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Activation of the NLRP3 inflammasome by cellular labile iron

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Cellular labile iron, which contains chelatable redox-active Fe^{2+}, has been implicated in iron-mediated cellular toxicity leading to multiple organ dysfunction. Iron homeostasis is controlled by monocytes/macrophages through their iron recycling and storage capacities. Furthermore, iron sequestration by monocytes/macrophages is regulated by pro-inflammatory cytokines including interleukin-1, highlighting the importance of these cells in the crosstalk between inflammation and iron homeostasis. However, a role for cellular labile iron in monocyte/macrophage-mediated inflammatory responses has not been defined. Here we describe how cellular labile iron activates the NLRP3 inflammasome in human monocytes. Stimulation of lipopolysaccharide-primed peripheral blood mononuclear cells with ferric ammonium citrate increases the level of cellular Fe^{2+} levels in monocytes and induces production of interleukin-1β in a dose-dependent manner. This ferric ammonium citrate-induced interleukin-1β production is dependent on caspase-1 and is significantly inhibited by an Fe^{2+}-specific chelator. Ferric ammonium citrate consistently induced interleukin-1β secretion in THP1 cells, but not in NLRP3-deficient THP1 cells, indicating a requirement for the NLRP3 inflammasome. Additionally, activation of the inflammasome is mediated by potassium efflux, reactive oxygen species-mediated mitochondrial dysfunction, and lysosomal membrane permeabilization. Thus, these results suggest that monocytes/macrophages not only sequestrate iron during inflammation, but also mediate inflammation in response to cellular labile iron, which provides novel insights into the role of iron in chronic inflammation.

Iron is the major transition metal in the body and plays essential roles in synthesis of DNA, mitochondrial ATP, and heme [1]. Under physiologic conditions, the majority of circulating iron exists in complex with transferrin, which maintains iron in a soluble and nontoxic form. The transferrin–iron complex is taken up by cells via receptor-mediated endocytosis and is then used for biological processes including hematopoiesis. However, when the iron-binding capacity of transferrin is overwhelmed, non-transferrin-bound iron (NTBI), which is bound mainly by citrate, appears in the plasma, as seen in patients with iron overload conditions including hemochromatosis, thalassemia, and myelodysplasia [2]. NTBI is rapidly taken up by cells through unknown mechanisms and is subsequently incorporated into chelatable, redox-active iron pools called labile iron pools, leading to generation of reactive oxygen species (ROS) through the Fenton reaction [3]. The oxidative stress generated by cellular labile iron is thought to play a central role in the pathogenesis of organ dysfunction associated with iron overload, including that of the heart, liver, and endocrine systems. However, the effects of cellular labile iron on immune responses remain unclear.

Monocytes/macrophages play a crucial role in systemic iron homeostasis, in which they phagocytize and degrade damaged or senescent erythrocytes and recycle heme-associated iron [4]. The recycled iron can be either stored as ferritin in monocytes/macrophages [5] or released into plasma via the iron export protein ferroportin [6]. Under inflammatory conditions, pro-inflammatory cytokines, including interleukin (IL)-6 and IL-1, stimulate production

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of hepcidin from hepatocytes, which strongly inhibits the expression of ferroportin on monocytes/macrophages [7], leading to iron retention within these cells. Thus, the hepcidin–ferroportin axis is responsible for anemia of chronic inflammation [8].

The pro-inflammatory cytokine IL-1β is secreted predominantly by monocytes/macrophages through a cytosolic protein complex called the inflammasome [9]. The NOD-like receptor family, pyrin domain-containing 3 (NLRP3) protein is the best characterized subtype and plays a pivotal role in host defense against infection, as well as in a variety of sterile inflammatory diseases including gout [10], atherosclerosis [11], diabetes [12], and Alzheimer's [13]. The NLRP3 inflammasome is assembled in response to various danger signals, leading to activation of caspase-1, which, in turn, catalytically cleaves the inactive IL-1β precursor protein (pro-IL-1β) into biologically active IL-1β. Several mechanisms have been proposed for activation of the NLRP3 inflammasome, including cytosolic potassium efflux [14], lysosomal damage [15], and reactive oxygen species (ROS)-dependent and -independent mitochondrial dysfunction [16,17]. Given that excess cellular labile iron could be toxic to many mammalian cells because of its redox activity, this may be recognized as a danger signal in monocytes/macrophages. In this study we addressed whether cellular labile iron activates the inflammasome.

Methods

Human blood samples

Human peripheral blood mononuclear cells were isolated from healthy donors with informed consent. The study was approved by the ethics committee of Tohoku University Graduate School of Medicine.

Isolation and stimulation of mononuclear cells

The PBMC fraction was isolated by density-gradient centrifugation using SEPARATE-L (Muto chemical, Tokyo, Japan). Monocytes were purified by negative selection using the EasySep Human Monocyte Enrichment Kit (STEMCELL Technologies, Vancouver, BC, Canada). These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. After 4 hours of priming with 10 ng/mL LPS (from Salmonella Minnesota R595, Enzo Life Sciences, Farmingdale, NY), cells were stimulated with FAC (Wako, Osaka, Japan) at the indicated doses for 4 hours or with nigericin (20 μmol/L, Invivogen, San Diego, CA) for 30 min. For the inhibition assay, LPS-primed PBMCs were pretreated with the following reagents for 30 min before stimulation: the Fe^{3+} chelator deferiprone (400 μmol/L, Sigma); the Fe^{2+}-specific chelator BIP (400 μmol/L, Sigma); the pan-caspase inhibitor Z-VAD-FMK; the caspase-1/ICE inhibitor Z-WEHD-FMK (100 μmol/L, R&D Systems, Minneapolis, MN); glyburide (200 μmol/L, Sigma-Aldrich); *N*-acetyl-L-cysteine (5 or 25 mmol/L, Wako); CytoD (10 μmol/L Wako); and CA074-ME (40 nmol/L, Enzo). The amount of IL-1β in cell culture supernatants was measured with a human IL-1β ELISA (enzyme-linked immunosorbent assay) kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. Cell viability was measured with propidium iodide (2 μg/mL, Sigma), using FACSCantoII (BD Biosciences, San Jose, CA).

Cell lines and transfectants

THP1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin). THP1-defNLRP3 cells were purchased from Invivogen. These cells were differentiated by phorbol 12-myristate 13-acetate (PMA) (5 ng/mL, Sigma-Aldrich) 48 hours before stimulation on 12-well plates (10^6 cells/well). After overnight priming with LPS (100 μg/ml), cells were stimulated with the indicated doses of FAC for 6 hours. The amount of IL-1β in cell culture supernatants was measured.

Immunoblot analysis

Lipopolysaccharide (10 ng/mL)-primed PBMCs were stimulated with the indicated doses of FAC for 4 hours. As a positive control, LPS-primed PBMCs were stimulated with ATP (2.5 mmol/L, Sigma) for 30 min. Culture supernatants and total cell lysates were collected and then clarified by centrifugation. Proteins in culture supernatants were precipitated with Strataclean Resin (Stratagene, La Jolla, CA). Anti-human caspase-1 p10 Ab (C-20, Santa Cruz), anti-human NLRP3 monoclonal antibody (mAb) (D2P5E, Cell Signaling Technology, Boston, MA), anti-IL-1β mAb (D3U3EH, Cell Signaling Technology), and anti-α-tubulin antibody (DM1A, Santa Cruz) were used for detection.

Quantification of intracellular labile iron pools

Intracellular labile Fe^{2+} pools were quantitated using the iron-sensing fluorescent probe calcein-AM as previously reported [18]. Briefly, FAC-loaded cells were stained with calcein-AM (250 nmol/L, Dojin Chemical, Tokyo, Japan) for 15 min. These cells were washed twice with phosphate-buffered saline and then treated with or without BIP (400 μmol/L, Sigma) for 1 hour. Intracellular Fe^{2+} levels were defined as the difference of MFI (FL-1 channel) in the presence or absence of BIP using FACSCantoII.

Quantitative polymerase chain reaction

Lipopolysaccharide-primed or unprimed PBMCs were stimulated with or without FAC (20 mmol/L) for 4 hours. Total RNA from PBMCs was extracted using the RNeasy Lipid Tissue Kit (Qiagen, Hilden, Germany) after stimulation. Total RNA was reverse transcribed into cDNA using SuperScript III with oligo$(dT)_{12–18}$ (Invitrogen, Carlsbad, CA). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed in a DNA chromo 4 (Bio-Rad Laboratories, Hercules, CA). The following PCR primers were used: *IL-1B*: sense 5′-TTA CAG TGG CAA TGA GGA TGA C-3′, antisense 5′-GTC GGA GAT TCG TAG CTG GAT-3′; *NLRP3*: sense 5′-GGA GAG ACC TTT ATG AGA AAG CAA-3′, antisense 5′-GCT GTC TTC CTG GCA TAT CAC A-3′; *ACTB*: sense 5′-ATT GCC GAC AGG ATG CAG AA -3′, antisense 5′-GCT GAT CCA CAT CTG CTG GAA -3′. SYBR green (Qiagen) was used for quantification of the amplified DNA. Data were analyzed with Opticon Monitor Version 3

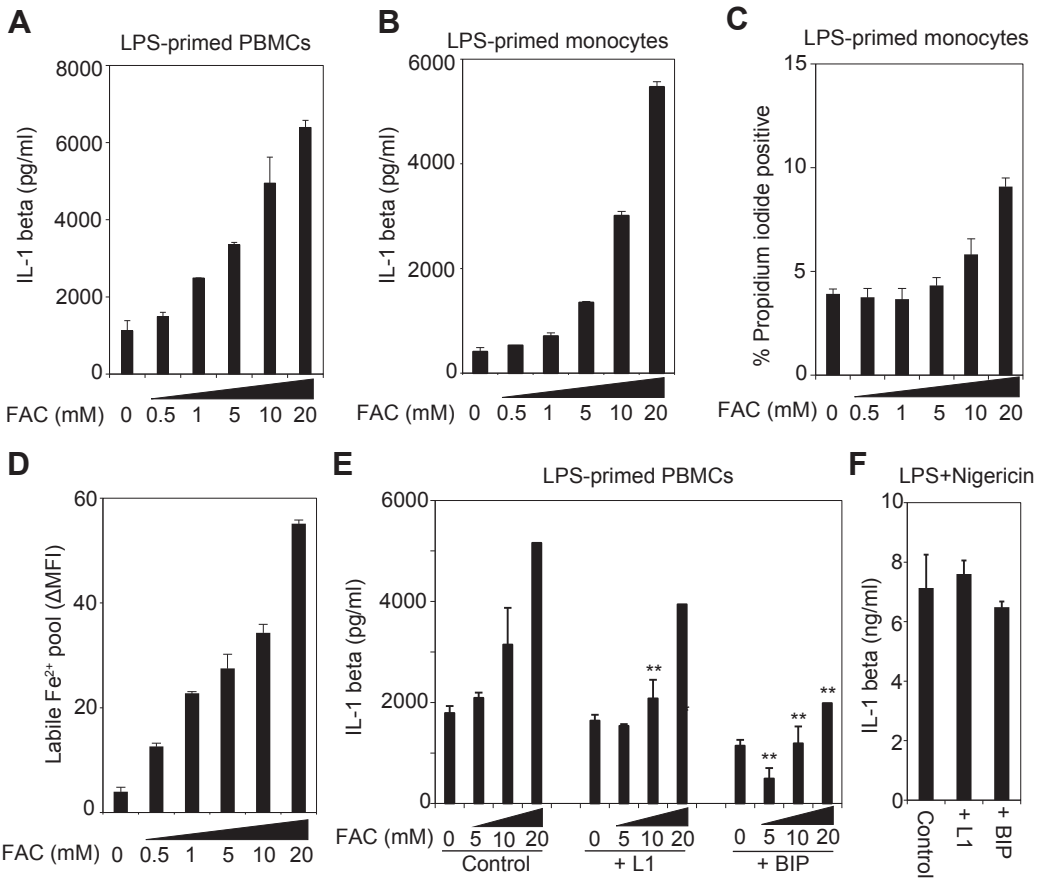

Figure 1. Cellular labile iron induces IL-1β production in human monocytes. (**A**) LPS-primed PBMCs (1×10^6/mL) and (**B**, **C**) LPS-primed monocytes (3×10^5/mL) were stimulated with the indicated doses of FAC for 4 hours. IL-1β in the culture supernatant was measured with ELISA. (**C**) Cell viability of monocytes was measured with propidium iodide. (**D**) After stimulation with the indicated doses of FAC, PBMCs were stained with calcein-AM (250 nmol/L), and then cells were incubated with or without the permeant Fe^{2+}-specific chelator BIP (400 μmol/L) for 1 hour. Cells were stained with anti-CD14 mAb, and subsequently the difference in fluorescence intensity of $CD14^+$ cells in the presence and absence of BIP was determined. (**E**, **F**) LPS-primed PBMCs (1×10^6/mL) were incubated with the Fe^{3+} chelator deferiprone (L1, 400 μmol/L) or BIP (400 μmol/L) for 30 min, and then cells were stimulated with the indicated doses of FAC for 4 hours (**E**) or nigericin for 30 min (**F**). The amount of IL-1β in culture supernatant was measured with ELISA. Data are means ± SD of triplicates. Similar results were obtained in at least three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with control.

software (Bio-Rad Laboratories), according to manufacturer's instructions.

ROS detection assay

Peripheral blood mononuclear cells (5×10^5) were stimulated with the indicated doses of FAC for 4 hours, and then cells were stained with allophycocyanin (APC)-conjugated anti-CD14 mAb (Biolegend). The level of ROS in the CD14-gated population was measured with the Total ROS Detection Kit (Enzo Life Sciences) according to the manufacturer's instructions. Mean fluoro-intensity (MFI) of the FL-1 channel in the CD14-gated population was determined by FACSCantoII.

Mitochondrial membrane potential assay

To evaluate mitochondrial damage, JC-10 assay (Abcam, Cambridge, UK) was used according to the manufacturer's instructions. Briefly, PBMCs (5×10^5) were treated with different doses of FAC for 4 hours. After being stained with APC-conjugated anti-CD14 mAb, cells were stained with JC-10 dye loading solution for 30 min. The percentage of CD14-positive cells with depolarized mitochondria (green fluorescence) was determined by FACSCantoII.

Fluorescence microscopy

To evaluate lysosomal membrane permeabilization, PMA-differentiated THP1 cells were loaded with Lysotracker Red (200 nmol/L, Invivogen) and Hoechst 33342 (1 μg/mL, Dojin Chemical) for 15 min. After being washed twice with phosphate-buffered saline, THP1 cells pretreated with 25 mmol/L NAC or not pretreated were incubated with various doses of FAC for the indicated periods. Fluorescence images were acquired using an Olympus IX81 microscope (Olympus, Tokyo, Japan) with a 20× objective lens. Merged images were obtained using Lumina Vision software (Mitani, Fukui, Japan).

Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student *t*-test: $*p < 0.05$; $**p < 0.01$. All data are represented as means ± SD.

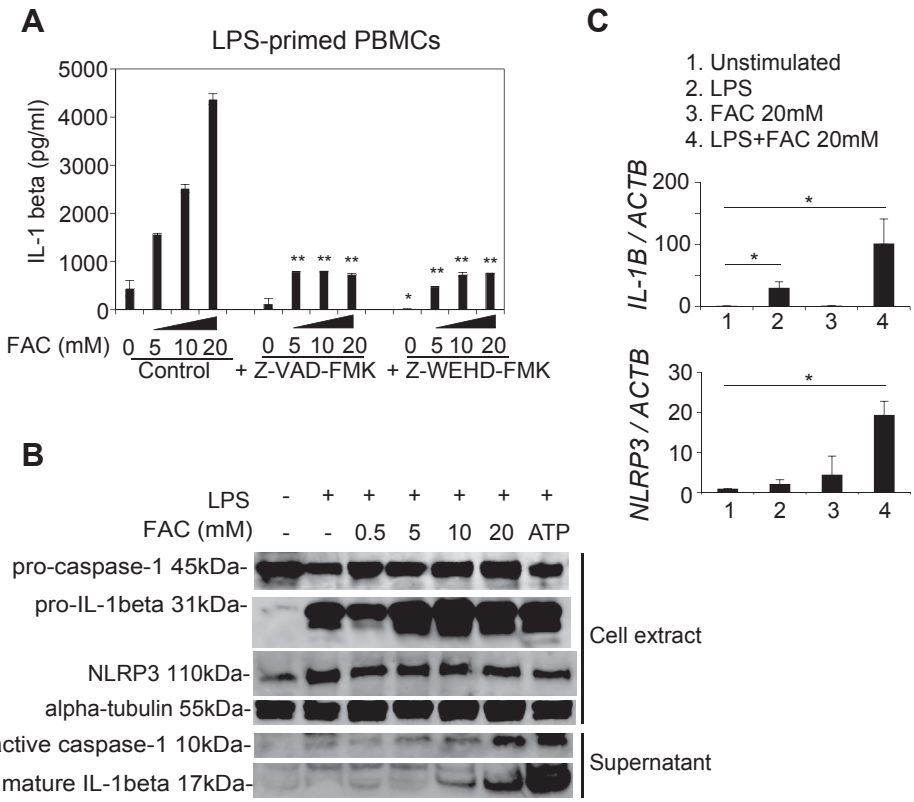

Figure 2. Cellular labile iron activates the inflammasome. (**A**) LPS-primed PBMCs (1×10^6/mL) were incubated with the pan-caspase inhibitor Z-VAD-FMK (100 μmol/L) or caspase-1 inhibitor Z-WEHD-FMK (100 μmol/L) for 30 min, and cells were then stimulated with the indicated doses of FAC for 4 hours. The amount of IL-1β in culture supernatant was measured with ELISA. Data are means ± SD of triplicates. $^{**}p < 0.01$ compared with control. (**B**) LPS-primed PBMCs were stimulated with the indicated doses of FAC for 4 hours. The rightmost lane represents LPS-primed PBMCs stimulated with 2.5 mmol/L ATP for 30 min (positive control). Maturation of caspase-1 and IL-1β in pooled supernatants and in cell extracts was analyzed by immunoblot. Anti-human α-tubulin antibody was used as a loading control. (**C**) Inflammasome-related gene mRNA levels were evaluated by quantitative RT-PCR after stimulation. The expression level of each gene relative to that of *ACTB* was calculated. The expression level of unstimulated cells was set to 1. Data are means ± SD of triplicates. $^{*}p < 0.05$ compared with unstimulated cells. Similar results were obtained in at least three independent experiments.

Results

Cellular labile iron induces IL-1β production in human monocytes

Non-transferrin-bound Fe^{3+} can rapidly enter cells and increases intracellular chelatable iron, known as labile iron pools, which contain redox-active Fe^{2+}. We first investigated whether excess cellular labile iron induces IL-1β production. To this end, we treated lipopolysaccharide (LPS)-primed peripheral blood mononuclear cells (PBMCs) with ferric ammonium citrate (FAC) as a source of non-transferrin- bound Fe^{3+} and evaluated the levels of IL-1β in culture supernatants. FAC induced IL-1β production in a dose-dependent manner in LPS-primed PBMCs (Fig. 1A) and LPS-primed monocytes (Fig. 1B). FAC also decreased cell viability of monocytes in a dose-dependent manner (Fig. 1C). To evaluate the labile Fe^{2+} pools within FAC-treated monocytes, we performed calcein-acetoxymethyl ester (calcein-AM) experiments [18]. We observed that FAC increased the amount of labile Fe^{2+} within monocytes in a dose-dependent manner (Fig. 1D). Furthermore, FAC-induced IL-1β production was inhibited not only by a Fe^{3+}-specific chelator, deferiprone (L1), but also by a Fe^{2+}-specific chelator, 2,2'-bipyridyl (BIP) (Fig. 1E), whereas these chelators did not inhibit the IL-1β production by nigericin (Fig. 1F). Thus, cellular Fe^{2+}, namely cellular labile iron, is involved in FAC-induced IL-1β production.

Cellular labile iron activates the NLRP3 inflammasome

Activation of caspase-1, formally known as IL-1-converting enzyme, is required for processing of pro-IL-1β into the mature form of IL-1β [19]. As illustrated in Figure 2A, IL-1β production is significantly inhibited by the pan-caspase inhibitor Z-VAD-FMK. In addition, IL-1β production is completely abrogated by the Z-WEHD-FMK caspase-1 inhibitor, indicating that caspase-1 is required for FAC-induced IL-1β production. Active caspase-1 constitutively exists in human monocytes, which allows the monocytes to produce IL-1β in response to priming signal alone [20]. We consistently found that LPS alone stimulated the production of IL-1β in a caspase-1-dependent manner, whereas FAC markedly

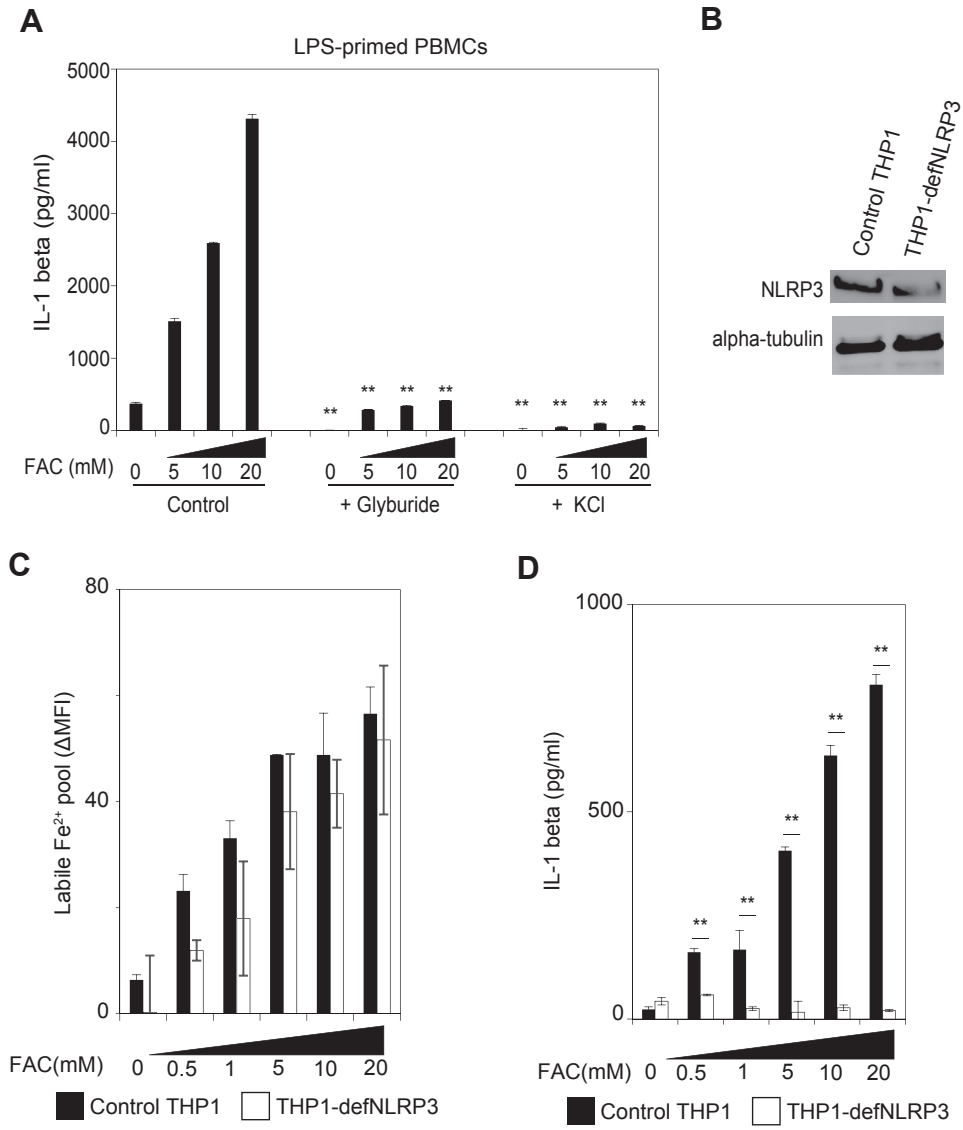

Figure 3. Cellular labile iron activates the NLRP3 inflammasome. (**A**) LPS-primed PBMCs (1×10^6/mL) were incubated with 200 μmol/L glyburide or 130 mmol/L potassium chloride (KCl) for 30 min and then stimulated with the indicated doses of FAC for 4 hours. IL-1β in the culture supernatant was measured with ELISA. (**B**) The NLRP3 protein levels of PMA-differentiated, mock-transfected THP1 cells and THP1-defNLRP3 cells. (**C**, **D**) PMA-differentiated THP1 cells and THP1-defNLRP3 cells (1×10^6/mL) were stimulated with the indicated doses of FAC for 6 hours. The labile iron pool in these cells was assessed as described in Figure 2B (**C**). IL-1β in the culture supernatant was measured with ELISA (**D**). Data are means ± SD of triplicates. $^{**}p < 0.01$ compared with control. Similar results were obtained in at least three independent experiments.

augmented IL-1β production (Figs. 1A, B and 2A). To confirm the involvement of inflammasome-mediated caspase-1 activation, we next performed Western blot analysis. As illustrated in Figure 2B, the mature form of IL-1β and activated form of caspase-1 (p10 subunit) were detectable in culture supernatant, indicating that inflammasome-mediated caspase-1 activation occurs in response to cellular iron. FAC stimulation alone failed to increase the mRNA of *IL-1B*, suggesting that it acts chiefly as an activation signal, not a priming signal (Fig. 2C).

Among the members of the inflammasome family, the NLRP3 inflammasome, in particular, is known to be activated in response to various sterile stimuli [21]. Thus, we hypothesized that cellular labile iron specifically activates the NLRP3 inflammasome. To this end, we incubated LPS-primed PBMCs with the NLRP3 inflammasome inhibitor glyburide [22] and observed significant inhibition of FAC-induced IL-1β production (Fig. 3A). Potassium efflux is a common trigger of NLRP3 inflammasome activation [14,23], and as expected, FAC-induced IL-1β production was completely abrogated in high-potassium media (Fig. 3A). To further define the involvement of NLRP3, we used PMA-differentiated macrophage-like THP1 cells and NLRP3-deficient THP1 cells (THP1-defNLRP3 cells) (Fig. 3B). As illustrated in Figure 3C,

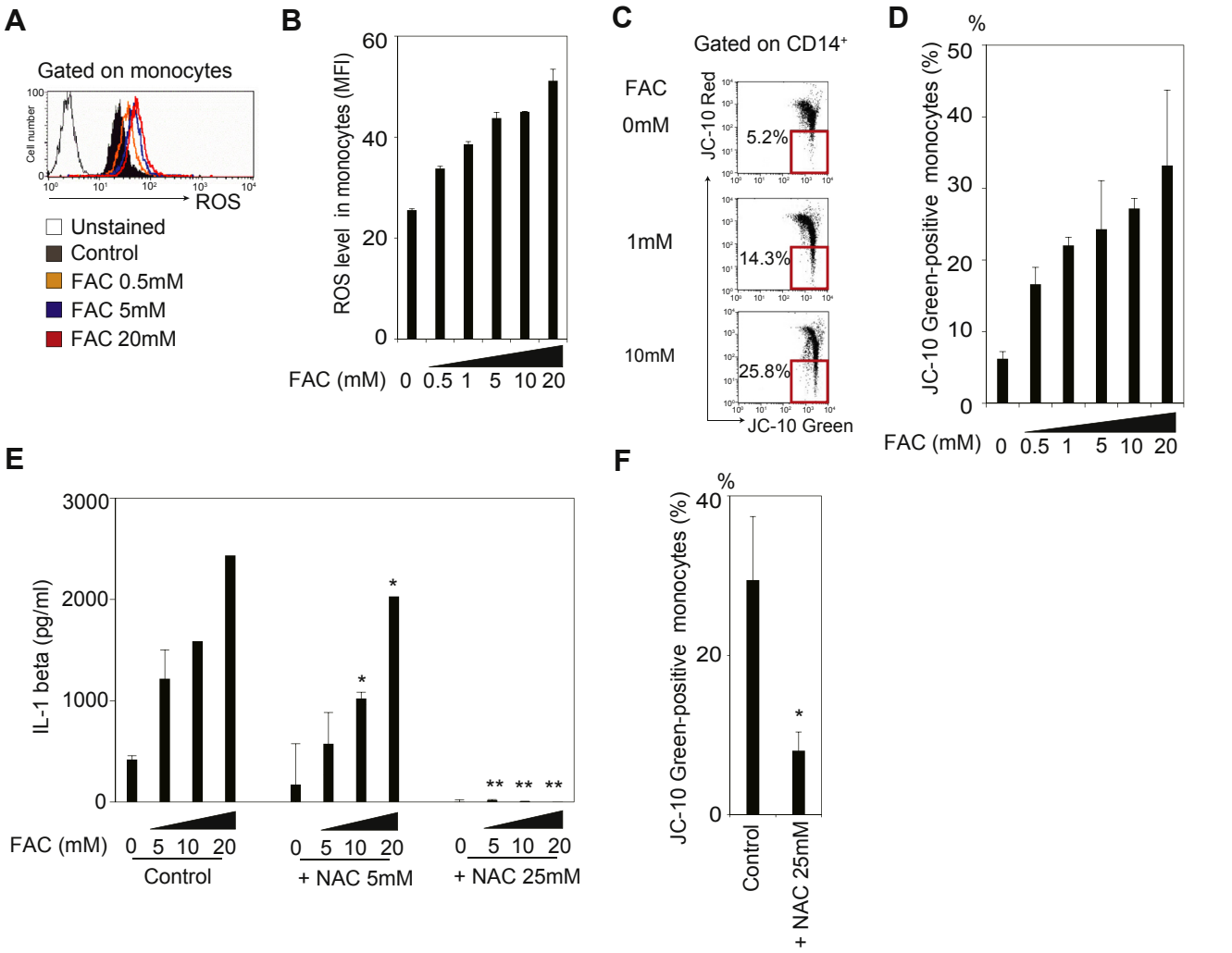

Figure 4. Cellular labile iron activates the NLRP3 inflammasome through ROS-dependent mitochondrial dysfunction. (**A**, **B**) PBMCs (5×10^5/mL) were stimulated with the indicated doses of FAC for 4 hours, and then cells were stained with anti-CD14 mAb. ROS levels in monocytes were determined by flow cytometry using the Total ROS Detection Kit. (**C**, **D**) LPS-primed PBMCs (5×10^5/mL) were stimulated with the indicated doses of FAC for 4 hours, and then cells were stained with anti-CD14 mAb. The percentage of monocytes with low mitochondrial membrane potential was determined as illustrated in (**C**), using the JC-10 assay. (**E**) LPS-primed PBMCs (5×10^5/mL) were incubated with 5 or 25 mmol/L NAC for 30 min, and then cells were stimulated with the indicated doses of FAC for 4 hours. The amount of IL-1β in culture supernatant was measured with ELISA. (**F**) LPS-primed PBMCs (5×10^5/mL) were incubated with or without 25 mmol/L NAC for 30 min and then stimulated with 20 mmol/L FAC for 4 hours. The percentage of monocytes with low mitochondrial membrane potential was determined. $*p < 0.05$, $**p < 0.01$ compared with control. Data are means ± SD of triplicates. Similar results were obtained in at least three independent experiments.

FAC treatment increased the level of cellular labile Fe^{2+} pools in both control-transfected THP1 cells and THP1-defNLRP3 cells; however, FAC-induced IL-1β production was observed in PMA-differentiated THP1 cells, but not in THP1-defNLRP3 cells (Fig. 3D). Taken together, these results indicate that cellular labile iron activates the NLRP3 inflammasome.

Cellular labile iron induces ROS production and mitochondrial dysfunction leading to NLRP3 inflammasome activation

Several studies have reported that ROS-dependent and -independent mitochondrial dysfunction activates the NLRP3 inflammasome [16,17]. Because cellular labile iron is known to catalyze ROS production [3], it is possible that ROS-mediated mitochondrial dysfunction is involved in NLRP3 inflammasome activation mediated by cellular labile iron. To address this possibility, we evaluated ROS generation in FAC-treated monocytes. We found that FAC treatment increased the ROS level in a dose-dependent manner (Fig. 4A and B). Similarly, FAC treatment increased the percentage of monocytes with decreased mitochondrial membrane potential (Fig. 4C and D). Furthermore, pretreatment with *N*-acetyl-L-cysteine (NAC) inhibited FAC-induced IL-1β production (Fig. 4E) and mitochondrial dysfunction (Fig. 4F). Collectively, we found that ROS-dependent mitochondrial dysfunction is involved in the activation of the NLRP3 inflammasome by cellular labile iron.

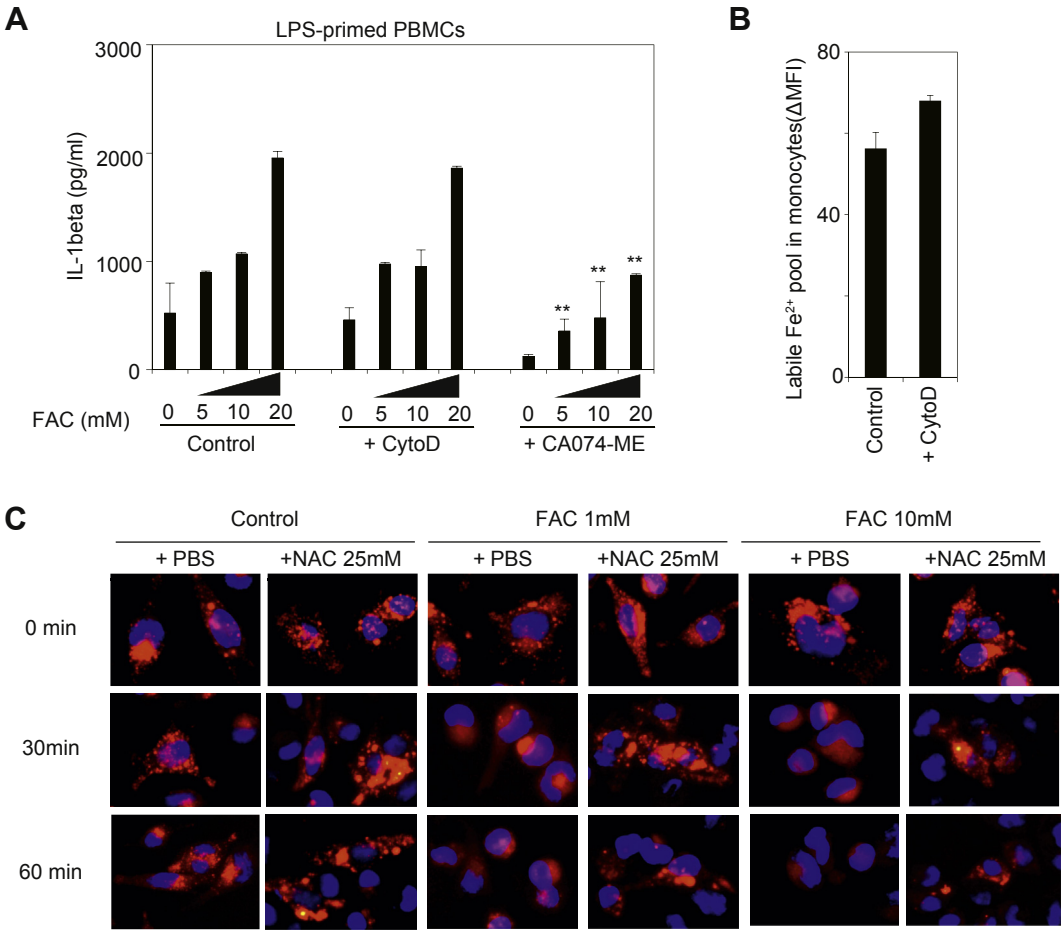

Figure 5. Reactive oxygen species-mediated lysosomal membrane permeabilization is involved in NLRP3 inflammasome activation by cellular labile iron. (**A**) LPS-primed PBMCs (5×10^5/mL) were incubated with 10 μmol/L CytoD or 40 nmol/L CA074-ME for 30 min, and then cells were stimulated with the indicated doses of FAC for 4 hours. IL-1β in the culture supernatant was measured with ELISA. (**B**) LPS-primed PBMCs (5×10^5/mL) were incubated with or without 10 μmol/L CytoD, and then cells were stimulated with 20 mmol/L FAC for 4 hours. The labile Fe^{2+} pools in monocytes were assessed as described in Figure 2B. Data are means ± SD of triplicates. $^{**}p < 0.01$ compared with control. (**C**) PMA-differentiated THP1 cells were loaded with 200 nmol/L Lysotracker Red and 1 μg/mL Hoechst 33342 for 15 min. Cells were incubated with or without 25 mmol/L NAC for 30 min, and then cells were stimulated with 1 or 10 mmol/LFAC for the indicated periods. Lysosomal membrane permeabilization was analyzed by fluorescence microscopy. Similar results were obtained in at least three independent experiments.

ROS-mediated lysosomal membrane permeabilization is involved in cellular labile iron-mediated activation of the NLRP3 inflammasome

The NLRP3 inflammasome has been reported to be activated by lysosomal damage following phagocytosis of crystalline and particulate materials [11,24]. Thus, we investigated the involvement of phagocytosis in FAC-induced IL-1β production. We found that cytochalasin D (CytoD), an inhibitor of actin polymerization, failed to inhibit IL-1β production by FAC-treated LPS-primed PBMCs (Fig. 5A). Furthermore, CytoD did not inhibit the increase in cellular Fe^{2+} in FAC-treated monocytes (Fig. 5B). These results indicate that actin-mediated phagocytosis is not required for the generation of cellular Fe^{2+} pools or NLRP3 inflammasome activation. In contrast, FAC-induced IL-1β production was significantly inhibited by the cathepsin B-specific inhibitor CA-074ME (Fig. 5A), suggesting the involvement of phagocytosis-independent lysosomal damage. ROS are known to induce lysosomal membrane permeabilization (LMP), leading to release of cathepsin B [25,26]. Thus, we evaluated whether cellular labile iron induces LMP through ROS generation by using PMA-differentiated THP1 cells stained with LysoTracker Red. Although FAC dramatically induced LMP in THP1 cells (seen as decreased fluorescence), pretreatment with NAC partially attenuated FAC-induced LMP (Fig. 5C). These results suggest that cellular labile iron activates the NLRP3 inflammasome through ROS-dependent LMP and subsequent release of cathepsin B.

Discussion

In this study, we found that excess cellular labile iron activates the NLRP3 inflammasome, leading to secretion of

IL-1β in human monocytes. The generation of ROS has been considered to be an upstream event for NLRP3 inflammasome activation in response to a wide range of stimuli [16,27]. Moreover, cellular labile iron is known to be a potent inducer of ROS through the Fenton reaction [28]. We consistently found that cellular labile iron activates the NLRP3 inflammasome through ROS-dependent mitochondrial dysfunction. The NLRP3 inflammasome is reportedly activated in response to lysosomal damage following phagocytosis of crystalline and particulate materials, including silica, alum, and cholesterol crystals [11,24]; however, we found that phagocytosis was not required for activation of the NLRP3 inflammasome by cellular iron. Instead, ROS-dependent LMP was involved in this process. Multiple pathways have been implicated for NTBI uptake, such as those including stimulator of Fe transport (SFT), DMT-1, ZIP14, and the L-type Ca^{2+} channel [29,30], which might contribute to generation of cellular labile Fe^{2+} pools in monocytes. Because our results are based on an in vitro study using PBMCs and the THP1 cell line with chemical inhibitors, there are limitations to our study. However, our results suggest a novel link between iron and inflammation.

Interleukin-1β, together with IL-6, plays key roles in iron homeostasis during inflammation. In response to IL-1β and IL-6, hepatocytes produce hepcidin, which downregulates the iron exporter channel ferroportin on monocytes/macrophages, leading to iron retention within these cells [7,8]. The role of cellular iron during innate immune reposes has been studied in mice with a myeloid lineage-specific ferroportin deficiency. These mice exhibit iron accumulation in reticuloendothelial macrophages of liver, spleen, and bone marrow and produce higher levels of tumor necrosis factor (TNF)-α in response to LPS than do control mice [31]. Conversely, low intracellular iron levels impair TRAM/TRIF-dependent TLR4 signaling in murine macrophages [32]. Moreover, iron chelation therapy ameliorates disease progression in experimental autoimmune encephalomyelitis [33] and animal models of rheumatoid arthritis [34], highlighting the importance of iron in inflammatory responses. However, the possibility that excess cellular iron may be recognized by an intracellular danger sensor, namely, the inflammasome, had not previously been addressed. Given that IL-1β stimulates production of hepcidin from hepatocytes, iron-mediated NLRP3 inflammasome activation, together with the hepcidin–ferroportin axis, might constitute a positive feedback loop that augments inflammation. Further study is necessary to understand the crosstalk between the hepcidin–ferroportin axis and NLRP3 inflammasome activation by cellular iron.

Iron-mediated organ dysfunction is a main cause of the mortality and morbidity in patients with thalassemia major, which causes iron overload as a result of increased intestinal iron absorption and/or transfusion dependency [35]. Notably, serum IL-1β levels in patients with thalassemia major are higher compared with those of asymptomatic carriers and heathy controls [36]. Moreover, several studies have found that iron replacement therapy stimulates production of pro-inflammatory cytokines including IL-1β in both healthy subjects and patients receiving hemodialysis [37,38]. Our results raise the possibility that NLRP3 inflammasome activation might be involved in the production of pro-inflammatory cytokines in patients with iron overload. However, it should be noted that plasma NTBI levels in patients with iron overload patients are <20 μmol/L [39], which is considerably lower than the concentration we used in this study. Given that iron deposition in the reticuloendothelial system is commonly observed in patients with iron overload, NLRP3 inflammasome activation might occur in reticuloendothelial cells that are exposed to locally high concentration of iron. Because inflammasome plays a critical role in inflammation-associated organ fibrosis [40,41], it is imperative to investigate the role of inflammasome activation in iron-mediated organ dysfunction, including liver cirrhosis, cardiomyopathy, and endocrinopathies. Overall, activation of the NLRP3 inflammasome by cellular labile iron might have broad implications for iron overload and chronic inflammation, and future study of this system is warranted.

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Author contributions

KN designed and performed experiments, analyzed data, and wrote the article. TK, NY, and MT performed experiments and analyzed data. TF, TI, and HH provided technical support and discussed experimental strategy. KO wrote the article and supervised experiments.

Conflict of interest disclosure

The authors declare that they have no competing interests.

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