Microglia are immune cells of the central nervous system and are implicated in brain inflammation. However, how brain microglia modulate transport and metabolism of the essential metal iron in response to pro- and anti-inflammatory environmental cues is unclear. Here, we characterized uptake of transferrin (Tf)-bound iron (TBI) and non-Tf-bound iron (NTBI) by immortalized microglial (IMG) cells. We found that these cells preferentially take up NTBI in response to the proinflammatory stimulus lipopolysaccharide (LPS) or β-amyloid (Aβ). In contrast, the anti-inflammatory cytokine interleukin 4 (IL-4) promoted TBI uptake. Concordant with these functional data, levels of the Tf receptor (TIR) in IMG cells were up-regulated in response to IL-4, whereas divalent metal transporter-1 (DMT1) and ferritin levels increased in response to LPS or Aβ. Similar changes in expression were confirmed in isolated primary adult mouse microglia treated with pro- or anti-inflammatory inducers. LPS-induced changes in IMG cell iron metabolism were accompanied by notable metabolic changes, including increased glycolysis and decreased oxidative respiration. Under these conditions, the extracellular acidification rate was increased, compatible with changes in the cellular microenvironment that would support the pH-dependent function of DMT1. Moreover, LPS increased heme oxygenase-1 (HO1) expression in IMG cells, and iron released because of HO1 activity increased the intracellular labile free-iron pool. Together, this evidence indicates that brain microglia preferentially acquire iron from Tf or from non-Tf sources, depending on their polarization state; that NTBI uptake is enhanced by the proinflammatory state; and that under these conditions microglia sequester both extra- and intracellular iron.

The brain is the most metabolically active organ in the body and has a high demand for iron. Iron serves as a cofactor in myelination, neurotransmission, oxygen transport, cellular division, and mitochondrial energy generation (1, 2). Left unchecked, a disruption in normal iron transport can lead to toxic side effects via the production of reactive oxygen species by excess iron. For example, high levels of iron have been associated with β-amyloid (Aβ) plaques that accumulate in Alzheimer’s disease and in microglial cells that are associated with these areas in brain (3). Microglia are the immune cells of the central nervous system (CNS), and brain inflammatory status is thought to be largely determined by their action (4). Microglia are dynamic cells that respond to changes in the local microenvironment by polarizing across a spectrum of pro- or anti-inflammatory states in an effort to maintain a stable neural environment (5). Polarization results in changes to microglial cell function, morphology, motility, glycolytic metabolism, and mitochondrial respiration (6, 7). Stimuli that induce a proinflammatory M1 state include the bacterial endotoxin lipopolysaccharide (LPS), proinflammatory cytokines such like interleukin 1β (IL-1β), and the Alzheimer’s disease protein Aβ. Conversely, cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13) act as anti-inflammatory stimuli in the context of microglial cell polarization to promote an M2 polarization state associated with resolution of inflammation and tissue repair (8, 9). A major goal to understand brain iron metabolism and neuroinflammation is to define the patterns of iron uptake by microglia when they are exposed to pro- or anti-inflammatory stimuli.

Many of the proteins involved in iron uptake, storage, and efflux in peripheral tissues and systemic circulation have been identified within the brain. Proteins involved in iron metabolism identified in microglia include divalent metal transporter-1 (DMT1), transferrin receptor (TfR), ferritin, and ferroportin (Fpn) (10–12). Cellular iron acquisition involves two distinct pathways, each of which is defined by the iron transport substrate: 1) the nontransferrin-bound iron uptake (NTBI) pathway and 2) the canonical transferrin-bound iron (TBI) uptake pathway. In the NTBI uptake pathway, Fe$^{3+}$ is reduced...
Microglial iron transport

at the cell surface to Fe^{2+} by an endogenous ferrireductase and transported through the plasma membrane and into the cytosol via a divalent cation transporter such as DMT1. In the TBI uptake pathway, iron is bound to transferrin as Fe^{3+}. This complex binds to TfRs and becomes internalized by endocytosis. Inside the endosome, the acidic environment promotes release of Fe^{3+} from Tf–TfR. Iron is then reduced to Fe^{2+} and translocated into the cytosol by DMT1 or other transporters (13, 14).

Microglia likely interact with NTBI and TBI as both forms of iron have been identified in cerebral spinal fluid (15). Relative to systemic levels circulating in plasma (25 μM), the amount of Tf in brain interstitial fluid is thought to be quite low (0.21–0.28 μM), whereas NTBI levels are quite high (0.3–1.2 μM). Although Tf moves iron across the blood–brain barrier, there is evidence that Tf-binding sites are saturated in the brain such that NTBI accumulates; this idea is supported by the presence of ferrous iron in cerebral spinal fluid (16). Thus, the actual form(s) of iron that may be taken up by microglia in the brain remains poorly characterized. A previous study of primary rat microglia suggested a correlation between microglial polarization, DMT1 expression, and microglial iron uptake (11). To better address these questions, we utilized an immortalized adult murine microglial (IMG) cell line (17). First, we demonstrate that IMG cells can acquire iron provided as either NTBI or TBI. Second, we show that transcript and protein levels of iron transport genes are differentially altered in response to pro- and anti-inflammatory stimuli and confirm that these changes accurately reflect the response of primary mouse microglia under the same conditions. Finally, we determine that utilization of NTBI or TBI pathways for IMG cell iron uptake depends upon microglial cell polarization. In particular, both LPS and the Alzheimer’s disease protein Aβ increase uptake of NTBI and expand the ferritin storage pool. The response to proinflammatory mediators serves to limit oxidative stress and potentially damaging ROS in the neural environment. Under proinflammatory conditions, IMG cells have increased glycolysis and extracellular acidification, supporting changes in the microenvironment favoring NTBI uptake by DMT1. LPS-treated IMG cells have decreased oxidative respiration, heme oxygenase-1 (HO1) is induced, and intracellular heme levels are reduced. Our study shows that the labile iron pool (LIP) is also increased under these conditions, suggesting that microglia sequester both intracellular iron released by heme catabolism and extracellular iron taken up by DMT1. Based on these data, we propose a model by which microglia actively modify the iron status of their environment in response to inflammation.

Results

IMG cell TBI uptake depends upon cellular iron status

TBI has been measured in the brain interstitial fluid albeit at lower amounts than systemic circulation (15). To examine whether microglia take up iron from this source, we incubated IMG cells with ^{55}Fe-Tf at 37 or 4 °C and monitored cell-associated ^{55}Fe over time. These results demonstrate that IMG cell ^{55}Fe-Tf uptake is both time- and temperature-dependent (Fig. 1A).

TfR is required for canonical Tf–TfR endosomal cycling and import of TBI into the cell. TfR expression is post-transcriptionally regulated by cellular iron status by the binding of iron-responsive proteins (IRPs) to iron-responsive elements (IREs) in the 3’-untranslated region (UTR) of the receptor transcript. High intracellular iron diminishes IRP–IRE interactions and increases nucleolytic turnover of the TfR transcript, resulting in a subsequent decrease in TfR protein level to diminish the cell’s ability to acquire iron from Tf (19). To determine whether IMG cell TfR is regulated by cellular iron content under these conditions, we examined TfR transcript and protein expression in IMG cells loaded for 18 h with or without ferric ammonium citrate (FAC). IMG cell iron loading resulted in a significant decrease in TfR transcript expression, protein expression, and ^{55}Fe-TBI uptake (Fig. 1, B–D). These data indicate that IMG cells can acquire iron as presented as TBI and that TBI transport is down-regulated when cellular iron status increases.

Characteristics of IMG cell NTBI uptake

Because significant levels of NTBI are found in cerebral spinal fluid and thought to be present in brain interstitial fluid (15), we examined characteristics of NTBI uptake by IMG cells. To first determine whether IMG cells could acquire extracellular NTBI, cells were treated for 18 h with or without 50 μM FAC as described above. The total steady-state intracellular ^{57}Fe content was then determined by inductively coupled plasma MS (ICP-MS). IMG cell iron content was more than 2-fold greater in cells treated with FAC compared with untreated control cells (Fig. 2A). Increased intracellular iron is typically sequestered in the iron storage protein ferritin. L- and H-ferritin are post-transcriptionally regulated by IRP binding to an IRE in the 5’-UTR of mRNA. Increased intracellular iron will disrupt this IRP–IRE interaction to promote protein synthesis (20). Western blot analysis confirmed that L-ferritin levels increase in FAC-treated IMG cells (Fig. 2B). To study iron flux under these conditions, IMG cells were incubated with ^{55}Fe-NTBI, and uptake was determined as described above for ^{55}Fe-Tf transport assays. The results of these experiments show that IMG cells take up NTBI in a time- and temperature-dependent manner and that similar levels of cellular uptake occur at pH 6.0 and 7.4 (Fig. 2C).

In addition to ferrous iron, several known divalent cation transporters will also transport manganese and zinc. Therefore, we examined divalent metal competition for ^{55}Fe-NTBI uptake by IMG cells. Both manganese and zinc blocked ^{55}Fe-NTBI uptake by IMG cells, irrespective of the pH of the assay buffer (Fig. 2D). The observed competition for uptake by these divalent cations suggests that Fe^{2+} is the actual transport substrate for NTBI uptake. To determine whether an endogenous ferrireductase might be limiting for transport, ^{55}Fe-NTBI uptake assays were performed at pH 6.0 and 7.4 with or without excess ascorbate present to reduce ferric to ferrous iron. The results of these experiments indicate that reduction is not rate-limiting for uptake of ^{55}Fe-NTBI by IMG cells (Fig. 2E). Although IMG cells appear to have sufficient endogenous ferrireductase activity to support NTBI uptake, ascorbate was added to all subsequent transport assays to avoid any potentially confounding effects. Taken together, these data indicate that IMG
cells can acquire non–Tf–bound iron present in their extracellular environment.

**Opposing pro- and anti-inflammatory stimuli dictate iron transport substrate preference by IMG cells**

We have previously demonstrated that LPS and IL-4 polarize IMG cells to more proinflammatory (M1) or anti-inflammatory (M2) states, respectively (17). To test the hypothesis that polarization of IMG cells would change the expression levels of transport-associated factors, cells were incubated for 18 h with or without LPS (10 ng/ml) or IL-4 (10 ng/ml), and mRNA was isolated for analysis by quantitative PCR (qPCR). LPS-treated IMG cells displayed increased DMT1 and H-ferritin transcript levels, whereas IL-4 treatment increased TfR mRNA abundance (Fig. 3A). Similar experiments confirmed the pattern of LPS- and IL-4–induced changes in primary adult mouse microglia (Fig. 3B).

To correlate changes in transcript levels with protein, Western blot analysis was carried out using lysates of IMG cells treated for 18 h with or without LPS or IL-4. Immunoblots were analyzed for DMT1, TfR, H-ferritin, and Fpn; β-tubulin was used as a loading control (Fig. 3C). Densitometry analysis revealed significant increases in LPS-treated cells for DMT1 and H-ferritin, whereas IL-4 treatment up-regulated levels of TfR but none of these other factors. A lower-molecular-weight DMT1 species that was detected most likely reflects newly synthesized unprocessed protein induced by LPS. We were unable to detect levels of two other divalent metal transporters, Zip8 and Zip14, in IMG cells (Fig. S1). Control experiments confirmed specific detection of Fpn (Fig. S1), but no changes in levels of the iron exporter were observed in response to LPS or IL-4.

To examine the functional consequences of LPS- and IL-4–induced changes in iron transporter expression (DMT1 and TfR, respectively), cellular NTBI and TBI uptake was measured after IMG cell polarization to the M1 or M2 state. For these experiments, IMG cells were first treated with LPS or IL-4 for 18 h prior to 55Fe uptake assays as described above. A significant increase in 55Fe uptake was noted in LPS-treated IMG cells when 55Fe-NTBI was used as a transport substrate, whereas uptake by IL-4–treated cells was similar to untreated control cells (Fig. 4A). These results are consistent with LPS-induced changes in cellular NTBI uptake reported for isolated primary rat microglia (11). To confirm a role for DMT1 in the LPS-induced transport pathway, ebselen was used as a pharmacological inhibitor of this transporter (21). Incubation with ebselen for 30 min prior to the start of and during the 55Fe-NTBI uptake assay reduced LPS-stimulated 55Fe-NTBI uptake by IMG cells (Fig. 4B). Unlike other divalent metal transporters, including Zip8 and Zip14 (22), DMT1 activity is proton-coupled. Therefore, we also determined the pH dependence of NTBI uptake by measuring the extent of activity in LPS-treated IMG cells at pH 6.0 and 7.4 compared with untreated control.
cells. Although increased uptake was observed under low-pH conditions, less $^{55}$Fe-NTBI was taken up at pH 7.4 (Fig. 4C).

These combined data support the idea that DMT1 plays a significant role in LPS-induced $^{55}$Fe uptake by IMG cells, and under proinflammatory conditions microglial cell NTBI uptake becomes markedly dependent on pH.

In contrast to the results obtained for NTBI uptake, when $^{55}$Fe-Tf was presented as a transport substrate, a significant increase in $^{55}$Fe uptake by IL-4–treated IMG cells was observed relative to both control or LPS-treated cells (Fig. 4D). To determine the role of TfR in this response, we used siRNA to knock down receptor levels induced by IL-4 treatment. TfR knockdown was confirmed in immunoblots of lysates from IMG cells transfected with control siRNA or TfR-specific siRNA and then treated for 18 h with IL-4 (Fig. 4E). Densitometric analyses confirmed reduction in TfR expression due to siRNA transfection by $\sim$56%. IMG cells transfected with control or TfR-specific siRNA and treated for 18 h with IL-4 were subsequently assayed for $^{55}$Fe-TBI uptake. Transfection with TfR-specific siRNA reversed the effect of IL-4 treatment compared with control
probed for the indicated proteins and the loading control

cells treated for 18 h with or without LPS (10 ng/ml) or IL-4 (10 ng/ml) were
treated for 18 h with or without LPS (10 ng/ml) or IL-4 (10 ng/ml) were
controlled (untreated) cells (Fig. 5).

Figure 3. Expression of iron-related factors in both primary microglia
and IMG cells treated with LPS or IL-4. Quantitative PCR was used to deter-
mine levels of iron-related transcripts in IMG cells (A; n = 9) or primary micro-
glia (B; n = 9) treated for 18 h with 10 ng/ml LPS or 10 ng/ml IL-4. The line
indicates control ΔCt set to 1. C, immunoblots of whole-cell lysates from IMG
cells treated for 18 h with or without LPS (10 ng/ml) or IL-4 (10 ng/ml) were
probed for the indicated proteins and the loading control β-tubulin. Shown are
representative blots with band densities relative to control determined
for at least three biological replicates. For DMT1, changes in the upper band
density were determined (arrow). Data are means ± S.D. One-way ANOVA or
a Student’s two-tailed t test was used to determine significance of LPS- and
IL-4–treated cells relative to control (untreated cells). *, p < 0.05; **, p < 0.01;
***, p < 0.005; %, p < 0.0005; #, p < 0.0001. Error bars represent S.D.

siRNA–transfected IMG cells (Fig. 4F). These data support the model that increased levels of TFR are responsible for enhanced
$^{55}$TBI uptake by IMG cells induced by IL-4. Thus, M1 and M2
polarized IMG cells enlist distinct transport mechanisms with
different substrate preferences for NTBI and TBI, respectively.

Aβ up-regulates DMT1 and ferritin to increase NTBI uptake by
IMG cells

We have previously shown that Aβ induces a proinflamma-
tory response in IMG cells (17). The Alzheimer’s disease pro-
tein appears to play an important role in microglial cell activa-
tion during neurodegeneration (3) and therefore is a relevant
physiological agonist known to induce a proinflammatory
response. To determine whether IMG cells regulate iron trans-
port under these conditions, we compared transcript levels in
cells treated with LPS or Aβ (Fig. 5A). Increased levels of DMT1
transcript were observed along with up-regulation of H-ferritin
message, similar to the pattern observed in IMG cells and pri-
mary microglia treated with LPS (Fig. 3). No change in TIR
transcript levels was observed, consistent with the idea that
NTBI rather than TBI uptake would be enhanced by M1
polarization. To confirm this idea, transport assays were car-
rried out after LPS or Aβ treatment. $^{55}$Fe-NTBI uptake by
IMG cells was enhanced under both conditions relative to
control (untreated) cells (Fig. 5B). These observations sup-
port the idea that microglia act to clear extracellular free iron
when recruited to Aβ-containing plaques during the neuro-
inflammatory response to disease.

IMG cell metabolic switch occurs in response to LPS

In many different cell types, the proinflammatory M1
response is associated with changes in cellular metabolism
reflected in increased glycolysis and decreased oxidative
metabolism (8, 9, 24). To examine whether similar metabolic
changes occur in IMG cells treated with LPS, we used Seahorse
XF extracellular flux assays to measure the glycolytic response
and rates of extracellular acidification along with mitochond-
drial stress and oxygen consumption rates. For these experi-
ments, cells were exposed to 10 ng/ml LPS for 4 h prior to flux
measurements performed as described under “Experimental
procedures.” As shown in Fig. 6A, LPS exposure was associated
with enhanced glucose-induced glycolysis and glycolytic cap-
city. Extracellular acidification due to increased glycolysis
would provide a cellular microenvironment compatible with
increased pH-dependent DMT1 activity (Fig. 4C). Additionally,
mitochondrial stress tests showed that LPS-treated IMG cells
had reduced respiration capacity (Fig. 6C). Reduced mitochon-
drial respiration further supports the idea that IMG cells
undergo metabolic reprogramming concurrent with changes in
iron transport substrate preference in response to proinflam-
matory stimuli. IMG cells do not appear to have significant
levels of spare mitochondrial respiratory capacity, and LPS-
stimulated IMG cells have further reduced oxygen consump-
tion when treated with the uncoupler carbonyl cyanide 4-(trif-
luoromethoxy)phenyldiazene (FCCP). LPS stimulation is
known to trigger complex I–mediated ROS production. ROS
further contribute to mitochondrial membrane depolarization
and network fragmentation. Dysregulated complex II activities
and/or reduced electron transport function may explain why
LPS-treated IMG cells have lower oxygen consumption rate (OCR)
upon FCCP addition.

M1 polarization induces heme degradation to increase the
intracellular LIP

Mitochondria are responsible not only for oxidative metab-
olism but also for heme synthesis, an important component
of cellular iron metabolism. Given the metabolic changes
observed with LPS stimulation, we further examined cellular
levels of heme and the heme-degrading enzyme HO1. LPS
induced both protein and transcript levels of HO1 (Fig. 7, A and
B). These changes were associated with a corresponding
decrease in cellular heme content in LPS-treated cells (Fig. 7C).
To determine whether iron released by HO1 heme degradation
entered the “free” LIP, the iron-binding dye calcein-AM was
used to determine changes elicited in response to LPS. These
measurements revealed that LIP was increased in LPS-treated
IMG cells and show that under proinflammatory conditions
levels of cellular free iron become elevated. This response
appears to be caused by iron released due to heme degrad-
because zinc protoporphyrin, an inhibitor of HO1, blocks this
response.

Microglial iron transport
Our investigation focused on identifying the major proteins and pathways involved in iron acquisition by microglia and how iron uptake and metabolism might be modulated under pro- or anti-inflammatory conditions. To address this question, we analyzed transcript expression, protein levels, and iron transport function in IMG cells treated with LPS and IL-4. Previous studies from our group demonstrated that IMG cells recapitulate the major functions of brain microglia and polarize to both proinflammatory (M1) and anti-inflammatory (M2) activation states (17). The results of this study show that, under resting conditions, IMG cells can take up iron presented as either the TBI or NTBI form.

**Figure 4.** DMT1- and TfR-mediated iron uptake by IMG cells is enhanced by LPS and IL-4, respectively. A, IMG cells were treated with LPS or IL-4 for 18 h and then subsequently incubated with 1 μM 55Fe-NTBI in pH 6 uptake buffer for 20 min at 37 °C to determine uptake. B, to test inhibition of NTBI uptake by ebselen, IMG cells were treated overnight with LPS and then incubated with 50 μM inhibitor for 30 min prior to the start of the 55Fe uptake assay. Total cell-associated 55Fe content was determined. C, IMG cells were treated with or without LPS for 18 h, and then NTBI uptake was determined at pH 6.0 or 7.4 as indicated in the panel. D, IMG cells were treated with LPS or IL-4 for 18 h and subsequently incubated with 0.5 μM 55Fe-Tf in serum-free medium for 1 h at 37 °C. Total cell-associated 55Fe content was determined. E, the immunoblot of whole-cell lysates from IMG cells transfected with control or TfR siRNA and then treated for 18 h with IL-4 prior to lysis. The blot was probed for TFR and loading control β-tubulin (β-tub). F, IMG cells were transfected for 48 h with control siRNA or TfR siRNA as indicated. Transfected IMG cells were then treated for 18 h with IL-4, and 55Fe-Tf uptake assays were performed. Data are means ± S.D. (n = 3). One-way ANOVA or Student’s t test was used to determine significance. *, p < 0.05; **, p < 0.005; ***, p < 0.0001. Error bars represent S.D.

**Figure 5.** Aβ stimulates NTBI uptake and increases DMT1 and ferritin levels. IMG cells were incubated for 18 h with or without LPS (10 ng/ml) or Aβ (1 μM). A, RNA was isolated from IMG cells after treatments, and qPCR was performed (n = 6). B, 55Fe-NTBI uptake assays were performed in triplicate using 1 μM 55Fe, 175 μM citrate, and 50 μM ascorbate in pH 6.75 uptake buffer. Total cell-associated 55Fe content was determined. Data are means ± S.D. One-way ANOVA or Student’s t test was used to determine significance of LPS-treated cells and Aβ-treated cells relative to control (untreated cells). **, p < 0.0005, ***, p < 0.0001. Error bars represent S.D.

**Discussion**

Our investigation focused on identifying the major proteins and pathways involved in iron acquisition by microglia and how iron uptake and metabolism might be modulated under pro- or anti-inflammatory conditions. To address this question, we analyzed transcript expression, protein levels, and iron transport function in IMG cells treated with LPS and IL-4. Previous studies from our group demonstrated that IMG cells recapitulate the major functions of brain microglia and polarize to both proinflammatory (M1) and anti-inflammatory (M2) activation states (17). The results of this study show that, under resting conditions, IMG cells can take up iron presented as either the TBI or NTBI form. Transport of both substrates was time- and temperature-dependent. Iron loading induced by exposure to FAC reduced Tf-mediated transport, corresponding to decreased TFR. Our 55Fe-TBI uptake studies reflect the known attributes of the canonical Tf–TfR pathway. Although TBI is a constituent of brain interstitial fluid, levels of NTBI are much higher, suggesting that it is also available to act as a substrate for iron transport by microglia (15). Using both ICP-MS analysis of steady-state iron levels and an isotopic tracer (55Fe) to monitor transport flux, our study shows that IMG cells can acquire and store NTBI. Thus, microglia take up extracellular iron from their environment from either Tf-bound or non-Tf-bound sources.

Polarization of brain microglia occurs in response to environmental cues from the local microenvironment. Such responses can be elicited from invading pathogens, protein aggregates...
such as those formed from Aβ peptides, extracellular cytokines, and other cell-signaling responses. In response to bacterial invasion in the periphery, systemic macrophages of the reticuloendothelial system will sequester iron from the blood in an effort to deplete the bacterium of the essential element and limit infection (25, 26). In contrast, bacterial pathogens have very limited access to the brain due to the blood–brain barrier. However, microglia are known to respond under conditions that break down the blood–brain barrier, for example pneumococcal meningitis as a result of encephalitis (27). We have shown previously that IMG cells respond to LPS by increasing their production of inducible NOS, an upstream component of the antimicrobial nitric oxide (NO) burst (6, 17). Here, we extend those findings to show that LPS induces changes in microglial cell iron uptake. Notably, LPS increased transcript and protein expression of DMT1 and ferritin in IMG cells; similar changes were confirmed in isolated primary mouse microglia. Correspondingly, NTBI uptake function was enhanced by LPS. This substrate selectivity induced by inflammation would limit extracellular iron to restrict pathogen growth. Entry of NTBI into IMG cells was associated with induction of the storage protein ferritin, which would safely sequester iron to limit its availability and to prevent oxidative damage. This neuroinflammatory response contrasts with the systemic inflammatory response of macrophages that lowers extracellular iron by down-regulation of iron export by Fpn in response to the regulatory hormone hepcidin (28). We have not observed induction of hepatic mRNA, any transcriptional or post-transcriptional changes in Fpn expression, or significant iron export function by IMG cells (results not shown). The lack of hepatic expression and regulation of Fpn function are consistent with in situ hybridization studies that have defined a rather limited expression of the hormone within the endothelium of blood vessels and the choroid plexus (29). These features suggest that, unlike macrophages of systemic iron metabolism, brain microglia exert metabolic influence over the distribution of pools of iron by uptake and sequestration rather than through regulation of cellular export. Because our data indicate that the Alzheimer’s disease protein Aβ induces similar inflammatory responses in IMG cell iron uptake and storage, it will be important to determine how microglial activity influences neurodegeneration.


**Microglial iron transport**

The up-regulation of pH-dependent DMT1 function is consistent with metabolic changes induced by LPS treatment of IMG cells, which include increased glycolysis and extracellular acidification. NTBI uptake in LPS-treated IMG cells appears to be limited under neutral pH and can be blocked by the DMT1 inhibitor ebselen, implicating the role of this transporter. Although we cannot rule out contributions of other divalent metal transporters like Zip8 and Zip14 to NTBI uptake by IMG cells, changes detected in transcript levels were minimal, and neither of these proteins were detected by Western blot analysis. These transporters are not proton-dependent and might contribute to NTBI transport, but we propose the model that uptake of iron is primarily mediated by DMT1 under proinflammatory conditions. Our results show that, under these conditions, NTBI uptake becomes strongly dependent on pH as cells condition their medium to become acidic. We also observed that oxidative metabolism is diminished in IMG cells treated with LPS. Correspondingly, HO1 is up-regulated, and cellular heme levels decrease. The degradation of heme and release of free iron appear to be reflected by increased cellular LIP. Thus, under proinflammatory conditions, microglia sequester both extra- and intracellular iron. We do not know how much of the labile cellular iron pool is ultimately targeted to be stored in ferritin, but our results show that LPS and Aβ both increase levels of the storage protein in IMG cells. It is interesting that the iron chaperone poly(C)-binding protein 1/2 has been shown to deliver iron to ferritin (30) and more recently has been implicated in iron transfer from DMT1 (31) and HO1 (32). Further studies to explore the function of iron chaperones and iron trafficking during inflammation are warranted.

IL-4 induces an alternative (M2) anti-inflammatory state in microglia to enhance resolution and repair, tissue remodeling, etc. Anti-inflammatory stimuli are known to limit microglial production of NO by increasing the expression of arginase-1, an enzyme that converts L-arginine to L-ornithine and urea. Arginine is an essential component for the production of NO by nitric-oxide synthase (33). We have reported that anti-inflammatory cytokines will induce IMG cells to polarize to an anti-inflammatory (M2-like) state associated with up-regulation of arginase-1 (17). The present study shows that IMG cells preferentially enhance acquisition of TBI when polarized by IL-4. Increased uptake by this pathway is associated with up-regulation of TRX transcript and protein levels. These changes may help to accommodate metabolic shifts to support mitochondrial respiration and limit the glycolytic response. In reticulocytes, it has been shown that the canonical TRX-mediated iron uptake pathways deliver iron directly to mitochondria (23). We do not yet know whether substrate utilization might direct transport substrate or whether changes in metabolism reflect altered cellular pools, but experiments are underway to determine how microglial cell iron transport and metabolism help to accommodate the anti-inflammatory response.

Our results demonstrate that microglial cell iron transport substrate preference depends upon cues from the environment. We hypothesize that, under pro- and anti-inflammatory conditions, polarized IMG cells have different metabolic stresses and requirements for iron that reflect a necessary dependence on NTBI versus TBI uptake. It is possible that iron may traffic to different cellular compartments via different chaperones determined by entry from either the NTBI or TBI pathway. We further speculate that microglia coordinate the iron uptake pathway to accommodate changes in energy metabolism elicited by M1/M2 polarization.

**Experimental procedures**

**Cell culture and reagents**

IMG cells were characterized previously, and the generation and characteristics of this cell line have been described in detail (17). IMG cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (4.5 g/liter), 10% (v/v) fetal bovine serum (FBS), and 100 units/ml penicillin/streptomycin. LPS was purchased from Sigma-Aldrich. IL-4 was purchased from Peprotech (Rocky Hill, NJ). Amyloid-β(1–42) was from rPeptide (Watkinsville, GA).

**TF-bound 55Fe uptake assays**

The loading of 55Fe onto Tf was performed as described previously (18). Briefly, 55FeCl (PerkinElmer Life Sciences) was incubated with Tf loading buffer (0.1 M HEPES, pH 7.5, 0.15 M NaCl) containing 20 mM NaHCO3 and 88 µM nitritolactric acid for 5 min at room temperature after which 20 µM apo-Tf was added and incubated for 1 h at room temperature. Non-Tf-bound 55Fe was removed from the 55Fe-Tf solution by buffer exchange using a Nanosep 10,000-molecular-weight-cutoff Omega spin column (PALL Corp., Port Washington, NY) 3 × 10 min at 5,500 rpm. This procedure yielded a 55Fe-Tf purity of >95%. IMG cells were incubated with or without LPS (10 ng/ml) or IL-4 (10 ng/ml) as indicated. Cells were washed three times with prewarmed phosphate-buffered saline (PBS) containing 0.5 mM MgCl2 and 1 mM CaCl2 (PBS+) and counted, and 2 × 106 IMG cells were used per replicate. IMG cells were incubated for 1 h at 37 °C with serum-free growth medium containing 55Fe-Tf, 1 mg/ml BSA, and 175 µM citrate. Cells were chilled on ice and washed twice with ice-cold PBS++. Cells were incubated with serum-free medium containing 1 µM mouse holo-Tf for 1 h at 4 °C. Cells were washed twice with PBS++ and lysed with 600 µl of solubilization buffer (0.1% (w/v) Triton X-100, 0.1% (w/v) NaOH) overnight at 37 °C. Whole-cell lysates were incubated at 85 °C for 10 min, and then 400 µl of this lysate was assayed for 55Fe using a HIDEX 300SL β counter (LabLogic, Brandon, FL). Remaining lystate was used for quantification of protein content. Counts were converted to pmol of 55Fe using a 55Fe standard curve and normalized for protein content.

**Non-Tf-bound 55Fe uptake assays**

IMG cells grown to semiconfluence in 6-well poly-d-lysine–coated tissue culture plates were used for 55Fe uptake assays. Cells were washed twice with prewarmed (37 °C) or prechilled (4 °C) PBS++ followed by incubation with 1 µM 55Fe and 175 µM citrate with or without 50 µM ascorbate in pH 6.0 or 7.4 uptake buffer (25 mM Tris, 25 mM MES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5 mM glucose) for 20 min at 37 or 4 °C as indicated. Cells were chilled on ice for 5 min, then washed three times with ice-cold quencher buffer (37.5 mM suc-
Clinical acid, 62.5 mM Tris, 12.8 mM EDTA, pH 6.0), and lysed with 600 µl of solubilization buffer for 1 h at 37 °C. Whole-cell lysates were incubated at 85 °C for 10 min, and then 400 µl of this lysate was assayed for 55Fe content. Remaining lysate was used for quantification of protein content. The amount of 55Fe taken up was normalized to protein content.

**ICP-MS**

IMG cells grown on 100-mm tissue culture dishes were used for ICP-MS analysis of 57Fe content. Cells were incubated for 18 h with or without FAC (50 µM) at 37 °C in 5% CO2. Cells were washed three times with ice-cold PBS + 1 mM EDTA to chelate cell surface–bound iron. Cells were removed from the dish into a 15-ml conical centrifuge tube, pelleted, and weighed. Cell pellets were digested for 24 h at room temperature with nitric acid (1 ml/g of cells). Samples were analyzed at the Harvard School of Public Health Trace Metals Laboratory using a PerkinElmer Life Sciences 6100 ICP-MS with a dynamic reaction cell.

**Immunoblotting**

IMG cells were incubated for 18 h with LPS (10 ng/ml) or IL-4 (10 ng/ml). IMG cells were lysed with radioimmunoprecipitation assay buffer plus protease inhibitors (Calbiochem, catalog number 539134; 1:100 dilution) for 60 min on ice. Protein concentration was determined, and 20–30 µg of protein/sample was heated for 10 min at 75 °C, cooled on ice, and then resolved by 10% (w/v) SDS-PAGE (HO-1) or 4–15% (w/v) SDS-PAGE (TfR, Fpn, and ferritin). The protein was transferred onto a nitrocellulose membrane (0.2 µm) using a Trans-Blot Turbo transfer system (Bio-Rad). The resulting membrane was blocked for 1 h at room temperature in TBST (TBS plus 0.05% (v/v) Tween 20) and then incubated for 1 h at room temperature with IRDye 800CW donkey anti-rabbit or anti-mouse IgG (1:5000 dilution; LI-COR Biosciences, Lincoln, NE, catalog number 926-32212/3, lot number C60322-02/4) or IRDye 680RD donkey anti-rabbit IgG (1:5000 dilution; LI-COR Biosciences, catalog number 926-68073, lot number C60217-06) in TBST plus 3% milk. The membrane was washed three times with TBST and imaged using LI-COR Odyssey 2.1 IR detection technology.

**Quantitative RT-PCR**

Total RNA was extracted from IMG cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was purified and on-column DNase-treated using the Direct-zol RNA Miniprep kit from Zymo Research (Irvine, CA) according to the manufacturer’s instructions. Purified RNA was then reverse transcribed using the SuperScript® III First-Strand Synthesis System (Invitrogen) with oligo(dT)20 primers and random hexamers. Quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and the StepOnePlus Real-Time PCR System (Life Technologies). In all cases, 36B4 was used as an internal control. Primers used for qPCR are listed in Table 1.

**Seahorse XF extracellular flux assays**

IMG cells were plated at 85,000 cells/well into XF 24 cell culture microplates in regular growth medium with 10% FBS and grown overnight at 37 °C in 5% CO2. The next morning, control vehicle or LPS (10 ng/ml) was added to the appropriate wells and incubated for 4 h at 37 °C in 5% CO2. For glycolytic stress test experiments, wells were washed and incubated with pH 7.4 DMEM (Corning Cellgro, catalog number 90-113-PB) plus 1 mM glutamine (Gibco, catalog number 25030-081) for 1 h at 37 °C minus CO2. The ECAR was measured at baseline and after sequential additions of d-glucose (10 mM; Sigma, G615), oligomycin (2 µM; Abcam, ab141829), and 2-deoxy-d-glucose (50 mM; Sigma, D8375). After each run, cells were lysed, and protein content was determined. Nonglycolytic acidification determined in the absence of glucose and in the presence of 2-deoxy-d-glucose was subtracted to determine glucose-induced glycolysis; glycolytic capacity was determined in the presence of oligomycin. For mitochondrial stress test experiments, wells were washed and incubated with pH 7.4 DMEM plus 1 mM D-glucose and 1 mM pyruvate (Gibco, catalog number 11360070) for 1 h at 37 °C minus CO2. The OCR was measured after sequential additions of oligomycin (2 µM), FCCP (5 µM; Sigma, C2920), and antimycin A (1 µM; Alfa Aesar, J63522) and rotenone (1 µM; Sigma, R8875). After each run, cells were lysed, and protein content was determined. Maximal respiration was calculated as the difference in OCR measured in the presence of FCCP and in the presence of antimycin A/rotenone.

### Table 1

**Primer list for qPCR**

| Transcript                | Forward primer | Reverse primer |
|---------------------------|----------------|----------------|
| Mouse 36B4                | TCATCCAGCGAGGTGTTGAC | TACCCGATTCGAGACACAC |
| Mouse DMT1                | GCGGGACGCGAGATCTATT | TCCTTGTAGCGGGCTGTGTO |
| Mouse Zip8                | CTGACAGACATGACTCTCTACG | CTGGACGAGACTTGGAGAG |
| Mouse TIR                 | GACAAGCCAGCTTATGTTGCT | GGATAGCAGACACTTGGAG |
| Mouse L-ferritin          | AAGCGGAGCCCTTGTGGTC | CTTGGCCCAACACACAG |
| Mouse H-ferritin          | CCTCTCTACCTCTACTTGT | CGGCTTGGTGGGAGAGAG |
| Mouse Fpn                 | TGGTCCCTGTCCACTCTCGT | TGGTAGGAGAGAGAGAG |

**Microglial iron transport**
**Microglial iron transport**

**Cellular heme measurements**

IMG cells were grown on 10-cm plates and incubated overnight with or without LPS (10 ng/ml). The next day, cells were washed three times with ice-cold PBS++ and lysed on ice with 1% Nonidet P-40, 50 mM Tris, pH 8.5, 150 mM NaCl (500 μl/dish) for 5 min. Cell debris was pelleted at 14,000 X g for 10 min, and 50 μl of each supernatant was mixed with 450 μl of 2 M oxalic acid. Serial dilutions of heme were used to develop a standard curve. Standards and samples were heated at 100 °C for 30 min; controls were incubated with oxalic acid but not heated. Samples and controls were transferred to a black walled 96-well plate, and fluorescence was measured (excitation, 400 nm; emission, 662 nm). Heme content was normalized to cellular protein content.

**Calcein-AM assays for LIP**

IMG cells were grown in a black walled 96-well plate and incubated overnight with or without LPS (10 ng/ml). Medium was removed, and cells were washed three times with PBS and then incubated with serum-free medium plus 5 M calcein-AM (Life Technologies) for 40 min at 37 °C in 5% CO2. Cell-associated fluorescence was measured (excitation, 495 nm; emission, 515 nm). Reduced fluorescence intensity reflects quenching due to free iron binding to calcein.

**Statistical analyses**

All statistical analyses were performed using Prism GraphPad version 7.01 for Windows (GraphPad Software, La Jolla, CA). One-way ANOVA followed by Tukey’s multiple comparison test was used where indicated. Student’s t test statistical analysis was used where indicated. Results shown are means ± S.D.

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