement between these studies is unclear but may be related to differing routes and timing of iron administration.

Iron homeostasis is tightly controlled to minimize the risk of toxicity. Hepcidin is a key regulator of iron homeostasis.[12,13] Hepcidin binds to and down-regulates ferroportin, the only known iron exporter in mammals. Acute inflammation has been shown to increase hepcidin levels,[14] inducing systemic hypoferremia with an increase in intracellular iron.

Mitochondria have an important role in the acute inflammatory response. Pathogen-associated molecular patterns such as lipopolysaccharide, a Toll-like receptor 4 (TLR4) agonist, induce inflammation in part by increasing the production of mitochondrial reactive oxygen species (mtROS).[15] Mitochondria are a major site of iron utilization within the cell[16] and exist in a dynamic equilibrium between biogenesis and mitophagy (removal of dysfunctional mitochondria by autophagy).[17] Perturbations in mitochondrial homeostasis (defined here as the balance between mitochondrial biogenesis and mitophagy) may increase the proportion of damaged or nonfunctional mitochondria, increasing mtROS production.[18]

In this study, we examined the effect of acute iron loading on the inflammatory response using *in vivo* and *in vitro* models of inflammation. We hypothesized that iron loading would exaggerate the proinflammatory effect of lipopolysaccharide by increasing mtROS production.

Materials and Methods

Reagents and Chemicals

Escherichia coli lipopolysaccharide (O55:B5), a TLR4 agonist, was purchased from List Biologicals (USA). The TLR2 and TLR3 agonists Pam-3-Cys (P3C) and poly(I:C) (PIC), respectively, were obtained from Invivogen (USA). Formyl peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF), iron dextran, 20% dextran, ferric ammonium citrate, deferoxamine, antimycin, and 3-methyladenine were purchased from Sigma-Aldrich (USA). Calcein-acetoxymethyl (AM), MitoSOX, MitoTracker Deep Red, MitoTracker Green, and SYTOX Blue were purchased from Molecular Probes (USA). Antibodies, isotype controls, and reagents for flow cytometry were obtained from BD Biosciences (USA). MitoTEMPO, a mitochondria-specific antioxidant,[19] was purchased from Enzo Life Sciences (USA).

Animals

The Institutional Animal Care and Use Committee at the Massachusetts General Hospital approved the animal studies. Male C57BL/6 mice (6 to 8 weeks old) were purchased from Jackson Laboratories (USA). The mice were fed a standard, iron-replete diet and were injected intraperitoneally with one dose of iron dextran (1 g/kg in a volume of 10 μl/g) or normal saline (control) for iron loading experiments. Pilot experiments were performed with a range of iron doses (0.5 to 2.0 g/kg), and serum iron levels and liver hepcidin messenger RNA (mRNA) were measured at various time points (1, 3, 4, and 7 days; data not shown). Mice injected with 1 g/kg iron dextran demonstrated both a sustained increase in serum iron levels and a strong induction of hepcidin after 3 days, leading us to choose this 72-h time point for further investigation. A separate group of wild-type mice was injected with normal saline or 7.5% dextran (10 μl/g, the same volume as iron dextran). Three days later the mice were injected intraperitoneally with lipopolysaccharide (5 mg/kg) or an equal volume of normal saline (control). The mice were sacrificed 6 h later, and blood and organs (lungs and liver) were collected. Serum and organs were stored at –80°C until use.

Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from American Type Cell Collection (USA). RAW 264.7 (hereafter referred to as "RAW") cells were cultured in 6-well (at 8×10^5 cells/well) or 96-well tissue culture plates (at 1.28×10^5 cells/well) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum with glutamine, penicillin, and streptomycin. For iron supplementation experiments, the cells were incubated with ferric ammonium citrate (final concentration of elemental iron, 200 μM) overnight and then treated with lipopolysaccharide (150 ng/ml) or medium alone for 6 h. The concentration of iron was chosen so as to approximate the serum iron concentrations used in pilot, *in vivo* dose-response experiments.

Human Monocyte Isolation

The Institutional Review Board at the Massachusetts General Hospital approved the collection of whole blood from volunteers for the purpose of monocyte isolation (Institutional Review Board protocol No. 2014P001656). Mononuclear cells were isolated from human whole blood collected in EDTA using Polymorphprep density gradient (Axis-Shield, Norway) according to the manufacturer's instructions. Mononuclear cells were incubated (at 8×10^5 cells/well) in 6-well tissue culture plates in serum-free DMEM for 4 h. The cells were then washed with serum-free DMEM to remove nonadherent cells and incubated overnight in DMEM with 10% fetal calf serum. The cells were incubated with iron and/or lipopolysaccharide as in the RAW cell experiments.

Mouse Serum Studies

Serum interleukin-6 and tumor necrosis factor (TNF) levels were measured using mouse interleukin-6 and TNF Quantikine enzyme-linked immunosorbent assay kits (R&D Systems, USA). Serum iron levels were measured using an Iron-SL assay (Japan).

Quantitative Reverse Transcription-Polymerase Chain Reaction

TaqMan primers for quantitative reverse transcription (RT)-polymerase chain reaction (PCR) were purchased from ThermoFisher Scientific (USA). SYBR Green primers were synthesized by the Massachusetts General Hospital DNA core facility. The sequences of SYBR Green and TaqMan primers used in this study are listed in supplemental table 1 (Supplemental Digital Content 1, http://links.lww.com/ALN/B431). Total RNA was extracted from mouse liver and lung tissues or RAW cells using TRIzol (Invitrogen, ThermoFisher Scientific, USA). Reverse RNA transcription was accomplished using Moloney murine leukemia virus RT (Promega, USA). Quantitative RT-PCR was performed using Applied Biosystems SYBR Green or TaqMan master mix (ThermoFisher Scientific) and an Eppendorf MasterCycler RealPlex2 (ThermoFisher Scientific). The level of target transcripts was normalized to the level of 18S rRNA using the relative CT method.

Intracellular Labile Iron Measurement

Intracellular labile iron was measured as described previously.[20] Briefly, cells were incubated with calcein-AM, which was transported across the cell membrane by viable cells and deesterified, producing intracellular, fluorescent, free calcein. Calcein binds with intracellular labile (or free) iron, a reaction that quenches calcein fluorescence. The concentration of labile iron in a cell is inversely proportional to the intensity of calcein fluorescence. SYTOX Blue was used to identify and exclude nonviable cells, which do not take up calcein-AM, and may thereby confound results.

Flow Cytometry

Mouse whole blood was incubated in erythrocyte lysis buffer for 3 min and washed twice in flow cytometry buffer (phosphate-buffered saline with 2% fetal calf serum). The cells were then incubated with PerCP-Cy5.5-conjugated mouse monoclonal anti-CD11b antibody, allophycocyanin-conjugated mouse monoclonal anti-Ly6G antibody, or isotype controls. The cells were then incubated for 30 min with calcein-AM (0.125 μM). RAW cells incubated with iron and/or lipopolysaccharide were treated with calcein-AM (0.125 μM), MitoSOX (2.5 μM), MitoTracker Deep Red (50 nM), or MitoTracker Green (50 nM) for 30 min at 37°C in DMEM. Flow cytometry was performed using a FACS Aria III machine (BD Biosciences, USA), and the results were analyzed using FlowJo software (TreeStar, USA). In all cases, the gating parameters were set to exclude doublets and nonviable cells.

Determination of the Ratio of Mitochondrial to Nuclear DNA

Total genomic DNA was isolated from RAW cells with a DNeasy blood and tissue kit (Qiagen, USA). Quantitative PCR was used to measure the amounts of cytochrome c oxidase I (CO1, a mitochondrial gene) and 18S ribosomal DNA (18S, a nuclear gene), as previously described.[21] The ratio of CO1 to 18S was used as a measure of the relative proportions of mitochondrial DNA (mtDNA) and nuclear DNA.

Statistics

For *in vitro* studies, the data are expressed as mean and SD of individual experiments replicated thrice. The data were tested for a normal distribution by the Shapiro–Wilk test and analyzed using Student's *t* test (or Mann–Whitney U test if the data were not normally distributed) or two-way ANOVA (iron × lipopolysaccharide). If the iron × lipopolysaccharide interaction was statistically significant, we applied all possible pairwise comparisons (Bonferroni *post hoc* tests). If the interaction was not statistically significant, we interpreted the main effects only and refrained from *post hoc* testing. For data that were not normally distributed, we used the Kruskal–Wallis test (with Dunn *post hoc* tests for all possible pairwise comparisons). For the sake of clarity, not all pairwise comparisons have been reported in the figures. Hypothesis testing was two-tailed. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism 7.0 (USA). Sample sizes for *in vivo* experiments were based on our prior experience with lipopolysaccharide injection without *a priori* power calculations. Conditions in the *in vivo* experiments were nonsequential, and processing of samples for the *in vivo* experiments was performed by investigators who were blinded to the experimental conditions.

Results

Iron Dextran Administration Increases Serum Iron and Intracellular Iron in Circulating Neutrophils and Monocytes

Injection of mice with iron dextran increased serum iron levels 10-fold (fig. 1A). Lipopolysaccharide injection alone reduced serum iron levels by more than 50%, consistent with previous reports.[22] Mice that were treated with both iron and lipopolysaccharide had iron levels similar to those of mice injected with iron alone. Iron loading or lipopolysaccharide administration each increased hepatic hepcidin gene expression, as has been described by others[23] (fig. 1B). Intracellular labile iron levels in circulating neutrophils (Ly6G-positive cells) and circulating monocytes (CD11b-positive cells) were elevated as shown by decreased calcein fluorescence in iron-treated mice (fig. 1, C and D). These observations demonstrate that parenteral administration of iron dextran induces hepcidin production and increases intracellular iron levels in circulating neutrophils and monocytes.

Iron Administration Potentiates the Inflammatory Effects of Lipopolysaccharide

Iron administration alone did not induce inflammation in mice, as determined by the absence of increase in either

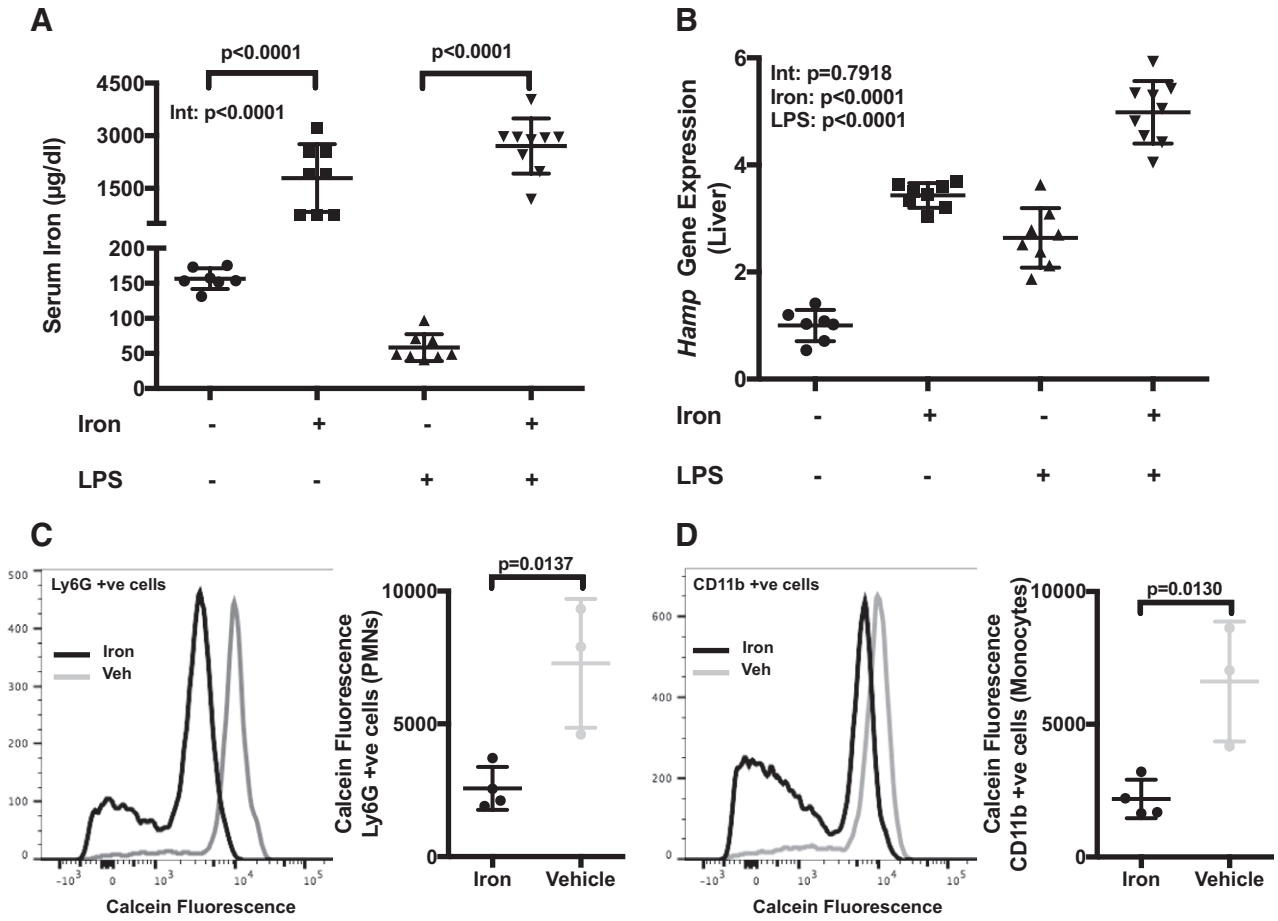

Fig. 1. Effects of parenteral iron overload in mice. (*A*) Mice treated with iron had significantly higher serum iron levels, while mice treated with lipopolysaccharide (LPS) alone became hypoferremic (two-way ANOVA with Bonferroni *post hoc* tests performed on $\log_{10}$-transformed values; *P* values adjusted for all possible comparisons). (*B*) Iron and lipopolysaccharide independently induce hepcidin (*Hamp*) mRNA levels in the liver (two-way ANOVA, interaction between iron and lipopolysaccharide not significant, therefore only main effects reported). N = 7 to 9 mice/group for (*A*, *B*). (*C*, *D*) Iron injection increases intracellular labile iron in circulating neutrophils and monocytes. Both neutrophils (Ly6G-positive cells) and monocytes (CD11b-positive cells) from the iron-treated mice had lower calcein fluorescence intensities, consistent with higher intracellular iron levels. Representative histograms are from one vehicle-treated (Veh) and one iron-treated mouse. The dot plots show mean fluorescent intensities of 3 to 4 mice/group (unpaired *t* test). Int = interaction *P* value; PMN = polymorphonuclear leukocytes.

serum protein levels or mRNA levels (in liver and lungs) of the cytokines interleukin-6 and TNFα (fig. 2, A–F). A nonlethal lipopolysaccharide challenge induced a marked increase in serum interleukin-6 and TNFα levels, as well as the corresponding mRNA levels in mouse liver and lungs. Iron-treated mice challenged with lipopolysaccharide showed more than 5-fold higher serum cytokine levels than mice challenged with lipopolysaccharide alone. Similarly, the mRNA levels of *interleukin-6* and *Tnfα* in lungs and liver of mice treated with iron and lipopolysaccharide were between 1.5- and 2.5-fold greater than in mice treated with lipopolysaccharide alone. Iron treatment and subsequent lipopolysaccharide challenge did not alter mRNA levels of the antiinflammatory cytokine interleukin-10 in the lung compared to lipopolysaccharide challenge alone (supplemental fig. 1, Supplemental Digital Content 1, http://links.lww.com/ALN/B431). In control studies, we showed that 7.5% dextran (the vehicle for iron) did not have an independent proinflammatory effect on a subsequent stimulation with 5 mg/kg lipopolysaccharide (supplemental fig. 2, A and B, Supplemental Digital Content 1, http://links.lww.com/ALN/B431). Taken together, these observations indicate that parenteral iron administration strongly augments the proinflammatory response to lipopolysaccharide in mice.

Preincubation with Iron Augments Lipopolysaccharide-induced Cytokine mRNA Induction in Human Monocytes and RAW Cells

Human monocytes were found to have a more intense response to lipopolysaccharide stimulation after being iron-loaded (fig. 3, A and B). *In vitro* incubation of RAW cells with iron increased intracellular labile iron (nonferritin-bound, catalytically active iron) concentration in RAW cells. Deferoxamine, an iron chelator, was used as an assay control, demonstrating

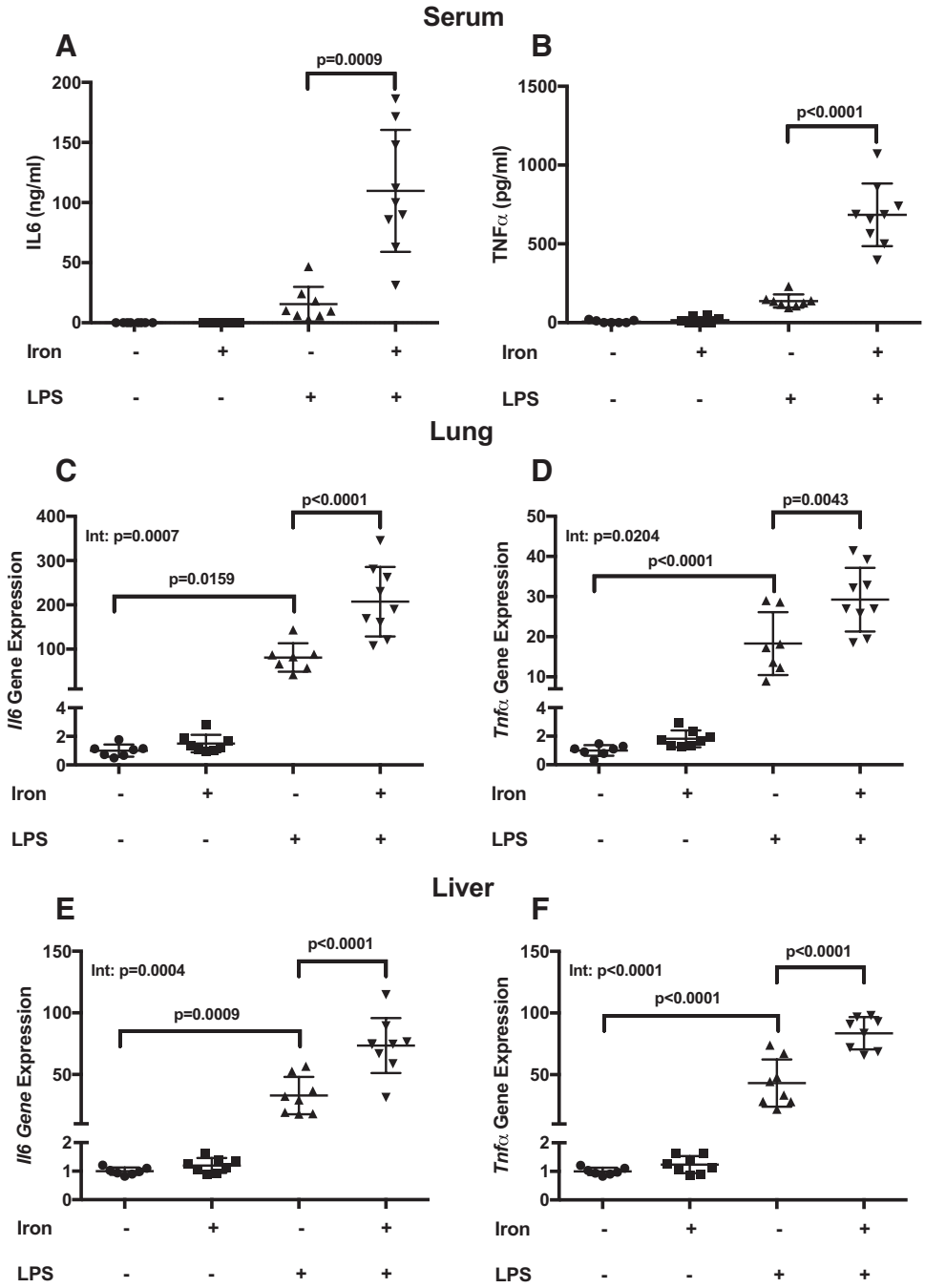

Fig. 2. Effect of parenteral iron administration on inflammatory gene expression and serum cytokine levels. Serum, lung, and liver samples were harvested from C57Bl/6 mice treated with iron and/or lipopolysaccharide (LPS) and analyzed for serum *Il6* (*A*), serum *Tnfα* (*B*), and mRNA levels of the two cytokines in the lungs (*C*, *D*) and livers (*E*, *F*). In each case, iron supplementation by itself did not induce inflammation. Lipopolysaccharide alone induced increases in cytokine protein levels, as well as mRNA levels. Serum cytokine levels in the control and iron-treated groups were below the detection level of the assay in many cases. Iron administration significantly enhanced the effect of lipopolysaccharide-induced inflammation in the mice. N = 7 to 9 mice per group (Mann–Whitney U test [*A*, *B*] and two-way ANOVA with Bonferroni *post hoc* tests [*C–F*]; *P* values adjusted for all possible comparisons). Il = interleukin; Int = interaction *P* value; TNF, tumor necrosis factor.

the inverse relationship between cellular labile iron levels and calcein fluorescence (fig. 4A). Iron-loaded RAW cells stimulated with lipopolysaccharide had significantly higher mRNA levels of interleukin-6 and TNFα than cells treated with lipopolysaccharide alone (fig. 4, B and C). Iron-loaded RAW cells stimulated with lipopolysaccharide also produced significantly more interleukin-6 protein than cells stimulated with lipopolysaccharide alone (supplemental fig. 3, Supplemental

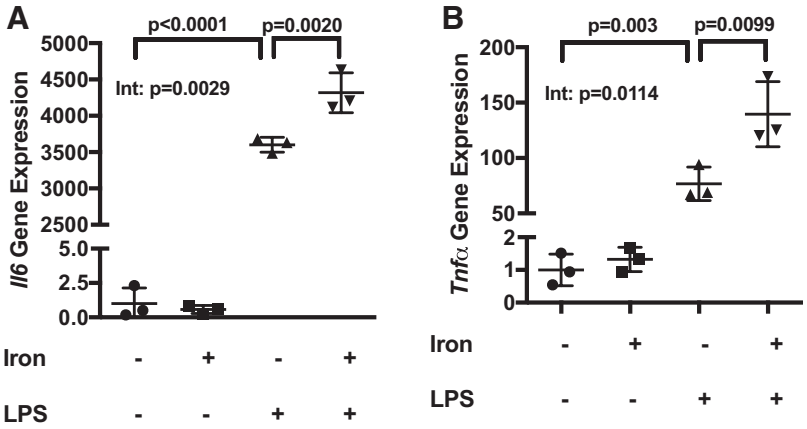

Fig. 3. Effect of iron loading on human monocytes *in vitro*. Human monocytes were isolated from peripheral blood and incubated in six-well plates. The cells were incubated with iron (200 μM) or control overnight and then stimulated for 6 h with lipopolysaccharide (LPS; 150 ng/ml) or medium. Lipopolysaccharide alone induced *Il6* mRNA (*A*) and *Tnfα* mRNA (*B*). Iron loading significantly increased mRNA levels in response to LPS (two-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons; n = 3 replicates/condition). Int = interaction *P* value; Il = interleukin.

Digital Content 1, http://links.lww.com/ALN/B431). Iron-treated RAW cells challenged with lipopolysaccharide showed increased mRNA expression of the chemokine monocyte chemotactic protein 1 (*Mcp1*) compared to cells treated with lipopolysaccharide alone (fig. 4D). Iron pretreatment with subsequent lipopolysaccharide reduced the expression of the antiinflammatory cytokine *interleukin-10* compared to cells treated with lipopolysaccharide alone (fig. 4E). Conversely, RAW cells preincubated with 30 μM deferoxamine showed a blunted response to lipopolysaccharide (fig. 5, A and B). Of note, incubation of cells with 200 μM iron had no adverse effects on cell viability, as determined by flow cytometric detection of nonviable cells (data not shown). These results demonstrate that iron loading augments the proinflammatory effect of lipopolysaccharide on RAW cells *in vitro* and that the use of an iron chelator blunts the cytokine response to lipopolysaccharide. Similarly, pretreatment of human monocytes with iron augments the proinflammatory effect of a subsequent exposure to lipopolysaccharide.

Response to Iron Loading In Vitro *Differs Depending on the Type of Inflammatory Stimulus*

We examined the effect of iron loading on the response of RAW cells to three additional proinflammatory mediators: P3C, PIC, and fMLF. P3C is a TLR2 agonist found in Gram-positive bacteria, PIC is a viral TLR3 agonist, and fMLF is a formylated peptide found in bacteria and mitochondria and an example of a damage-associated molecular pattern (DAMP). In contrast to the 2.5-fold increase in *interleukin-6* mRNA levels induced by iron and lipopolysaccharide, compared to lipopolysaccharide alone, iron-loaded cells stimulated with PIC did not show any significant increase in *interleukin-6* mRNA compared to cells treated with PIC alone. The combination of iron and P3C produced a 1.5-fold increase in *interleukin-6* mRNA compared to P3C alone. Iron together with the mitochondrial DAMP fMLF produced a 2.5-fold increase in *interleukin-6* mRNA compared to DAMP alone (fig. 5C). These results suggest that the effects of iron loading on enhancing the inflammatory response are pathway specific and depend on the type of proinflammatory stimulus.

mtROS Contribute to the Proinflammatory Effect of Iron Loading

To determine the effect of iron loading on mtROS levels, RAW cells were incubated with iron and stained with MitoSOX, a fluorescent dye that specifically detects mitochondrial superoxide.[24] Compared to untreated RAW cells, RAW cells exposed to iron had a 40% increase in fluorescence intensity, suggesting that iron loading results in an increased level of intracellular mtROS. The combination of iron and lipopolysaccharide increased RAW cell mtROS levels by 50% compared to cells treated with lipopolysaccharide alone (fig. 6, A and B).

To consider the possibility that an antioxidant might blunt the proinflammatory effect of iron, RAW cells were incubated with MitoTEMPO (100 μM), a mitochondria-specific antioxidant,[19] before stimulation with lipopolysaccharide. Preincubation with MitoTEMPO significantly blunted (but did not abolish) the inflammatory response to lipopolysaccharide compared to similarly treated cells that were not exposed to MitoTEMPO (fig. 6, C and D), showing *interleukin-6* and *Mcp1* mRNA levels, respectively). These results demonstrate that iron induces mtROS in RAW cells and that inhibiting mtROS production diminishes the inflammatory response in iron-loaded cells treated with lipopolysaccharide. However, increased mtROS alone (as caused by iron loading) is not sufficient to increase inflammatory mRNA levels, as cells treated with iron alone did not express increased cytokine mRNA levels (fig. 4, B and C).

Iron Administration Modifies mRNA Levels of Genes Involved in Mitochondrial Homeostasis

Compared to control mice, mice treated with lipopolysaccharide had lower mRNA levels of genes associated with

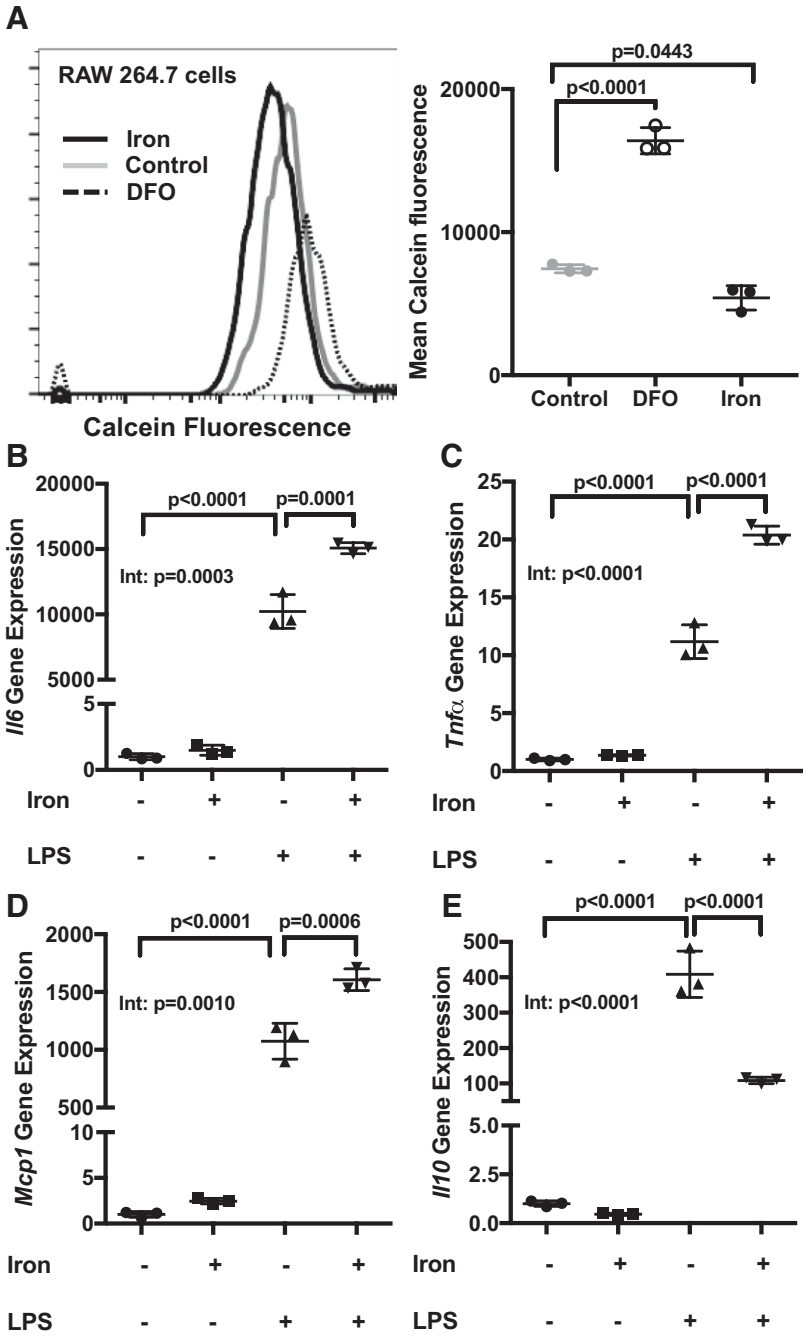

Fig. 4. Effect of iron loading on RAW 264.7 cells *in vitro*. (*A*) RAW cells treated with lipopolysaccharide (LPS), iron, or deferoxamine (DFO, an iron chelator) were incubated with calcein and subjected to flow cytometry, and calcein fluorescence was quantified. Calcein fluorescence decreased in iron-treated cells (one-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons). A representative histogram from one of three independent experiments is shown. The dot plots show mean fluorescent intensities of three replicates/condition. (*B*, *C*) RAW cells were incubated with iron (200 μM) or control overnight and then stimulated for 6 h with lipopolysaccharide (150 ng/ml). Lipopolysaccharide alone induced *Il6* and *Tnfα* mRNA. Iron loading significantly increased mRNA levels in response to lipopolysaccharide. (*D*) The combination of iron and lipopolysaccharide increased *Mcp1* mRNA levels to a greater extent than lipopolysaccharide alone. (*E*) Conversely, the combination of lipopolysaccharide and iron resulted in less *Il10* mRNA levels than lipopolysaccharide alone (two-way ANOVA with Bonferroni *post hoc* tests [*B*–*E*]; *P* values adjusted for all possible comparisons; n = 3 replicates/condition). Il = interleukin; Int = interaction *P* value.

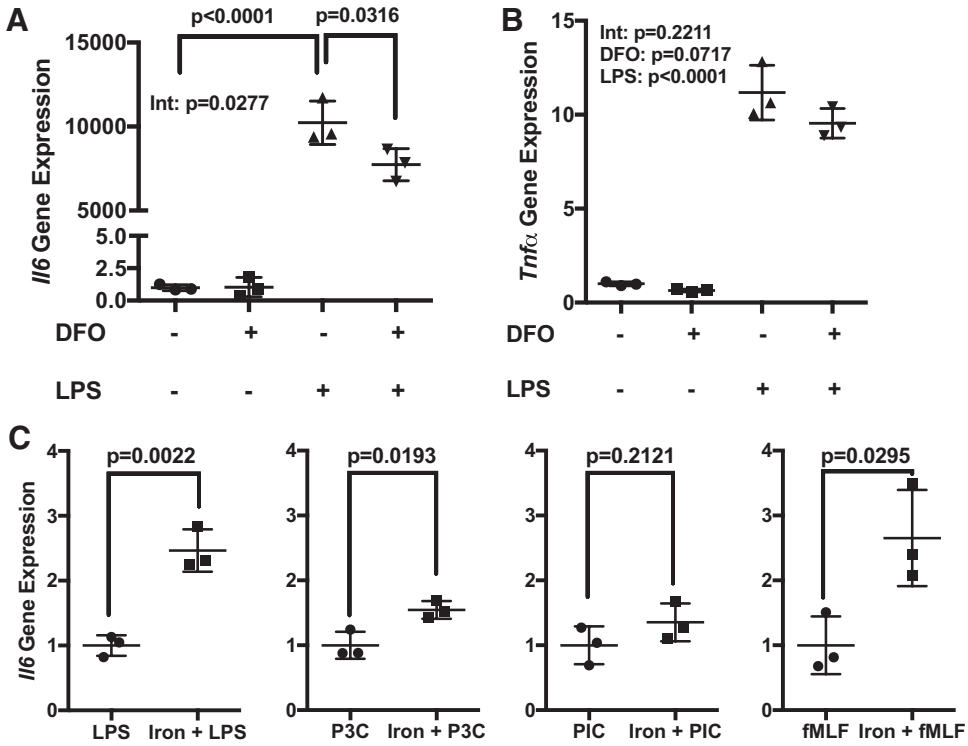

Fig. 5. (*A*, *B*) Effect of an iron chelator on RAW 264.7 cells *in vitro*. RAW cells were pretreated with 30 μM deferoxamine (DFO, an iron chelator) and subsequently stimulated with lipopolysaccharide (LPS) for 6 h. Deferoxamine pretreatment decreases *Il6* mRNA induced by lipopolysaccharide significantly. There is a trend towards decreasing TNF mRNA in deferoxamine-pretreated cells that did not reach statistical significance (two-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons; n = 3 replicates/condition). (*C*) RAW cells were incubated with iron (200 μM) or control overnight and then stimulated for 6 h with lipopolysaccharide (150 ng/ml), Pam-3-Cys (P3C; 1 μg/ml), poly(I:C) (PIC; 25 μg/ml), or *N*-formyl-L-methionyl-L-leuyl-L-phenylalanine (fMLF; 20 μM). *Il6* gene expression was normalized to the response to lipopolysaccharide alone. The response to iron loading is not uniform across different proinflammatory agents. Iron loading augments the inflammatory response to lipopolysaccharide (a TLR4 ligand) and P3C (a TLR2 ligand), but not to PIC (a TLR3 ligand). Iron loading also augments the response to *N*-formyl-L-methionyl-L-leuyl-L-phenylalanine, a formylated peptide that is considered a damage-associated molecular pattern (unpaired *t* tests; n = 3 replicates/condition). Il = interleukin; Int = interaction *P* value.

mitochondrial biogenesis (*Pgc-1α* and *Ampk*; fig. 7, A and B) in the liver, but higher expression of *Lc3b*, a gene involved in mitophagy (fig. 7C). Conversely, iron-treated mice that were challenged with lipopolysaccharide had greater expression of *Pgc-1α* and *Ampk* than mice treated with lipopolysaccharide alone (fig. 7, A–C), suggesting a shift towards either greater mitochondrial biogenesis or decreased mitophagy. We found similar changes in *Ampk* gene expression in mouse lungs and in RAW cells (supplemental fig. 4, A and B, Supplemental Digital Content 1, http://links.lww.com/ALN/B431). These results suggest that lipopolysaccharide alone induces a relative shift towards mitophagy, while iron pretreatment before lipopolysaccharide administration tilts the balance towards mitochondrial biogenesis.

Iron Loading Increases the Proportion of Nonfunctional to Total Mitochondria in RAW Cells

To examine the effect of iron treatment on the functional status of mitochondria, we stained RAW cells that were treated with iron and/or lipopolysaccharide with MitoTracker Deep Red (a dye that stains functional mitochondria only) and MitoTracker Green (a dye that stains all mitochondria, functional and otherwise).[18] The proportion of RAW cells stained with MitoTracker Green alone (*i.e.*, [MT Green – MT Deep Red]/MT Green) was significantly increased in RAW cells treated with iron compared to control cells and was also significantly higher in iron-loaded cells treated with lipopolysaccharide than in lipopolysaccharide-only cells (fig. 8, A and B). This suggests that iron loading increases the proportion of nonfunctional mitochondria in RAW cells.

Mitochondrial genomic DNA content, a measure of mitochondrial mass, was higher in iron-loaded RAW cells treated with lipopolysaccharide than in cells treated with lipopolysaccharide alone (fig. 8C). The mtDNA content in iron-loaded cells that were not treated with lipopolysaccharide was similar to lipopolysaccharide-treated cells that were not iron-loaded. Taken together, these data suggest that iron-loaded cells stimulated with lipopolysaccharide have both a higher mitochondrial mass and a greater proportion

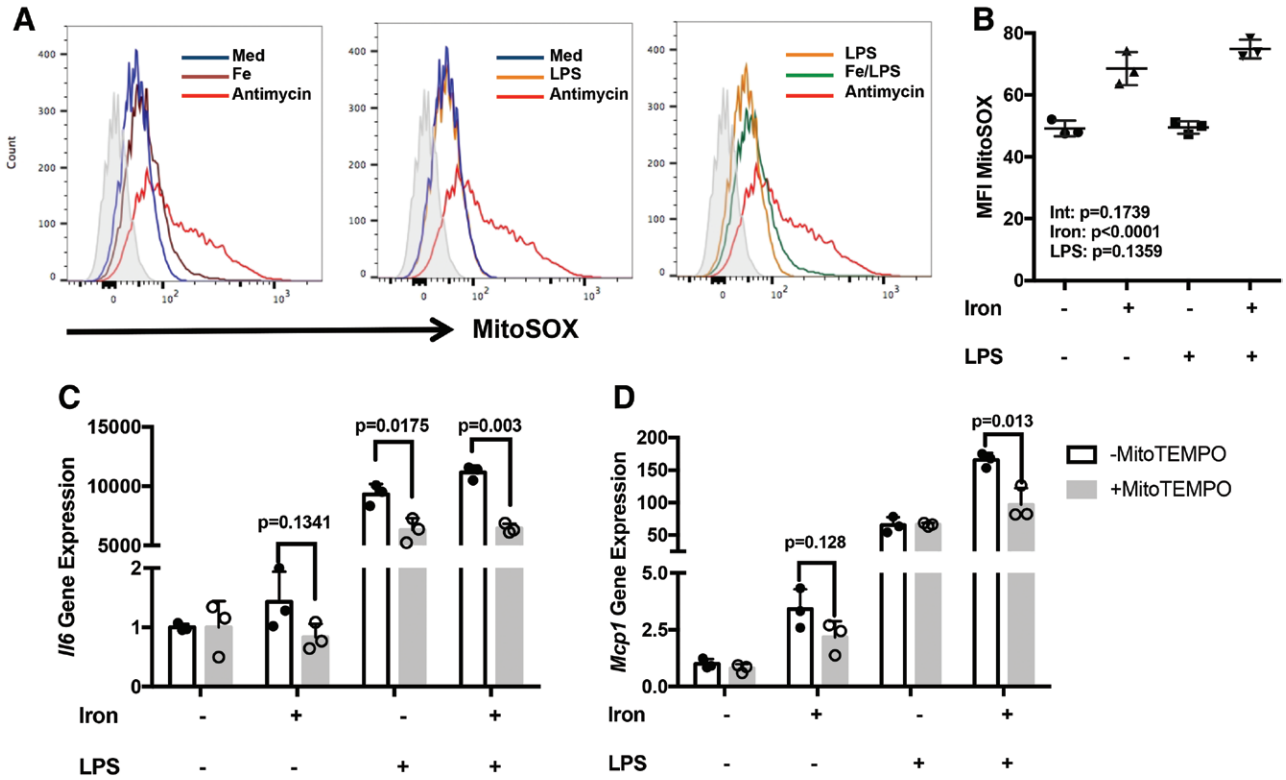

Fig. 6. The effect of iron and lipopolysaccharide (LPS) on mitochondrial reactive oxygen species production. (*A*) Effect of iron supplementation on mitochondrial reactive oxygen species production. The shaded histogram depicts unstained cells. The histogram in red shows the effects of antimycin (20 μg/ml), an inhibitor of mitochondrial complex III used as a positive control. Iron-naïve cells treated with lipopolysaccharide do not show increased MitoSOX fluorescence compared to controls (*middle*). However, iron-pretreated RAW cells exposed to lipopolysaccharide have more mitochondrial reactive oxygen species production compared to cells treated with lipopolysaccharide alone (*right*), although the increase is similar to that caused by iron incubation alone (*left*). Data are representative of three experiments. (*B*) Summary bar graphs showing relative MitoSOX fluorescence (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported; n = 3 replicates/condition). (*C*, *D*) Incubating RAW cells with 100 μM of the mitochondrion-specific antioxidant MitoTEMPO blunted the mRNA response to lipopolysaccharide (*Il6* and *Mcp1*) in iron-loaded macrophages (unpaired *t* tests; n = 3 replicates/condition). Il = interleukin; Int = interaction *P* value; Med = medium; MFI = mean fluorescent intensity.

of nonfunctional mitochondria than cells that were treated with lipopolysaccharide alone.

Discussion

In this study, we observed a strong proinflammatory effect of iron loading on a subsequent challenge with lipopolysaccharide, *in vivo* and *in vitro*. Parenteral iron loading increased intracellular labile iron in circulating neutrophils and monocytes and strongly increased the cytokine response to a subsequent lipopolysaccharide challenge. Similarly, the addition of iron to RAW cells increased intracellular labile iron and further augmented the lipopolysaccharide-induced increase in mRNA levels of proinflammatory genes. As with RAW cells, iron-loaded human peripheral blood monocytes also had higher inflammatory cytokine mRNA levels after lipopolysaccharide stimulation. Iron loading induced mtROS, and inhibition of mtROS formation blunted the augmented response to lipopolysaccharide in iron-loaded RAW cells. Iron loading in conjunction with lipopolysaccharide stimulation also increased the expression of genes associated with mitochondrial biogenesis *in vivo* (in liver and lungs) and increased mitochondrial genomic DNA *in vitro* (in RAW cells), suggesting that iron loading alters mitochondrial homeostasis. Iron loading increased the proportion of nonfunctional mitochondria in RAW cells. Taken together, the data suggest that a combination of iron loading together with an inflammatory stimulus results in an increased proportion of defective mitochondria and increased mtROS production.

Anesthesiologists often provide care for patients given parenteral iron supplements before major surgery and for critically ill patients in intensive care units, who may be iron-loaded from blood transfusion or iron therapy. Thus the effect of acute iron loading on the inflammatory response is of particular relevance in perioperative medicine, especially because there are few studies that have examined the effects of iron infusions on outcomes.[25]

Wang *et al.*[26] used a mouse model of hemochromatosis (*Hfe* knockout mice) to show that low intracellular labile iron in *Hfe*

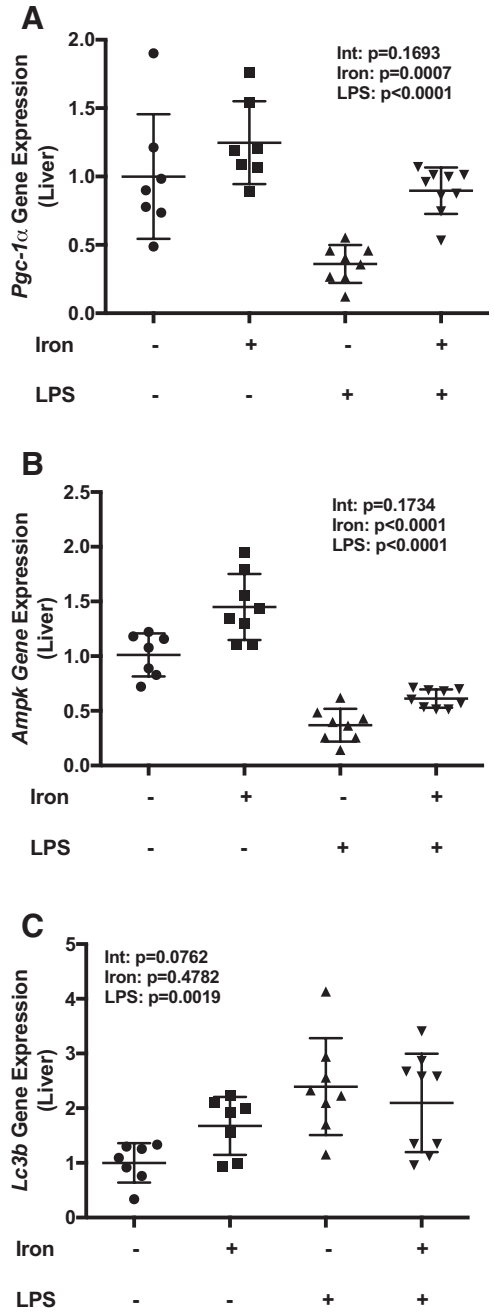

Fig. 7. Iron loading changes the balance between genes responsible for mitochondrial biogenesis and mitochondrial autophagy (mitophagy). C57Bl/6 mice were treated with iron and/or lipopolysaccharide (LPS); N = 7 to 9 mice per group. Total RNA was extracted from mouse liver, and quantitative reverse transcription-polymerase chain reaction was performed using primers for *Pgc-1*α (a mitochondrial biogenesis-associated gene [*A*]), *Ampk* (a mitochondrial biogenesis-associated gene [*B*]), and *Lc3b* (a mitophagy-associated gene [*C*]). Lipopolysaccharide alone increases *Lc3b* mRNA levels, while decreasing *Pgc-1*α and *Ampk* mRNA levels. Iron-loaded mice stimulated with lipopolysaccharide had the opposite profile, with an increase in the mRNA levels of the mitochondrial biogenesis-associated genes *Pgc-1*α and *Ampk* (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported). Int = interaction *P* value.

KO macrophages caused a diminished inflammatory response to lipopolysaccharide. In addition, pretreatment of macrophages from wild-type mice with an iron chelator reduced the inflammatory response to lipopolysaccharide.[26] Other studies showed that the iron chelator deferoxamine attenuated the inflammatory response to lipopolysaccharide in RAW cells and decreased inflammation in mouse models of endotoxemia[9] and peritonitis.[27] In contrast, Pagani *et al.*[10] found that iron-deficient mice (with low macrophage iron levels) had a greater inflammatory response to lipopolysaccharide compared to iron replete mice. De Domenico *et al.*[11] found that oral iron supplementation followed by a lipopolysaccharide challenge blunted the response of mice to lipopolysaccharide. In both of the last two studies, the authors attributed their findings to antiinflammatory effects of the iron-regulating hormone hepcidin. Iron supplementation increases hepcidin production, while low hepcidin production in iron-deficient mice induces an exaggerated response to lipopolysaccharide. However, the mechanism proposed for the presumptive antiinflammatory effect of hepcidin (activation of the janus kinase-signal transducer and activator of transcription pathway by hepcidin-ferroportin binding[11]) has since been questioned.[28] In this study, we demonstrated a robust proinflammatory response to iron loading in spite of an increase in hepcidin gene expression (fig. 1B). The mode of iron supplementation (enteral and parenteral), as well as the formulation of iron, may impact the bioavailability of iron and hence intracellular iron concentrations,[7,29] possibly accounting for differences between our study and that of De Domenico *et al.*

We found that the response to iron loading differs depending on the type of proinflammatory stimulus, suggesting the presence of specific pathways that are influenced by intracellular iron. Indeed, Wang *et al.*[26] found that intracellular iron influences lipopolysaccharide signaling specifically by modifying the MyD88-independent adaptor toll/interleukin-1 receptor domain-containing adapter inducing interferon beta-related adaptor molecule-related response to lipopolysaccharide.

To further examine the mechanisms responsible for the proinflammatory effects of iron loading, we measured mtROS production. Iron loading increased mtROS production in RAW cells, and a mitochondrial-specific antioxidant (Mito-TEMPO) blunted the proinflammatory effect of iron on RAW cells, reducing cytokine (*interleukin-6*) and chemokine (*Mcp1*) mRNA levels to those of macrophages treated with lipopolysaccharide alone. These findings suggest that iron-induced mtROS may have a "priming" effect on macrophages, augmenting the response to a subsequent lipopolysaccharide challenge. In spite of the lack of a demonstrated increase in mtROS production with lipopolysaccharide, lipopolysaccharide stimulation appears to increase mtROS production, because treatment with Mito-TEMPO blunts the inflammatory response to lipopolysaccharide alone. These results are consistent with findings reported by Bulua *et al.*,[30] who found that mouse embryonic fibroblasts have a decreased response to lipopolysaccharide when pretreated with a different mitochondrial superoxide inhibitor, MitoQ. Bulua

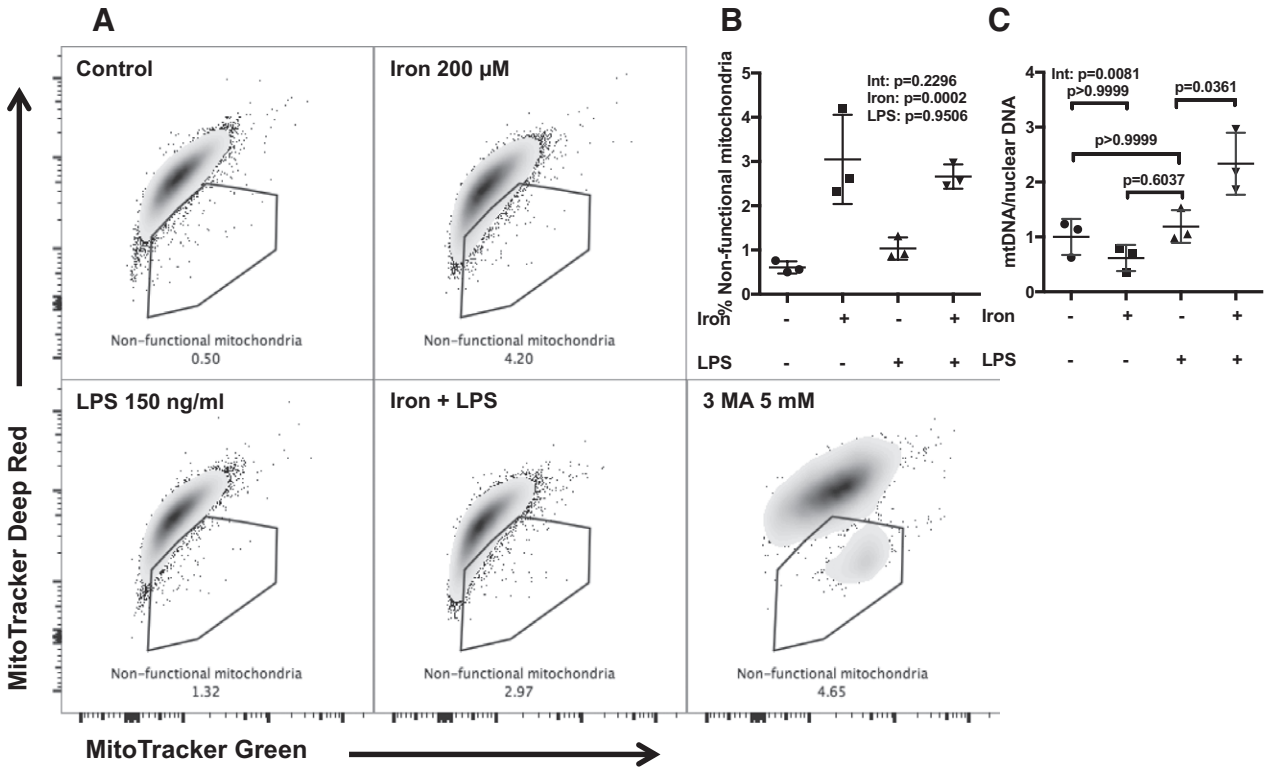

Fig. 8. Iron loading increases the proportion of nonfunctional mitochondria in RAW 26.7 cells. RAW cells were treated with iron and/or lipopolysaccharide (LPS), then incubated with MitoTracker Deep Red and MitoTracker Green, and subjected to flow cytometry. (*A*) Density plots for each condition, representative of three independent experiments. The gate shown contains the cells that are MitoTracker DeepRed–negative/MitoTracker Green–positive cells (nonfunctional mitochondria). 3-Methyladenine (3 MA), an inhibitor of autophagy, was used as a positive control. (*B*) A summary histogram of the cytometry results (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported; n = 3 replicates/condition). (*C*) Iron-loaded, lipopolysaccharide-treated RAW cells have higher mitochondrial mass defined by mitochondrial gene: nuclear gene ratio (two-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons; n = 3 replicates/condition). Int = interaction *P* value; mtDNA = mitochondrial DNA.

et al.[30] also did not find increased MitoSOX staining in the fibroblasts treated with lipopolysaccharide alone.[3] We speculate that while lipopolysaccharide does increase mtROS production, the effects of lipopolysaccharide on mtROS levels may be opposed by increased mitophagy, resulting in no change in net mtROS levels.

Other studies have found that increased intracellular iron increased mtROS production and that mtROS was associated with increased inflammation in different cell types, such as cardiomyocytes[31] and macrophages.[32] The results of this study therefore add to the existing literature by showing that iron loading potentiates inflammation by augmenting mtROS production.

Although the *in vitro* data in our study were derived from monocytes and macrophages, it is possible that iron loading may impact ROS production by neutrophils, which are a major source of ROS *in vivo*.[33] Sampaio *et al.*[34] showed that iron dextran administration in a streptozotocin-induced model of diabetes in rats was associated with a strong increase in neutrophil ROS production. Iron loading may therefore be proinflammatory in both monocytes and neutrophils.

Iron-loaded cells exposed to lipopolysaccharide had a greater mitochondrial mass, as determined by the relative abundance of mitochondrial DNA to nuclear DNA. Of note, we did not find a significant difference in the proportion of mtDNA to nuclear DNA between iron-loaded cells and lipopolysaccharide-treated cells. Iron loading RAW cells increased the proportion of nonfunctional mitochondria relative to total mitochondria. Others have shown that damaged or nonfunctional mitochondria produce more mtROS.[18,35] Mitochondrial mass can be increased by increasing mitochondrial biogenesis, decreasing mitophagy, or both. In this study, the combination of iron and lipopolysaccharide increased mRNA levels of genes involved in mitochondrial biogenesis, *Pgc-1α* and *Ampk in vivo*. Lipopolysaccharide-treated mice had higher expression of hepatic *Lc3b*, a gene involved in mitophagy, which is consistent with prior data reporting that lipopolysaccharide increases mitophagy.[36] The data therefore suggest that the combination of iron loading and lipopolysaccharide stimulation increases mitochondrial biogenesis *in vivo*. Of note, increased mitochondrial biogenesis is not necessarily deleterious; some studies have shown

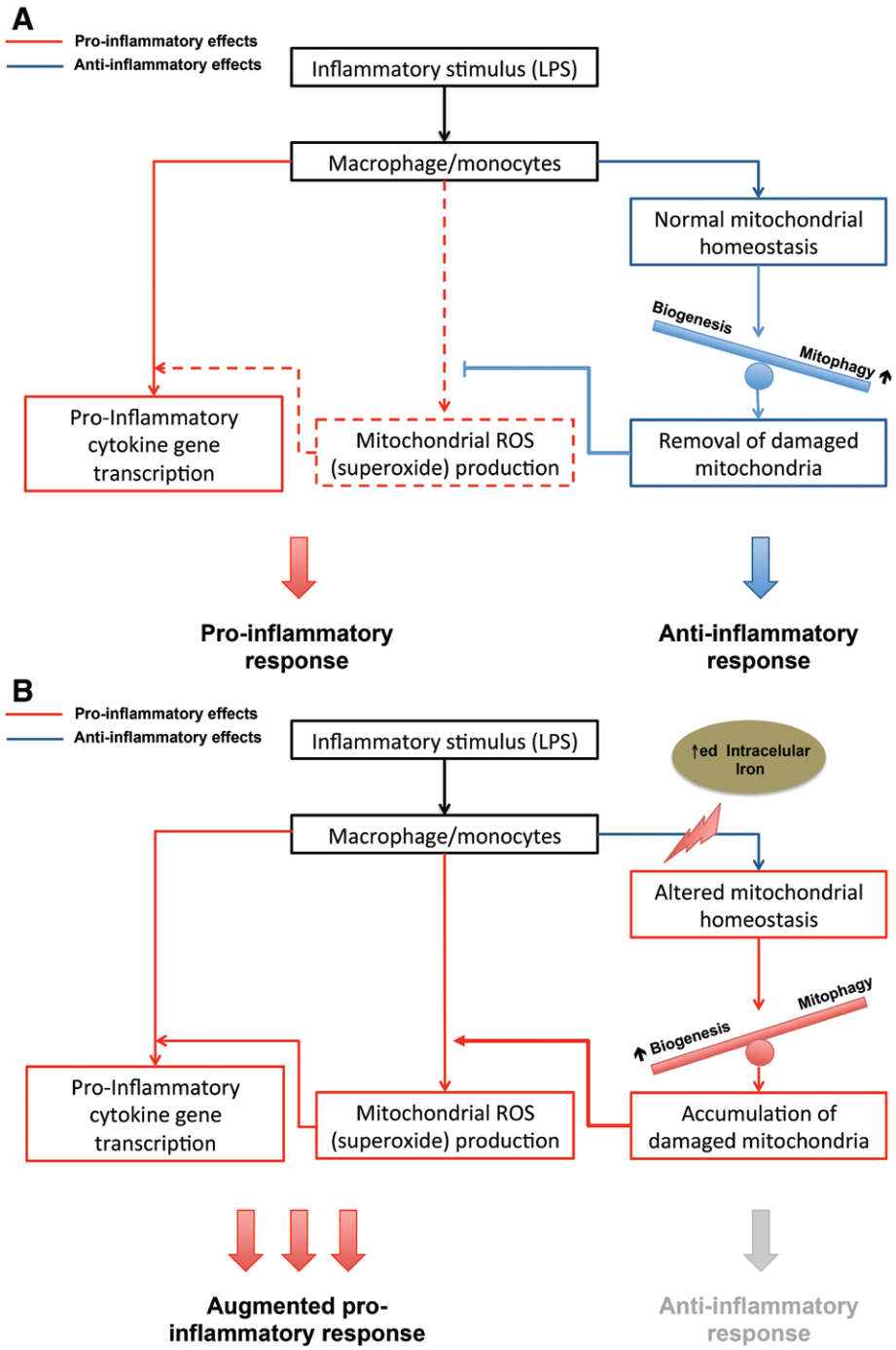

Fig. 9. A schematic view of the role of mitochondrial homeostasis (the balance between mitochondrial biogenesis and mitophagy) in the acute inflammatory response. (*A*) Lipopolysaccharide (LPS)-induced inflammation induces inflammatory gene transcription. Simultaneously, maintenance of mitochondrial homeostasis allows removal of damaged mitochondria by mitophagy (mitochondrial "quality control"), resulting in a blunted inflammatory response, which may be why mitochondrial reactive oxygen species (ROS) does not appear to increase in lipopolysaccharide-stimulated cells. (*B*) In the presence of excess intracellular iron, the balance of mitochondrial homeostasis shifts towards increased biogenesis and less effective mitophagy, resulting in an accumulation of damaged mitochondria, increased mitochondrial reactive oxygen species, and an augmented proinflammatory effect.

that mitochondrial biogenesis imparted a prosurvival phenotype in acute inflammatory states.[37]

Studies using mouse models of defective mitophagy showed that accumulation of nonfunctional mitochondria potentiated mtROS formation and induced a more potent inflammatory response to innate immune stimulants, including lipopolysaccharide.[18,21,38] Duvigneau *et al.*[39] showed that endotoxin-induced iron accumulation in cells was associated with altered mitochondrial respiration and mitochondrial dysfunction. Lowering intracellular

iron levels using iron chelators induced mitophagy in a *Caenorhabditis elegans* model of *Pseudomonas* infection,[40] while iron loading promoted mitochondrial biogenesis in osteoclasts.[41] These reports are consistent with our finding that intracellular iron levels modulate mitochondrial homeostasis.

This study has some limitations. We used iron dextran, rather than iron sucrose or iron gluconate, because iron dextran was previously shown to be the least likely to cause direct iron-induced toxicity.[42] Because we did not test other forms of iron *in vivo*, we cannot comment on the effects of other iron formulations. A second potential limitation is that we examined the effects of increased intracellular iron on the early inflammatory response and thus cannot comment on the effect of iron loading on the temporal course of inflammation. In addition, while we have found that iron loading has a proinflammatory effect on macrophages, we did not investigate the effect of iron loading on macrophage phenotype, although others have shown that iron loading induced an M1 phenotype in macrophages.[43] In RAW cells, we observed a decrease in *interleukin-10* mRNA in response to iron loading, but a similar effect was not observed in murine lungs *in vivo* (supplemental fig. 1, Supplemental Digital Content 1, http://links.lww.com/ALN/B431). Finally, our data demonstrating the effects of iron loading on mitochondria function are limited to *in vitro* assays. Further work is needed to determine whether iron loading alters mitochondrial function *in vivo*.

The results of this study suggest that increased intracellular iron leads to an increased proinflammatory response to the TLR4 ligand lipopolysaccharide, raising the possibility of targeting intracellular iron as a therapeutic modality in acute inflammatory states. However, many questions need to be addressed before intracellular iron can be considered a viable biologic target. Critically ill patients are often hypoferremic, and iron chelators will likely exacerbate hypoferremia. Therapy with iron chelators[44,45] may increase the risk of bacterial infections by organisms that can extract iron from the iron-chelator complex. Finally, we do not currently have reliable assays for monitoring intracellular iron levels to guide therapy.

Conclusions

Our results suggest that iron loading alters mitochondrial homeostasis, leading to the accumulation of defective mitochondria and increasing production of mtROS. The production of mtROS primes macrophages for a "second hit," such as exposure to lipopolysaccharide, greatly augmenting the inflammatory response to the second stimulus. Figure 9 presents a schematic of the role increased intracellular iron plays in modulating the acute inflammatory response. The data presented here highlight the proinflammatory effects of iron loading in acute inflammation and suggest that clinicians should consider the risks of treatments that result in iron loading in acutely ill patients.

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Competing Interests

Dr. Bagchi is a consultant for Lungpacer Medical Inc., Burnaby, British Columbia, Canada. The other authors declare no competing interests.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Laughing Gas in Baltimore, Hagerstown, and Smithsburg: Maryland's Dr. D. W. Crowther

Son of an English army officer, David William Crowther, D.D.S. (1834 to 1916), was born in Devonshire but relocated as a baby with his family to Drummondville, Canada. As a young man, he subsequently moved to New York and then Alabama to serve from Mobile in the Confederacy's First Alabama Battery. Following the Civil War, he trained in dentistry, earning his D.D.S. in Maryland in 1868 from the Baltimore College of Dental Surgery. Around 1874 he moved 74 miles northwest in Maryland to Hagerstown, where he established his dental practice on North Potomac Street. According to this lovely trade card from the Ben Z. Swanson Collection of the Wood Library-Museum, Dr. Crowther extracted teeth "with [laughing] gas" just a "door above Clapp's Junior Hall Store." Never forsaking Maryland professionally, Crowther moved in 1890 about 8 miles east to Smithsburg. He retired from dentistry in 1896 and passed away a decade later. All told, during his 28 yr of practice as a degreed dentist, Crowther administered laughing gas in Baltimore, Hagerstown, and finally Smithsburg, Maryland.

George S. Bause, M.D., M.P.H., Honorary Curator and Laureate of the History of Anesthesia, Wood Library-Museum of Anesthesiology, Schaumburg, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.