Iron overload results in hepatic oxidative stress, immune cell activation, and hepatocellular ballooning injury, leading to nonalcoholic steatohepatitis in genetically obese mice

Priya Handa,1 Vicki Morgan-Stevenson,1 Bryan D. Maliken,2 James E. Nelson,2 Shenna Washington,2 Mark Westerman,3 Matthew M. Yeh,4 and Kris V. Kowdley1,2

1Liver Care Network and Organ Care Research, Swedish Medical Center, Seattle, Washington; 2Benaroya Research Institute, Seattle, Washington; 3Intrinsic Life Sciences, La Jolla, California; and 4Department of Pathology, University of Washington, Seattle, Washington

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Handa P, Morgan-Stevenson V, Maliken BD, Nelson JE, Washington S, Westerman M, Yeh MM, Kowdley KV. Iron overload results in hepatic oxidative stress, immune cell activation, and hepatocellular ballooning injury, leading to nonalcoholic steatohepatitis in genetically obese mice. Am J Physiol Gastrointest Liver Physiol 310: G117–G127, 2016. First published November 12, 2015; doi:10.1152/ajpgi.00246.2015.—The aim of this study was to determine the effect of iron overload in the development of nonalcoholic steatohepatitis (NASH) in a genetically obese mouse model (Leprdb/db). Leptin receptor-deficient mice were fed a normal or an iron-supplemented chow for 8 wk and switched to normal chow for 8 wk. All dietary iron (DI)-fed mice developed hepatic iron overload predominantly in the reticuloendothelial system. Hepatocellular ballooning injury was observed in the livers of 85% of DI mice, relative to 20% of chow-fed Leprdb/db. Hepatic malondialdehyde levels and mRNA levels of antioxidant genes (Nrf2, Gpx1, and Hmox1) were significantly increased in the DI mice. Hepatic mRNA levels of mitochondrial biogenesis regulators Pgc1α, Tfam, Cox4, and Nrf1 were diminished in the DI mice. In addition, gene expression levels of cytokines (Il6, Tnfα) and several innate and adaptive immune cell markers such as Tlr4, Ifn, CD11c, CD4, CD8, and Ifny were significantly increased in livers of the DI group. Strikingly, Nlrp3, a component of the inflammasome and Il1β, a cytokine elicited by inflammasome activation, were significantly upregulated in the livers of DI mice. In addition, RAW 264.7 macrophages loaded with exogenous iron showed significantly higher levels of inflammatory markers (Inos, Tufa, Mcp1, Tlr4). Thus dietary iron excess leads to hepatic oxidative stress, inflammasome activation, induction of inflammatory and immune mediators, hepatocellular ballooning injury, and therefore NASH in this model. Taken together, these studies indicate a multifactorial role for iron overload in the pathogenesis of NASH in the setting of obesity and metabolic syndrome.

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) is the most prevalent chronic liver disease in the world and is strongly associated with obesity and the attendant metabolic syndrome (17). NAFLD encompasses a broad spectrum of liver disorders, ranging from simple steatosis to its more severe form, nonalcoholic steatohepatitis (NASH), which progresses to cirrhosis or hepatocellular carcinoma in 15–25% of the patients (17). Oxidative stress is believed to be an important contributor to the pathogenesis of NASH (7, 6, 25). Reactive oxygen species (ROS) initiate an oxidative stress cascade, causing lipid peroxidation, which damages mitochondria, causing cellular and membranous injury and cell death (7, 6, 25). Iron is known for catalyzing the production of ROS through Fenton’s reaction (7, 6, 25).

In a large human study utilizing 849 patients, we reported that >34% of US patients enrolled in the NASH Clinical Research Network had stainable hepatic iron in liver biopsies (26). We showed in a separate study that elevated levels of serum ferritin, a cytokine associated with iron overload, is an independent predictor of histological severity and advanced fibrosis among patients with NAFLD (14). In particular, iron in reticuloendothelial system cells (RES) (consisting predominantly of cell types such as Kupffer cells, recruited hepatic macrophages, and other immune cells) was associated with advanced disease, including increased steatosis, portal and lobular inflammation, and advanced fibrosis in patients with NAFLD (26). In yet another study, we demonstrated that iron deposition in a RES compartment was significantly associated with increased levels of oxidative stress and apoptosis in patients with NAFLD (20).

However, despite the strong association between hepatic RES iron deposition and the worsening of NAFLD, it remains unclear as to whether iron deposition in hepatic RES cells is a cause or consequence of a more severe NAFLD. Given the prevalence of increased iron stores in patients with NASH (25, 26, 14, 20), the supplementation of iron in foods, and the safety and efficacy of reducing iron overload via diet or iron depletion (7, 6, 25), understanding the relationship between hepatic iron loading and NASH carries high significance and broad clinical impact.

In this study, we examined the effect of dietary iron overload in the context of genetic obesity, type 2 diabetes, and accompanying metabolic syndrome in a previously described murine model that was shown to recapitulate many of the features of human NASH (11). The goal of the present study was to test the hypothesis that dietary iron overload would lead to a severe NASH phenotype in an obese, diabetogenic mouse model, to
characterize the underlying mechanisms in vivo and to confirm these observations in vitro in murine macrophage cells.

MATERIALS AND METHODS

Animals and diet. Five-week-old male B6.BKS(D)-Lepr+/J diabetic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Lepr+/+ mice were maintained under standardized conditions of temperature (68–72°F) and humidity (30–70%), with light from 0600 to 1800 h. At 6–8 wk of age, mice were fed a normal chow diet (iron content: 280 ppm) or a chow supplemented with 2% carboxyl iron (iron content: 20,000 ppm; Test Diets, St. Louis, MO) ad libitum for 8 wk. After the 8 wk of iron loading, the mice were allowed to feed on the standard rodent chow for an additional 8 wk. The body weights for 8 wk of iron loading, the mice were allowed to feed on the standard rodent chow for an additional 8 wk. The body weights of all mice were determined weekly. At the study end point, fasting mice were killed, and serum and plasma samples were collected by cardiac puncture for assays of glucose, iron parameters, and liver enzymes. Livers were rapidly dissected, and pieces of the left and medial lobes were fixed in formaldehyde saline (10%) solution for histological analysis; the rest were snap frozen in liquid nitrogen and stored at −80°C until further use. All animal protocols were approved by the Benaroya Research Institute Animal Care and Use Committee.

Cell culture of RAW 264.7 macrophage cell line. RAW 264.7 cells were obtained from ATCC. They were maintained in DMEM, 10% FBS. Cells were grown in complete RAW cell media till 80% confluent. Just before the experiment, they were treated overnight in RAW cell media containing 1% FBS. They were then treated for 4 h with 250 μM ferric ammonium citrate (FAC) from 50 mM stock of FAC made in sterile water; Sigma Aldrich, St. Louis, MO) in the same medium. Treated cells were harvested and assessed for changes in gene expression by quantitative RT-PCR.

Serum and hepatic biochemical assays. Serum and plasma were separated by centrifugation at 4°C and stored at −80°C. Serum glucose, ferritin, and liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined using the Beckman DXC 800 Analyzer (Brea, CA). Serum hepcidin levels were measured in the serum using the Murine Hepcidin Compete™ ELISA developed by Intrinsc Lifesciences (La Jolla, CA) (10). Hepatic malonyldialdehyde (MDA) levels and hepatic triglyceride levels were quantified using the TBARS assay Kit (Cayman, Ann Arbor, MI) and the Triglyceride Quantiification Kit (MBL International, Woburn, MA), respectively, following the manufacturers’ instructions. Hepatic non-heme iron content was measured as previously described (2). Semiquantitative grading of iron was performed on liver sections using Perl’s Prussian Blue staining as previously described (2, 13, 14, 20, 26).

Histological analysis of liver tissues. Liver tissues were fixed in formaldehyde saline (10%) solution and stained with hematoxylin-eosin, Perl’s Prussian Blue for iron staining, and Masson’s Trichrome to assess fibrosis. Histological scoring was performed by a hepatopathologist (MMY) blinded to treatment assignment for steatosis

![Table 1. NASH histology and hepatic iron grade and distribution](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00246.2015)

Table 1. NASH histology and hepatic iron grade and distribution

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<thead>
<tr>
<th>Parameter</th>
<th>Chow</th>
<th>Dietary Iron</th>
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<td>Steatosis</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Inflammation</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Ballooning</td>
<td>0</td>
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<td>Hepatic HC iron grade</td>
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<td>Hepatic RES iron grade</td>
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<td>Hepatic HC iron distribution</td>
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<td>Hepatic RES iron distribution</td>
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Nonalcoholic Steatohepatitis (NASH) Clinical Research Network grading criteria were used to determine NASH histology and the iron grade and distribution in the hepatocellular (HC) and reticuloendothelial system (RES) compartments, in the livers of the chow- and dietary iron-fed mice and reported as median scores; n=5 chow-fed mice and n=8 dietary iron-fed mice.

qRT-PCR analysis of liver. The isolation of RNA, the preparation of cDNA, and the RT-PCR reaction were performed as previously described (11). Briefly, total RNA was isolated from liver, adipose tissue, and RAW 264.7 macrophage cells using the RNeasy kit (Qiagen, Valencia, CA). cDNA synthesis was then performed using 1–2 μg of total RNA with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The PCR reaction mixture contained 125–150 ng cDNA (total volume 5 μl), 1 μl of each of the primers (20 μM), and 10 μl SYBR Green mix (Invitrogen). RT-PCR was performed using an ABI 7900HT instrument (AB Systems, Foster City, CA). Levels of the target mRNAs were calculated relative to the reference gene, GAPDH, using the ΔΔCt formula. Several RT-PCR primer sequences have been described previously (11), and the results are available upon request.

Western blot analysis. Procedures related to protein separation and Western blotting have been described (11). Briefly, protein was extracted from liver samples and quantified (Micro BCA Protein Assay Kit; Pierce, Rockford, IL). Equal amounts of protein lysates (~20 μg) were used for immunoblotting using gradient 4–20% Tris-glycine gels (Bio-Rad, Hercules, CA). Blots were probed using the following primary antibodies: phospho-thr-172-AMPKα (Cell Signaling Technologies, Danvers, MA), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) antibodies (1:500; Abcam, Cambridge, MA), MAC-2 (1:1,000; Cedarlane, Burlington, Ontario, Canada), CD68 (Abcam), GAPDH at 1:1,000 dilution (Cell Signaling Technologies) or phospho-ser-727-STAT-3 (Cell Signaling Technologies). Corresponding secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

![Fig. 1. Dietary iron (DI) causes metabolic dysfunction. Chow (normal chow, NC) and DI-fed mice were examined with respect to glucose, serum alanine amino transferase (ALT), and serum aspartate amino transferase (AST) levels; n= 5 chow-fed mice, n = 8 DI-fed mice. *P < 0.05.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00246.2015)
Statistical analysis. Statistical analysis was performed using GraphPad version 5.0 (Palo Alto, CA). All values below \( P < 0.05 \) were considered significant. Values are represented as means ± SE, unless otherwise mentioned. For the normally distributed populations involving two group comparisons, Student’s \( t \)-test was performed; for multiple comparisons, one-way ANOVA followed by post hoc tests was performed; for nonnormally distributed populations, Mann-Whitney tests were performed.

RESULTS

Effect of dietary iron on body and liver weight. Relative to chow-fed animals whose average weight was 59.42 ± 1.19 g, the average weight of the DI-fed mice was 56.56 ± 1.13 g. Despite the reduced body weights in the DI-fed mice, they were not significantly different (\( P = 0.06 \)). There was no significant change in the liver mass (chow fed = 4.65 ± 0.12, iron fed = 4.58 ± 0.14).

Dietary iron overload exacerbates hyperglycemia and leads to elevation of serum liver enzymes. Relative to the chow-fed animals, DI-fed mice had significantly higher serum glucose levels (126.6 ± 13.4 vs. 201.1 ± 22.10 mg/dl, Fig. 1). Furthermore, serum liver enzymes AST and ALT were higher in the DI group. ALT levels were significantly increased relative to chow-fed controls (Fig. 1).

Dietary iron excess leads to elevated levels of serum and hepatic markers of iron overload. Serum hepcidin and serum ferritin levels were significantly enhanced in the DI mice relative to controls (Fig. 2, A and B). Furthermore, hepatic iron content, hepatic hepcidin (\( \text{Hamp} \)), and transferrin receptor 1 mRNA expression were significantly higher in the DI group compared with chow-fed controls (Fig. 2, C and D). Staining with Perl’s Prussian Blue revealed hepatic iron overload in the DI mice, whereas chow-fed control

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**Fig. 2.** Effect of DI overload on serum and hepatic markers of iron homeostasis. A: representative Perl’s Prussian Blue staining for iron on chow- and DI-fed mice at a magnification of \( \times 630 \); n = 5 chow-fed mice, n = 8 DI-fed mice. B: serum hepcidin was determined by performing serum ELISA. C: serum ferritin was determined using the Beckman Bioanalyzer. D: hepatic iron concentration in the chow- and DI-fed mice was determined using a biochemical assay. E: gene expression levels of hepatic hepcidin and transferrin receptor 1 in the livers of chow- and DI-fed mice. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).
mice did not demonstrate iron staining (Fig. 2E). The distribution of hepatic iron was largely in the RES compartment with a much lower hepatocellular (or parenchymal) distribution (Table 1, Fig. 2E). In addition, the grade of iron staining in the RES compartment was significantly greater than the hepatocellular compartment (Table 1, Fig. 2E).

**Dietary iron overload results in NASH histology.** We next asked whether dietary iron excess led to NASH histology. Dietary iron overload caused significant hepatocellular ballooning in 87.5% of the DI-fed mice compared with 20% chow-fed controls (Table 1, Fig. 3, A–C), whereas the scores for steatosis and inflammation were not significantly altered between DI and chow-fed mice (Table 1). The mean histology score for ballooning showed a significant elevation in the DI mice relative to chow-fed controls (Fig. 3C). As a result of the heightened ballooning injury in seven out of eight DI mice, the percentage of NASH diagnosis was 75% for the DI mice compared with 20% for the chow-fed controls (Table 1, Fig. 3).

**Dietary iron overload causes hepatic oxidative stress and NLRP3 inflammasome activation.** Because iron is a known contributor of oxidative stress (7, 6, 25), we next examined the effect of iron overload on hepatic oxidative stress. We observed that the levels of MDA (a derivative of lipid peroxidation) were significantly higher in the DI mice relative to chow-fed mice (Fig. 4A). Consistent with these findings, hepatic gene expression levels of antioxidant response genes such as heme oxygenase-1 (Hmox1), glutathione peroxidase-1 (Gpx1), and nuclear response factor 2 (Nrf2) were significantly augmented (Fig. 4B). In addition, we observed a decrease in the unfolded protein response (UPR)/ER stress-related transcription factor/oxidative stress regulator, X-box binding protein 1 (Xbp1) mRNA expression (16).

Furthermore, because oxidative stress is known to activate the nucleotide binding domain and leucine-rich repeat-containing (NLR) protein, NLRP3 inflammasome (32), we examined gene expression of Nlrp3 and the cytokine Il18 and found that they were significantly upregulated in the DI mice.

**Excess iron results in impaired hepatic mitochondrial biogenesis and fatty acid oxidation pathway.** Because oxidative stress is closely linked to mitochondrial dysfunction (7, 6, 25), we next interrogated hepatic gene expression levels of signature fatty acid β-oxidation/lipolytic transcripts such as carnitine palmitoyltransferase 1A (Cpt1a), Acyl-CoA oxidase 1 (Acox1), and lipoprotein lipase (Lpl) and found them to be significantly reduced in the livers of DI mice relative to the controls (Fig. 5A). Furthermore, the mRNA levels of additional regulatory mitochondrial biogenesis and fatty acid β-oxidation genes such as Pgc1α, Nrf1, mitochondrial transcription factor A (Tfam), and cytochrome c oxidase subunit IV (Cox4) were significantly diminished in the DI-fed mice (Fig. 5B). Consis-

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![Figure 3](http://ajpgi.physiology.org/)  
**Fig. 3.** Effect of DI overload on hepatocellular ballooning. A: hematoxylin and eosin (H&E)-stained liver histological images of representative chow-fed (left) and DI-fed mice (right) at ×200 magnification. The arrows point toward ballooning injury in the liver of the DI-fed mouse. B: representative H&E staining for chow- and DI-fed mice, at a magnification of ×630. The arrows point toward ballooning injury in the liver of the DI-fed mouse. C: graph represents average ballooning score from chow-fed mice (n = 5) and DI-fed mice (n = 8). **P < 0.01.
tent with these findings, Western blots performed with protein lysates prepared from the livers of chow-fed and DI mice revealed that phosphorylation status of AMPK-α at threonine-172 and PGC-1α protein levels (master regulators of lipid metabolism) were diminished (data not shown), indicative of reduced mitochondrial function/fatty acid oxidation in the livers of the mice with iron excess. Consistent with decreased lipolysis, we found hepatic triglycerides significantly elevated in the DI mice relative to chow-fed controls (Fig. 5C).

Iron overload leads to inflammatory activation in the epididymal white adipose tissue. We next assessed the effect of iron on the epididymal white adipose tissue (EWAT) with respect to lipid metabolism and proinflammatory activation. Dietary iron caused significant upregulation in mRNA expression of several lipid metabolism genes such as stearoyl COA desaturase (Scd1), Acox1, Lpl, and diacylglycerol O-acyltransferase 1, a rate-limiting triglyceride synthetic enzyme (Dgat1) (Fig. 6A). Furthermore, dietary iron caused inflammatory activation in the EWAT, as indicated by increased expression levels of chemokine Mcp1, its cognate receptor Ccr2, hypoxia inducible factor (Hif1α), and reduced levels of the M2 marker, arginase 1 (Fig. 6B).

Iron excess leads to increase in hepatic inflammatory mediators and immune cell activation. We next examined whether iron overload had an effect on inflammatory activation in the liver. We found that the livers of DI-fed mice displayed increased gene expression levels of several proinflammatory cytokines such as Il6, Tnfα, Tlr4, and Inos relative to chow-fed mice, associated with an increased M1 macrophage phenotype (Fig. 7A). In contrast, the levels of anti-inflammatory or M2-related macrophage markers such as Mgl1 and Tgfβ were reduced in the livers of DI mice (Fig. 7B). Western blots performed with liver lysates from chow-fed and DI-fed mice showed that the protein levels of macrophage markers MAC-2

Fig. 4. Dietary iron leads to oxidative stress and inflammasome activation in the liver. A: lysates from the livers from chow-fed mice and DI-fed mice were subjected to a TBARS assay to determine the presence of reactive oxygen species evidenced by the production of malonyldialdehyde. B: mRNA expression levels of oxidative stress genes such as Gpx1, Hmox1, Nif2, and Xbp1 and inflammasome markers such as Nlrp3 and Il18 were determined by RT-PCR in the chow-fed and DI-fed mice; n = 5 chow-fed mice, n = 5–8 DI-fed mice. *P < 0.05, **P < 0.01.
and CD68 were elevated in DI mice compared with chow-fed mice, indicating an increase in the macrophage activation levels in response to dietary iron excess (Fig. 7C). In keeping with these observations, the phosphorylated levels (Ser727) of STAT3 protein, a mediator of inflammatory activation, were elevated in the liver lysates from DI mice (data not shown). In addition, we found that the gene expression level of several immune cell markers such as CD4, CD8, and interferon (Ifn)-γ were upregulated in the livers of the DI mice. Additionally, mRNA levels of a dendritic cell (DC) marker, CD11c (gene name Itgax) were enhanced, whereas the expression levels of CD1d, a natural killer T (NKT) cell marker, were downregulated by iron administration (Fig. 7D).

Iron loading of RAW 26.7 cells leads to upregulation of inflammatory mediators. We utilized the RAW 264.7 cell line as a surrogate for the macrophages in the liver and asked whether iron administration led to inflammatory/immune cell activation, as was observed in the livers of the DI mice. RAW macrophages were treated with 250 μM FAC as described MATERIALS AND METHODS. Relative to untreated controls, we found that the gene expression levels of several chemokine, cytokine, and antioxidant mediators such as Inos, Tnfα, Mcp1, hepcidin (gene name Hamp), Hmox1, and Tlr4 were upregulated in response to exogenously administered iron (Fig. 8).

DISCUSSION

The goal of this study was to assess the effect of iron overload on NASH development in a murine model of obesity and type 2 diabetes. Our previous studies in human subjects with NAFLD have demonstrated a strong relationship between hepatic iron deposition, especially in RES cells to NASH and advanced fibrosis in NAFLD, but these have been cross-sectional studies (7, 14, 26). The goal of the present study was to determine whether hepatic iron loading in the setting of obesity and steatosis causes NASH and to demonstrate the underlying mechanisms of RES iron-mediated NASH.

We found that dietary iron overload in the setting of obesity and diabetes led to NASH by causing hepatocellular ballooning injury in the livers of DI mice. Ballooning is a hallmark of human NASH (3, 34) and has been difficult to recapitulate in most experimental animal models of NASH (11). Hepatocellular ballooning is accompanied by significantly increased hepatic oxidative stress and inflammasome activation in the DI mice. There are additional crucial pathways on which dietary iron exerted an effect in this
model. The DI mice displayed increased hepatic inflammatory immune cell activation and impaired hepatic mitochondrial fatty acid β-oxidation.

We observed that, whereas the NASH histology scores for steatosis and inflammation were largely unaltered between control and DI-fed mice, hepatic ballooning injury increased significantly in the DI mice. Although it is not clear what cellular mechanisms contribute to hepatic ballooning, oxidative stress, impaired proteasomal function, and cytoskeletal degradation have been thought to be involved (3, 34). Iron-induced oxidative stress and inflammasome activation could be contributing to the ballooning injury in our model.

DI mice experienced oxidative stress, as evidenced by increased levels of MDA and elevated hepatic gene expression levels of known antioxidant stress response genes such as Hmox1, Gpx1, and Nrf2. This appears to be a compensatory response to the ROS generated by iron. NRF2 activators have been demonstrated to prevent iron- or diet-induced NASH (22, 30). Additionally, heme oxygenase-1 has also been shown to play a protective role in concert with adiponectin against iron-induced injury in hepatocytes (15). Furthermore, the mRNA levels of XBP1, the UPR regulator, were downregulated in the DI mice. XBP1, in addition to its role in UPR/ER stress, has recently been found to be an oxidative stress regulator (16). Furthermore, NLRP3, an inflammasome danger sensor, thought to be involved in NASH pathogenesis, is believed to be linked closely to oxidative stress (32). Thus the upregulation of antioxidant genes in our model is indicative of RES iron-mediated oxidative stress-related liver injury. This might have contributed to the ballooning degeneration that was evident in the DI mice. We observed that seven out of eight DI mice had ballooning injury, relative to one out of five in chow-fed controls. Furthermore, the reduced levels of mitochondrial biogenesis and fatty acid β-oxidation genes such as Pgc1α and Nrf1 and reduced hepatic levels of activated AMPKα and PGC1α proteins in our model suggest that the mice experienced impaired mitochondrial function. Oxidative stress is known to lead to mitochondrial dysfunction. Iron-induced oxidative stress, inflammasome activation, and mitochondrial dysfunction could be the primary injury in this mouse model, contributing to NASH.

Iron overload had additional crucial effects on the iron-fed mice, in that DI mice showed immune cell activation in the liver, as evidenced by the activated status of macrophages, T cells, and DC marker(s), but reduced expression of a known beneficial NKT cell marker, CD1d. Although the exact role of NKT cells in NASH is not yet completely elucidated, CD1d-null mice have been reported to be predisposed to diet-induced metabolic disorders (21). Interestingly, although we did not see an alteration in the inflammation score in the NASH histology, there were significant changes in the gene expression levels and the protein levels of key immune cell markers, which have been shown to be important for NASH progression, demonstrating that the immune cells get activated or altered by iron overload in our model (Fig. 9). These observations suggest that gene expression analysis might be a more sensitive readout of immune cell activation, rather than histological analysis. Consistent with these in vivo findings, RAW macrophages treated with iron showed increased proinflammatory gene expression changes.

Iron was restricted to predominantly RES localization in the livers of DI mice. We have previously demonstrated in several cross-sectional human studies that RES localization of iron is associated with an increased severity of NASH and advanced fibrosis (26) and increased oxidative stress and apoptosis (20), important features of NASH (14, 20, 26). Furthermore, in vitro studies with iron-loaded Kupffer cells have previously shown that iron causes the activation of NF-κB, a potent inflammatory mediator, implicated in the
pathogenesis of NASH (4). Thus, in our model, the predominant localization of iron in the macrophages and the increased inflammatory activation of RAW macrophages upon iron-loading point to the role of iron in mediating its effect on hepatic macrophages by causing and augmenting hepatic inflammation.

Kupffer cells and recruited hepatic macrophages have been shown to be critical for the pathogenesis of NASH in several key rodent studies (23, 31). An increase in the polarization of macrophages toward the more "proinflammatory M1, or classically activated" phenotype and the reduction in the anti-inflammatory M2 phenotype have been causally linked to adipose tissue inflammation and obesity (18). Furthermore, studies are emerging that indicate a similar phenotype switch promoting proinflammatory M1 macrophage polarization and limiting M2 macrophage populations occurs in the liver as it progresses from benign fatty liver to NASH (9, 23, 31). In our model, iron appears to be the trigger to cause the switch in polarization toward the M1 macrophages. In addition, CD8 T cells, which are one of the main producers of the potent cytokine, interferon γ, were upregulated in the DI mice and have been implicated in the mechanisms leading to NASH (19, 28). Additionally, DCs, a type of antigen-presenting myeloid immune cell population (characterized by the presence of CD11c marker, which was elevated in the livers of the DI mice), have also been proposed to contribute to the pathogenic process that governs NASH (12). The livers of DI mice may have experienced hepatocellular injury and necroinflammation, not only as a result of hepatic oxidative damage, inflammasome activation, and accompanying mitochondrial dysfunction, but also attributable to the activation and alterations in key

Fig. 7. Effect of iron excess on hepatic inflammation and immune cell activation. A: gene expression levels of M1 markers such as Inos, Tlr4, Il6, and Tnfa and M2 markers such as Mgl1 and Tgfb in chow-fed mice and DI-fed mice were examined. B: liver lysates were assessed for the expression of CD68 and MAC-2, 2 macrophage-specific markers. C: mRNA levels of immune markers such as CD11c (Itgax), CD1d, CD4, CD8, and Ifnγ; n = 5 chow-fed mice, n = 5–8 DI-fed mice. *P < 0.05, **P < 0.01.
hepatic immune cell populations, the associated production of signature proinflammatory M1 cytokines and chemokines, and diminishing M2 markers in our model.

In addition to the above findings, we observed that the iron-supplemented mice displayed increased metabolic impairments. Whereas the genetically obese and diabetic Lepr
db/db mice are hyperglycemic at baseline, the mice supplemented with iron showed a significant increase in their fasting glucose levels, elevated liver function tests, increased oxidative stress levels as evidenced by increased MDA, and elevated serum

Fig. 8. Effect of iron excess on RAW 264.7 macrophages. RNA from untreated and iron-treated RAW cells was assessed for gene expression changes related to signatures of inflammation and oxidative stress such as Inos, Tifα, Mcp1, hepcidin (Hamp), Hmox1, and Tlr4; n = 3–5. *P < 0.05, **P < 0.01.

Fig. 9. Proposed model for the mechanisms by which iron could influence the adipose tissue and the liver in the genetically obese diabetic mice to cause nonalcoholic steatohepatitis (NASH). Iron overload leads to impaired lipid metabolism and inflammatory activation in the visceral adipose tissue. In the liver, dietary iron causes alteration in lipid metabolism, oxidative stress, M1 inflammatory activation, alteration in hepatic immune cells leading to hepatocellular ballooning injury, and thereby NASH.
hepcidin and ferritin levels. These findings are consistent with previous iron overload studies, which have demonstrated systemic effects of iron on several metabolic parameters (5–7, 25). These data suggest that dietary iron exacerbates systemic inflammation and metabolic impairments in this genetic model of “diabesity,” contributing to the worsening of the metabolic syndrome and the NAFLD in these mice. Increased serum ferritin has been associated with advanced NAFLD with severe fibrosis in our studies on patients (14). Furthermore, we have also demonstrated that increased serum hepcidin has been associated with increased RES iron in patients with NAFLD (24). We believe that the increased levels of iron deposition in the RES compartment could be a consequence of the elevated serum hepcidin levels in response to iron-induced inflammation in our model. This predominantly hepatic RES deposition in response to 2% carbonyl iron is consistent with a previous report (5). Increased serum hepcidin has also been associated with metabolic syndrome in humans (29).

We also observed that the hepatic and adipose lipid metabolism was impaired in our DI mice. Increased expression of lipolytic and triglyceride synthetic genes in the visceral adipose tissue in our model may have contributed to enhanced levels of free fatty acids. Consistent with these observations, we found that the hepatic triglyceride levels were elevated in the dietary iron-supplemented mice. Although iron overload has been demonstrated in a rodent model to cause oxidative stress and fibrogenesis (1), and obesity has been shown to alter iron homeostasis in the adipose tissue macrophages (27), and the interplay between iron overload/homeostasis, metabolic syndrome, and NASH has been reviewed previously (8, 33), to our knowledge, this is the first study examining the effect of iron overload on hepatic immune cell activation and its relation to NASH. In this study, we show that iron overload leads to alterations in liver immune cell populations such as recruited and resident macrophages, lymphocytes (CD4 and CD8), DCs, and NK cells. Our study is unique also in demonstrating that iron in and of itself causes the hepatic and adipose tissue macrophages to be M1 polarized.

In summary, we have demonstrated that dietary iron in the context of genetic obesity, with the attendant constellation of metabolic dysfunction, can accelerate the progression of NAFL to NASH because of adipose tissue dysfunction, RES deposition of hepatic iron, hepatic oxidative stress, inflammation associated with immune cell activation, and ballooning injury as described in the proposed model. Studies aimed at better understanding molecular mechanisms underlying and mitigating iron overload will be important next steps toward developing effective therapeutic interventions for iron-mediated NASH.

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