


Original Research

Adenosine Acts Through A2AR to Alleviate the Inflammatory Response Caused by Activation of Perivascular-Resident Macrophage-Like Melanocytes

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Academic Editor: Gabriella Lupo

Submitted: 24 September 2025 Revised: 10 November 2025 Accepted: 21 November 2025 Published: 22 December 2025

Abstract

Background: Disruption of the blood labyrinth barrier (BLB) is considered a pathological cause of diverse hearing impairments. Perivascular-resident macrophage-like melanocytes (PVM/Ms) play a critical role in maintaining inner ear homeostasis and BLB integrity. Activation of PVM/Ms leads to decreased production of pigment epithelium-derived factor (PEDF), contributing to BLB breakdown. This study investigated the role of the adenosine A2A receptor (A2AR) pathway in lipopolysaccharide (LPS)-induced inflammation in PVM/Ms and elucidated the underlying mechanisms. **Methods:** The anti-inflammatory effects of adenosine and its specific receptor A2AR were evaluated in LPS-induced PVM/Ms. The levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) were measured by quantitative real-time PCR (qRT-PCR) and enzyme linked immunosorbent assay (ELISA). Additionally, matrix metalloproteinase-9 (MMP-9) and PEDF were quantified using western blot and ELISA, respectively. An endothelial cell (EC)-PVM/M co-culture model exposed to LPS was established and treated with adenosine and SCH58261 to assess effects on BLB permeability. **Results:** LPS treatment significantly changed the production of inflammatory factors, including IL-6 and TNF- α , as well as MMP-9, TIMP-1, and PEDF. These changes were abrogated by adenosine, which also reduced the production of reactive oxygen species (ROS) and inhibited the activation of PVM/Ms. SCH58261 partially reversed the effects of adenosine following LPS treatment. The p38 MAPK pathway was found to be involved in adenosine regulation of LPS-induced PVM/Ms. **Conclusions:** Adenosine attenuates the inflammatory activation of PVM/Ms and enhances their ability to maintain endothelial barrier integrity by binding to A2AR. The findings support adenosine and A2AR as potential therapeutic targets for treating hearing impairments.

Keywords: adenosine; adenosine A2A; lipopolysaccharides; stria vascularis; cochlea

1. Introduction

The cochlear blood labyrinth barrier (BLB) tightly regulates the cochlear micro-environment for auditory function [1–5]. Disruption of the BLB is strongly associated with a number of hearing disorders, including noise-induced hearing loss and age-related hearing loss [1]. Progress has recently been made in detailing the structural complexity of this barrier. This has highlighted the critical role of accessory cells in BLB integrity, such as perivascular-resident macrophage-like melanocytes (PVM/Ms) [1,2]. The authors previously demonstrated that PVM/Ms are activated in animals exposed to loud sound, in aged animals, and in animals with induced inflammation [3–5]. Previous research has also demonstrated that lipopolysaccharide (LPS)-induced PVM/M activation, through its induction of cytokine production, affects the expression of tight junction-associated proteins (zonula occludens-1 [ZO-1], occludin, and vascular en-

dothelial cadherin) and is one of the main causes of BLB disruption [5].

LPS-induced damage of the blood brain barrier (BBB) is associated with the expression of pro- and anti-inflammatory cytokines, including interleukin-1 (IL-1), reactive oxygen species (ROS), and tumor necrosis factor- α (TNF- α) [5,6]. These cytokines further activate matrix metalloproteinases (MMPs), alter transporter function, and significantly affect vascular permeability [7–9]. Among them, matrix metalloproteinase-9 (MMP-9) has been shown to destroy tight junctions and degrade vascular basement membranes, leading to breakdown of the BBB [10–13]. The levels of ROS and MMP-9 were therefore evaluated in the present study. Additionally, the authors previously found that targeting PVM/M-mediated dysfunction of the BLB helps to improve the function of damaged vascular integrity and hearing sensitivity [2].

Adenosine is an effective endogenous immunomodulator and plays a key role in the functional regulation of



various cells and molecules in the central nervous system (CNS) [14]. Adenosine receptors consist of four subtypes: adenosine A1 receptor, adenosine A2A receptor (A2AR), adenosine 2b receptor (A2BR) and adenosine A3 receptor, all of which belong to the G protein coupled receptor family [15,16]. Adenosine regulates various cellular functions, such as inflammation, angiogenesis, and wound healing, by interacting with corresponding adenosine receptors on the cell surface. Among these, the balance of A2AR is also considered to be necessary for oxidative stress in the cochlea, which is the most common underlying mechanism of inner ear pathology [15,17]. In recent years, the potent anti-inflammatory effects of adenosine have attracted increasing attention. Activation of A2AR has been shown to play a critical anti-inflammatory role in maintaining blood retinal barrier homeostasis by decreasing microglial activation and reducing inflammatory cytokine levels [18]. However, to the best of our knowledge, the role of adenosine in regulating the integrity of the BLB has yet to be investigated.

The present study demonstrates that adenosine plays a central role in LPS-induced BLB dysfunction. We examined the effect of adenosine on PVM/Ms in LPS-induced BLB injury, as well as the underlying mechanism. The findings presented herein may inform more suitable drug therapeutic strategies for the management of hearing loss.

2. Materials and Methods

2.1 Animals

C57BL/6 mice aged 10–15 days were obtained from inbred C57BL/6J mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The protocol for the care and use of these animals was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2024-KY-0035-002).

2.2 Primary Cell Culture and Treatment

Mice were anesthetized by intraperitoneal injection of tribromoethanol (0.2 mL/10 g body weight of 1.25% solution, TG-Avertin-M, Tigergene, Wuxi, Jiangsu, China) and then euthanized by cervical dislocation. The protocols for generating the cell lines used in these experiments were reported previously [2,19]. All cell lines were validated by STR profiling and tested negative for mycoplasma. Purified primary PVM/Ms or endothelial cells (ECs) were pre-treated with 1 μ M adenosine (T0853, TopScience, Shanghai, China) for 1 h, then with 1 μ g/mL LPS (L4391, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. PVM/Ms or ECs were treated with 1 μ M SCH58261 (T6979, TopScience, China) for 1 h to inhibit A2AR activity. For the suppression of p38 mitogen-activated protein kinases (MAPK) activity, PVM/Ms or ECs were treated with 10 μ M SB203580 (S1076, Selleck, Houston, TX, USA) for 30 min.

Table 1. Primer sequences used for qRT-PCR analysis.

Gene	Sequence (5'-3')
<i>IL-6</i>	F: TTCTTGGGACTGATGCTG
	R: CTGGCTTTGTCTTTCTTGT
<i>TNF-α</i>	F: ACGGCATGGATCTCAAAGAC
	R: AGATAGCAAATCGGCTGACG
<i>TIMP-1</i>	F: TCTGGCATCCTCTTGTGCTAT
	R: CCACAGCGTCGAATCCTT
<i>PEDF</i>	F: TCGCATAGACCTTCAGGAGATTA
	R: ATCAGAGTCCAAGCCATATCGTA
<i>GAPDH</i>	F: GTGAGGCCGGTGTGCTGAGTAT
	R: GTGCAGGATGCATTGCTGAC

qRT-PCR, quantitative real-time PCR; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; TIMP-1, tissue inhibitor of matrix metalloproteinase 1; PEDF, pigment epithelial-derived factor.

2.3 Cell Viability Assay

The cytotoxicity of 1 μ M adenosine (with or without 1 μ M SCH58261) for PVM/Ms was measured within 48 h of treatment by cell counting kit-8 assay (CX001S, Cellorlab™, Shanghai, China). PVM/Ms were seeded into 96-well plates and treated with LPS (1 μ g/mL) for different times, then incubated with 10 μ L of CCK-8 solution at 37 °C and 5% CO₂ for 3 h. The absorbance of surviving cells was measured at 450 nm using a microplate reader.

2.4 Quantitative Real-Time PCR (qRT-PCR)

The procedure was reported previously [20]. Briefly, total RNA (300–500 ng) was extracted from different groups of PVM/Ms using an RNA extraction kit (RNAiso Plus 9108, Takara, Kusatsu, Japan) and quantified with an ND-2000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA (500 ng) from each sample was then reverse-transcribed using an all-in-one, 1st-strand cDNA synthesis supermix (E047, Novoprotein, Beijing, China). Following cDNA synthesis, quantitative real-time PCR (qRT-PCR) was performed using SYBR qPCR Supermix Plus (E096, Novoprotein, China). The real-time PCR cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Triplicate measurements were made for each gene. GAPDH was used as the internal reference gene, and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of mRNA. The primer sequences are shown in Table 1.

2.5 Western Blot

Total protein was extracted from PVM/Ms in different groups using RIPA lysis buffer (PC101, Epizyme, Shanghai, China) and subjected to Western blot as previously described by our group [2]. PVDF (WJ001, Epizyme, China) membranes were incubated overnight at 4 °C with the following primary antibodies: MMP-9 rabbit polyclonal anti-

body (1:1000, A0289, ABclonal, Wuhan, Hubei, China), p38 MAPK rabbit antibody (1:1000, A0227, ABclonal, China), p-p38 MAPK rabbit antibody (1:1000, AP0526, ABclonal, China), and GAPDH (1:5000, K200057M, Solarbio, Beijing, China) as the loading control. After washing three times with TBST (G0001, Servicebio, Wuhan, Hubei, China), the membranes were incubated with corresponding secondary antibodies for 1 h at room temperature. The protein bands were visualized using a chemiluminescence reagent (SQ202, Epizyme, China), with some images equivalently enhanced for ease of visualization.

2.6 Enzyme Linked Immunosorbent Assay (ELISA)

The concentrations of specific proteins in the culture medium of PVM/Ms were measured with the following commercial ELISA kits according to the manufacturer's instructions: pigment epithelial-derived factor (PEDF) (CSB-E08820, CUSABIO, Wuhan, Hubei, China), interleukin-6 (IL-6) (RK00008, ABclonal, China), TNF- α (SEA133Mu, Cloud-Clone, Wuhan, Hubei, China), and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) (E-EL-M0641c, Elabscience, Wuhan, Hubei, China).

2.7 Measurement of Reactive Oxygen Species (ROS)

ROS in PVM/Ms was evaluated with a ROS assay kit (S0033S, Beyotime, Shanghai, China). PVM/Ms were seeded into 6-well plates and incubated with LPS (1 μ g/mL) for 24 h after different pretreatments. Subsequently, the cells were incubated at 37 °C in the dark for 30 min with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) at a final concentration of 10 μ M. Cells were subsequently treated with trypsin and washed three times with PBS. The production of ROS was measured by flow cytometer (BD Biosciences, San Jose, CA, USA) at emission and excitation wavelengths of 535 and 488 nm, respectively.

2.8 Cell Staining

Purified primary PVM/Ms (passage 3–4) were seeded into glass well dishes and treated with LPS (1 μ g/mL) for 24 h after different pretreatments. The PVM/Ms were subsequently fixed in 4% PFA (Solarbio, P1110, China) for 15 min, permeabilized in 0.25% Triton X-100 (Solarbio, T8200, China) in PBS for 5 min, and blocked with 10% goat serum. They were then incubated overnight at 4 °C with rat monoclonal antibody F4/80 (14-4801-85, eBioscience, San Diego, CA, USA) diluted 1:50 in 1% PBS-bovine serum albumin. After three washes in PBS, the samples were incubated with 4',6-diamidino-2-phenylindole (C0065, Solarbio, China) and with the secondary antibodies Dylight 488 goat anti-rat IgG (A23240, Abbkine, Wuhan, Hubei, China) at 1:200 and Griffonia simplicifolia-IB4 (GS-IB4) conjugated to alexa fluor 568 (I21412, Life Technologies, Eugene, OR, USA) at 1:100 for 1 h.

2.9 Permeability Assay

ECs were seeded onto polyethylene terephthalate membranes of transwell inserts (353095, Corning Falcon, Corning, NY, USA) and cultured for 2–3 days to form an EC-only monolayer. For coculture models, PVM/Ms were seeded in advance in the lower well surface of the transwell receiver trays for 1–2 days, and then co-cultured with ECs that had formed a confluent monolayer. Chambers were examined microscopically for EC monolayer confluence, integrity, and uniformity. The permeability of EC monolayers was assessed by quantifying the flux of FITC-dextran (FD10S, Sigma-Aldrich, St. Louis, MO, USA) in the upper and lower chambers. FITC-dextran was added to the upper chamber at a final concentration of 1 mg/mL. After 20 min incubation in darkness, 100 μ L of medium was collected from the receiver tray and transferred to 96-well plates for measurement. The permeability of EC monolayers was assessed by the detection of FITC-dextran fluorescence using a plate reader (SpectraMax M5, Molecular Devices, San Jose, CA, USA), with filters for 485 nm excitation and 535 nm emission.

2.10 Statistical Analysis

SPSS 26.0 software (SPSS Inc., Chicago, IL, USA) was used to perform all statistical analyses. Data were presented as the mean \pm standard deviation (SD) of three samples. Differences between groups were analyzed by analysis of variance (ANOVA) or *t* test, with a *p*-value of <0.05 considered statistically significant.

3. Results

3.1 Adenosine Decreases the Expression of Inflammatory Factors (IL-6 and TNF- α) in LPS-Treated PVM/Ms by Activating A2AR

The levels of inflammatory factors IL-6 and TNF- α are closely associated with disruption of the BBB [21]. Activation of astrocytes and microglia can alter the BBB function in various brain diseases via the production of inflammatory cytokines [22–24]. PVM/Ms play a critical role in maintaining the integrity of the cochlear BLB, which is essential for normal hearing function [2]. Activation of PVM/Ms has been observed in response to LPS-induced middle ear infection [5]. The present study investigated the effects of adenosine on IL-6 and TNF- α levels in PVM/Ms. First, the cytotoxicity of adenosine toward PVM/Ms was examined. The results from CCK-8 assay revealed that treatment with adenosine did not alter the viability of PVM/Ms. SCH58261, a potent and selective A2AR antagonist, also had no effect on cell viability (Fig. 1A). The mRNA levels of IL-6 and TNF- α were markedly decreased following LPS treatment of adenosine-treated PVM/Ms. Concurrent with the decreased mRNA expression, the protein levels of IL-6 and TNF- α were also markedly decreased, which was reversed by treatment with the A2AR antagonist SCH58261 (Fig. 1B–E).

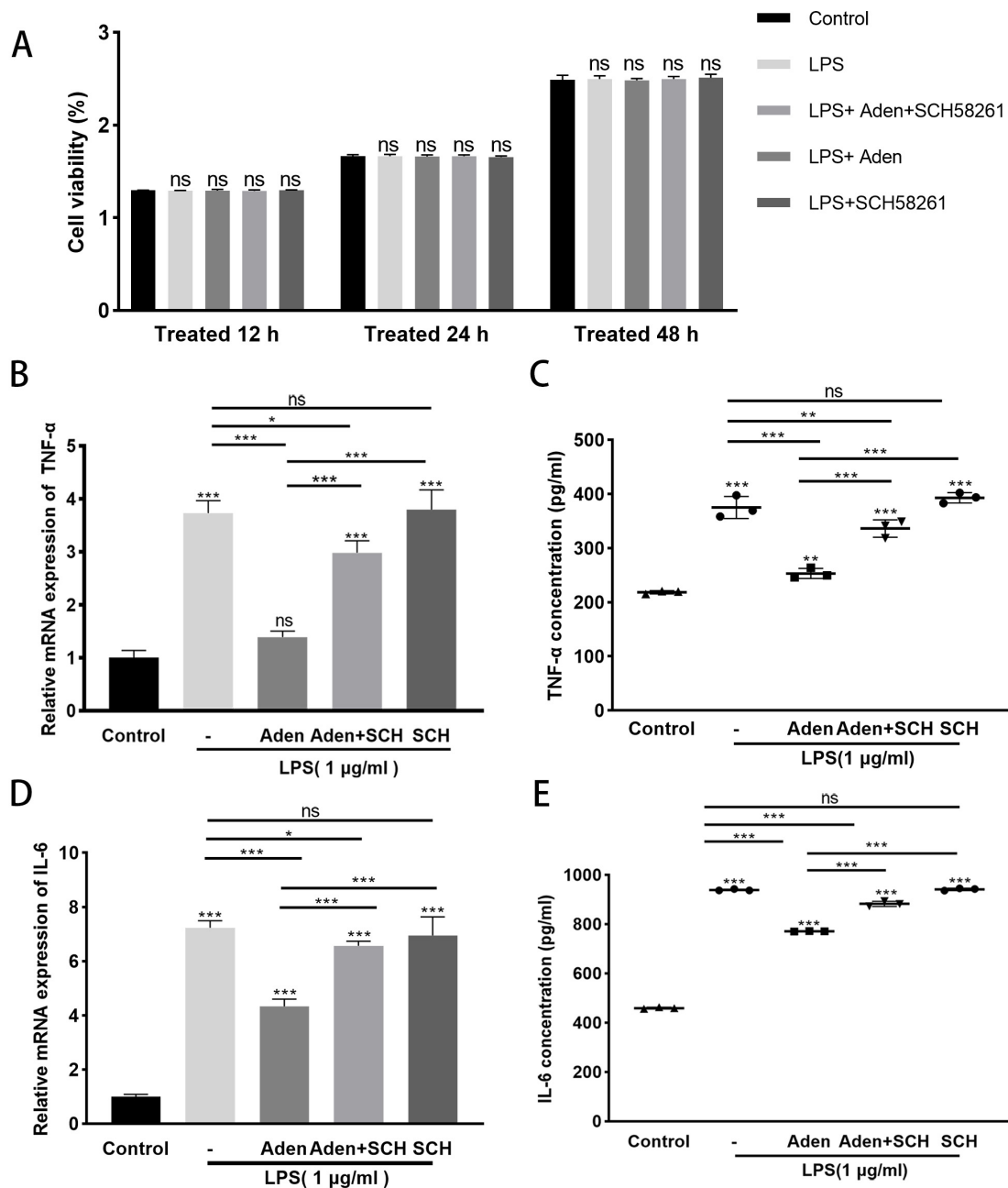


Fig. 1. Adenosine decreases expression of the inflammatory factors IL-6 and TNF- α in LPS-treated PVM/Ms by activating A2AR. (A) No change in cell viability of PVM/Ms treated with LPS was apparent following treatment with adenosine or SCH58261 for up to 48 h. (B–E) Changes in the mRNA and protein expression of TNF- α and IL-6 in PVM/Ms. Adenosine reduced the LPS-induced expression of inflammatory factors IL-6 and TNF- α by PVM/Ms. The A2AR antagonist SCH58261 reversed this decrease. Values shown are the mean \pm SD (n = 3), * p < 0.05, ** p < 0.01, *** p < 0.001, ns, non-significant; one-way ANOVA. Statistical comparisons are made with the control group, except where specifically indicated between two groups. LPS, lipopolysaccharide; PVM/Ms, perivascular-resident macrophage-like melanocytes; A2AR, adenosine A2A receptor.

3.2 By Activating A2AR, Adenosine Decreases ROS Levels and MMP-9 Protein Expression, and Increases TIMP-1 Expression in LPS-Treated PVM/Ms

The overproduction of ROS is closely associated with disruption of the BBB [18,25]. Furthermore, several studies have reported that tissue damage is closely related to an increased level of MMP-9 [26–29]. The present study investi-

gated the effects of adenosine on ROS and MMP-9 levels in PVM/Ms. ROS levels were found to be markedly increased following LPS treatment of PVM/Ms. Adenosine treatment reduced the level of ROS, but this effect was reversed by treatment with the A2AR antagonist SCH58261 (Fig. 2A). In addition, Western blot, qRT-PCR and ELISA assays revealed that MMP-9 expression in PVM/Ms increased fol-

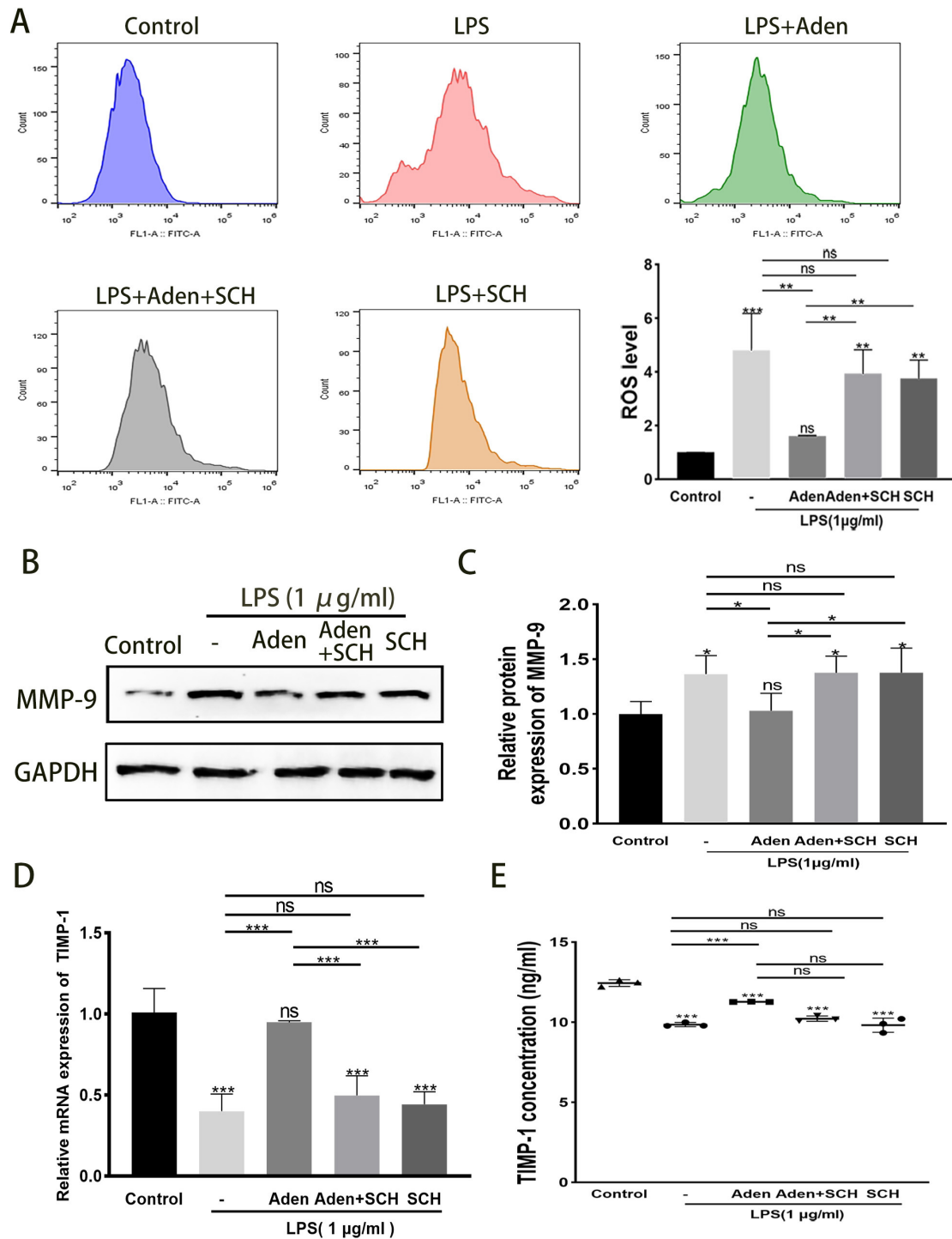


Fig. 2. Adenosine decreases ROS levels and MMP-9 protein expression and increases TIMP-1 expression in LPS-treated PVM/Ms by activating A2AR. (A) ROS levels were measured by flow cytometry. Adenosine decreases ROS levels in LPS-treated PVM/Ms by activating A2AR. (B–E) Changes in mRNA and protein expression of MMP-9 and TIMP-1 in PVM/Ms. Values shown are the mean \pm SD (n = 3), * p < 0.05, ** p < 0.01, *** p < 0.001, ns, non-significant; one-way ANOVA. ROS, reactive oxygen species; MMP-9, matrix metalloproteinase-9; SCH, SCH58261.

lowing LPS treatment, whereas TIMP-1 expression decreased. These changes were prevented by adenosine treatment, while SCH58261 partially counteracted the effects of adenosine (Fig. 2B–E). In summary, adenosine decreased

ROS levels and MMP-9 protein expression, and increased TIMP-1 expression in LPS-treated PVM/Ms. These effects appear to be mediated via A2AR.

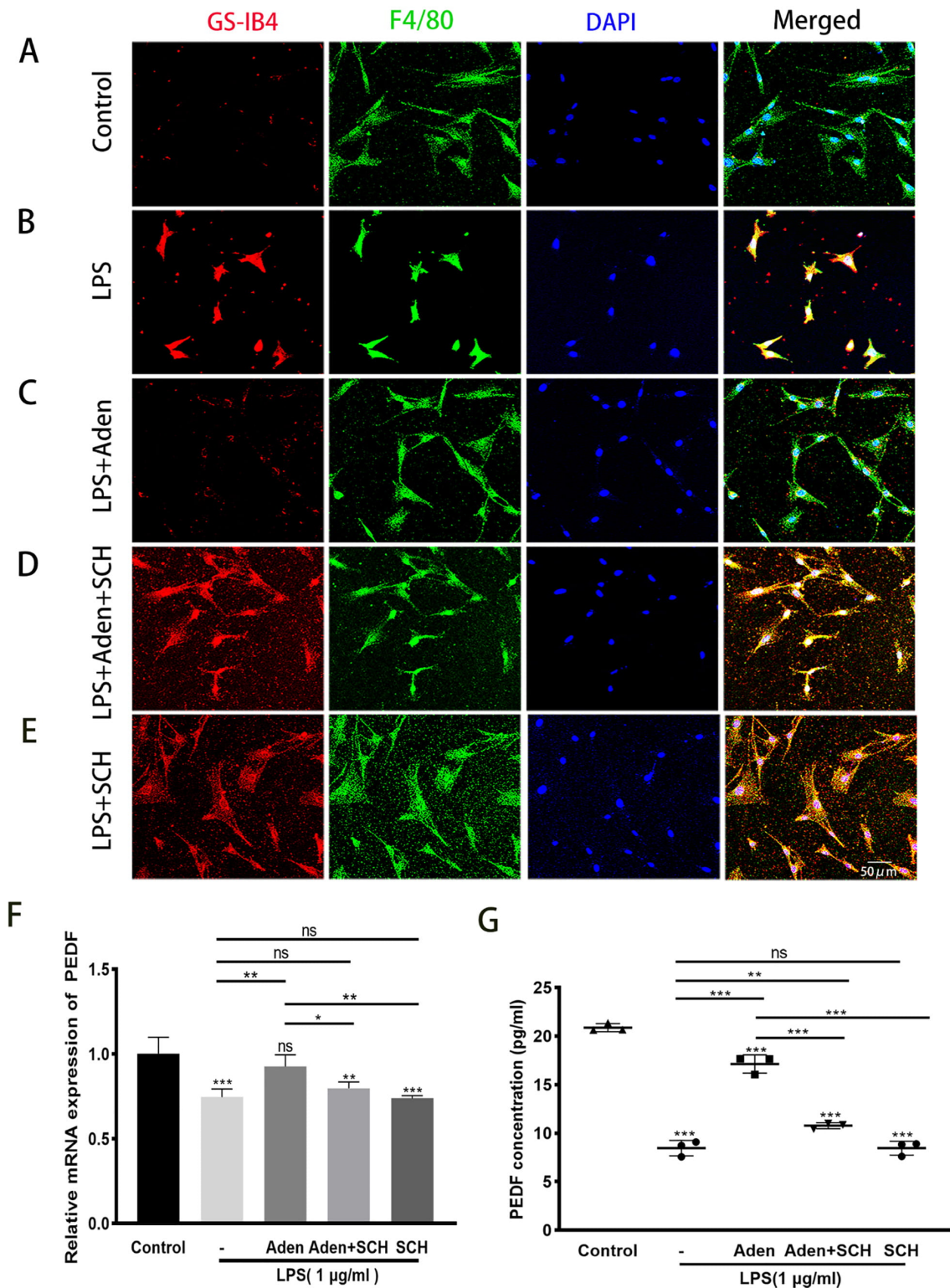


Fig. 3. Adenosine attenuates LPS-treated PVM/M activation and increases PEDF secretion by activating A2AR. (A–E) Adenosine inhibited the inflammatory activation of PVM/Ms and increased dendritic length compared to the LPS group. These changes were significantly attenuated following inhibition of A2AR. PVM/Ms were labeled with an antibody for F4/80 (green), while the nucleus was labeled with DAPI (blue). Activated PVM/Ms show red fluorescence due to the presence of a terminal galactopyranosyl group on the membrane surface which binds the lectin GS-IB4. Scale bar = 50 μ m. (F,G) mRNA and protein expression of PEDF in the PVM/Ms. Adenosine increased the expression of PEDF in LPS-induced PVM/Ms. This effect was reversed by SCH (SCH58261). Values shown are the mean \pm SD (n = 3), * p < 0.05, ** p < 0.01, *** p < 0.001, ns, non-significant; one-way ANOVA.

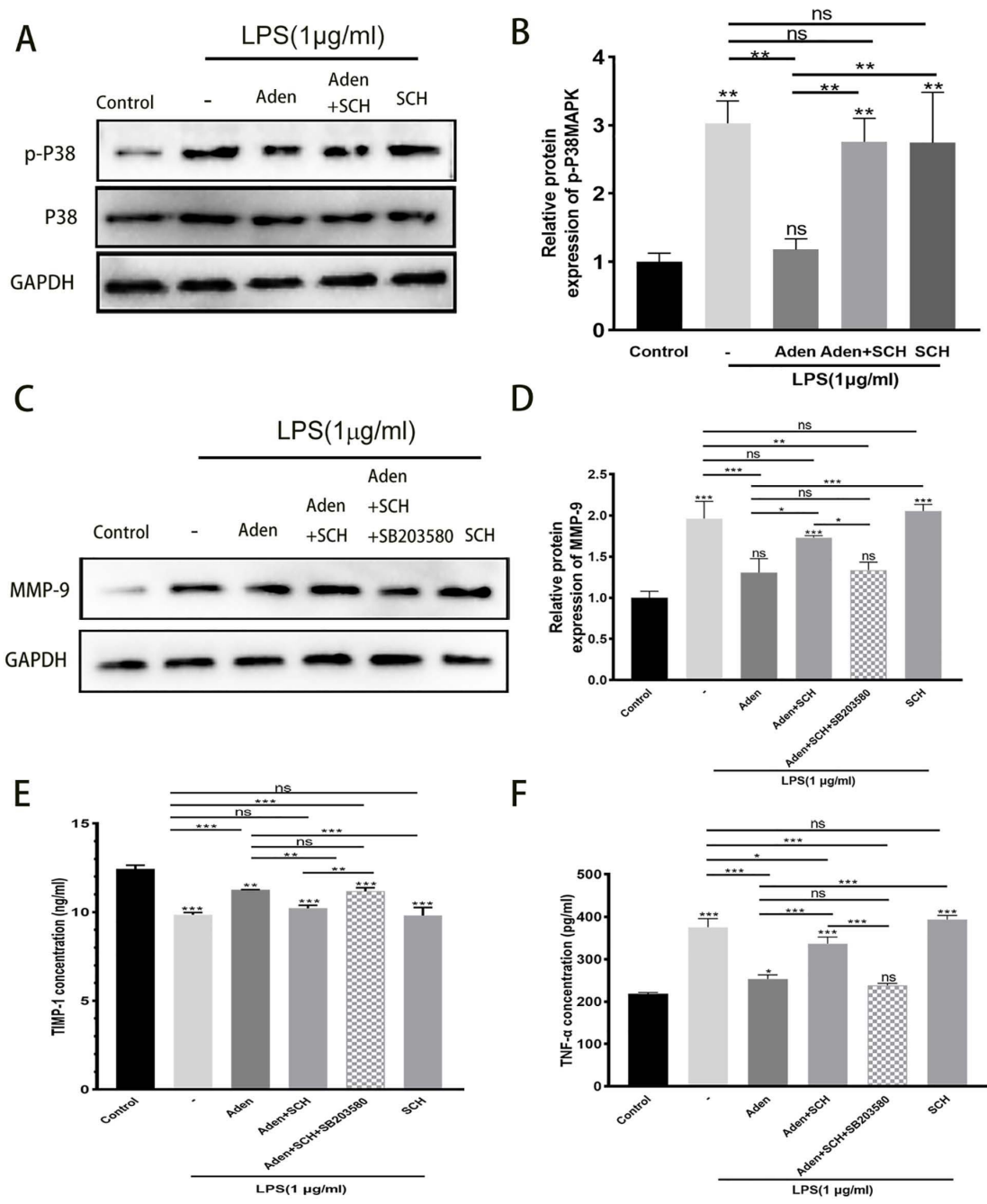


Fig. 4. Adenosine reverses the increase in MMP-9 and TNF- α expression, and decreases in TIMP-1 expression, in LPS-treated PVM/Ms by inhibiting phosphorylation of the p38 MAPK pathway. (A,B) The protein expression of p38 MAPK and p-p38 MAPK in PVM/Ms. (C–F) Protein expression of MMP-9, TIMP-1, and TNF- α in PVM/Ms. Values shown are the mean \pm SD (n = 3). * p < 0.05, ** p < 0.01, *** p < 0.001, ns, non-significant; one-way ANOVA.

3.3 Adenosine Attenuates LPS-Induced Activation of PVM/Ms and Increases PEDF Secretion by Activating A2AR

PEDF, a key signaling molecule secreted by PVM/Ms, regulates barrier integrity by modulating the expression of tight junction-related proteins [2,3]. Inflammatory damage to cochlear BLB was shown to be closely related to the activation of PVM/Ms [3,5]. Zhang *et al.* [5] previously

reported that LPS activated PVM/Ms, resulting in significant morphological changes and positive GS-IB4 staining. In agreement with these earlier results, the present study also found that LPS indeed activated PVM/Ms. For example, these showed altered morphology, with some becoming flattened and amoeboid in shape. Moreover, the dendrites of PVM/Ms were observed to be significantly shortened (Fig. 3A,B). A significant decrease in the activation

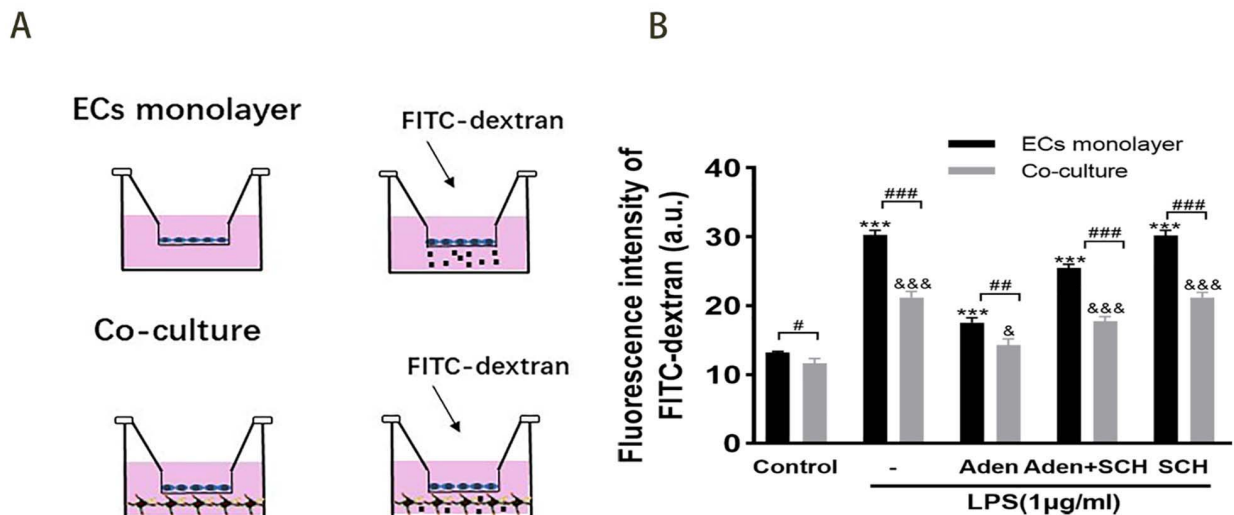


Fig. 5. Adenosine reduces the permeability of the BLB following LPS treatment by activating A2AR. (A) Schematics of the BLB models and *in vitro* experimental setup to detect paracellular permeability of the EC monolayer using a Transwell chamber. The arrows indicate FITC-dextran dropped in the upper chamber. (B) Quantitative analysis of FITC-dextran in the lower transwell chamber in the presence or absence of PVM/Ms. There was significantly less leakage of FITC-dextran in the adenosine-treated group than in the LPS-treated group. SCH was able to reverse this change. Values shown are the mean \pm SD (n = 3). EC-monolayer groups: *** p < 0.001; one-way ANOVA. Co-culture groups: & p < 0.05, && p < 0.001; one-way ANOVA. EC-monolayer vs co-culture groups: # p < 0.05, ## p < 0.01, ### p < 0.001; student's *t* test. BLB, blood labyrinth barrier; EC, endothelial cell; SCH, SCH58261.

of PVM/Ms was observed following treatment with adenosine, with the dendritic shape becoming slightly longer than in the LPS group. The aforementioned changes were significantly mitigated by inhibition of A2AR (Fig. 3C–E). Similarly, adenosine treatment resulted in the upregulation of PEDF expression in LPS-treated PVM/Ms, which could be prevented by SCH58261 treatment (Fig. 3F,G). The above results indicate that adenosine attenuates LPS-induced PVM/Ms activation and increases PEDF secretion by activating A2AR.

3.4 Adenosine Reverses MMP-9, TIMP-1 and TNF- α Expression in LPS-Treated PVM/Ms by Inhibiting Phosphorylation of the p38 MAPK Pathway

Previous research has demonstrated that anti-inflammatory drug DMH-CBD activated macrophage A2AR targets NF- κ B activity and suppresses TNF- α production via the inhibition of p38 MAPK [30]. Moreover, the inhibition of MAPK, particularly p38 MAPK, in brain astrocytes was shown to significantly prevent increased levels of MMP-9 and ROS, thus mitigating the occurrence of brain edema [31]. Therefore, the present study investigated the effects of adenosine on the p38 MAPK pathway in PVM/Ms. The total protein expression of p38 MAPK in the PVM/Ms was unchanged following the different treatments. However, adenosine decreased the expression of p-p38 MAPK in LPS-treated PVM/Ms, which was reversed by the A2AR inhibitor SCH58261 (Fig. 4A,B). To further determine whether the effects of adenosine on MMP-9, TIMP-1 and TNF- α expression were also

dependent on p38 MAPK signaling, SB203580 was used to inhibit p38 MAPK activity. Of note, SB203580 was found to exert the same salvage effect as adenosine treatment (Fig. 4C–F). These results demonstrate that adenosine reverses the increased expression of MMP-9 and TNF- α , and the decreased expression of TIMP-1, in LPS-treated PVM/Ms by inhibiting phosphorylation of the p38 MAPK pathway.

3.5 Adenosine Reduces Permeability of the BLB Following LPS Treatment by Activating A2AR

We next established *in vitro* models of EC monolayers and EC-PVM/M co-culture to further examine the effects of adenosine on BLB integrity. Whereas previous research demonstrated protective effects of adenosine on ECs [20], the present study examined its effects on PVM/Ms. A schematic diagram of the BLB model and *in vitro* experimental setup is illustrated in Fig. 5A. The permeability of the EC monolayer barrier was determined by evaluating the fluorescence intensity of FITC-dextran crossing the cell monolayer paracellularly into the inferior chamber of a Transwell. FITC-dextran leakage was observed to be lower in the co-culture group than in the EC monolayer group (Fig. 5B), suggesting that PVM/Ms play a beneficial role in maintaining the integrity of the endothelial barrier, in accordance with the findings by Zhang *et al.* [2]. However, following LPS-treatment the EC monolayer barrier was found to be more permeable than the EC-PVM/M co-culture (one-way ANOVA). Pre-treatment with adenosine significantly decreased the LPS-induced FITC-dextran leakage in both

groups, which was abrogated by treatment with SCH58261. These *in vitro* findings demonstrate that adenosine reduces the permeability of the BLB following LPS treatment by activating A2AR, with a positive effect on PVM/Ms as well as ECs.

4. Discussion

The authors have previously demonstrated that PVM/Ms, the resident macrophages of the stria vascularis, play a critical role in maintaining tissue homeostasis and BLB integrity [2]. PVM/Ms are activated in animals exposed to loud sound or in aged animals, and are closely associated with BLB dysfunction [3,4]. In the present study, LPS treatment of PVM/Ms was used to mimic the inflammatory response *in vivo*. Adenosine was found to attenuate LPS-induced PVM/M activation and to increase PEDF secretion by activating A2AR. It is well known that LPS can provoke macrophages to differentiate into the inflammatory phenotype via the p38 MAPK pathway [32]. The present study found that adenosine attenuated the phosphorylation of p38 MAPK in PVM/Ms, and this effect was reversed by an A2AR inhibitor.

Dysfunction of the BLB is associated with a number of hearing disorders [1]. The essential role of PVM/Ms in regulating BLB integrity is mainly due to their communication with ECs in the capillary network skeleton that control microvascular permeability [2]. In another previous study, the authors reported that LPS activation of PVM/Ms resulted in significant morphological changes, as well as the disruption of BLB integrity by reducing the expression of tight junction proteins between ECs, including ZO-1 and occludin [5]. Adenosine is an ancient extracellular signalling molecule that can regulate almost all aspects of tissue function [32,33]. A number of studies have shown that A2AR plays a favorable role in brain barrier integrity [10,21]. The potential protective role of adenosine in the auditory system was also discussed recently, although this was limited to sensory hair cells [34]. The extensive range of neuroprotective action suggests that adenosine may also exert a protective effect in the cochlea. The authors previously demonstrated that adenosine partially reversed the LPS-induced decrease in tight junction-related proteins (ZO-1 and occludin) to protect BLB integrity in ECs by acting on A2AR [20]. The present study further demonstrated that LPS induced the activation of PVM/Ms, thereby losing the capacity to produce PEDF. These changes were significantly mitigated following the inhibition of A2AR. PVM/Ms are known to regulate the integrity of the BLB through secretion of the signaling molecule PEDF [2], which upregulates the expression of tight junction-associated proteins between ECs [2]. The present study found that adenosine increased the expression of PEDF in LPS-treated PVM/Ms, and this effect was blocked by the adenosine A2AR antagonist SCH58261 (Fig. 3F,G). These results indicate that adenosine attenuates LPS-induced PVM/M activation and

increases PEDF secretion by activating A2AR. The integrity of the barrier is critical for maintaining inner ear homeostasis [1,35], suggesting that adenosine may be a promising target for hearing impairment caused by BLB dysfunction.

MAPKs are involved in the ERK, p38 and JNK pathways, with p38 MAPK being a key enzyme in cell signaling pathways responsible for the production of inflammatory cytokines [36]. The activated macrophage A2A receptor was recently reported to target NF- κ B activity, thereby inhibiting TNF- α production via the suppression of p38 MAPK [30]. Secondly, inhibition of p38 MAPK in brain astrocytes was shown to suppress the increase in MMP-9 and ROS levels, thus mitigating the occurrence of brain edema [31]. Further research found that Cannabidiol exerts anti-inflammatory effects on macrophages in an A2AR-dependent manner by inhibiting LPS-induced TNF production and NF- κ B activity, which is closely related to decreased phosphorylation of p38 MAPK [30]. Based on these previous findings, the present study investigated the effects of adenosine on the p38 MAPK pathway in PVM/Ms. Our results suggest the MAPK pathway was activated by LPS. Adenosine inhibited phosphorylation of the p38 MAPK pathway, and this effect was reversed by the A2AR inhibitor SCH58261, suggesting the protective effect of adenosine in PVM/Ms is achieved by acting on A2AR. This finding is in accordance with regulation of the p38 MAPK pathway by A2AR in macrophages [30]. However, some scholars have reported that the physiological activation of A2AR leads to increased phosphorylation of p38 MAPK in human ECs and cardiomyocytes [37,38]. The inhibition of MAPKs, particularly p38 MAPK, was found to markedly suppress the increase in MMP-9 and ROS levels in astrocytes, with similar findings reported in the aorta [31,39]. The present study confirmed that activation of p38 MAPK was also involved in upregulating pro-inflammatory mediators in an LPS-induced PVM/M model of BLB injury. These pro-inflammatory mediators can then further activate MAPKs, forming a positive feedback loop to amplify inflammatory signals. LPS enhances oxidative damage by promoting the generation of a number of toxic substances, such as ROS, nitric oxide, and cytokines [18,40]. The present study also found that LPS treatment enhanced ROS levels in PVM/Ms and induced EC monolayer barrier injury. Adenosine treatment attenuated the LPS-induced production of ROS and increase in MMP-9 expression, thereby mitigating BLB injury. These effects were abrogated by an A2AR inhibitor. Thus, it is reasonable to assume that adenosine may act as a scavenger of ROS to protect the BLB from LPS-induced injury in PVM/Ms, and that p38 MAPK mediates this process to regulate downstream PEDF and ROS. Furthermore, a recent study suggested that activation of A1AR suppresses ROS generation by inhibiting signal transducer and activator of transcription (STAT1) and suppressing the ERK1/2, p38, and JNK MAPK pathways

[41]. Thus, adenosine may act on A2AR to attenuate LPS-induced PVM/M activation associated with STAT1 signaling, although this requires further exploration.

In the brain, adenosine inhibits inflammatory cytokine production by activated primary murine microglia via A2A receptors [42]. Similarly, adenosine was found to target PVM/M activation, thereby decreasing the release of inflammatory cytokines such as IL-6, TNF- α and MMP-9, and increasing TIMP-1 secretion. This suggests that adenosine prevents the LPS-induced activation of PVM/Ms, thus suppressing cytokine production and attenuating LPS-induced inner ear inflammation. Of note, previous studies have reported the anti-inflammatory effects of adenosine were associated with suppression of the release of inflammatory cytokines [43–46]. In the present study, we investigated the molecular mechanisms underlying the regulatory effects of adenosine on PVM/Ms and BLB permeability. The A2AR-specific antagonist SCH58261 was used to further examine the protective effects of adenosine on PVM/Ms and the BLB *in vitro*. The protective effects were found to be mediated via the A2AR. In contrast, A2AR has been found to mediate high BBB permeability under physiological conditions [12,47,48]. We hypothesize that in the early stage of inflammatory injury, A2AR activation could increase the permeability of the BLB. This allows a large number of inflammatory factors to invade the endothelial barrier and subsequently activate the surrounding PVM/Ms. With the development of inflammation, activation of A2AR alters the communication between ECs and PVM/Ms, ultimately protecting BLB integrity. However, several limitations of this study should be acknowledged. First, validation of the protective effects of adenosine was confined to *in vitro* cell models only, with no *in vivo* animal experiments (e.g., LPS-induced mouse models). Second, there was insufficient investigation of the chronic effects of LPS exposure. Finally, the detailed internal mechanisms have yet to be fully explored. Further studies, including *in vivo* validation, are required to clarify the effects of adenosine.

5. Conclusions

In conclusion, the present study demonstrates that adenosine acts via A2AR to attenuate LPS-induced PVM/M injury. This maintains integrity of the endothelial barrier by modulating the inflammatory activation of PVM/Ms and promoting PEDF expression. Inhibition of p38 phosphorylation in the MAPK pathway may be one of the underlying mechanisms. These findings suggest potential targets for the development of adenosine-based therapeutics for hearing disorders associated with PVM/M activation.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

WF and JL contributed equally to the study. Specifically, WF, JL, ZX, and LN did the immunohistochemistry, qRT-PCR and WB. BC and BZ were involved in data analysis. JZ designed the research study, analyzed the experimental results and wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures involving animals in this study complied with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of First Affiliated Hospital of Zhengzhou University (2024-KY-0035-002).

Acknowledgment

Not applicable.

Funding

This study was supported by the Natural Science Foundation of Henan Province of China (Grant no. 242300420078), the Henan Province Higher Education Institutions Key Scientific Research Project Plan (Grant no. 26A320041), funds from the Science Foundation for The Excellent Youth Scholars of Zhengzhou University (Grant no. JC23862072).

Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Shi X. Pathophysiology of the cochlear intrastrial fluid-blood barrier (review). *Hearing Research*. 2016; 338: 52–63. <https://doi.org/10.1016/j.heares.2016.01.010>.
- [2] Zhang W, Dai M, Fridberger A, Hassan A, Degagne J, Neng L, *et al.* Perivascular-resident macrophage-like melanocytes in the inner ear are essential for the integrity of the intrastrial fluid-blood barrier. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109: 10388–10393. <https://doi.org/10.1073/pnas.1205210109>.
- [3] Zhang F, Dai M, Neng L, Zhang JH, Zhi Z, Fridberger A, *et al.* Perivascular macrophage-like melanocyte responsiveness to acoustic trauma—a salient feature of strial barrier associated hearing loss. *FASEB Journal*. 2013; 27: 3730–3740. <https://doi.org/10.1096/fj.13-232892>.
- [4] Neng L, Zhang J, Yang J, Zhang F, Lopez IA, Dong M, *et al.* Structural changes in the strial blood-labyrinth barrier of aged C57BL/6 mice. *Cell and Tissue Research*. 2015; 361: 685–696. <https://doi.org/10.1007/s00441-015-2147-2>.
- [5] Zhang J, Chen S, Hou Z, Cai J, Dong M, Shi X. Lipopolysaccharide-induced middle ear inflammation disrupts the cochlear intra-strial fluid-blood barrier through down-regulation of tight junction proteins. *PLoS ONE*. 2015; 10: e0122572. <https://doi.org/10.1371/journal.pone.0122572>.

- [6] Quintanilla-Dieck L, Larrain B, Trune D, Steyger PS. Effect of systemic lipopolysaccharide-induced inflammation on cytokine levels in the murine cochlea: a pilot study. *Otolaryngology–Head and Neck Surgery*. 2013; 149: 301–303. <https://doi.org/10.1177/0194599813491712>.
- [7] Erickson MA, Hansen K, Banks WA. Inflammation-induced dysfunction of the low-density lipoprotein receptor-related protein-1 at the blood-brain barrier: protection by the antioxidant N-acetylcysteine. *Brain, Behavior, and Immunity*. 2012; 26: 1085–1094. <https://doi.org/10.1016/j.bbi.2012.07.003>.
- [8] Scaini G, Morais MOS, Galant LS, Vuolo F, Dall’Igna DM, Pasquali MAB, *et al*. Coadministration of branched-chain amino acids and lipopolysaccharide causes matrix metalloproteinase activation and blood-brain barrier breakdown. *Molecular Neurobiology*. 2014; 50: 358–367. <https://doi.org/10.1007/s12035-013-8618-0>.
- [9] Jaeger LB, Dohgu S, Sultana R, Lynch JL, Owen JB, Erickson MA, *et al*. Lipopolysaccharide alters the blood-brain barrier transport of amyloid beta protein: a mechanism for inflammation in the progression of Alzheimer’s disease. *Brain, Behavior, and Immunity*. 2009; 23: 507–517. <https://doi.org/10.1016/j.bbi.2009.01.017>.
- [10] Kim JY, Ko AR, Hyun HW, Kang TC. ETB receptor-mediated MMP-9 activation induces vasogenic edema via ZO-1 protein degradation following status epilepticus. *Neuroscience*. 2015; 304: 355–367. <https://doi.org/10.1016/j.neuroscience.2015.07.065>.
- [11] Pan P, Zhang X, Li Q, Zhao H, Qu J, Zhang JH, *et al*. Cyclosporine A alleviated matrix metalloproteinase 9 associated blood-brain barrier disruption after subarachnoid hemorrhage in mice. *Neuroscience Letters*. 2017; 649: 7–13. <https://doi.org/10.1016/j.neulet.2017.03.050>.
- [12] Kim DG, Bynoe MS. A2A adenosine receptor modulates drug efflux transporter P-glycoprotein at the blood-brain barrier. *The Journal of Clinical Investigation*. 2016; 126: 1717–1733. <https://doi.org/10.1172/JCI76207>.
- [13] Li Q, Han X, Lan X, Hong X, Li Q, Gao Y, *et al*. Inhibition of tPA-induced hemorrhagic transformation involves adenosine A2b receptor activation after cerebral ischemia. *Neurobiology of Disease*. 2017; 108: 173–182. <https://doi.org/10.1016/j.nbd.2017.08.011>.
- [14] Bynoe MS, Viret C, Yan A, Kim DG. Adenosine receptor signaling: a key to opening the blood-brain door. *Fluids and Barriers of the CNS*. 2015; 12: 20. <https://doi.org/10.1186/s12987-015-0017-7>.
- [15] Vlajkovic SM, Housley GD, Thorne PR. Adenosine and the auditory system. *Current Neuropharmacology*. 2009; 7: 246–256. <https://doi.org/10.2174/157015909789152155>.
- [16] Nguyen DK, Montesinos MC, Williams AJ, Kelly M, Cronstein BN. Th1 cytokines regulate adenosine receptors and their downstream signaling elements in human microvascular endothelial cells. *Journal of Immunology*. 2003; 171: 3991–3998. <https://doi.org/10.4049/jimmunol.171.8.3991>.
- [17] Vlajkovic SM, Guo CX, Telang R, Wong ACY, Paramanathasivam V, Boison D, *et al*. Adenosine kinase inhibition in the cochlea delays the onset of age-related hearing loss. *Experimental Gerontology*. 2011; 46: 905–914. <https://doi.org/10.1016/j.exger.2011.08.001>.
- [18] Ahmad S, Fatteh N, El-Sherbiny NM, Naime M, Ibrahim AS, El-Sherbini AM, *et al*. Potential role of A2A adenosine receptor in traumatic optic neuropathy. *Journal of Neuroimmunology*. 2013; 264: 54–64. <https://doi.org/10.1016/j.jneuroim.2013.09.015>.
- [19] Neng L, Zhang W, Hassan A, Zemla M, Kachelmeier A, Fridberger A, *et al*. Isolation and culture of endothelial cells, pericytes and perivascular resident macrophage-like melanocytes from the young mouse ear. *Nature Protocols*. 2013; 8: 709–720. <https://doi.org/10.1038/nprot.2013.033>.
- [20] Zhang J, Fan W, Neng L, Chen B, Wang Y, Zuo B, *et al*. Adenosine improves LPS-induced ROS expression and increasing in monolayer permeability of endothelial cell via acting on A2AR. *Microvascular Research*. 2022; 143: 104403. <https://doi.org/10.1016/j.mvr.2022.104403>.
- [21] Liu Y, Alahiri M, Ulloa B, Xie B, Sadiq SA. Adenosine A2A receptor agonist ameliorates EAE and correlates with Th1 cytokine-induced blood brain barrier dysfunction via suppression of MLCK signaling pathway. *Immunity, Inflammation and Disease*. 2018; 6: 72–80. <https://doi.org/10.1002/iid3.187>.
- [22] Hunter CA, Jennings FW, Kennedy PG, Murray M. Astrocyte activation correlates with cytokine production in central nervous system of *Trypanosoma brucei* infected mice. *Laboratory Investigation*. 1992; 67: 635–642.
- [23] Ahmad MH, Fatima M, Mondal AC. Influence of microglia and astrocyte activation in the neuroinflammatory pathogenesis of Alzheimer’s disease: Rational insights for the therapeutic approaches. *Journal of Clinical Neuroscience*. 2019; 59: 6–11. <https://doi.org/10.1016/j.jocn.2018.10.034>.
- [24] Abbott NJ, Rönnbäck L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nature Reviews. Neuroscience*. 2006; 7: 41–53. <https://doi.org/10.1038/nrn1824>.
- [25] Yi H, Huang H, Meng Q, Liu Q. Mechanism of Endothelial Cell Death in Cerebral Ischemic Stroke and Application of Protective Drugs. *Frontiers in bioscience (Landmark edition)*. 2025; 30: 26034. <https://doi.org/10.31083/FBL26034>.
- [26] Mitropoulou G, Kompoura V, Saffioti F, Mavroeidis VK. The Role of Matrix Metalloproteinases in Liver Function and Disease. *Frontiers in bioscience (Landmark edition)*. 2025; 30: 27127. <https://doi.org/10.31083/FBL27127>.
- [27] Toba H, Cannon PL, Yabluchanskiy A, Iyer RP, D’Armiento J, Lindsey ML. Transgenic overexpression of macrophage matrix metalloproteinase-9 exacerbates age-related cardiac hypertrophy, vessel rarefaction, inflammation, and fibrosis. *American Journal of Physiology. Heart and Circulatory Physiology*. 2017; 312: H375–H383. <https://doi.org/10.1152/ajpheart.00633.2016>.
- [28] Kim S, Bong N, Kim OS, Jin J, Kim DE, Lee DK. Lithium chloride suppresses LPS-mediated matrix metalloproteinase-9 expression in macrophages through phosphorylation of GSK-3 β . *Cell Biology International*. 2015; 39: 177–184. <https://doi.org/10.1002/cbin.10352>.
- [29] Hanania R, Sun HS, Xu K, Pustynnik S, Jeganathan S, Harrison RE. Classically activated macrophages use stable microtubules for matrix metalloproteinase-9 (MMP-9) secretion. *The Journal of Biological Chemistry*. 2012; 287: 8468–8483. <https://doi.org/10.1074/jbc.M111.290676>.
- [30] Silva RL, Silveira GT, Wanderlei CW, Cecilio NT, Maganin AGM, Franchin M, *et al*. DMH-CBD, a cannabidiol analog with reduced cytotoxicity, inhibits TNF production by targeting NF- κ B activity dependent on A_{2A} receptor. *Toxicology and Applied Pharmacology*. 2019; 368: 63–71. <https://doi.org/10.1016/j.taap.2019.02.011>.
- [31] Ralay Ranaivo H, Hodge JN, Choi N, Wainwright MS. Albumin induces upregulation of matrix metalloproteinase-9 in astrocytes via MAPK and reactive oxygen species-dependent pathways. *Journal of Neuroinflammation*. 2012; 9: 68. <https://doi.org/10.1186/1742-2094-9-68>.
- [32] Haskó G, Pacher P, Deitch EA, Vizi ES. Shaping of monocyte and macrophage function by adenosine receptors. *Pharmacology & Therapeutics*. 2007; 113: 264–275. <https://doi.org/10.1016/j.pharmthera.2006.08.003>.
- [33] Hixson EA, Borker PV, Jackson EK, Macatangay BJ. The Adenosine Pathway and Human Immunodeficiency Virus-Associated Inflammation. *Open Forum Infectious Diseases*. 2021; 8: ofab396. <https://doi.org/10.1093/ofid/ofab396>.

- [34] Shukla M, Roy K, Kaur C, Nayak D, Mani KV, Shukla S, *et al.* Attenuation of adverse effects of noise induced hearing loss on adult neurogenesis and memory in rats by intervention with Adenosine A_{2A} receptor agonist. *Brain Research Bulletin*. 2019; 147: 47–57. <https://doi.org/10.1016/j.brainresbull.2019.02.006>.
- [35] Shi X. Physiopathology of the cochlear microcirculation. *Hearing Research*. 2011; 282: 10–24. <https://doi.org/10.1016/j.heares.2011.08.006>.
- [36] Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*. 2002; 298: 1911–1912. <https://doi.org/10.1126/science.1072682>.
- [37] Seidel MG, Klinger M, Freissmuth M, Höller C. Activation of mitogen-activated protein kinase by the A(2A)-adenosine receptor via a rap1-dependent and via a p21(ras)-dependent pathway. *The Journal of Biological Chemistry*. 1999; 274: 25833–25841. <https://doi.org/10.1074/jbc.274.36.25833>.
- [38] Ribé D, Sawbridge D, Thakur S, Hussey M, Ledent C, Kitchen I, *et al.* Adenosine A_{2A} receptor signaling regulation of cardiac NADPH oxidase activity. *Free Radical Biology & Medicine*. 2008; 44: 1433–1442. <https://doi.org/10.1016/j.freeradbiomed.2007.12.035>.
- [39] Xu J, Wang P, Wang T, Wang M, Chen S, Yu P, *et al.* Effects of reduced β 2-glycoprotein I on the expression of aortic matrix metalloproteinases and tissue inhibitor matrix metalloproteinases in diabetic mice. *BMC Cardiovascular Disorders*. 2014; 14: 114. <https://doi.org/10.1186/1471-2261-14-114>.
- [40] Hung TH, Shyue SK, Wu CH, Chen CC, Lin CC, Chang CF, *et al.* Deletion or inhibition of soluble epoxide hydrolase protects against brain damage and reduces microglia-mediated neuroinflammation in traumatic brain injury. *Oncotarget*. 2017; 8: 103236–103260. <https://doi.org/10.18632/oncotarget.21139>.
- [41] Kaur T, Borse V, Sheth S, Sheehan K, Ghosh S, Tupal S, *et al.* Adenosine A₁ Receptor Protects Against Cisplatin Ototoxicity by Suppressing the NOX3/STAT1 Inflammatory Pathway in the Cochlea. *The Journal of Neuroscience*. 2016; 36: 3962–3977. <https://doi.org/10.1523/JNEUROSCI.3111-15.2016>.
- [42] Newell EA, Exo JL, Verrier JD, Jackson TC, Gillespie DG, Janesko-Feldman K, *et al.* 2',3'-cAMP, 3'-AMP, 2'-AMP and adenosine inhibit TNF- α and CXCL10 production from activated primary murine microglia via A_{2A} receptors. *Brain Research*. 2015; 1594: 27–35. <https://doi.org/10.1016/j.brainres.2014.10.059>.
- [43] Zheng X, Wang D. The Adenosine A_{2A} Receptor Agonist Accelerates Bone Healing and Adjusts Treg/Th17 Cell Balance through Interleukin 6. *BioMed Research International*. 2020; 2020: 2603873. <https://doi.org/10.1155/2020/2603873>.
- [44] Ansari MA, Attia SM, Nadeem A, Bakheet SA, Raish M, Khan TH, *et al.* Activation of adenosine A_{2A} receptor signaling regulates the expression of cytokines associated with immunologic dysfunction in BTBR T⁺ Ipr3^{tf}/J mice. *Molecular and Cellular Neurosciences*. 2017; 82: 76–87. <https://doi.org/10.1016/j.mcn.2017.04.012>.
- [45] Zhao L, Liu YW, Yang T, Gan L, Yang N, Dai SS, *et al.* The mutual regulation between miR-214 and A_{2A}R signaling plays an important role in inflammatory response. *Cellular Signalling*. 2015; 27: 2026–2034. <https://doi.org/10.1016/j.cellsig.2015.07.007>.
- [46] Liu C, Shang Q, Bai Y, Guo C, Zhu F, Zhang L, *et al.* Adenosine A_{2A} receptor, a potential valuable target for controlling reoxygenated DCs-triggered inflammation. *Molecular Immunology*. 2015; 63: 559–565. <https://doi.org/10.1016/j.molimm.2014.10.012>.
- [47] Carman AJ, Mills JH, Krenz A, Kim DG, Bynoe MS. Adenosine receptor signaling modulates permeability of the blood-brain barrier. *The Journal of Neuroscience*. 2011; 31: 13272–13280. <https://doi.org/10.1523/JNEUROSCI.3337-11.2011>.
- [48] Kim DG, Bynoe MS. A_{2A} Adenosine Receptor Regulates the Human Blood-Brain Barrier Permeability. *Molecular Neurobiology*. 2015; 52: 664–678. <https://doi.org/10.1007/s12035-014-8879-2>.