

SHORT REPORT

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The inhibitory effect of LPS on the expression of GPR81 lactate receptor in blood-brain barrier model in vitro

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Abstract

Background: Lipopolysaccharide (LPS) is one of the main constituents of the cell wall of gram-negative bacteria. As an endotoxin, LPS induces neuroinflammation, which is associated with the blood-brain barrier impairment. Lactate is a metabolite with some significant physiological functions within the neurovascular unit/blood-brain barrier (BBB). Accumulation of extracellular and cerebrospinal fluid lactate is a specific feature of bacterial meningitis. However, the role of lactate production, transport, and sensing by lactate receptors GPR81 in the pathogenesis of bacterial neuroinflammation is still unknown.

Methods: In this study, we analyzed effects of LPS on the expression of GPR81 and MCT-1 and proliferation of cerebral endothelial cells in the BBB model in vitro. We used molecular profiling methods to measure the expression of GPR81, MCT-1, IL-1 β , and Ki67 in the cerebral endothelium after treatment with different concentrations of LPS followed by measuring the level of extracellular lactate, transendothelial electric resistance, and permeability of the endothelial cell layer.

Results: Our findings showed that exposure to LPS results in neuroinflammatory changes associated with decreased expression of GPR81 and MCT-1 in endothelial cells, as well as overproduction of IL-1 β and elevation of lactate concentrations in the extracellular space in a dose-dependent manner. LPS treatment reduced JAM tight junction protein expression in cerebral endothelial cells and altered BBB structural integrity in vitro.

Conclusion: The impairment of lactate reception and transport might contribute to the alterations of BBB structural and functional integrity caused by LPS-mediated neuroinflammation.

Keywords: Lipopolysaccharide, Neuroinflammation, Blood-brain barrier, Lactate

Introduction

Bacterial meningitis is a life-threatening disease of the central nervous system (CNS), particularly, in children. Despite the progress in development of novel antibiotic-based treatment methods in early diagnosis of bacterial meningitis, the mortality and morbidity rates of this disease are still very high. Bacterial meningitis is recognized as the main cause of the top ten infection-associated death worldwide [1, 2]. The disease is an inflammation of meninges (arachnoid and

pia mater) caused by the invasion of bacteria (*Haemophilus influenzae*, *Neisseria meningitides*, streptococci, pneumococci) into the subarachnoid space [3]. Among these pathogens, *Neisseria meningitides* is the leading cause of the infection in children and young adults [4]. Lipopolysaccharide (LPS) is a major endotoxin found in Gram-negative bacteria with strong pro-inflammatory effects [5]. LPS is a major component of the *Neisseria meningitides* outer membrane, responsible for inflammatory responses in meningitis and sepsis [6, 7]. LPS is usually used for bacterial meningitis modeling in vivo and in vitro due to its well-known effects on immune cells and cerebral microvessel endothelial cells [8, 9].

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Several pathogenic mechanisms lead to the development of brain injury in bacterial meningitis, including leukocyte transmigration and microglia activation. Such events cause cytotoxicity, neuronal cell death, and local production of pro-inflammatory cytokines and chemokines. Overall, these effects lead to acute neuroinflammation, remodeling of extracellular matrix, BBB breakdown, and progression of neurological deficits [10, 11].

BBB structural and functional integrity is controlled by various mechanisms, such as coordinated expression of tight junction and adherence junction proteins in brain microvessel endothelial cells (BMECs), functional activity of perivascular cells (pericytes, astrocytes), and complex intercellular interactions within the neurovascular unit (NVU) [12]. Loss of BBB integrity in bacterial meningitis can be primary (due to the direct effect of pathogens on NVU cells) or secondary (due to the overwhelming production of pro-inflammatory cytokines by microglial and astroglial cells, diminished metabolism of BMECs, and impaired reparative angiogenesis in cerebral microvessels) [13–16]. Interaction of bacteria with the brain endothelium alters the junctional machinery in BMECs, thereby resulting in neuroinflammation associated with pathological overproduction of cytokines with pro-inflammatory action (i.e., IL-1 β , IL-6, TNF α , chemokines) and elevated permeability of the barrier [17]. Particularly, LPS induces a significant increase of BBB permeability by changing the RhoA signaling and cytoskeletal rearrangements in BMECs [18], stimulating the cyclooxygenase activity [19] and activating matrix metalloproteinase [20, 21]. Previous *in vitro* studies showed that LPS mediates the BBB breakdown at 24 h. This phenomenon is characterized by tight junction deregulation, reactive oxygen species production in BMECs, and cytokine production by activated microglia [22, 23]. However, some other reports suggested that BBB breakdown is not necessarily correlated with the pathogenesis of bacterial meningitis [2].

Inflammation in bacterial meningitis is accompanied by a prominent increase of lactate concentrations in cerebrospinal fluid (CSF) and can be used as a reliable criterion for differential diagnosis of bacterial and aseptic meningitis [24], and/or viral meningitis [25]. Local production and transport of lactate within the NVU supports integration and normal functions of BBB [12] whereas elevated concentrations of lactate might be associated with neuroinflammation and BBB impairment [26]. Within the neurovascular unit, lactate is produced by neurons, perivascular astroglial cells, or BMECs. Monocarboxylate transporters (MCTs) carry on the influx and efflux of lactate and contribute to effective metabolic coupling of NVU cells [27]. However, in some other cell types, inhibition of MCT-1 activity results in the suppression of angiogenesis [28]. GPR81 receptors transport lactate into BMECs. These receptors are expressed at luminal and

abluminal membranes that act as metabolic sensors. As we demonstrated before, elevation of extracellular lactate or activation of GPR81 stimulate mitochondrial biogenesis and support angiogenic properties of BMECs *in vitro* [12, 29]. However, there are no data on the role of GPR81 or MCT-1 in the pathogenesis of BBB impairment in bacterial meningitis. Therefore, in this study, we analyzed the *in vitro* effects of LPS on the expression of GPR81 and MCT-1 in BMECs associated with neuroinflammation and BBB breakdown.

Materials and methods

Isolation and culture of BMECs within the BBB model *in vitro*

This study was performed on Wistar rats cells ($n = 5$ for isolation of astroglial and neuronal cells; $n = 5$ for isolation of cerebral endothelial cells). All experiments and animal use were conducted in compliance with the European Community Directive (2010/63/EC) and were approved by the Local Ethic Committee of the Krasnoyarsk State Medical University named after Prof. V.F. Voino-Yasenetsky. We isolated rat BMECs from P10 rats using the protocol of Liu et al. with minimal modifications [30]. Astroglial and neuronal cells were isolated from P4 neonatal rats according to a standard protocol [31]. The cells were cultured at 37 °C, 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM/F12 + 20% FBS, 3 mg/ml glucose, 0.58 mg/ml glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin) in gelatin-coated vials (seeding density 1×10^5 cell/ml). Culture medium was changed every 2–3 days. When the cells reached 90% confluence, they were separated by trypsinization and seeded in culture on gelatin-coated cups. Phase-contrast microscopy was continuously used to evaluate cell morphology (Additional file 1).

Assessment of BBB integrity and marker expression

BBB model *in vitro* was reconstructed according to an original protocol described in details elsewhere [32]. Briefly, cells (endothelial, neuronal, and astroglial) were co-cultured in plates where neurons and astrocytes placed on wells bottom and endothelial cells placed on inserts while covered with semi-permeable membrane (incubated at 37 °C, 5% CO₂).

Monitoring of BBB permeability was carried out using TEER measurement system known as an Epithelial Voltohmmeter (EVOM2) with chopstick electrode STX2 (World Precision Instrument, USA). Achieving a TEER of $\geq 120 \Omega$ was used as a criterion to obtain BBB model with the permeability characteristics suitable for further studies.

Immunohistochemistry was used to assess the expression of GPR81 receptors, lactate transporter MCT-1, pro-inflammatory cytokine IL-1 β , and cell proliferation marker Ki67 with appropriate primary and secondary

antibodies. We used primary antibodies at 1:400 and secondary at 1:600 (Abcam, USA) followed by visualization with confocal laser microscopy (Olympus FV10i-W, Japan) and Olympus Fluoview software. The relative numbers of antigen-positive cells were counted in at least 10 fields.

Lactate extracellular concentrations were evaluated spectrophotometrically with the Lactate assay kit (Sigma-Aldrich, USA). Cell viability was tested with Trypan blue.

Analysis of BMEC monolayer permeability for Lucifer yellow

Lucifer yellow (LY) assay allows measuring the endothelial layer permeability in vitro [22]. For transport experiments, all media were removed from the upper chamber of the insert and replaced with pre-warmed media with solutions of LY (50-μM working concentration). Samples were collected at 30, 60, and 90 min. At the end of each experiment (5 replicates), the concentration of the fluorescent compounds accumulated in the chamber was measured using a spectrofluorimeter (CM2203, “SOLAR”, Belarus) with excitation at 430/485 nm and emission at 535 nm.

We used the following equation to measure the permeability coefficients:

$$\text{Permeability coefficients (Pe)} = dC/dT \times V/(A \times C)$$

[V = sample volume, dC = concentration variations, dT = time variations, C = the initial concentration in the donor compartment, A = exposed surface (cellular monolayer in cm²).]

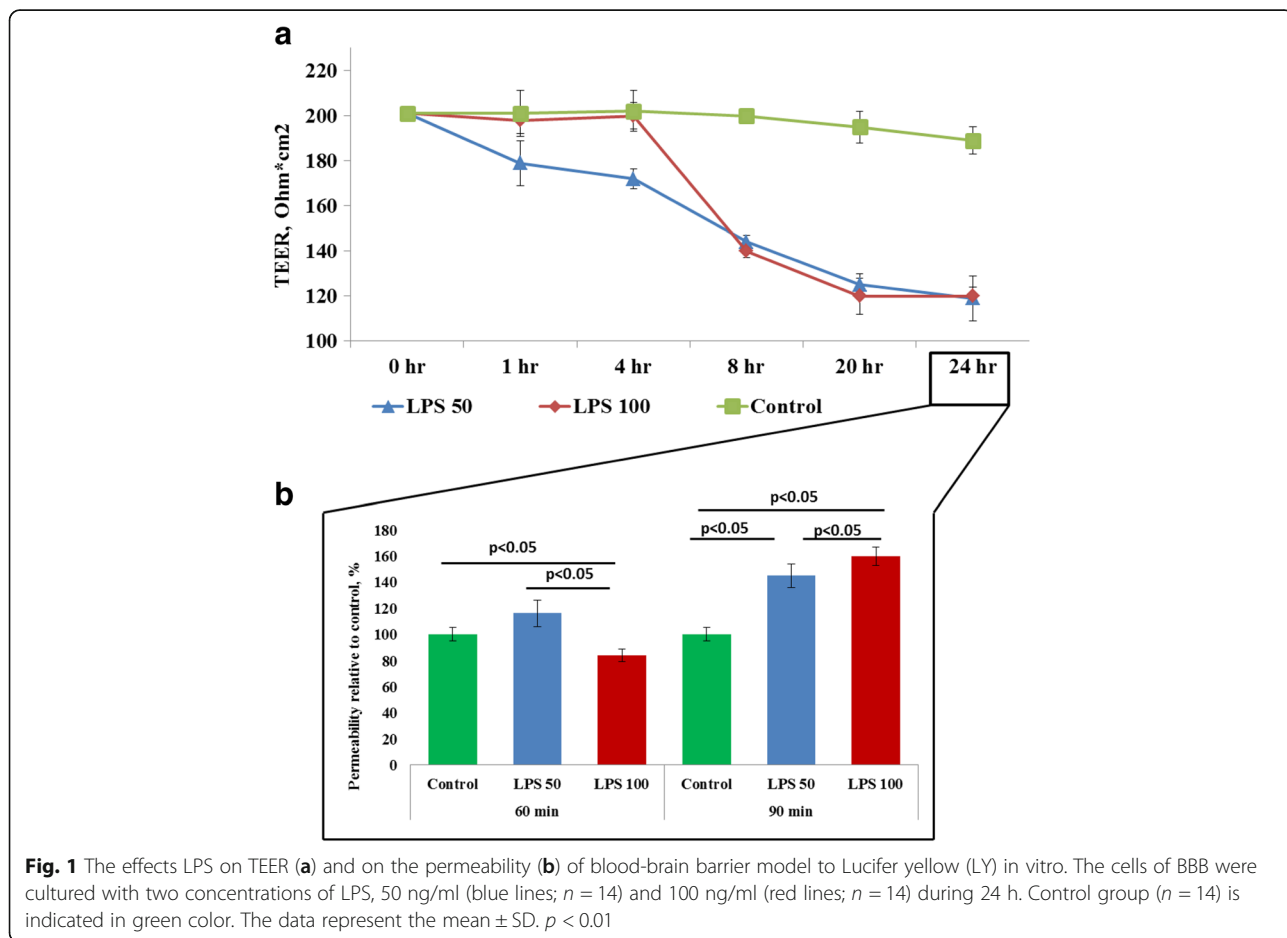
LPS-induced inflammation in BBB model in vitro

We tested the effects of LPS on BMECs and BBB in vitro at 24 h of LPS exposure (*Escherichia coli* strain 0111:B4 LPS lot no L4391, Sigma, USA), concentrations 50 and 100 ng/ml (indicated as LPS₅₀ and LPS₁₀₀, respectively).

Statistical analysis was performed using the nonparametric data χ² tests, Kruskal-Wallis test result with pairwise comparisons for the groups. P values below 0.05 were considered significant.

Results and discussion

In vitro exposure of rat BBB model to LPS in two tested concentrations during 24 h resulted in a progressive



decrease of TEER and elevated permeability of the barrier in LY-test (Fig. 1a, b). Thus, LPS-induced breakdown was evident in BBB model. Incubation of BMECs (within the BBB model) with LPS exhibited a reduction in GPR81 expression after 24 h of incubation with no clear difference between two tested LPS concentrations (Fig. 2a). Similarly, expression of lactate transporter MCT-1 in brain microvessel endothelial cells was dramatically suppressed after incubation with LPS for 24 h (Fig. 2b). The observed changes in the expression of lactate transporters were associated with elevated concentrations of lactate in the culture medium at 24 h of BBB model exposure to LPS (Fig. 2f). However, these changes in extracellular lactate concentrations were not caused by cytotoxicity. We evaluated the viability of the cells in each group before measuring the expression of the target molecules and lactate levels in the medium. The average number of viable cells was comparable in all tested groups (95% in control versus 93% in LPS₅₀-treated group and 94% in LPS₁₀₀-treated group). Thus, elevated

extracellular lactate is actively produced rather than passively released from damaged cells. The origin of lactate produced in the LPS-treated BBB model remains to be evaluated, i.e., astroglial production is rather possible.

We found a reduced expression of junctional adhesion molecule (JAM) proteins in BMECs (Fig. 2d) and elevated expression of pro-inflammatory cytokine IL-1 β in BBB model at 24 h of LPS exposure (Fig. 2e). Thus, LPS-induced changes suggest establishment of a pro-inflammatory micro-environment, altered transport and reception of extracellular lactate, and deregulation of tight junction protein expression in BMECs (Fig. 3). Neuroinflammation and loss of BBB integrity are well-known triggers of BMEC proliferation [33]. Moreover, after being cultured within the BBB model, BMECs demonstrated visible angiogenic potential. Taking into the consideration that activation of glycolysis and lactate production is a prerequisite for BMEC activation and proliferation [34], we further tested the proliferative activity of BMECs which were exposed to LPS for 24 h. We assessed the expression of Ki67 protein in

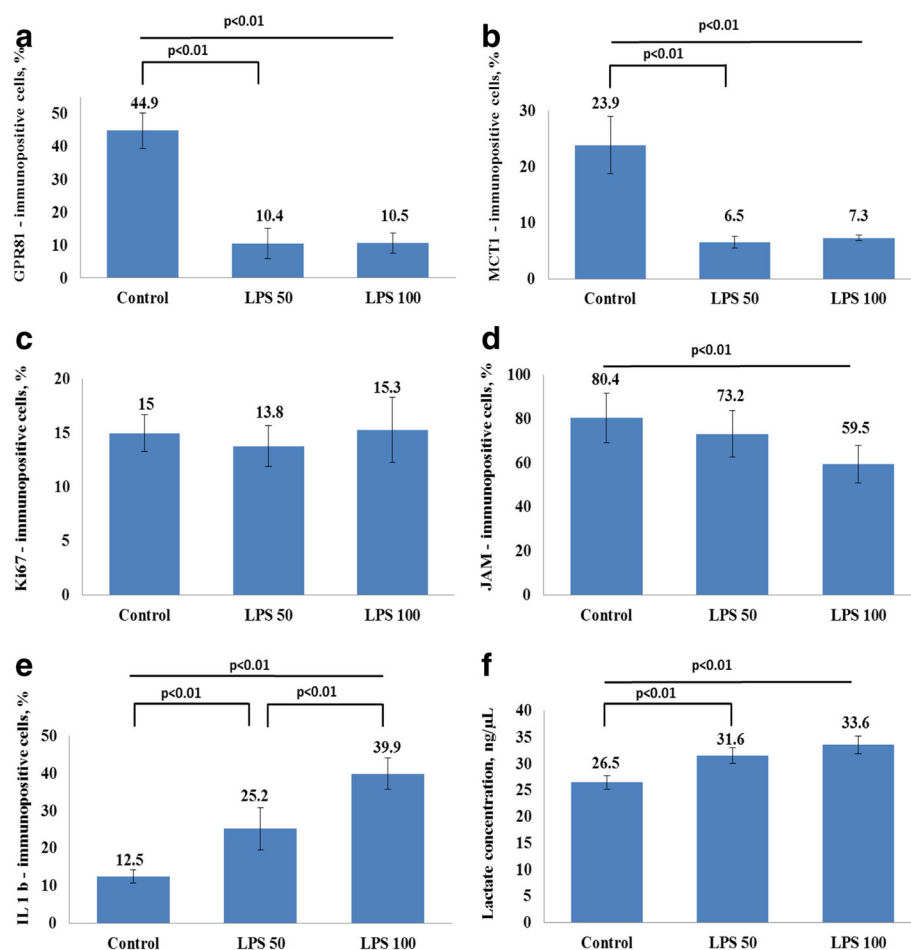


Fig. 2 The effect of LPS on the expression of **a** GPR81, **b** MCT-1, **c** Ki67, **d** JAM, **e** IL-1 β , and **f** lactate concentrations in the BBB in vitro model. The cells of BBB were cultured with LPS concentration 50 and 100 ng/ml during 24 h. The control group, $n = 8$; LPS₅₀, $n = 8$; LPS₁₀₀, $n = 8$. The data represent the mean \pm SD. $p < 0.01$

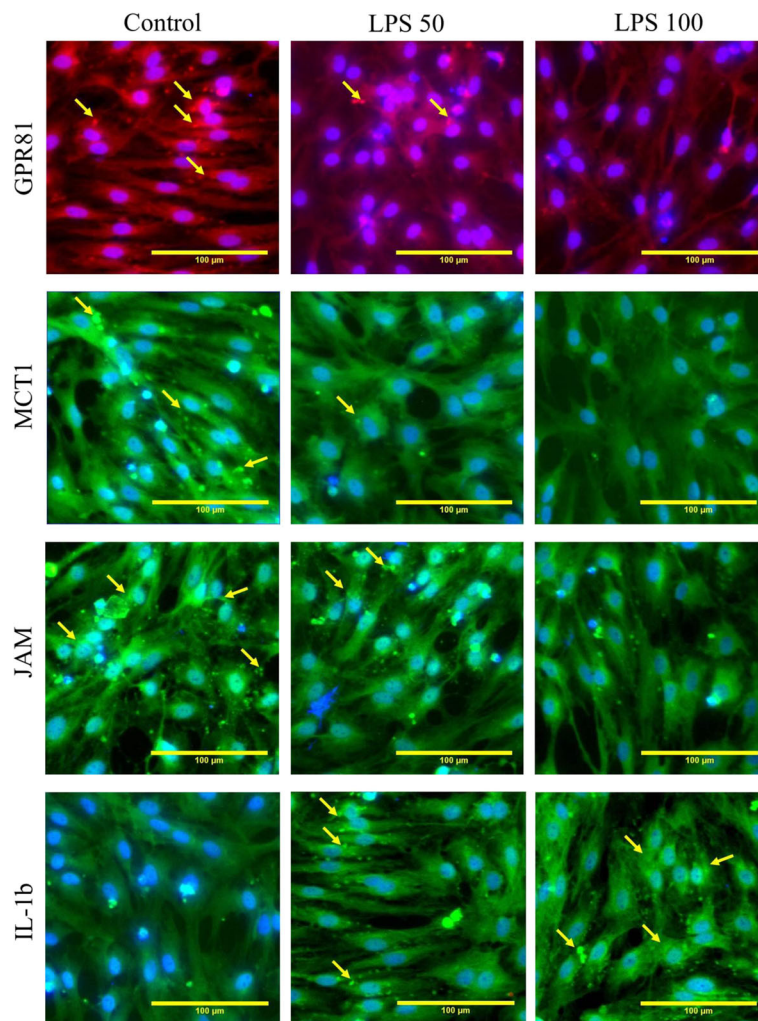


Fig. 3 Microphotographs of endothelial monolayer in the model of BBB in vitro. The photos show GPR81, MCT-1, JAM, and IL-1 β expression after incubations of BMECs with LPS₅₀ ($n = 8$), LPS₁₀₀ ($n = 8$), or in the control group ($n = 8$). Scale bars 100 μ m

BMECs as a marker of cell proliferation. Moreover, altered expression of Ki67 was evident in human BMECs affected by *N. meningitis* [35]. We found that Ki67 expression in rat BMECs exposed to LPS was not significantly changed at 24 h (Fig. 2c). However, Ki67 expression in BMECs was significantly reduced at 72 h (16.5% Ki67-immunopositive cells in the control versus 10.6% in LPS₅₀-treated group and 7.8% in LPS₁₀₀-treated group ($p < 0.01$)). Thus, BBB breakdown and impaired transport and reception of lactate in BMECs were not associated with the stimulation of their proliferation in LPS-induced neuroinflammation, but rather resulted in long-lasting suppression of BMEC angiogenic potential.

Expression of GPR81 receptors have been previously reported in BMECs [36], but their role in the regulation of BBB integrity or neurovascular coupling is mainly unknown. Proposed involvement of GPR81 and MCT-1 in the pathogenesis of BBB alterations in cerebral malaria

[26] suggests that similar mechanisms might be active in a case of acute meningitis. Excessive glycolysis in brain cells is a characteristic of bacterial meningitis [37]; thus, BMECs should be equipped with effective machinery for transporting and sensing elevated brain tissue lactate concentrations. However, observed changes in GPR81 and MCT-1 expression in LPS-treated cells of BBB in vitro could reflect impaired lactate clearance through the BBB in bacterial meningitis. Recently, we found that stimulation of GPR81 receptors in brain microvessel endothelial cells resulted in the stimulation of mitochondrial biogenesis [29] which is a prerequisite for cell proliferation and highly controlled permeability of endothelial cell layer [38, 39]. Therefore, reduced expression of lactate transporters and GPR81 in BMECs exposed to LPS could prevent lactate-mediated effects on mitochondrial dynamics, thus resulting in the suppression of endothelial metabolism. Given into the consideration that endothelial cells could use extracellular

MCT-1-transported lactate as a “fuel” for mitochondrial respiration, we may propose that reduced expression of MCT-1 in LPS-treated BMECs within the BBB model would negatively affect their mitochondrial energy production and metabolic status. The latter could lead to impairment of tight junctions and barrier function of BMECs as well as suppression of angiogenesis. Thus, elevated production of lactate due to the presence of pro-inflammatory concentrations of LPS is associated with reduced transport and reception of lactic acid by endothelial cells, impaired lactate clearance mechanism, and loss of BBB structural integrity.

Conclusions

Action of LPS in the BBB model in vitro results in endothelial layer impairment which is coupled to the accumulation of extracellular lactate and reduced ability of brain microvessel endothelial cells to sense or transport lactic acid. Reduced expression of GPR81 lactate receptors and MCT-1 lactate transporters in brain microvessel endothelial cells corresponds to the loss of BBB integrity and elevated permeability of the barrier in vitro.

Additional file

Additional file 1: Figure S4. Phase contrast microphotograph (ZOE Fluorescent Cell Imager, Bio-rad, USA) of endothelial cells in the in vitro model of BBB, showing the monolayer status of cultured cells. Figure magnification $\times 20$. Scale bar represents 100 μm . (DOCX 333 kb)

Abbreviations

BBB: Blood-brain barrier; BMECs: Brain microvessel endothelial cells; CSF: Cerebrospinal fluid; GPR81: G protein-coupled receptor 81; IL-1 β : Interleukin 1-beta; JAM: Junctional adhesion molecule; LPS: Lipopolysaccharide; MCT-1: Monocarboxylate transporter 1; NVU: Neurovascular unit; TEER: Transendothelial electric resistance

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Availability of data and materials

Please contact the author for data requests.

Authors' contributions

EB, EO, AM, GM, OF, RO, YG, and YP carried out experimental works. EB, AM, EO, AT, OF, EP, and AS participated in the design of the study and performed the statistical analysis. EB, EP, AM, OF, AT, and AS conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval

All experiments and animal use were conducted in compliance with the European Community Directive (2010/63/EC) and were approved by the Local Ethic Committee of the Krasnoyarsk State Medical University named after Prof. V.F. Voino-Yasenetsky.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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