Activation of C6 glioblastoma cell ceruloplasmin expression by neighboring human brain endothelia-derived interleukins in an in vitro blood–brain barrier model system

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Abstract

Background: Iron transport across the blood–brain barrier (BBB) involves the cooperation of brain microvascular endothelial cells (BMVEC) and their neighboring astrocytes. Astrocytes secrete a soluble form of ceruloplasmin (sCp) which, in turn, acts to export iron from ferroportin (Fpn) on the basolateral surface of BMVEC. Although regulation of astrocyte sCp gene expression has been demonstrated to be influenced by interleukin-1 beta (IL-1β) and interleukin-6 (IL-6), the role of neighboring BMVEC in this regulation has yet to be determined and is the basis for this work.

Results: We provide evidence that human BMVEC (hBMVEC) IL-1β and IL-6 positively influence the expression of sCp transcript by neighboring C6 glioma cells (astrocytes). The effect of hBMVEC on C6 glioma sCp expression at the level of transcript and protein was repressed via the addition of IL-1 receptor antagonist protein and SC144, respectively. Stimulation of hBMVEC interleukin gene expression by apical exposure to bacterial endotoxin lipopolysaccharide significantly enhanced hBMVEC-mediated C6 glioma sCp gene expression.

Conclusion: hBMVEC influence the gene expression of neighboring C6 glioma sCp. This change in gene expression is mediated by the secretion of IL-1β and IL-6 from hBMVEC. Furthermore, the hBMVEC-induced increase in neighboring C6 glioma sCp gene expression leads to an increased rate of hBMVEC iron efflux. Taken together, our results indicate that hBMVEC-secreted cytokine activity increases the gene expression of neighboring C6 glioma sCp, which reciprocally acts on basolateral hBMVEC Fpn to enhance brain iron import.

Keywords: IL-1β, IL-6, Ceruloplasmin, Glial cells, Brain microvascular endothelial cells, Blood–brain barrier, Iron, Cytokines, Inflammation

Introduction

Iron, a first-row transition metal, is essential for cellular respiration [1,2]. As the major user of metabolic energy (on a per-weight basis) the central nervous system (CNS) strongly relies on iron while at the same time is highly vulnerable to iron-induced oxidative stress. Indeed, progressive accumulation of iron in a normal aging brain [3] or pathologic alterations of its homeostasis can be the cause of or contribute to the cellular degeneration observed in many neurologic disorders [3-6]. The export of iron from the basolateral surface of the brain microvascular endothelial cells (BMVEC) of the blood–brain barrier (BBB) is mediated by several different layers of regulation by neighboring astrocytes [7]. In a model BBB system, C6 glioblastoma cells (astrocytes) inhibit the export of iron from BMVEC via secretion of the peptide hormone hepcidin which when bound to its receptor ferroportin (Fpn), induces its internalization and degradation [7-9]. Alternatively, C6 cells increase the export of iron from the basolateral surface of BMVEC (and thus import into brain) via the secretion of the soluble multicopper oxidase ceruloplasmin (sCp) [7,10].

C6 glioma Cp expression can be modulated by interleukin-1β (IL-1β) [11,12]. IL-1β binding to its
receptor, IL-1 receptor 1 (IL-1R1), modulates the activity of mitogen-activated protein kinases (MAPK) in mammalian cells [13]; this activity has been demonstrated in C6 cells in culture [11]. Furthermore, IL-1β has been shown to increase production of C6 glioma-secreted sCp protein [14]. IL-1β is produced by monocytes, macrophages, fibroblasts, epidermal cells, and also by endothelial cells [13,15-17].

Alternatively, cellular Cp gene expression can be modulated by IL-6 [18,19]. Signal transduction by IL-6 requires the binding to its receptor, the IL-6 receptor (IL-6R) on the cell surface. This binding event leads to association of the receptor with the signal transducer gp130 which initiates a downstream signaling cascade leading to the modulation of gene expression [20]. Reports from the literature suggest that human endothelial cells express and secrete IL-6 [21,22].

A direct link between IL-1β or IL-6 secretion from BMVEC and the downstream effect of increasing astrocyte sCp gene expression has not been established; this link is likely part of the cell communication and signaling network that results in iron flux across the BBB. Here, we investigate this potential link in the context of brain iron influx. We use a human BMVEC line (hBMVEC) to demonstrate that hBMVEC increase the gene expression of C6 glioma sCp. Furthermore, we confirm that hBMVEC express and secrete IL-1β and IL-6 into their extracellular space. We show that the increase in C6 glioma sCp gene expression in the presence of hBMVEC can be repressed through the inhibition of either the IL-1R1 or the gp130 signal transducer molecule involved in IL-6 modulated gene activation. Finally, we demonstrate that hBMVEC cytokine release, and thus stimulation of astrocyte sCp gene expression, can be enhanced by apical exposure of hBMVEC to bacterial endotoxin lipopolysaccharide. These data are the first to define a likely cell communication and signaling network between BMVEC and astrocytes at the BBB that enhances iron transport across the BBB and into the brain.

Results

hBMVEC increase C6 glioma ceruloplasmin mRNA abundance

We have demonstrated previously that C6 glioma cells grown distal to hBMVEC in a transwell culture system (top well: hBMVEC; bottom well, bottom surface: C6 cells) significantly enhance hBMVEC iron efflux into the basal chamber via secretion of C6 cell sCp [7]. Also observed, was an increase in C6 cell sCp transcript mediated by neighboring hBMVEC [7]. We took advantage of this transwell model system to determine the mechanism by which hBMVEC increase C6 cell sCp gene expression in co-culture. Two distinct cellular orientations were used for our experiments which included C6 glioma cells seeded either alone (C6 only) or spatially adjacent but distal to hBMVEC (EC/-/C6) (Figure 1A).

After the cells were grown to confluency in their respective orientations, the media in the basal (bottom) chamber was switched to RPMI 1640 minus serum to mimic the interstitial fluid of the brain. Cells were incubated with this new media for an additional 24 h to be consistent with previous work [7]. Total RNA was extracted from the hBMVEC and the C6 glioma cells under the different orientations as indicated. Transcripts for both splice variants of Cp (sCp and GPI-Cp) were identified in C6 glioma cells (Figure 1B) and their relative abundance under each orientation was assessed quantitatively using qPCR. We observed a 7-fold increase in the transcript abundance of splice variant non-specific Cp abundance in C6 glioma cells seeded spatially adjacent to hBMVEC compared to their respective C6 only control (Figure 1C). The increases in sCp and GPI-Cp mRNA were 5- and 10-fold (Figures 1D and 1E). The effect on C6 glioma sCp transcript expression was not influenced by serum from the apical chamber (Additional file 1: Figure S1). Similarly, there was only a small increase in the abundance of sCp transcript in hBMVEC grown distally to C6 cells (Additional file 1: Figure S2). Note that we do not make comparisons between varying transcript targets (sCp vs. GPI-Cp) or between hBMVEC and C6 glioma sCp message as this is not possible with qPCR methodology. Previous data has shown there is no significant difference in sCp message in C6 cells grown either in the distal (EC/-/C6) and proximal (EC/C6) orientations [7].

We tested if the increase in sCp transcript abundance within C6 glioma cells required co-culture with hBMVEC or whether this increase was due to a stable hBMVEC exocrine factor retained in hBMVEC conditioned media. To test the latter model, hBMVEC were seeded alone in transwell and grown to confluence. Their basal media was exchanged with serum-free media to mimic the brain interstitial fluid, and the hBMVEC were incubated for 24 h to “condition” the basolateral media. This basolateral hBMVEC-conditioned media (ECM) was then applied to C6 glioma cells grown in 66-mm dishes and allowed to incubate for 24 h. Total RNA was isolated from the C6 glioma cells and qPCR revealed a significant, 3-fold increase in C6 glioma sCp message (Figure 1F). These data suggest the secretion of the putative hBMVEC factor into the basal chamber that stimulated C6 glioma sCp gene expression did not require but was enhanced by signaling from C6 glioma cells to hBMVEC.

hBMVEC express cytokines IL-1β and IL-6 which are secreted from their basolateral surface

Data indicate that Cp transcript abundance in astrocytes can be increased through exposure to the cytokines IL-1β or IL-6 [11,12,14,18,19] suggesting the model that either
or both of these cytokines were the factor(s) secreted by hBMVEC into the basal chamber. Thus, total RNA was isolated from hBMVEC; RT-PCR analysis revealed the presence of transcripts for both factors (Figure 2A). We demonstrated also the presence of the IL-1β and IL-6 protein in basolateral hBMVEC-conditioned media. To do this hBMVEC were grown alone in transwell. The basolateral media was switched to media without serum and the cells were incubated for an additional 24 h to condition the media as above. Western blot analysis of this hBMVEC-conditioned media (ECM) demonstrated the presence both IL-1β and IL-6 protein, confirming that hBMVEC secrete both cytokines from their basal surface into the extracellular space (Figure 2B). Of note is that the abundance of hBMVEC IL-6 and IL-1β transcripts were increased by co-culture in transwell with C6 glioma cells (Figures 2C and 2D, respectively). This effect correlates with the 7-fold increase C6 glioma sCp transcript when grown in transwell together with hBMVEC (Figure 1D) in comparison to the 3-fold increase in C6 cells incubated with basal ECM (not in transwell; Figure 1F).

IL-1β and IL-6 act additively to increase C6 glioma sCp mRNA and protein

Since both IL-1β and IL-6 are secreted from the basolateral surface of hBMVEC, we examined the direct effect of added recombinant cytokine on C6 glioma sCp abundance. C6 glioma cells were incubated for 6 h in the absence of either cytokine (control) and in the presence of IL-1β, IL-6, or both. Total RNA was isolated from the C6 cells and analyzed for sCp transcript abundance by qPCR. The cytokines IL-1β (10 ng/mL) and IL-6 (10 ng/mL) had an additive effect with respect to the increase of C6 cell sCp transcript abundance (Figure 3A). These data are fully consistent with literature data that show IL-6 and IL-1β mediate increases in sCp gene expression [11,12,18,19].

SB203580 and interleukin-1 receptor antagonist protein (IRAP) repress the induction of C6 glioma sCp gene expression by hBMVEC

Although we have demonstrated that IL-1β is expressed and secreted by hBMVEC, and confirmed in our system that addition of this cytokine to C6 cells induced sCp expression, these two findings do not specifically show that in the hBMVEC/C6 cell co-culture, the increase in C6 cell sCp was due to hBMVEC IL-1β. Thus we examined if pharmacological inactivation of the IL-1 receptor (IL-1R) or the downstream MAPK pathway in C6 cells would repress the effect of hBMVEC on sCp expression. Interleukin-1 receptor antagonist protein (IRAP) inhibits IL-1R activation by IL-1β [13,23] and IRAP has been effectively used in C6 cells to inhibit sCp gene expression.
induced by IL-1β [24]. Furthermore, in binding its receptor, IL-1R, the IL-1β activates three independent MAPK pathways, one of which involves the p38 MAP kinase [13]; SB203580 is a specific inhibitor of p38 MAP kinase activated by IL-1β [11,25]. Addition of SB203580 to C6 cells has been documented to repress the IL-1β-induced increase in Cp transcript in those cells [11]. Thus, either 1 μg/mL IRAP or 20 μM SB203580 were added to separate basolateral chambers of transwells with hBMVEC grown distal to C6 glioma cells (EC/-/C6). After a 24 h incubation period, total RNA was isolated from the C6 glioma cells and qPCR was performed. The addition of either IRAP or SB203580 to the basolateral chamber partially repressed the hBMVEC-induced increase in C6 cell sCp message (Figure 3B). These data are consistent with the model that IL-1β secreted from hBMVEC enhances C6 sCp gene expression at least in part by activating the IL-1R-dependent signaling pathway.

The hBMVEC-mediated induction of C6 glioma sCp gene expression is repressed by pharmacological agents that block the activation of IL-1β and IL-6 receptors

Blocking the IL-1R pathway only partially repressed the activation of sCp expression in hBMVEC co-cultures. Thus, we hypothesized IL-6, secreted from hBMVEC, also contributed to this activation. Promotion of C6 gene expression by IL-6 has been observed previously [18,19]. Induction of gene expression by IL-6 begins with the cytokine binding to the IL-6R. This ligand binding event induces the association of gp130 which is required for signal transduction and downstream gene activation [20]. Phosphorylation of serine residue 782 on gp130 down-regulates gp130 glycosylation and leads to suppression of its downstream signaling pathway [20]. SC144, a recently identified inhibitor of gp130, was described to induce this phosphorylation of gp130 Ser782 and thus inhibit gp130 signal transduction [20]. We used SC144 examine whether hBMVEC-secreted IL-6 contributed to C6 glioma sCp gene expression.
C6 glioma cells were grown in 6-well tissue culture dishes to confluence. The media was exchanged with serum-free media plus or minus either IRAP (1 μg/mL), SC144 (20 μM), or both and the cultures were allowed to incubate for 1 h. The media was removed and replaced by hBMVEC-conditioned media as above (cells grown alone) and the C6 glioma cells were incubated for an additional 1 h, maintaining the initial concentration(s) of the two drugs. The incubation period was shortened in comparison to Figures 3A and 3B due to SC144 cytotoxicity at 24 h. Total RNA was isolated from the C6 glioma cells and qPCR was performed to determine the transcript abundance of sCp in each condition. In the 1-h incubation period, hBMVEC basal conditioned media (Basal ECM) caused a 2-fold induction in C6 glioma sCp gene expression (Figure 3C). This induction was repressed by both IRAP and SC144 but was completely inhibited with the addition of both drugs together (Figure 3C).

Since IRAP and SC144 treatment diminished the hBMVEC-induced increase in C6 glioma sCp transcript expression, we predicted that this observation would translate to the level of protein expression. First, we confirmed by western blot that IL-1β and IL-6 incubated with C6 glioma cells for 6 h yielded an increase in the secretion of sCp from C6 glioma cells into the media (Figure 3D). Purified human sCp (20 ng) is used as a positive control in the top blot. (E) Total RNA isolated from C6 glioma cells treated as described in (D) were analyzed for sCp transcript abundance by qPCR. One-way ANOVA analyses of the variance parameters were used to determine the significance of the data. *P < 0.05, **P < 0.01, ***P < 0.001, ns is not significant. Data are represented as means ± s.d. (n = 3, technical replicates).

Figure 3 hBMVEC IL-6 and IL-1β enhance C6 glioma sCp gene expression. (A) qPCR of C6 glioma sCp transcript from total RNA isolated from C6 cells incubated with either nothing, IL-1β (10 ng/mL), IL-6 (10 ng/mL), or both for 24 h. (B) SB203580 and IRAP, which inhibit the action of hBMVEC-secreted IL-1β, repress the ability of hBMVEC-secreted IL-1β to enhance C6 glioma sCp gene expression. Relative C6 glioma sCp transcript abundance grown either alone (C6), or distal to hBMVEC (EC/C6) with or without 20 μM SB203580 or 1 μg/mL IRAP for 24 h. (C) Relative abundance of C6 sCp from total RNA of C6 glioma cells seeded alone with or without the addition of basal hBMVEC-conditioned media (Basal ECM) and the IL-1β inhibitor IRAP (1 μg/mL) or the IL-6 inhibitor SC144 (20 μM). Drugs were added to C6 glioma cells for 1 h prior to the addition of Basal ECM for an additional 1 h before total RNA was isolated. (D) Immunoblot probing for Cp in C6-conditioned media in which the cells had been incubated with either nothing, IRAP (1 μg/mL), SC144 (20 μM), or both for 1 h prior to the addition of both IL-1β (10 ng/mL) and IL-6 (10 ng/mL), or nothing for an additional 6 h. Purified human sCp (20 ng) is used as a positive control in the top blot. (E) Total RNA isolated from C6 glioma cells treated as described in (D) were analyzed for sCp transcript abundance by qPCR. One-way ANOVA analyses of the variance parameters were used to determine the significance of the data. *P < 0.05, **P < 0.01, ***P < 0.001, ns is not significant. Data are represented as means ± s.d. (n = 3, technical replicates).
After this induction period, the media was analyzed for the relative abundance of sCp by immunoblotting. We found that IRAP, and to a greater extent SC144 reduced the amount of sCp present in the C6 glioma-conditioned media (Figure 3D); furthermore, no sCp was detected in the media when IRAP and SC144 were added together (Figure 3D). Analysis of the total RNA collected from C6 glioma cell samples processed in parallel with those used for the western blot data as in Figure 3D demonstrated a corresponding decrease in sCp transcript abundance (Figure 3E).

**Induction of C6 glioma sCp gene expression by hBMVEC leads to an increase in the rate of hBMVEC $^{59}$Fe-efflux**

Our data indicate that hBMVEC-secreted cytokines IL-1β and IL-6 activate sCp gene expression in C6 glioma cells. We have previously demonstrated that C6 glioma-secreted sCp enhances basolateral hBMVEC $^{59}$Fe efflux from Fpn [7]; immunodepletion experiments were used to confirm that sCp was indeed the species in the C6 secretome that supported the increase in hBMVEC iron mobilization [7]. Therefore, we tested the hypothesis that cytokine-dependent increased C6 glioma sCp expression would lead to an increase in iron efflux activity from hBMVEC Fpn. Thus, we prepared transwells with hBMVEC alone (EC Only), C6 glioma cells alone (C6 Only), or both cells seeded distal to one another (EC/-/C6) (Figure 4A). After the cells had grown to confluence the media in the apical chamber was replaced with fresh RPMI1640 with serum while the basal chamber media was replaced with serum-free RPMI1640. Transwells were incubated for 24 h to allow the basal chamber media to become “conditioned”. After 24 h, the basal chamber media was collected and used as efflux media for hBMVEC monolayers that had been loaded for 24 h with $^{59}$Fe$^{2+}$-citrate plus Asc in RPMI1640 without serum. After a 24 h efflux period, we observed a significant enhancement of hBMVEC $^{59}$Fe-efflux when the efflux media was conditioned with EC/-/C6 as compared to medium that was conditioned by either cell type alone (Figure 4B). These data are consistent with the model that hBMVEC-induced up-regulation of C6 glioma sCp gene expression has a functional role in stimulating Fpn-dependent iron efflux from hBMVEC. The hBMVEC cytokine-mediated increase in glial cell sCp described herein is not functionally adequate to override the effect of glial cell secreted hepcidin on hBMVEC iron efflux when hBMVEC are seeded proximal to C6 glioma cells in a model BBB system [7].

![Image](image_url)
Lipopolysaccharide positively influences the hBMVEC-induced increase in C6 glioma sCp transcript abundance

Lipopolysaccharide (LPS) is a bacterial endotoxin used experimentally as a positive modulator of inflammation (i.e. interleukin up-regulation) in mammalian cell culture [27,28]. An increase in the polarized release of cytokines (most notably IL-6) from BMVEC via the addition of 100 μg/mL LPS has been reported [28]. We tested the hypothesis that addition of LPS at this concentration to hBMVEC would further increase C6 glioma sCp transcript abundance via increased cytokine secretion from hBMVEC. Indeed, an increase in the transcript abundance of IL-6 and IL-1β in hBMVEC treated for short time periods with LPS (100 μg/mL) was noted (Figure 5A). LPS addition (100 μg/mL) to the apical chamber of hBMVEC grown distal to C6 glioma cells induced a 40-fold increase in sCp transcript abundance in the C6 glioma cells over 24 h (Figure 5B). To demonstrate that the effect of LPS on C6 glioma cell sCp expression was dependent upon hBMVEC, we examined the effect of LPS addition to the apical chamber of transwells in which C6 glioma cells were grown alone in the bottom chamber; in this experiment, LPS had no positive effect on C6 cell sCp transcript abundance (Additional file 1: Figure S3).

We hypothesized that hBMVEC IL-1β and/or IL-6 were likely responsible for the increase in C6 glioma sCp gene expression when hBMVEC were stimulated apically with LPS as in Figure 5B. To test this, we grew C6 glioma cells either alone (C6) or distal to hBMVEC in transwell (EC/-/C6). Fresh RPMI 1640 with serum was added to the apical chamber and fresh RPMI 1640 without serum was added to the basal chamber 24 h prior to the collection of C6 glioma RNA. Twenty hours after the media was exchanged, LPS (100 μg/mL) was added to the apical chamber and the inhibitors IRAP (1 μg/mL) and SC144 (20 μM) were added to the basal chamber as

![Figure 5](http://www.biosignaling.com/content/12/1/65)
indicated. The cells were incubated for an additional 4 h before total RNA was isolated from the C6 glioma cells and analyzed for the relative transcript abundance of sCp by qPCR (Figure 5C). As shown, the IL-6 inhibitor SC144 blocked the action of LPS-induced hBMVEC on C6 glioma cell sCp gene expression, while the IL-1β inhibitor IRAP had no notable effect (Figure 5C). Note that complete inhibition of the LPS effect is represented by the level of sCp transcript abundance in the EC/-/C6 without LPS condition (i.e. approx. 5) and not by the level of sCp transcript abundance in the C6 alone condition (i.e. approx. 1). The absence of an IRAP effect (that is, a down-regulation of the IL-1 β pathway) in this instance could be the result of a dominant IL-6 dependent activation of sCp transcript production.

Discussion

In the brain parenchyma, a tissue closed off from the peripheral circulation, cell-to-cell communication is imperative for the transport of essential metabolites. Without paracrine signaling within the brain, cells would have to rely on the steady diffusion of metabolites through the brain interstitial fluid. Using this transwell BBB model we previously have provided evidence in support of a model that direct communication between BMVEC and their neighboring astrocytes is required for proper regulation and transport of iron into the brain [7]. Here, we have extended these findings to understand the role BMVEC play in self-regulating their own iron transport through manipulation of a neighboring glial cell type.

In this model system regulation of C6 cell sCp expression is modulated by BMVEC-secreted IL-6 and IL-1β (Figure 3). This modulation is enhanced further when the apical (blood-side) of the brain capillary is exposed to bacterial endotoxin (LPS) (Figures 5 and 6A). Acute exposure to LPS induces increased gene expression of the cytokines IL-1β and IL-6 by hBMVEC (Figure 6B). The eventual increase in hBMVEC IL-6, but apparently not IL-1β, secretion into the interstitial space between hBMVEC and C6 cells yields activation of IL-6R endogenous to the glial cells. Transduction of this signal leads to downstream activation of Cp gene expression (Figure 6C). The soluble Cp splice variant gene product is secreted by the glial cells to act on Fpn endogenous to hBMVEC (Figure 6D). In theory, this model provides a mechanism by which inflammation may negatively affect BBB integrity via oxidative stress to the BMVEC caused by excessive extracellular iron in the interstitial space at the BBB. Previous data from the literature indicates that chronic inflammation may lead to a loss of BBB integrity [29]. Pathogenic organisms such as Streptococcus pneumonia (pneumococcal meningitis) may induce or take advantage of encephalitis and the breakdown of the BBB as a mechanism of entering the brain and CNS [30]. Thus, our data suggest that inflammation and/or pathogens in the brain cause hBMVEC to modulate their own basolateral iron efflux by supporting a cytokine-mediated activation of sCp expression in neighboring astrocytes.

While the mechanism of iron efflux from hBMVEC during inflammation described in Figure 6 is probable, a number of alternative mechanisms could be playing a role. For example, increased iron uptake by hBMVEC may lead to an IRE-IRP mediated increase in Fpn expression and iron efflux. Alternatively, inflammation may enhance the generation of nitric oxide leading to an increase in the expression of hBMVEC Fpn [31]. These hypotheses might be incorporated into future studies regarding
hBMVEC response to inflammatory signals as it pertains to iron metabolism.

The model described in Figure 6 may also have implications in progressive neurodegeneration with aging. There is evidence that age correlates with increased brain expression of IL-1 protein and transcript [32]. Amyloid-β precursor protein (APP) present in both astrocytes and endothelial cells [33], may contribute to the Alzheimer’s disease pathology, and is positively regulated, in part, by IL-1 [34]. Also, data suggest that iron plays a role in the disease progression associated with Aβ plaque formation [35]. Reciprocally, a fragment of APP (FTP, for ferroportin-targeting peptide) stabilizes Fpn in the membrane of hBMVEC allowing for activation of brain iron import by exocyttoplasmic ferroxidases [36]. In as much as we’ve now demonstrated that IL-1β and IL-6 increase sCp production in the brain, this sCp could act in concert with sAPP to increase the brain interstitial iron pool, thus exacerbating the progression of Alzheimer’s disease pathology, and is positively regulated, in part, by IL-1 [34]. Also, data suggest that iron plays a role in the disease progression associated with Aβ plaque formation [35]. Reciprocally, a fragment of APP (FTP, for ferroportin-targeting peptide) stabilizes Fpn in the membrane of hBMVEC allowing for activation of brain iron import by exocyttoplasmic ferroxidases [36]. In as much as we’ve now demonstrated that IL-1β and IL-6 increase sCp production in the brain, this sCp could act in concert with sAPP to increase the brain interstitial iron pool, thus exacerbating the progression of Alzheimer’s disease.

In summary, we have provided several lines of evidence demonstrating hBMVEC communicate with C6 glioma cells so as to provide the necessary exocrine factor, sCp, to hBMVEC in support of Fpn-dependent iron efflux and thus to brain iron accumulation. Our data indicate also that this cell signaling pathway appears to link system infection to brain iron metabolism in the context of our model BBB system.

Experimental procedures

**Cell culture and reagents**

hBMVEC and C6 glioma cells were cultured in RPMI 1640 with 10% FBS and 10% NuSerum as previously described [37]. Experiments were performed in ThinCert tissue culture inserts (Transwells) (Greiner bio-one) or in 24-well tissue culture dishes as previously described [7]. Serum-free media was RPMI-1640 with the addition of sodium selenite and insulin as described previously [7]; no cytotoxicity or loss of adherence was noted in cells incubated with serum-free media for prolonged periods [7]. IL-1β, IL-6, and IRAP were purchased from Peprotech (Rocky Hill, NJ), SB203580 (Santa Cruz Biotechnology), SC144 and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich.

**RT-PCR and qPCR**

Total RNA was extracted from hBMVEC or C6 glioma cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. RNA was purified and DNase treated using the Direct-zol RNA Miniprep kit (Zymo-research) as per the manufacturer’s instructions. Pure RNA was then reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen) along with genespecific primers, and PCR was performed using the Qiagen OneStep RT-PCR kit. Quantitative PCR was performed as previously described [7] using SsoAdvanced SYBR Green Supermix (Bio-rad, Hercules, CA) and the Bio-rad CFX-96 real-time PCR instrument (Bio-Rad). In all cases β-actin was used as an internal control. Endpoint qPCR reactions were separated on a 1.7% agarose gel to confirm product size. Primers used for RT-PCR and qPCR are listed in Additional file 1: Table S1. Where applicable, 24 h incubations were used in an effort to maintain consistency with previous work utilizing this in vitro model BBB system [7].

**Immunoblots**

Immunoblots were performed as previously described with minor changes [37]. Briefly, hBMVEC-conditioned basolateral media was concentrated and SDS-PAGE (12%) was used to separate the concentrate for the examination of IL-1β or IL-6. A 10% SDS-PAGE was used to separate proteins from C6-conditioned media for the examination of sCp. The membrane was blocked, and incubated with rabbit anti-IL-1β (ab2105) (Abcam, Inc., Cambridge, MA), rabbit anti-IL-6 (ab6672) (Abcam), or goat anti-Cp (Bethyl Laboratories, Inc.) overnight at 4°C and then incubated with secondary anti-rabbit or anti-goat HRP antibody (Santa Cruz Biotechnology). Blots were adjusted for brightness and contrast using Adobe Photoshop software (version 7.0).

**59Fe efflux assay**

Assays were performed with hBMVEC grown in 24-well tissue culture dishes as previously described [7,26]. Briefly, hBMVEC were loaded for 24 h with 59Fe in the presence of citrate (250 μM) and ascorbate (5 mM). After loading, cells were washed and incubated for an additional 24 h with fresh RPMI 1640 plus the appropriate reagents as indicated in the text and figure legends. Post-efflux, cells were washed twice with quench buffer and were lysed for analysis of 59Fe (CPM) and protein content. Cell-associated 59Fe values (LKB Wallac CompuGamma) were normalized by protein concentration. Protein concentrations were determined using the Pierce BCA protein assay (Thermo Scientific, Pittsburgh, PA) as per the manufacturer’s instructions.

**Statistical analysis**

One-way ANOVA or paired t-test statistical analyses were used as indicated. Statistical analyses were performed using Prism Graphpad software version 5.0 (GraphPad Software, San Diego, CA),
Additional file

Additional file 1: Figure S1. Apical chamber assay has no effect on C6 glial sCp transcript abundance. Figure S2. hBMVEC sCp transcript abundance is positively regulated by C6 glial cells. Figure S3. Modulation of C6 glial sCp transcript abundance by LPS requires hBMVEC.

Table S1. Primer list used for qPCR.

Abbreviations
BBB Blood-brain barrier; BMVEC Brain microvascular endothelial cells; sCp Soluble ceruloplasmin; Fpn Ferroportin; IL-1β Interleukin-1β; IL-6 Interleukin-6; hBMVEC Human brain microvascular endothelial cell; IL-1RI1; Interleukin-1 receptor 1; MAPK Mitogen-activated protein kinase; IL-6R interleukin-6 receptor; GPC GRP Glycylglycophosphatidylinositol-linked ceruloplasmin; ECM hBMVEC-conditioned media; IRAP Interleukin-1 receptor antagonist protein; LPS Lipopolysaccharide; APP Amyloid-β precursor protein; FTP Ferroportin-targeting peptide; CPM Counts per minute.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RCM conceived the project and wrote the manuscript; DJK assisted in the planning of experiments, reviewed and revised the manuscript and analyzed the data; RCM planned and executed the experiments and analyzed the resulting data. Both authors read and approved the final manuscript.

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