Hepatic macrophage iron aggravates experimental alcoholic steatohepatitis

Shigang Xiong,1 Hongyun She,1 An-Sheng Zhang,2 Jiaohong Wang,1,3 Hasmik Mkrtchyan,1 Alla Dynnyk,1 Victor R. Gordeuk,4 Samuel W. French,5 Caroline A. Enns,2 and Hidekazu Tsukamoto1,3

1Department of Pathology, Keck School of Medicine of the University of Southern California, Los Angeles, California; 2Department of Cell and Developmental Biology, Oregon Health and Science University, Portland, Oregon; 3Department of Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California; 4Center for Sickle Cell Disease, Howard University, Washington, DC; and 5Department of Pathology, Harbor-UCLA Medical Center, Torrance, California

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Xiong S, She H, Zhang A-S, Wang J, Mkrtchyan H, Dynnyk A, Gordeuk VR, French SW, Enns CA, Tsukamoto H. Hepatic macrophage iron aggravates experimental alcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 295: G512–G521, 2008. First published July 3, 2008; doi:10.1152/ajpgi.90327.2008.—One prime feature of alcoholic liver disease (ALD) is iron accumulation in hepatic macrophages/Kupffer cells (KC) associated with enhanced NF-κB activation. Our recent work demonstrates a peroxynitrite-mediated transient rise in intracellular labile iron (ILI) as novel signaling for endotoxin-induced IKK and NF-κB activation in rodent KC. The present study investigated the mechanism of KC iron accumulation and its effects on ILI response in experimental ALD. We also tested ILI response in human blood monocytes. Chronic alcohol feeding in rats results in increased expression of transferrin (Tf) receptor-1 and hemochromatosis gene (HFE), enhanced iron uptake, an increase in nonheme iron content, and accentuated ILI response for NF-κB activation in KC. Ex vivo treatment of these KC with an iron chelator abrogates the increase in iron content, ILI response, and NF-κB activation. The ILI response is evident in macrophages derived from human blood monocytes by PMA treatment but not in vehicle-treated monocytes, and this differentiation-associated phenomenon is essential for maximal TNF-α release. PMA-induced macrophages load iron dextran and enhance ILI response and TNF-α release. These effects are reproduced in KC selectively loaded in vivo with iron dextran in mice and more importantly aggravate experimental ALD. Our results suggest enhanced iron uptake as a mechanism of KC iron loading in ALD and demonstrate the ILI response as a function acquired by differentiated macrophages in humans and as a priming mechanism for ALD. IKK; NF-κB; labile iron; TNF-α; transferrin receptor-1; HFE

ENHANCED TNF-α EXPRESSION BY hepatic macrophages/Kupffer cells (KC) underlies chronic inflammation and cytotoxicity in alcoholic liver disease (ALD) (5, 11, 13, 21). In search for the mechanism responsible for abnormal TNF-α upregulation, we discovered that an increased chelatable pool of nonheme iron is causally associated with enhanced NF-κB activation and TNF-α expression by KC in experimental ALD (32). As we further dissected the mechanistic link, we identified the novel signaling for activation of IKK involving a transient rise in the intracellular level of labile iron (ILI) in LPS or TNF-α-stimulated KC (37). The ILI signaling takes place at or within 2 min after agonist stimulation and is transient much like the well-known intracellular calcium concentration ([Ca2+]i) response (37). This study also identifies peroxynitrite as an upstream effector that evokes the ILI response (37). Exogenous iron is also rapidly taken up by macrophages within 2 min (1) and substitutes for the endogenous ILI response to activate IKK in cultured KC (28) in a manner requiring activation and protein-protein interactions of TGF-β-activated kinase 1, p21ras, and phosphatidylinositol 3-kinase in caveolae (4). In fact, we propose that this activation of IKK by extracellular ferrous iron is an important and endotoxin-independent mode of NF-κB activation and proinflammatory gene expression in chronic liver disease.

Our findings also demonstrate that the ILI response is upregulated by an increased nonheme and chelatable pool of iron resulting from genetic or environmental manipulations. For instance, a deficiency in natural resistance-associated macrophage protein-1 (N ramp1), the macrophage-specific iron transporter responsible for iron efflux from late endosomes, results in an increased chelatable pool of nonheme iron, accentuated LPS-induced ILI response, and consequent enhancement of NF-κB activation and TNF-α expression (37). In experimental ALD, a similar association between the increased iron pool and NF-κB activation is noted (32). However, why nonheme iron content in KC is increased in ALD and whether this results in augmented ILI response are yet to be determined. Furthermore, it is unknown whether the ILI response is relevant in humans. In this study, we examined the mechanism and consequence of the increased nonheme iron content in KC in the rodent models of ALD. We also tested whether the ILI response is evident in human macrophages and enhanced after iron loading.

MATERIALS AND METHODS

Materials. PMA, LPS (Escherichia coli 055:B5), iron dextran, and dextran 500 were purchased from Sigma Chemical (St. Louis, MO). NycoPrep (AXIS-SHIELD POS AS) was obtained from Accurate Scientific (Delaware, OH). FeSO4 and Fe3O4 were from Calbiochem (San Diego, CA). Ultrafree-MC centrifugal filters (size-exclusion column) were purchased from Millipore (Bedford, MA) and SYBR GREEN real-time PCR master mixture from Applied Biosystems (Foster City, CA). Anti-mouse hepcidin and anti-mouse ferroportin-1 (FPN1) antibodies were purchased from Alpha Diagnostics International (San Antonio, TX), anti-human transferrin receptor (TIR) from Zymed Laboratories (South San Francisco, CA), and anti-mouse α-smooth muscle actin from Sigma Chemical.

Address for reprint requests and other correspondence: H. Tsukamoto, Keck School of Medicine of the Univ. of Southern California, 1333 San Pablo St., MMR 402, Los Angeles, CA 90033-9141 (e-mail: htsukamo@usc.edu).

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Cell isolation and culture. The use of animals for this study was approved by the Institutional Animal Care and Use Committee of the University of Southern California. KC were isolated from normal Wistar rats, alcohol-fed, pair-fed control rats, and iron dextran-injected mice by the Non-Parenchymal Liver Cell Core of Research Center for Alcoholic Liver and Pancreatic Diseases and Cirrhosis by in situ sequential digestion of the liver with pronase and collagenase followed by arabinogalactan gradient ultracentrifugation and adherence purification method as previously described (28, 32, 37). The cells were routinely cultured in DMEM containing 5% fetal calf serum (FCS) on plastic dishes for 2 days before experiments. KC isolated from alcohol-fed, pair-fed, and iron dextran-injected animals were cultured for 3 h or overnight in 2% FCS and studied immediately thereafter. For assessment of ILI signaling and TNF-α release, the cells were incubated in serum-free PBS and stimulated with peroxynitrite (10 μM) or LPS (500 ng/ml). For isolation of peripheral blood monocytes (PBMs), venous blood samples (40 ml) were withdrawn from healthy volunteer subjects who signed informed consent forms; and monocytes were isolated using NycoPrep 1.068 according to the manufacturer’s directions. Briefly, the blood was first diluted 1:10 with a solution of dextran 500 (8.0%, wt/vol, ~300 mOs m/kg) in 0.9% (wt/vol) NaCl and left at room temperature for 30–45 min. Leukocyte-rich plasma was removed and layered over 3 ml NycoPrep in a 15-ml Falcon tube and centrifuged at 600 g at 20°C for 15 min. PBMs were collected at the interface and washed twice by mixing with a solution of 0.9% (wt/vol) NaCl containing 0.13% (wt/vol) EDTA and 1% (vol/vol) FCS and centrifuging at 600 g for 7 min. Isolated PBMs were cultured in DMEM containing 10% FCS and antibiotics. The cells were treated with 100 nM PMA for 16 h at 37°C for induction of macrophage differentiation as determined by macrophage-specific gene expression by real-time PCR and fluorescence-activated cell sorting (FACS) described below.

Animal experiments. For chronic alcohol administration, male Wistar rats (14 wk old) and C57BL/6 mice (10 wk old) were aseptically implanted with gastrostomy catheters as described (33). A high-fat diet (35% calories as corn oil) was infused along with an isonutrient high-fat diet (35% calories as corn oil, 0.9% SDS, and complete protease inhibitor mixture). The rats and the incubation was stopped at the respective time points by removing PBS and adding 200 μl of lysis buffer (1.4 M NaCl, 0.1 M HEPES, pH 7.4, 1.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). Peroxynitrite obtained from the supplier had the concentration of 170–200 mM in 4.7% NaOH. We took the midpoint (185 mM) for our use. For addition to cell culture, the stock was first diluted 1:10 using 4.7% NaOH and added to media (e.g., 5.4 μl into 1 ml) to achieve the final concentration of 10 mM. For control, the same amount of 4.7% NaOH was added. A low molecular mass fraction (<5,000 Da) was used for a centrifugation of the lysate in a size-exclusion column (Millipore) at 8,600 g for 30 min at 4°C. Radioactivity of ultrafiltrate or total lysate was determined by a liquid scintillation counter as previously described (37). As an alternative method, ILI released by agonist stimulation was assessed by spectrophotometric detection at 430 nm of ILI chelated with

Table 1. Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>TR1</td>
<td>5'-CATGAGGAGGATTTGATGCCTTA and 5'-GCCCAAGAAGATAGTTCGGA</td>
</tr>
<tr>
<td>TR2</td>
<td>5'-CTACTTGCTGCTCGACACCCCT and 5'-TGAGATTGCATCTCCAGAA</td>
</tr>
<tr>
<td>HFE</td>
<td>5'-CTGAAAAAGGTTGCTATGATGTTG and 5'-AGGGCCATCTGACATTCTGCT</td>
</tr>
<tr>
<td>Zip14</td>
<td>5'-CTCAGGAAGGTTGCTATGATGTTG and 5'-ATTCGAGGAAACATATCGGTCA</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-GGAGGACAGACCTCAGAG and 5'-GCCATTGCAACAATCTTTT</td>
</tr>
<tr>
<td>FPN-1</td>
<td>5'-TGGCTCTACTTGCGGCGG and 5'-GACCAGCTGTAAGCACAC</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>5'-GCAATGAAAGACGTGGA and 5'-TGCGAGAAATTTGTGTC</td>
</tr>
<tr>
<td>N Brampl</td>
<td>5'-AGGCTTCCACATTCGTTTATC and 5'-GCAAGCGGACATACATT</td>
</tr>
<tr>
<td>DMT-1</td>
<td>5'-GCCTCGTGGTTTGTGTTT and 5'-GCCGGTCAACGAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-AGCAGCGGCTGCAAAC and 5'-GACCAGCGCAAGAAG</td>
</tr>
<tr>
<td>CD68</td>
<td>5'-TGCTCACCGGGGCAAC and 5'-CGGGGTCAGGAGAG</td>
</tr>
<tr>
<td>36B4</td>
<td>5'-TTTACGCGCTGTTATAC and 5'-GCCGGCGGCTGTC</td>
</tr>
</tbody>
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TR1, transferrin receptor; HFE, hemochromatosis gene; FPN-1, ferroportin-1; N Brampl, natural resistance-associated macrophage protein-1; DMT-1, divalent metal transporter-1.

Real-time PCR. Isolated cells were cultured in 0.5% FCS for 3 h, washed with PBS, and subjected to RNA extraction using TRIzol reagent following the manufacturer’s instruction. Two micrograms of total RNA were reverse transcribed and amplified by using the SYBR GREEN PCR master mixture (Applied Biosystems) in a Stratagene MX3000 real-time PCR. Each Ct value from the treated sample was standardized to that of 36B4 housekeeping gene and compared with control samples. The PCR primers used for real-time PCR are shown in Table 1. A single peak of the dissociation curve was always confirmed for each PCR reaction.

Immunoblot analysis and ELISA. Release of TNF-α into the media by cultured PBMs or KC was determined by commercially available immunoassay kits for rodent and human TNF-α following the manufacturer’s instruction (R&D Systems). Total KC lysates of control rats (Cont-KC) and alcohol-fed rats (Alc-KC) were prepared with an assay buffer (RIPA-PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and complete protease inhibitor mixture). The protein extracts were resolved on a 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were visualized by the enhanced chemiluminescence detection system (Pierce, IL). Plasma prohepcidin levels were determined by ELISA (DRG Instruments, Marburg, Germany).

ILI response assessment. Cultured rat or mouse KC or PMA-primed PBMs-derived macrophages were pretreated with or without iron dextran (1 μM) on a 60-mm dishes (1×10⁶ cells per dish) for 16 h at 37°C and 5% CO₂. The cells were then incubated with 5 μCi/ml FeCl₃ for 14–16 h in 5 ml of DMEM containing 10% FCS. Labeled cells were sequentially washed with 5 ml of warm PBS once, PBS containing 100 μM bathophenanthroline sulfonate once, and PBS twice. The washed cells were treated with LPS (500 ng/ml) or peroxynitrite (10 μM) in 5 ml of warm PBS for 2, 5, 10, or 20 min, and the incubation was stopped at the respective time points by removing PBS and adding 200 μl of lysis buffer (1.4 M NaCl, 0.1 M HEPES, pH 7.4, 1.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). Peroxynitrite obtained from the supplier had the concentration of 170–200 mM in 4.7% NaOH. We took the midpoint (185 mM) for our use. For additional cell culture, the stock was first diluted 1:10 using 4.7% NaOH and added to media (e.g., 5.4 μl into 1 ml) to achieve the final concentration of 10 mM. For control, the same amount of 4.7% NaOH was added. A low molecular mass fraction (<5,000 Da) was used for a centrifugation of the lysate in a size-exclusion column (Millipore) at 8,600 g for 30 min at 4°C. Radioactivity of ultrafiltrate or total lysate was determined by a liquid scintillation counter as previously described (37). As an alternative method, ILI released by agonist stimulation was assessed by spectrophotometric detection at 430 nm of ILI chelated with

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desferrioxamine and added to culture of Alc-KC and Cont-KC as previously described (37).

EMSA. EMSA for NF-κB was performed as previously described (28, 37). Briefly, nuclear proteins extracted from KC with and without prior treatment with L1 were incubated with P32-labeled double-strand κB consensus sequence and resolved on a 6% nondenaturing polyacrylamide gel for subsequent autoradiography. Two shifted bands detected by this method are previously shown to comprise p65/p50 heterodimer and p50/p50 homodimer by supershift assay (28).

**Results**

**Increased chelatable pool of iron causes enhanced ILI response in KC from ALD.** We have previously demonstrated that alcohol feeding to rats using the intragastric ethanol infusion model causes an increase in nonheme iron content, enhanced NF-κB activation, and cytokine expression in KC, all of which are abrogated by ex vivo treatment with the iron chelator L1 (1,2-dimethyl-3-hydroxypyridin-4-one) (32). Recently, we have also shown that agonist-stimulated IKK and NF-κB activation in KC requires novel ILI response as demonstrated as a peroxynitrite-mediated transient rise in ILI, which is positively regulated by increased iron content (37). Thus it is plausible that accentuated ILI response is a potential intermediary for the link between the increased nonheme iron content and enhanced NF-κB activation in KC in experimental ALD. To test this notion, we isolated KC from rats fed ethanol or control diet and assessed for the chelatable pool of nonheme iron and ILI signaling response. As previously shown, the nonheme iron content is increased 70% in Alc-KC compared with Cont-KC. After overnight treatment with the lipophilic iron chelator L1 (200 μM), the nonheme iron content in Alc-KC is normalized (a shaded portion of the bar), suggesting that the increment in the nonheme iron content in Alc-KC is due to an expansion of the chelatable pool of iron (Fig. 1A). These cells were also analyzed for the ILI response by the method involving chelation of liberated ILI with desferrioxamine and spectrophotometric determination of the iron chelator complex in cell suspension (37). This method was chosen over the Fe59-labeling technique because of a difficulty in performing a prolonged in vitro experiment of radioactive labeling for ILI analysis without altering in vivo changes of isolated cells. We have previously shown that a transient rise in ILI after LPS or peroxynitrite stimulation determined with Fe59 is also captured well by this spectrophotometric method (37). As shown in Fig. 1B, both Alc-KC and Cont-KC exhibit a transient peak of the iron chelator complex at 5–7 min after addition of peroxynitrite (10 μM), the effector molecule for the ILI signaling (37). Although the pattern of the ILI response is similar between the two groups, the peak for Alc-KC is significantly higher. Pretreatment of the cells with L1 completely abolishes the ILI response (Fig. 1B) and clearly attenuates enhanced NF-κB activation as assessed by EMSA (Fig. 1C) in Alc-KC. These results support the notion that enhanced ILI response constitutes the priming mechanism that bridges the increased chelatable pool of nonheme iron and enhanced NF-κB activation in Alc-KC.

**Increased FPN1 expression by Alc-KC.** Next, we analyzed the expression of iron transporters Alc-KC and Cont-KC to...
help understand the mechanism of iron accumulation in Alc-KC. As shown in Fig. 2A, the expression of FPN1, Nramp1, and divalent metal transporter-1 (DMT-1) mRNA are significantly increased in Alc-KC along with IL-6 and TNF-α, which serve as the positive controls for these cells (13). Zip14, which is a member of the SLC39A zinc transporter family, is also suggested to participate in nontransferrin-bound iron uptake (19). Expression of this transporter is unchanged by alcohol feeding. Hepcidin, which downregulates FPN1 expression via a posttranslational mechanism (7), is detected in Cont-KC and reduced in Alc-KC. Induction of FPN1 in Alc-KC is also confirmed at the protein level by immunoblot analysis. C: FPN1 mRNA level in cultured rat KC is markedly increased at 24 h after addition of iron sulfate following suppressive effects between 1 and 8 h. KC isolated from normal rats were cultured and treated with iron sulfate (50 μM) in serum-free media and RNA extracted at the indicated time points for real-time PCR analysis for FPN1. *P < 0.05 compared with the level at 0 h. D: treatment with the iron chelator L1 reduces FPN1 mRNA in cultured KC. Normal rat KC were isolated, cultured, and treated with L1 (100 μM), and RNA was extracted and analyzed as above. *P < 0.05 compared with the level at 0 h.

| Enhanced iron uptake by Alc-KC. Since the observed changes in the iron efflux transporters cannot explain iron loading in Alc-KC,
we next examined the mRNA expression of TfR1 and TfR2, as well as hemochromatosis gene (HFE) that is shown to regulate Tf-mediated iron uptake (22, 39). As shown in Fig. 3A, TfR1 mRNA but not TfR2 is significantly upregulated 5.7-fold in Alc-KC. HFE mRNA is also significantly increased twofold in Alc-KC. Increased TfR1 expression in Alc-KC is also confirmed at the protein level by immunoblot analysis (Fig. 3B). Densitometric analysis shows a significant 9.5-fold increase in TfR1 protein expression in Alc-KC (0.195 ± 0.075 vs. 0.020 ± 0.007, P < 0.05). We next determined uptake of iron by Alc-KC and Cont-KC using Fe^{59}Cl_3 in serum-containing media. Iron uptake by Alc-KC is significantly enhanced compared with Cont-KC over a 90-min period (Fig. 3C). These results suggest that iron uptake by Alc-KC is enhanced due to induction of TfR1, a dominant receptor type in KC and concomitant induction of HFE, which is suggested to promote Tf-dependent iron uptake in macrophages (22). Interestingly, a similar finding on alcohol-induced TfR1 induction has recently been reported for hepatocytes (16).

**ILI response is an acquired function of differentiated macrophages.** All experiments on ILI response described to date have utilized KC from rodents or murine macrophage cell line (RAW264.7) (37) but not macrophages from humans. Thus whether this phenomenon takes place in humans is an important question. To address this question, PBMs were isolated from human volunteers and tested for peroxynitrite-stimulated ILI response using Fe^{59}. This experiment failed to detect ILI response in LPS or peroxynitrite-stimulated human PBMs. Next, we treated isolated PBMs with PMA (10–200 nM) overnight to force their differentiation to macrophages as assessed by mRNA expression of macrophage markers (CD14 and CD68) (Fig. 4A) and detection of CD68-positive cells by FACS (Fig. 4B). These results demonstrate that the PMA concentration of 100 nM achieves the maximal differentiation effect. Using this concentration, we repeated ILI analysis. The cells were washed after overnight PMA treatment and rested for 6 h before the treatment with peroxynitrite. As shown in Fig. 4C, PMA-treated cells but not vehicle (DMSO)-treated cells, exhibit ILI signaling at 2 min after peroxynitrite addition. Acquisition of this signaling event by differentiated macrophages is associated with a fourfold increase in the cells’ ability to produce TNF-α in response to peroxynitrite compared with vehicle-treated cells (Fig. 4D). These results demonstrate that ILI response occurs in macrophages from humans and is a function acquired by macrophage differentiation. This acquired function confers that macrophage maximal cytokine release as both ILI response (data not shown) and TNF-α expression (Fig. 4D) is abrogated by pretreatment with the iron chelator L1.

Iron dextran increases macrophage iron content and promotes ILI signaling and TNF production. Next, we increased nonheme iron content in cultured rat KC by treating the cells

**Fig. 3.** A: transferrin receptor-1 (TfR1) and hemochromatosis gene (HFE) mRNA levels are increased in Alc-KC. TfR1, TfR2, and HFE mRNA levels were analyzed for Alc-KC and Cont-KC as described for Fig. 2A. *P < 0.05 compared with Cont-KC. B: Alc-KC express higher levels of TfR1 protein as determined by immunoblot analysis. C: Alc-KC have significantly higher uptake of Fe^{59}Cl_3 compared with Cont-KC. The cells isolated from the animals were incubated with Fe^{59}Cl_3 in serum-containing media for the indicated periods, and the radioactivity in the cells was counted after washing. *P < 0.05 compared with Cont-KC.
with iron dextran to determine how this manipulation influences peroxynitrite-induced ILI response. Iron in this large complex with dextran is readily and preferentially taken up by macrophages and is therefore used to selectively induce iron loading in macrophages (23). Cultured rat KC were treated with iron dextran (1, 10, and 100 μg/ml) overnight, washed extensively, and stimulated with LPS (500 ng/ml) in serum-free medium for 6 h. Iron dextran treatment at 1, 10, and 100 μg/ml increases the nonheme iron content 2.2-, 5.4-, and 11.1-fold, respectively (data not shown). These treatments do not affect basal TNF-α release (Fig. 5A, left) but enhance LPS-stimulated TNF-α release 190, 151, and 138%, respectively (Fig. 5A, right). Using the 1-μg/ml concentration, LPS-stimulated ILI response was determined using Fe59. The Fe59-specific activity was not different in both untreated and iron dextran-treated cells after Fe59 labeling. As predicted, LPS-induced ILI response is significantly accentuated in the cells pretreated with iron dextran (Fig. 5B). Next we tested iron dextran treatment for PBMs from rats. Iron dextran treatment does not increase nonheme iron content in vehicle-treated PBMs until the concentration is raised to 100 μg/ml (Fig. 5C). On the other hand, PMA-induced, PBM-derived macrophages dose dependently increase their nonheme iron content in response to iron dextran (Fig. 5C), suggesting their enhanced pinocytic activity. PMA-treated PBMs with or without subsequent iron dextran treatment (1 μg/ml) were tested for peroxynitrite-induced ILI response. As shown for KC, iron dextran-treated, PBM-derived macrophages show an accentuated ILI response to the agonist stimulation (Fig. 5D), suggesting that the priming with iron dextran occurs in macrophages in general.

Iron dextran administration in mice promotes KC TNF-α production and aggravates experimental ALD. Next, we extended the manipulation with iron dextran in vivo by injecting it subcutaneously into mice at different doses (10–100 mg/kg). A single subcutaneous injection of iron dextran achieves a gradual uptake by macrophages over several weeks (23). After testing various doses, the doses of 25 and 75 mg/kg were shown to increase 1.7- and 3.8-fold the nonheme iron content of KC isolated from mice at 2 wk after the injection, respectively. KC isolated from the mice given 25 mg/kg were tested for TNF-α release without or with peroxynitrite (10 μM) following Fe59Cl3 labeling. Note PMA-induced macrophages but not monocytes exhibit the ILI response. D: peroxynitrite-induced TNF-α release by PMA-induced macrophages derived from PBMs is fourfold greater than vehicle-treated PBMs. L1 treatment abrogates this differentiation-associated, maximal TNF-α expression. *P < 0.05 compared with vehicle-treated PBMs (no PMA). +P < 0.05 compared with PMA-treated PBMs without L1 treatment.
Fig. 5. A: normal rat cultured KC were pre-treated with the different concentrations of iron dextran and stimulated with LPS to determine the effects of iron loading on TNF-α release. Note iron dextran treatment alone does not affect the basal TNF-α release (left) but enhances LPS-stimulated TNF-α release twofold at 1 μg/ml concentration and 40–50% at 10–100 μg/ml of iron dextran (right). *P < 0.05 compared with the cells without the iron dextran treatment. B: iron loading by incubation with 1 μg/ml iron dextran overnight accentuates the ILI response in cultured KC as determined by LPS-induced [LMW-Fe]. *P < 0.05 compared with the cells without prior iron dextran treatment (control). C: PMA-induced macrophages from rat blood monocytes take up iron dextran to increase iron storage more efficiently than vehicle-treated monocytes (no PMA). Normal rat blood monocytes were cultured with or without PMA (100 nM) and subsequently treated with iron dextran for 24 h before the cells were collected for determination of non-heme iron content. *P < 0.05 compared with vehicle-treated rat blood monocytes (no PMA). D: iron dextran loading enhances peroxynitrite-induced ILI response in rat blood monocyte-derived macrophages by PMA treatment. PMA-induced rat macrophages with or without iron dextran treatment (1 μg/ml) were analyzed for peroxynitrite-induced [LMW-Fe], using Fe59Cl. *P < 0.05 compared with the cells without iron dextran treatment.

els (an index of liver damage) from 241 ± 33.7 (U/l) in alcohol-fed mice without iron dextran treatment to 315.5 ± 30.1 and 522 ± 71.4 in alcohol-fed mice given the two respective doses (Fig. 6B). Blind histological analysis and scoring of the liver specimens were also performed, and the results show similar trends of aggravated liver pathology (Fig. 6C). Furthermore, a morphometric analysis reveals 40 and 220% increases in the number of inflammatory cells (both mononuclear and polymorphonuclear cells) in the livers of alcohol-fed mice given 25 mg/kg and 75 mg/kg iron dextran, respectively, compared with alcohol-fed mice without iron dextran treatment (Fig. 6D). In mice given alcohol and 75 mg/kg of iron dextran, liver necrosis and accompanying inflammation are evident. In these necrotic and inflammatory areas, KC stained for iron are noted (Fig. 6E), and these cells are also stained positively for active p65 (Fig. 6F), suggesting that KC with iron accumulation have enhanced NF-κB activation. Liver fibrosis is also evident in these animals as demonstrated by reticulin staining (Fig. 6G) and numerous activated hepatic stellate cells identified by α-smooth muscle actin immunostaining (Fig. 6H). Iron dextran administration to pair-fed control mice only slightly affect plasma ALT levels and liver histology in pair-fed control mice (data now shown). This absence of drastic effects is consistent with the lack of stimulation for basal TNF-α expression in cultured KC exposed to iron dextran (Fig. 5A, left). In summary, these results demonstrate that KC iron loading and consequent NF-κB priming by iron dextran administration accentuate alcoholic liver damage and further suggest the importance of the primed ILI signaling in the pathogenesis of ALD.

DISCUSSION

ALD is a chronic inflammatory disease much like atherosclerosis, pulmonary fibrosis, glomerulosclerosis, and neurodegenerative diseases, and its pathogenesis is largely mediated through induction of the iron regulatory hormone hepcidin by inflammatory cytokines and the ability of this iron regulatory hormone to reduce the expression of the iron exporter FPN (7). However, in our rat model of ALD, FPN1 expression is induced in KC whose nonheme iron content is increased. Thus reduced iron export is an unlikely cause of iron accumulation in these cells, and FPN1 induction may reflect a consequence of iron loading (15). Indeed, our results from cultured KC exposed to iron or iron chelator support this notion of a positive relationship between iron and FPN (Fig. 2, C and D). Instead, our research has identified induced expression of TIR1 and enhanced iron uptake as the most probable mechanism of KC iron loading in
alcohol-fed rats. TfR1 is a predominant receptor type expressed by KC (40) and is increased nearly sixfold in Alc-KC (Fig. 3A). We also observed twofold induction of HFE Alc-KC. Mechanisms by which HFE controls iron homeostasis is poorly understood. HFE appears to have different effects depending on the cell types studied. For instance, HFE expression causes reduced intracellular levels in HeLa and H1299 cell lines associated with decreased Tf-mediated iron uptake (26,
Although this inhibition is thought to be mediated by the ability of HFE to compete with Tf for binding to TIR1 (17), this effect is also evident in the cells lacking endogenous TIR expression (3). In contrast, in macrophage and colonic intestinal cell lines, HFE increases cellular iron content via inhibition of iron export (6, 8). Consistent with this notion, reconstitution of HFE-deficient mice with wild-type bone marrow results in increased splenic iron storage, whereas the reverse reconstitution (wild-type mice with HFE-deficient bone marrow) causes decreased splenic iron content (20). Furthermore, transduction of wild-type HFE in PBMs from patients with HFE C282Y mutation normalizes Tf-dependent iron uptake and ferritin iron accumulation (22). Thus HFE appears to facilitate iron accumulation in macrophages, and this effect might have also contributed to increased iron content in Alc-KC via HFE induction.

The second key finding of the present study is the relevance of the ILI response in humans. Using PBMs from healthy volunteers, we have shown PMA-treated, PBM-derived macrophages, but not vehicle-treated monocytes, exhibit peroxynitrite-provoked ILI response. A functional significance of this ILI response is suggested by a fourfold enhancement in peroxynitrite-induced TNF-α production by macrophages that have acquired the ILI response. This notion is supported by the concomitant abrogation of the ILI response and TNF-α production by the iron chelator treatment. These results reveal that the ILI response is relevant in humans and is an acquired function of macrophage differentiation. This conclusion is not surprising since macrophages are already known to utilize iron as a vital tool for their host defense mechanisms. Such examples include a bacteriostatic effect on intracellular pathogens by iron deprivation of late endosomes achieved by the transporter Nramp1, unique in macrophages and an antimicrobial action of iron released by macrophages against extracellular microbes (12).

Finally, our study demonstrates that an increase in nonheme iron content augments the ILI response. This finding has a significant implication in understanding the link between KC iron accumulation and chronic liver disease such as ALD at a mechanistic level. Alc-KC have increased nonheme iron content, augmented ILI response and enhanced NF-κB activation, and the treatment of these cells with a lipophilic iron chelator (L1) coordinately suppresses all these three parameters. This delayed ILI response at 5–7 min detected by the chelator method vs. <2 min by tracing with Fe59 appears to result from the fact that the chelator is not reaching liberated ILI immediately. Nevertheless, this method accurately detects and validates agonist-stimulated ILI response demonstrated by the Fe59 method (37). Iron dextran is a convenient tool to validate our notion because it is more readily taken up by macrophages and increases KC iron content. This manipulation was used in vitro and in vivo in our study to confirm the positive relationship between iron content, ILI signaling, and TNF-α expression in KC. Furthermore, this priming technique was extended to the mouse alcohol model to demonstrate that KC iron loading aggravates experimental ALD. The iron chelator treatment has previously been shown to reduce the severity of liver injury in the rat intragastric ethanol infusion model (27) and KC NF-κB activation and liver damage in a rat model of cholestatic liver injury (18). In patients with viral hepatitis (14, 38) or nonalcoholic steatohepatitis (NASH) (29, 34), iron reduction therapy by phlebotomy improves liver pathology. In a recent elegant study on the rabbit model of NASH, erythropagocytosis by KC was shown to promote liver inflammation and fibrosis (25). In addition to increased iron uptake demonstrated in the present study, this mechanism of KC iron loading may also contribute to KC iron overload in ALD in which abnormalities of red blood cells are common and enhanced erythropagocytosis by KC is expected. We propose that the link between accentuated ILI signaling and enhanced NF-κB activation revealed in the present study provides a cellular and molecular explanation for the role of KC iron in the pathogenesis of chronic liver disease.

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