(−)-Epigallocatechin gallate inhibits endotoxin-induced expression of inflammatory cytokines in human cerebral microvascular endothelial cells

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Abstract

Background: (−)-Epigallocatechin gallate (EGCG) is a major polyphenol component of green tea that has antioxidant activities. Lipopolysaccharide (LPS) induces inflammatory cytokine production and impairs blood–brain barrier (BBB) integrity. We examined the effect of EGCG on LPS-induced expression of the inflammatory cytokines in human cerebral microvascular endothelial cells (hCMECs) and BBB permeability.

Methods: The expression of TNF-α, IL-1β and monocyte chemotactic protein-1 (MCP-1/CCL2) was determined by quantitative real time PCR (qRT-PCR) and ELISA. Intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM) in hCMECs were examined by qRT-PCR and Western blotting. Monocytes that adhered to LPS-stimulated endothelial cells were measured by monocyte adhesion assay. Tight junctional factors were detected by qRT-PCR (Claudin 5 and Occludin) and immunofluorescence staining (Claudin 5 and ZO-1). The permeability of the hCMEC monolayer was determined by fluorescence spectrophotometry of transmembrane fluorescein and transendothelial electrical resistance (TEER). NF-κB activation was measured by luciferase assay.

Results: EGCG significantly suppressed the LPS-induced expression of IL-1β and TNF-α in hCMECs. EGCG also inhibited the expression of MCP-1/CCL2, VCAM-1 and ICAM-1. Functional analysis showed that EGCG induced the expression of tight junction proteins (Occludin and Claudin-5) in hCMECs. Investigation of the mechanism showed that EGCG had the ability to inhibit LPS-mediated NF-κB activation. In addition, 67-kD laminin receptor was involved in the anti-inflammatory effect of EGCG.

Conclusions: Our results demonstrated that LPS induced inflammatory cytokine production in hCMECs, which could be attenuated by EGCG. These data indicate that EGCG has a therapeutic potential for endotoxin-mediated endothelial inflammation.

Keywords: 67LR, endothelial, (−)-epigallocatechin gallate, LPS, NF-κB

Background

The brain endothelial cells interact with resident cells in the central nervous system (CNS), providing the protective blood–brain barrier (BBB) interface between the CNS and peripheral blood system. By controlling the access of blood components, including immune cells to the CNS, the BBB regulates the delicate milieu optimal for neuronal communication and helps to maintain the homeostasis of the CNS. Breakdown of BBB is an early and significant event in CNS inflammation induced by extrinsic or intrinsic stimuli, including endotoxins [1]. It has been shown that brain endothelial cells are a primary target of immunological attack in bacterial infection, and their injury can lead to vasculopathy and organ dysfunction associated with disruption of tight junctions in the brain endothelium [2].

Lipopolysaccharide (LPS), a product of bacterial infection, is known to induce inflammatory cytokines and impairs the BBB system. A central feature of the pathophysiology of acute inflammation and septic shock triggered by LPS is the production of multiple proinflammatory mediators such as cellular adhesion molecules, cytokines,
and chemokines by monocytes–macrophages as well as vascular endothelial cells [3]. LPS can directly elicit a variety of inflammatory cytokines in endothelial cells, such as inducible nitric oxide synthase (iNOS) [4], IL-1β [5] and IL-6 [6,7]. Furthermore, LPS, TNF-α, or IL-1β can significantly stimulate human microvascular endothelial cells (HBMECs) to release monocyte chemotactic protein-1 (MCP-1/CCL2), an important factor for monocyte migration bound to the apical endothelial surfaces [8].

Intact cerebral endothelial cells constitutively express low levels of intercellular adhesion molecule 1 (ICAM-1), which plays a vital role in the process of leukocyte transmigration through endothelial cell barriers and has been shown to mediate signal transduction events in endothelial cells induced either by its cross-linking or by the binding of T lymphocytes [9]. However, when stimulated by LPS or certain cytokines, these cells produce high levels of ICAM-1 [10,11]. The cell walls of Streptococcus pneumoniae (PCW), the most common cause of adult bacterial meningitis, also induces ICAM-1 expression in rat primary brain microvascular endothelial cell cultures [4]. This induction could be completely blocked by TNF-α antibody, suggesting that ICAM-1 expression is mediated by the inflammatory cytokine and that cerebral endothelial cells regulate critical steps in inflammatory BBB disruption of bacterial meningitis. Therefore, regulation of endothelial adhesion molecule and cyto-kine expression in brain endothelial cells is critical in maintaining BBB integrity.

(−)-Epigallocatechin gallate (EGCG), also known as epigallocatechin 3-gallate, is the most abundant catechin in green tea. EGCG as a potent antioxidant has been shown to have both anti-inflammatory and anti-atherogenic properties in experimental studies conducted in vitro and in vivo [12,13]. EGCG was found to inhibit TNF-α-induced production of MCP-1/CCL2 from bovine coronary artery endothelial cells, providing direct vascular benefits in inflammatory cardiovascular diseases [14]. Previous studies have also demonstrated that EGCG attenuated the increase in malondialdehyde levels caused by cerebral ischemia and reduced the formation of post-ischemic brain edema and infarct volume [15]. This study suggests that EGCG is a neuroprotective agent against excitotoxicity-related neurologic disorders such as brain ischemia. Further, the neuroprotective effect of EGCG against ischemia-induced brain damage was found, in part, to be due to modulation of NOS isoforms and preservation of mitochondrial complex activity and integrity [16]. Thus, the in vivo neuroprotecor effects of EGCG are not exclusively due to its antioxidant effects but involve more complex signal transduction mechanisms. In this study, we examined whether EGCG possesses the ability to protect the endothelial monolayer from loss of tight junction proteins and disruption of BBB permeability. We used a well-established in vitro model of the human BBB to monitor the effects of LPS and/or EGCG on the inflammatory responses and barrier permeability. We also investigated the mechanisms through which EGCG exerts its action on LPS-induced inflammation in human brain endothelial cells.

Materials and methods

(--)-Epigallocatechin gallate

EGCG (≥95%) was purchased from Sigma-Aldrich St. Louis, MO, USA (CAS#: 989-51-5; Cat# E4143). EGCG stock solution was prepared in sterile double distilled water at 20 mM.

Brain endothelial cell culture

Human cerebral microvascular endothelial cell (hCMEC) line, D3 clone, was developed by immortalization of the primary human brain vascular endothelial cells after transduction with lentiviral vectors encoding the catalytic subunit of human telomerase hTERT and SV40 T antigen, as described previously [17]. The hCMEC/D3 cell line recapitulates most of the unique properties of brain endothelium and may thus constitute a well-established in vitro model of the human BBB [17]. It has also been reported that D3 cell line maintains the in vitro physiological permeability barrier properties of the BBB, even in the absence of abluminal astrocytes [18]. D3 cells express typical endothelial markers (VE-cadherin and Occudin) and have been successfully used as an in vitro model of the BBB [19-21]. D3 cells were grown in endothelial cell growth medium (EGM<sup>®</sup>; Lonza, Walkersville, MD, USA) supplemented with vascular endothelial growth factor, insulin-like growth factor-1, epidermal growth factor, basic fibroblast growth factor, Gentamicin, ascorbic acid, heparin, fetal bovine serum and hydrocortisone (Lonza, Walkersville, MD, USA). Cells were cultured on collagen-coated (BD Biosciences, Rockville, IL, USA) tissue culture plate in a humidified atmosphere at 37°C in 5% CO₂.

Treatment of endothelial cells

hCMEC/D3 cells were treated with 100 ng/ml LPS for different time periods (3, 6, 24, and 48 h) or with different concentrations of LPS (1, 10, 100, and 1000 ng/ml) for 6 h. For the pretreatment, EGCG was added to the culture media (1, 5, and 25 μM) 1 h prior to LPS treatment and further incubated together for 6 h. To test the blockage effect of 67-kDa laminin receptor (67LR), cells were incubated with mouse monoclonal antibody against LR (clone MluC5; 5 μg/ml; NeoMarkers, Fermenton, CA, USA) or isotype control mouse IgM for 1 h before the addition of EGCG to the cell cultures. To test the direct
effect of EGCG on the expression of Toll-like receptor (TLR)4 and Myeloid differentiation primary response gene (88) (MyD88), hCMEC/D3 cells were treated with 5 μM of EGCG for 12 to 72 h or with 0 to 25 μM of EGCG for 24 h.

Reverse transcription and quantitative real time PCR
Total RNA was extracted with Tri-reagent (Sigma-Aldrich) and quantitated by spectrophotometric analysis. Reverse transcription was performed using the AMV transcriptase and RNasin (Promega Co., Madison, WI, USA) according to the manufacturer’s instruction. Quantitative real time PCR (qRT-PCR) was performed with Brilliant SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) described previously [22]. The primers that were used for the PCR amplifications are listed in Table 1. The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). All values were calculated using the delta delta Ct method and expressed as the change relative to the expression of glyceraldrye 3-phosphate dehydrogenase (GAPDH) mRNA.

ELISA
TNF-α, IL-1β and MCP-1/CCL2 gene expressions, identified from RT-PCR, were evaluated for protein expression using ELISA. After hCMEC/D3 cells were treated as indicated in the figure Figure. 1 and 2, conditioned medium was collected and levels of TNF-α, IL-1β and MCP-1/CCL2 were measured using conventional double sandwich ELISA kits from eBioscience Inc. (San Diego, CA, USA). Assays were performed according to the manufacturer’s instructions.

Immunofluorescence assay
hCMEC/D3 cells were cultured on chamber slides or glass coverslips in 24-well plate coated with collagen. After treatment, cells were washed with ice-cold PBS (with Ca2+ and Mg2+) twice, then fixed at 4°C in 4% paraformaldehyde plus 4% sucrose in PBS for 30 minutes. Subsequently, the cells were permeated with 0.2% Triton X-100 in PBS on ice for an additional 10 minutes. Cells were blocked in Block Solution (Pierce, Rockford, IL, USA) for 1 h at room temperature. The cells were then incubated at room temperature with mouse anti-Claudin 5 (1:50; Invitrogen, Grand Island, NY, USA) or mouse anti-ZO-1 (1:50; Invitrogen) for 1 h. After three washes with PBS, cells were incubated with Alexa488-conjugated goat anti-mouse IgG (1:250) for 1 h. Cells were then viewed under a fluorescence microscope (Olympus IX71, Japan).

Western blot analysis
The expression of the tight junctional proteins Claudin-5 and Occludin in hCMEC/D3 was evaluated by immunoblot analysis. Following incubation with specific antibodies and extensive washing in PBS containing 0.05% Tween-20, membranes were incubated with horseradish peroxidase-conjugated goat antimouse IgG (Pierce, Chester, UK) for 1 h at room temperature. Membranes were extensively washed in PBS containing 0.05% Tween-20, and immunoblots were visualized by enhanced chemiluminescence detection (ECL, Amersham, Bucks, UK).

Monocyte adhesion assay
Endothelial hCMEC/D3 cells were plated at 104 cells/well in 200 μl clonetics medium in collagen-coated black bottom 96-well plates for 3 days. Cell cultures were then maintained in partial clonetics medium (without growth factors) for an additional 72 h prior to the treatment with LPS and/or EGCG. Monocytes were isolated from peripheral blood mononuclear cells of a healthy donor as described previously [23] at the Path Bio Resource Human Immunology Core in accordance with protocols.

Table 1 Primer sequences for the quantitative real time PCR

| Gene name | Forward | Reverse |
|-----------|---------|---------|
| TNF-α     | 5’-CGA GTG ACA AGC CTG TAG C-3’ | 5’-GGT GTG GGT GAG GAG CAC AT-3’ |
| IL-1β     | 5’-AAG CTG AGC GGC CTA AAC AG-3’ | 5’-AGG TGC ATC GTG CAC ATG AG-3’ |
| MCP-1/CCL2| 5’-CAT AGC AGC CAC CTI CAT TCC-3’ | 5’-TCT GCA CTG AGA TCT TCC TAT TGG-3’ |
| ICAM-1    | 5’-CCT TCC TCA CGG TGT ACT GG-3’ | 5’-AGC GTA GGG TAA GGT TCC TGC-3’ |
| VCAM-1    | 5’-GGG AGC TCT GTC ACT GTA AG-3’ | 5’-ATG CGT ATC CTC AAA CT-3’ |
| Occludin  | 5’-AAG CAA GTG AAG GGA TCT GCT-3’ | 5’-GGG GTT ATG GTC CAA AGT CA-3’ |
| Claudin 5 | 5’-GTC TTT ACT CCA TCG GTA GG-3’ | 5’-TTT TTC TTT GTT GAG AGT TCA AAG C-3’ |
| TLR4      | 5’-CAT TGC TTC TTG CTA AAT GCT G-3’ | 5’-GGA TTA AAG CTC AGG GCC TTG-3’ |
| MyD88     | 5’-CCG CGC TGG CGG AGG AGA TGG AC-3’ | 5’-GCA GAT GAA GGG TCA GAA AGC CTC-3’ |
| GAPDH     | 5’-GGT GGT CTC TTC CTA GTT CAA CA-3’ | 5’-GTT GGT GTC AGA GTC AAA TTC GTT GT-3’ |

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; MCP, monocyte chemotactic protein; TLR, Toll-like receptor; VCAM, vascular adhesion molecule.
approved by the Human Subjects Research Committee of the University of Pennsylvania. The purity of isolated monocytes was higher than 95%. Cell viability was determined by trypan blue exclusion assay and monocyte viability above 95% was used. Briefly, monocytes were resuspended in serum-free DMEM at $5 \times 10^6$ cells/ml and labeled with 5 μM Calcein-AM (Molecular Probes, OR, USA) at 37°C for 30 minutes. After washing with serum-free medium twice, the monocytes were resuspended in clonetics medium at $10^6$ cells/ml.
The labeled monocytes (10^5 cells) were then added to the endothelial hCMEC/D3 monolayer and incubated for 15 minutes at 37°C to allow the adhesion [24]. The plate was then carefully rinsed twice with 1× PBS to remove non-adherent monocytes and read on a fluoroscent plate reader at 494/517 nm. The actual number of adherent monocytes was determined by comparing a standard plate with known numbers of labeled monocytes.

Transendothelial diffusion

The endothelial cell permeability was assessed by determining the flux of fluorescein through the hCMEC/D3 monolayer using a procedure described elsewhere with a slight modification [25,26]. Briefly, hCMEC/D3 cells were plated onto collagen-coated 0.4-μm Millicell hanging cell culture inserts (Millipore, MA, USA) and left to reach confluence for 7 to 10 days with a culture media change every 2 to 3 days. Cells were then treated with LPS in the presence or absence of EGCG for 24 h. After treatment, fluorescein sodium salt in clonetics medium was loaded to the apical filter compartment to produce an initial concentration of 1 μM. Subsequently, 100 μl medium was removed from the basolateral compartment after 60 minutes. The fluorescence was measured with a fluorescence microplate reader (PerkinElmer 1420 Multiplate Counter, Bridgeville, PA, USA) at 488/525 nm. The concentration of fluorescein sodium salt in the lower compartment represents the endothelial monolayer permeability [26].

Transendothelial electrical resistance

To determine the integrity of brain endothelial monolayers, transendothelial electrical resistance (TEER) measurements were performed using the 1600R ECIS system (Applied Biophysics, Troy, NY, USA). The ECIS system provides real-time monitoring of changes in TEER. Briefly, hCMEC/D3 at 10^5 per well were plated on collagen type I-coated 96W10E + electrode arrays (Applied Biophysics). The cells were then allowed to form monolayers reaching stable TEER values. After 4 days (with a media change every 2 days), the monolayers were exposed to various concentrations of LPS with or without EGCG pretreatment as indicated. The readings were acquired continuously for 72 h at 4000 Hz and at 30-minute intervals. Confluent hCMEC/D3 monolayers showed baseline TEER readings between 500 and 800 Ω/cm^2. The data are shown...
as a percentage change of baseline TEER along with the standard error of the mean of condition replicates.

Transfection and luciferase assays
The plasmid (pNF-κB-Luc) containing NF-κB promoter linked with a luciferase gene was developed by Dr Petrak [27]. Two copies of the mouse k light chain enhancer [28] were cloned into pBLCAT3 vector [29], and then the construct was modified by replacing the CAT reporter with the luciferase gene obtained from pGEM-Luc plasmid [27]. DNA was prepared by Miniprep techniques, according to the manufacturer’s instruction (Qiagen, Valencia, CA, USA) and used in the transfection experiments. For each transfection experiment, the hCMEC/D3 cells were seeded in a 12-well tissue culture plate at a density of $3 \times 10^5$ cells/well 1 day before transfection. The cells were transfected with the pNF-κB-Luc using FuGene HD Transfection Reagent (Roche Molecular Bilchemicals, Indianapolis, IN, USA) with a ratio of FuGene HD : plasmid 3:1 ($\mu$g:μg). Six hours after the transient transfection, the cells were incubated with or without EGCG (1, 5, and 25 $\mu$M) for 1 h, then treated with LPS for an additional 6 h. At the termination of the experiments, cells were washed twice with PBS, then lysed in 0.1 ml 1× Cell Culture Lysis Buffer (Promega, Madison, WI, USA). Cell-free lysates were obtained by centrifugation at 13,000 × g for 2 minutes at 4°C. The effects of LPS/EGCG on the activation of NF-κB promoter in these transiently transfected cells were determined by NF-κB promoter-driven luciferase activity. Luciferase activity in cell lysate was quantified using a luciferase assay system (Promega) and a luminometer. The results were presented as relative light units (RLU) and data were expressed as -fold of untreated cells. Measurements were performed by calculating the average of triplicate samples of two independent experiments.

Statistically analysis
Data are expressed as the mean ± SD of at least three independent experiments. Statistical significance was analyzed using a one-way analysis of variance (ANOVA) followed by post Newman-Keul’s test. It was considered statistically significant when the P-value was less than 0.05.

Results
(−) Epigallocatechin gallate inhibits lipopolysaccharide-induced expression of monocyte chemotactic protein and adhesion molecule
Endothelial cells express MCP and adhesion molecules that mediate the interactions between cells of the immune system and the endothelium system. We found that LPS treatment of hCMEC/D3 cells induced the expression of MCP-1 in both time-dependent (Figure 2A,D) and dose-dependent (Figure 2B,E) fashions. EGCG pretreatment at 5 and 25 $\mu$M significantly inhibited the upregulation of MCP-1 by LPS treatment (Figure 2C,F). In addition, LPS could time- and dose-dependently induce the mRNA expressions of two adhesion molecules, ICAM-1 (Figure 3A,B) and vascular adhesion molecule (VCAM)-1 (Figure 3D,E). The induction of ICAM-1 and VCAM-1 by LPS was also observed at the protein level as determined by western blotting (Figure 3G). It was found that 6 h stimulation was enough to potently induce the protein expression of ICAM-1 and VCAM-1 (Figure 3G), with no significant increase when the treatment was extended to 24 h or 48 h. Therefore, we used 6 h stimulation when performing monocyte adhesion experiments. We next examined whether EGCG pretreatment of hCMECs could attenuate the expression of ICAM-1 and VCAM-1. We observed that EGCG pretreatment of hCMEC/D3 cells inhibited LPS-mediated induction of ICAM-1 and VCAM-1 at both the mRNA (Figure 3C,F) and protein levels (Figure 3H).

Epigallocatechin gallate mediates monocyte adhesion to endothelial cells
Since MCP-1, ICAM-1 and VCAM-1 are the key elements in mediating the adhesion of leukocytes to vascular endothelia, we investigated the effect of EGCG on monocyte adhesion to hCMEC/D3 cells. Figure 4A shows that LPS treatment of hCMEC/D3 significantly increased monocyte adhesion to the endothelial cell monolayer. This LPS effect was in a dose-dependent manner (Figure 4B). EGCG alone has little effect on monocyte adhesion whereas EGCG pretreatment of hCMEC/D3 cells attenuated LPS-induced adhesion of monocytes to the endothelial monolayer (Figure 4A,B).
Epigallocatechin gallate blocks lipopolysaccharide-mediated suppression of Claudin 5 and Occludin

The tight junctional proteins, mainly Claudins and Occludins, are closely associated with the integrity of the BBB. We therefore examined the effect of EGCG on LPS-induced expression of these proteins. As shown in Figure 5A and B, LPS treatment suppressed the expression of Claudin 5 and Occludin in hCMEC/D3 cells at the mRNA level. This LPS effect, however, was inhibited in a dose-dependent fashion by EGCG pretreatment (Figure 5A, B). As it has been reported that Occludin was not consistently detected at cell-cell contacts of hCMEC/D3 cells [17], we only examined the protein expression of Claudin 5 and ZO-1 in hCMEC/D3 cells by immunofluorescence assay. Figure 5C and D shows that the LPS treatment compromised the expression of these two junctional proteins while EGCG pretreatment could protect the loss of Claudin 5 and ZO-1 at the cell-cell contacts.

(-)-Epigallocatechin gallate protects blood–brain barrier permeability compromised by lipopolysaccharide treatment

We next examined the effect of LPS with or without EGCG pretreatment on the BBB permeability. As shown in Figure 6A, LPS treatment enhanced the diffusion of sodium fluorescein across the hCMEC/D3 monolayer to the basal compartment, while the pretreatment...
with EGCG reduced LPS-mediated diffusion of sodium fluorescein in the hCMEC/D3 cells (Figure 6A). hCMEC/D3 cells form a relative low TEER across the monolayer as compared with primary human brain microvascular endothelial cells [30]. Figure 6A shows that LPS treatment reduced the TEER of the hCMEC/D3 monolayer while EGCG pretreatment retains the TEER at a comparatively high level. Thus, EGCG has protective activity against LPS-induced compromise of BBB permeability.

![Figure 4](image)

**Figure 4** Effect of (−)-epigallocatechin gallate on lipopolysaccharide-induced monocyte adhesion to endothelial cells. (A) Human cerebral microvascular endothelial cells/D3 (hCMEC/D3) were pretreated with or without (−)-epigallocatechin gallate (EGCG) (25 μM) for 1 h prior to lipopolysaccharide (LPS) (100 ng/mL) treatment for an additional 6 h. Calcein-AM-labeled fresh monocytes were then added to hCMEC/D3 cell cultures and incubated for 15 minutes at 37°C. The fluorescence was measured at 494/517 nm and the actual numbers of adherent monocytes were determined by comparing a standard plate with known numbers of labeled monocytes. (B) hCMEC/D3 cells were treated with LPS at the indicated concentrations for 6 h, or EGCG (1, 5, 25 μM) for 7 h, or pretreated with EGCG (1, 5 or 25 μM) for 1 h followed by LPS treatment (100 ng/mL) for 6 h. Data are expressed as mean ± SD of three different experiments (*P < 0.05, **P < 0.01).

![Figure 5](image)

**Figure 5** Effect of (−)-epigallocatechin gallate on the expression of Claudin-5/Occludin of human cerebral microvascular endothelial cells/D3. Human cerebral microvascular endothelial cells/D3 (hCMEC/D3) were pretreated with (−)-epigallocatechin gallate (EGCG) at the indicated doses for 1 h prior to lipopolysaccharide (LPS) treatment (100 ng/mL) for 24 h. RNA extracted was subjected to real time PCR for Claudin-5 and Occludin expression (A,B). Data are expressed as mean ± SD of three independent experiments. (C) hCMEC/D3 cells were pretreated with 5 μM EGCG for 1 h and then with 100 ng/ml LPS for 24 h. Cells were fixed and then incubated with anti-Claudin 5 or anti-ZO-1 antibody and observed under a fluorescence microscope (magnification ×200).
NF-κB is a key transcription factor for regulating the immune response to bacterial or viral infections. Dysregulations of NF-κB are associated with inflammatory and autoimmune diseases, viral infections and improper immune responses [31]. As shown in Figure 7, LPS treatment of hCMEC/D3 cells induced the activation of NF-κB. When the cells were pretreated with 25 μM EGCG, LPS-induced NF-κB activity was attenuated from 20-fold to 8-fold (Figure 7), while EGCG alone had little effect on NF-κB activation or inhibition (data not shown).

**67-kDa laminin receptor is involved in (−)-epigallocatechin gallate-mediated suppression of lipopolysaccharide-mediated inflammatory cytokine induction**

67LR, a cell-surface receptor for EGCG, has been shown to be involved in the EGCG-mediated inhibitory effect on the TLR4 signaling pathway in macrophages [32]. We therefore examined whether EGCG acts through 67LR on the inhibition of LPS-mediated inflammation. We found that, when hCMEC/D3 cells were pretreated with neutralization antibody to 67LR prior to EGCG and LPS treatment, the inhibitory effect of EGCG on LPS-induced inflammatory cytokine expression was abrogated (Figure 8A,B). The blockade of 67LR also attenuated the inhibitory effect of EGCG on LPS-mediated NF-κB activation (Figure 8C) and increased fluorescein sodium transmembrane diffusion (Figure 8D). EGCG alone had little effect on the expression of TLR4 and MyD88 (Figure 9).

**Discussion**

In the present study, we demonstrated that EGCG, a bioactive polyphenol in green tea, suppressed the expression of LPS-induced inflammatory cytokines in brain endothelial cells. This finding supports a previous report showing that EGCG attenuated LPS-mediated inflammation by suppressing the TNF-α and IL-1β expression in macrophages, leading to the downregulation of inflammatory responses [32].
EGCG pretreatment also inhibited the monocyte adhesion to the brain endothelial cell monolayer and blocked the negative impact of LPS on the tight junctional protein expression in brain endothelial cells. MCP-1/CCL2 plays a critical role in the recruitment of leukocytes to the site of inflammation elicited by LPS [33]. It has been reported that EGCG could decrease the MCP-1 and CCR2 gene expression, together with MCP-1 secretion and CCR2 expression at the cell surface in THP-1 monocytes, thus preventing the migration and adhesion of monocytes to fibronectin [34]. Our data showed that in unstimulated hCMEC/D3 cells, the basal expression of MCP-1/CCL2 was low but measurable, while LPS treatment greatly induced the expression of MCP-1/CCL2. This is consistent with a previous report [35] showing that hCMEC/D3 cells express lower levels of MCP-1/CCL2 than two primary human brain endothelial cells generated from multiple sclerosis brain tissue or from temporal lobe resections from epileptic patients.

In addition, we found that LPS treatment of hCMECs induced the expression of ICAMs and VCAMs, the key ligands for the beta2 integrin molecules present on leukocytes [36]. The expression of ICAM-1 in hCMEC/D3 cells is higher than that of VCAM-1 [17]. ICAM-1 and VCAM-1 facilitate the adhesion of monocytes to the endothelium. Endothelial ICAM-1 was shown to be essential for T cell diapedesis across the BBB under static conditions [37]. Adamson and colleagues [38] revealed that endothelial ICAM-1 was essentially involved in lymphocyte migration through brain endothelial cell monolayers by rearrangement of the endothelial actin cytoskeleton and functional endothelial cell Rho proteins. Treatment of HBMEC with TNF-α resulted in increased polymorphonuclear leukocyte adhesion that was significantly inhibited by blocking
antibodies to E-selectin and ICAM-1, but not VCAM-1 [39].

It is well known that NF-κB activation is required for the LPS-induced inflammatory cytokine production [40]. Endothelial-selective blockade of NF-κB activation repressed expression of multiple endothelial adhesion molecules and reduced neutrophil infiltration into multiple organs [41]. EGCG was found to have the ability to block NF-κB activation in the intestinal epithelial cell line IEC-6 [42]. In addition, theaflavin-3,3'-digallate (another polyphenol) from black tea was also reported to have even stronger suppression of LPS-induced NF-κB activity than other polyphenols through downregulation of IκB kinase activity in macrophages [43]. Thus, the suppression of NF-κB activation by EGCG justifies the inhibitory effect of EGCG on LPS-mediated endothelial inflammation.

It is known that TLR4 is involved in LPS-mediated inflammation. Sheth and colleagues [44] showed that LPS disrupts tight junctions in cholangiocyte monolayers by a c-Scr-, TLR4- and LBP-dependent mechanism. A recent report [32] showed that EGCG downregulated inflammatory responses by directly suppressing TLR4 mRNA and protein expression. In addition, EGCG treatment of macrophages was found to upregulate the expression of Tollip [32], a negative regulator of the TLR signaling pathway. However, our data demonstrated that EGCG had little effect on TLR4 expression in brain endothelial cells. Youn and colleagues [45] reported that EGCG could inhibit LPS- or PolyI:C-mediated activation of interferon regulatory factor 3. EGCG could modulate both MyD88- and TIR-domain-containing adapter-inducing interferon-(TRIF)-dependent signaling pathways of TLR3 and the subsequent inflammatory target gene expression in macrophages. However, we did not observe a modulatory effect of EGCG on the expression of MyD88, a key adaptor to mediate the TLR-MyD88-dependent signaling pathway. These discrepancies may be due to the different types of cells used to investigate EGCG activity, suggesting that other mechanisms are likely to be involved in EGCG-mediated inhibition of endothelial inflammation.

67LR is a receptor that presents on the eukaryotic cell membrane for the cellular prion proteins and also interacts with extracellular matrix components [46]. 67LR was also found to express on both rodent and human cerebral endothelial cells [47]. It has been suggested that 67LR serves as a co-receptor for bacterial pathogens that target the BBB. 67LR on brain endothelial cells interacts with three most commonly neuroinvasive bacteria: pneumococcus, H. influenzae, and meningococcus. The binding of 67LR to these pathogens initiates the bacterial interaction...
with the BBB and promote their CNS tropism, inducing cell signaling through the other receptors, such as the TLRs. The interaction of TLRs with 67LR may synergistically promote bacterial adherence and invasion of BBB [47]. Interestingly, 67LR was shown to be involved in the inhibitory effect of EGCG on the TLR4 signaling pathway in macrophages [32]. These findings promoted our interest in examining the role of 67LR in the anti-inflammatory effect of EGCG in brain endothelial cells. Our data that pretreatment of hCMEC cells with anti-67LR antibody significantly blocked the EGCG effect on LPS-mediated TNF-α and IL-1β induction, as well as on NF-κB activation, indicate that EGCG exerts its anti-inflammatory effect in endothelial cells at least partially through 67LR. It is likely that pretreatment of hCMEC/D3 cells with EGCG enables the binding of 67LR to EGCG and disrupts or modulates LPS interaction with 67LR. It has been suggested this disruption or modulation might engender unexpectedly broad protection against systemic infections [47]. Thus, our data support the notion that EGCG can be used as a potential therapeutic compound to treat CNS inflammation related to the BBB.

Abbreviations
67-LR: 67-kDa laminin receptor; ANOVA: analysis of variance; BBB: blood–brain barrier; CNS: central nervous system; DMEM: Dulbecco’s modified Eagle’s medium; EGCG: (–)-epigallocatechin gallate; ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HBMEC: human brain microvascular endothelial cells; hCMEC: human cerebral microvascular endothelial cell; ICAM: intercellular adhesion molecule; IL: interleukin; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; MCP: monocyte chemotactic protein; NF: nuclear factor; PBS: phosphate-buffered saline; PMN: polymorphonuclear leukocytes; qRT-PCR: quantitative real time polymerase chain reaction; TEER: transendothelial electrical resistance; TLR: Toll-like receptor; TNF: tumor necrosis factor; VCAM: vascular adhesion molecule.

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

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Authors’ contributions
JL and LY designed and performed experiments, and drafted the manuscript. JL, LY, XW, YW and YZ performed experiments. WZH conceived of the study, JL and LY designed and performed experiments, and drafted the manuscript. Authors’ contributions

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