

Effect of thymosin β 4 on lipopolysaccharide-stimulated brain microvascular endothelial cell remodeling: A possible role in blood-brain barrier injury

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Abstract. War veterans, in particular, are more prone to mental illness as they are more likely to have encountered multiple traumatic brain injuries (TBIs) whilst serving on active duty in war zone areas. A TBI is known to cause mortality or serious neurological disabilities among survivors and elicits a number of pathological processes, including neuroinflammation and blood brain barrier (BBB) disruption, leading to secondary brain damage and subsequent impairment of the neurovascular unit. Although several drugs exhibit promising effects for TBI, the repertoire of currently available therapeutic strategies remains limited. Thymosin 4 (T β 4) is a 43-amino acid G-acting sequestering peptide that confers neuroprotective potential in TBI models. However, its role in BBB function remains unclear. Further research into the mechanism of BBB disruption induced by TBI and its specific role in neurovascular pathophysiology is necessary. In the present study, the protective effects of T β 4 in lipopolysaccharide (LPS)-stimulated gene expression of several tight junction proteins, inflammatory genes, apoptotic genes, and adhesion genes in human brain microvascular endothelial cells (hBMVECs), one of the pivotal cell types in the BBB, were reported. The results suggested that pretreatment with T β 4 reversed the LPS-induced damage of BBB components in hBMVECs. Furthermore, these results identified neuregulin 1 as a possible target for T β 4. Therefore, it is proposed that T β 4-mediated cellular signaling in hBMVEC may be vital for understanding the association between the BBB and TBI pathophysiology, which warrants further investigation.

Introduction

Traumatic brain injury (TBI), caused by exposure to explosions (blast TBI), is common among war veterans who have served in war zones during their active duty. Some service members face the aftermath of the explosions instantaneously. However, the majority of veterans face the consequences further into their veteran status. The deleterious effects of blast TBI on mental health conditions in war veterans are becoming a matter of significant concern (1,2). It is established that blast TBIs may have long-term adverse effects on normal brain function, increasing the risk of memory loss, inducing post-traumatic stress disorder and reducing the quality of life for returning war veterans. This is particularly the case for those who served in Operation Enduring Freedom/Operation Iraqi Freedom/Operation New Dawn as these war veterans were exposed to blast TBI more frequently in the combat war zone (1,2). Although the cause of the TBI may have occurred in the past, the possibility of blood brain barrier (BBB) leakage persists, thus, exacerbating mental health conditions further (for example, memory loss or reduced cognitive function).

BBB disruption is a major pathophysiological feature of TBI and contributes to brain edema, structural protein breakdown and cell death. BBB leakage is also one of the major secondary effects after a blast that has a long-term effect on the brain (3,4). By contrast, it has also been demonstrated that transient and size-selective modulation of the BBB increases the movement of water from the brain parenchyma to blood vessels, leading to a decrease in the swelling of the brain (5). TBI can elicit a series of secondary injuries after the initial trauma, which is mainly mediated by microglial activation, resulting in the release of inflammatory molecules to induce BBB leakage (6-8). The consequent damage is critical and poses a major risk factor for high mortality or permanent cognitive dysfunction. The BBB maintains brain homeostasis by facilitating nutrient delivery whilst prohibiting the entry of toxic molecules and peripheral immune cells, thereby acting as a solute exchange barrier between the blood and the brain. The BBB is primarily comprised of brain microvascular endothelial cells (BMVECs) along with astrocytes, pericytes, microglia and basal membrane. Together, these cells and the extracellular matrix form the neurovascular unit which acts as

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a dynamic tissue barrier that selectively controls BBB permeability (9,10). All structural components in this neurovascular unit perform specific functions in the central nervous system (CNS) to maintain normal brain homeostasis (11-13). Typically, the BBB serves as a gatekeeper in the CNS and contributes a significant physiological role in permeability, ion balance and nutrient transport. The monolayer of BMVECs is pivotal in maintaining BBB function in the brain. Furthermore, the BBB is regulated by various types of tight junctions (TJs), such as zonula occludens (ZO) 1, ZO2, ZO3, occludin and claudins, which are present on cerebral endothelial cells and form transmembrane and cytosolic TJ-associated protein complexes (14,15). Following TBI, the breakdown of TJ proteins in the BBB endothelium causes leakage and increased permeability, contributing to cytotoxicity and neuronal damage (15). Therefore, BMVECs (BMVECs) are particularly important cell types in the microvasculature that are implicated in TBI-induced BBB disruption. This is a primary reason for their selection in the present study. Glial cells are another cell type in the neurovascular unit and are also major contributors to BBB disruption. Following activation, glial cells secrete an array of chemokines and cytokines, triggering microvascular remodeling and immune activation, thus resulting in inflammation and cellular apoptosis (7,16-19). Taken together, the adverse remodeling of human (h)BMVECs is highly likely to be involved in the impairment of the neurovascular unit. Since the BBB lies in an intricate network among hBMVECs and other cerebral cell types, it is also likely to be involved in the immune and inflammatory responses that occur following TBI. Previous studies have attempted to alleviate the secondary injury caused by BBB disruption in TBI or cerebral ischemia models by administering mesenchymal stem cells; however, these studies have demonstrated limited potential (20,21). The underlying mechanism of BBB damage after TBI remains to be fully elucidated and there is an urgent need to discover novel therapeutic strategies for the treatment of TBI.

The neurotrophic factor neuregulin-1 (Nrg1) regulates a wide variety of functions, including ensheathment and myelination in neurons and glia (22). Nrg1 is an endogenous growth factor that is encoded by four genes, Nrg1, Nrg2, Nrg3 and Nrg4. The neuroprotective effect of Nrg1 has been proposed to be associated with its anti-inflammatory action in murine brain ischemia models (23-25). Nrg1 signals are transduced through the ErbB family of receptor protein tyrosine kinases, namely ErbB1 (HER1)-ErbB4 (HER4). It has been demonstrated that the binding of Nrg1 to ErbB3 or ErbB4 results in the phosphorylation and dimerization of the ErbB receptors (26). Furthermore, it has been shown that the Nrg1/ErbB4 pathway can regulate visual cortical plasticity in a Cre recombinase parvalbumin expressing neuron knock-in mouse model (27). Although Nrg1 has been characterized extensively in terms of cardiac pathophysiology (28), the role of Nrg1 signaling in the BBB remains elusive. Thymosin β 4 (T β 4) is a major G-actin sequestering molecule that has been observed to serve various biological functions due to its actin-binding properties (29,30). Previous studies have demonstrated that T β 4 treatment results in myocardial damage repair, and that T β 4 promotes cardiomyocyte survival (31,32), reduces inflammation, stimulates angiogenesis (30-32), accelerates wound healing (33) and attenuates oxidative stress (34). This suggests that T β 4 can

exert a diverse range of physiological and pathological functions. Previously, T β 4 and its bioactive peptide, Ac-SDKP, have been found to mediate neuronal protection and improve neurological functions in a TBI rat model (35-38). However, the role of T β 4 in BBB function repair remains poorly understood.

In the present study, the hypothesis that T β 4 may mitigate lipopolysaccharide (LPS)-induced neurovascular remodeling in human BMVEC (hBMVECs) was investigated. Bacterial LPS is a bacterial endotoxin that is a potent stimulus of inflammatory molecule release and has been shown to affect the permeability and transport physiology of the BBB (39). Therefore, LPS-induced alterations in the expression of TJ proteins, production of inflammatory molecules/cytokines, apoptosis, and Nrg1 and vascular gene expression in hBMVECs were all examined in the present study.

To the best of our knowledge, the present study is the first to report that LPS-induced hBMVEC remodeling is associated with enhanced permeability, downregulation of TJ gene expression, enhanced inflammatory response, NF- κ B activation and downregulation of Nrg1 expression. However, all of these alterations were restored in LPS-stimulated hBMVECs following pretreatment with T β 4. These data suggest that T β 4 and Nrg1 can serve as targets for BBB protection against LPS stimulus, which may offer a promising therapeutic intervention method for TBI sequelae.

Materials and methods

Cell culture and treatment. hBMVECs (passage 3; cat no. ACBRI 376) were purchased from Cell Systems and cultured per the manufacturers' protocol using Complete Classic Medium with serum and CultureBoost™ (cat. no. 4Z0-500; Cell Systems; AnaBios) and Passage Reagent Group™ (cat. no. 4Z0-800; Cell Systems; AnaBios) as per their associated protocols. Passages 6-8 were used for all experiments. Cells were allowed 48 h for proliferation at 37°C in 5% CO₂ incubator to a field density of 2.5x10⁵ cells/cm² prior to serum starvation for 2 h with a Complete Serum-Free Medium Kit with RocketFuel™ (cat. no. SF-4Z0-500; Cell Systems; AnaBios). The cells were then pretreated with T β 4 (MilliporeSigma) at a concentration of 1 μ g/ml or Nrg1 antibody (cat. no. ab191139; Abcam,) at a concentration of 2.5 μ g/ml for 2 h prior to 100 ng/ml LPS (cat. no. L4391; Sigma-Aldrich) stimulation at 37°C for 24 h. Three separate experimental groups were used for gene expression studies: Control, LPS and T β 4 + LPS. For western blotting and immunofluorescence analyses, an extra group, T β 4, was used. The control cells were treated with PBS. This dose of T β 4 was based on previously published articles (37,38). Furthermore, similar doses were used by other investigators in experiments with HUVECs and hiPSC-ECs (40,41). The dose did not show any toxic effect or damage to the cells. The dose for Nrg1 antibody (2.5 μ g/ml) was published by Liu *et al* (42). The present study was conducted using a research protocol approved by the Research & Development Committee of the Central Texas Veterans Health Care System (Waco, USA), that includes the Institutional Biosafety Committee and the Institutional Review Board (approval no. 00711). The Research and Development Committee is also the human research ethics oversight committee of the institution.

Table I. Primers used for quantitative PCR.

Gene name	Cat. no.	Sequence
ZO1	HP206807	FP: GTCCAGAATCTCGGAAAAGTGCC RP: CTTTCAGCGCACCATACCAACC
ZO2	HP208078	FP: ATTAGTGCGGGAGGATGCCGTT RP: TCTGCCACAAGCCAGGATGTCT
Occludin	HP206202	FP: ATGGCAAAGTGAATGACAAGCGG RP: CTGTAACGAGGCTGCCTGAAGT
Claudin 5	HP206822	FP: ATGTGGCAGGTGACCGCCTTC RP: CGAGTCGTACACTTTGCACTGC
IL-6	HP200567	FP: AGACAGCCACTCACCTCTTCAG RP: TTCTGCCAGTGCCTCTTTGCTG
TNF- α	HP200561	FP: CTCTTCTGCCTGCTGCACTTTG RP: ATGGGCTACAGGCTTGCTCACTC
IL-1 β	HP200544	FP: CCACAGACCTTCCAGGAGAATG RP: GTGCAGTTCAGTGATCGTACAGG
ICAM1	HP200186	FP: AGCGGCTGACGTGTGCAGTAAT RP: TCTGAGACCTCTGGCTTCGTCA
VCAM1	HP230503	FP: GATTCTGTGCCACAGTAAGGC RP: TGGTCACAGAGCCACCTTCTTG
Caspase-3	HP207674	FP: GGAAGCGAATCAATGGACTCTGG RP: GCATCGACATCTGTACCAGACC
Bcl-2	HP200598	FP: ATCGCCCTGTGGATGACTGAGT RP: GCCAGGAGAAATCAAACAGAGGC
Nrg1	HP228585	FP: GATTCTACCGAGACTCTCCTC RP: TGGAAGGCATGGACACCGTCAT
GADPH	HP205798	FP: GTCTCCTCTGACTTCAACAGCG RP: ACCACCCTGTTGCTGTAGCCAA

FP, forward primer; RP, reverse primer; ZO, zonula occludens; IL, interleukin; TNF- α , tumor necrosis factor- α ; ICAM1, intercellular adhesion molecule 1; VCAM1, vascular CAM1; Bcl-2, B-cell lymphoma-2; Nrg1, neuregulin 1.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the hBMVECs was extracted using the RNEasy kit (Qiagen, Inc.) according to the manufacturer's instructions. For RT, 200 ng total RNA was reverse transcribed into cDNA using a cDNA synthesis kit (OriGene Technologies, Inc.) following the manufacturer's instructions. qPCR was performed as previously described (37). Change in gene expression was evaluated using the $2^{-\Delta\Delta C_q}$ method (43). All experiments and reactions were performed in triplicate with GAPDH used as the internal reference. The gene specific primers used for the present study were purchased from OriGene Technologies, Inc. The sequences for all primers are listed in Table I.

Immunofluorescence microscopy. The hBMVECs were seeded in 6-well plates coated with attachment factor (cat. no. 4Z0 210; Cell Systems; AnaBios) and coverslips were placed over each well. The cells were treated as aforementioned in the Cell culture and treatment subsection. Cells were washed with 1X PBS, fixed in 4% paraformaldehyde at room temperature for 20 min, before being washed and permeabilized in 0.1% Triton X-100 in PBS. The cells were then

blocked with 5% Blocker BSA (Thermo Fisher Scientific, Inc.) for 60 min at room temperature, before incubation with anti-CD31 (cat. no. 3528; Cell Signaling Technology, Inc.; 1:800), anti-ZO3 (cat. no. 3704; clone no. D57G7 XP; Cell Signaling Technology, Inc.; 1:1,600), anti-occludin (cat. no. 91131; clone no. E6B4R; Cell Signaling Technology, Inc.; 1:400) or anti-p65 (cat. no. 8242; clone no. D14E12; Cell Signaling Technology, Inc.; 1:200) antibodies overnight at 4°C, in an antibody dilution buffer (1% Blocker BSA in PBS and 0.01% Triton X-100). The cells were then washed three times with 1X PBS and then incubated in the dark with the corresponding HRP-linked anti-rabbit secondary antibodies (cat. no. 7074; Cell Signaling Technology, Inc.; 1:1,000) for 1 h at room temperature. After this incubation, the cells were again washed three times with 1X PBS. A cover glass was mounted on the slide containing a drop of ProLong Gold Anti-fade mounting media containing DAPI (cat. no. P36962; Thermo Fisher Scientific, Inc.) at room temperature for 15 min. Fluorescent images at x20 magnification were captured using a Leica DMi8 inverted LED fluorescence motorized microscope. Anti-ZO3 (cat. no. 3704), anti-occludin (cat. no. 91131), anti-p65 (cat. no. 8242) and

DAPI (cat. no. 4083) were purchased from Cell Signaling Technology, Inc. Immunofluorescence-positive cells were evaluated in 5-7 separate field images in all groups. The degradation and restoration of ZO1, ZO3 and occludin immunofluorescence-positive cells were counted based on immunofluorescence intensity.

Western blotting. The protein lysates were prepared from 3-5 independent cell culture preparations from each group. Cells homogenized with cell lysis buffer (cat. no. 9803; Cell Signaling Technology, Inc.) containing protease inhibitors were centrifuged at 5,200 \times g at 4°C for 15 min. Protein concentration estimation was conducted using the Bradford assay (Bio-Rad Laboratories, Inc.). Western blotting experiments were performed as described previously (37), with minor modifications. A stain-free 10-15% gradient Tris-Glycine eXtended™ gel (Bio-Rad Laboratories, Inc.) was used for protein separation. In total, 40 and 80 μ g cell lysates were used for occludin and caspase-3 analyses, respectively. After running the gel, the protein was transferred onto a PVDF membrane using the Trans Blot Turbo transfer system (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. The membrane was then blocked using EveryBlot Blocking Buffer (cat. no. 12010020; Bio-Rad Laboratories, Inc.) for 5 min at room temperature. Images were captured at each step using the ChemiDoc MP imaging system (Bio-Rad Laboratories, Inc.). Primary antibodies against occludin (cat. no. 91131) and cleaved caspase-3 (cat. no. 9664) were purchased from Cell Signaling Technology, Inc. Both occludin and cleaved caspase-3 antibodies were incubated with the membranes overnight with at 4°C at 1:1,000 dilutions. The corresponding HRP-conjugated secondary antibody (cat. no. 7074) used for the final immunoblotting process was purchased from Cell Signaling Technology, Inc. The dilution used for the secondary antibody was 1:1,000 and the membrane was incubated for 1 h at room temperature. The immunoreactive bands were visualized using Clarity Max Western enhanced chemiluminescence kit (cat. no. 1705062; Bio-Rad Laboratories, Inc.), before the density of the band was semi-quantified and analyzed using the ImageJ 4.1 software (National Institutes of Health). The parallel blot for the GAPDH (cat. no. 2118; Cell Signaling Technology, Inc.) control was conducted using the same protocol for the target proteins. The dilution used for the GAPDH antibody was 1:1,000.

Permeability assay. The permeability of the hBMVECs was assessed using the Endothelial Transwell Permeability Assay Kit (cat. no. CB6929; Cell Biologics, Inc.) according to manufacturer's protocols. Data from this assay were expressed as absorption readings collected at 450 nm, which was considered the absorbance of relative permeability. The appropriate dose of T β 4 for the permeability assay was determined using three separate doses, 250, 500 and 1,000 ng/ml.

Statistical analysis. Data were expressed as the mean \pm standard error of 3-5 separate experiments and were analyzed using one-way analysis of variance for multiple groups followed by Tukey's post hoc tests, when justified using GraphPad Prism 5.0 software (Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of T β 4 on LPS-induced TJ disruption in hBMVECs. To examine the effects of LPS on TJ gene expression, hBMVECs were stimulated with 100 ng/ml LPS for 24 h. This stimulatory dose of 100 ng/ml LPS did not exert toxic effects or damage the cells. Therefore, this dose was used for all subsequent studies. LPS treatment significantly decreased the mRNA expression of ZO1, ZO2 and occludin ($P < 0.05$; Fig. 1A-C) compared with that in the untreated cells. However, pre-treatment with T β 4 markedly prevented this reduction in mRNA expression, with the exception of ZO1 ($P < 0.05$). The change in mRNA expression of claudin 5 in LPS-treated cells was not significant, despite there being a slight reduction (Fig. 1D). Together, these data suggest that T β 4 pre-treatment protected against LPS-induced TJ gene expression in hBMVECs.

Since it has been frequently applied as a representative TJ protein marker, occludin was chosen for examination by western blotting. The results showed that occludin protein levels were reduced after LPS stimulation compared with that in untreated cells ($P < 0.05$; Fig. 1E and F). However, T β 4 pre-treatment markedly prevented this.

Effect of T β 4 on the LPS-induced inflammatory response in hBMVECs. Inflammation plays a pivotal role in BBB damage (17,18). Therefore, the mRNA expression levels of interleukin-6 (IL-6), tumor necrosis factor α (TNF α) and IL-1 β in LPS-treated hBMVECs were measured. LPS treatment was found to significantly increase the mRNA expression of IL-6, TNF α and IL-1 β ($P < 0.05$) compared with that in untreated cells. By contrast, cells pre-treated with T β 4 were significantly more resistant to this LPS-induced phenomenon ($P < 0.05$; Fig. 2A-C). Together, these data suggest that T β 4 pre-treatment can prevent the LPS-induced inflammatory response in hBMVECs.

Role of T β 4 in the LPS-induced activation of adhesion molecules in hBMVECs. Adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), are critical for BBB integrity and are activated during BBB injury (16,19). The present study demonstrated that both ICAM1 and VCAM1 mRNA expression levels were significantly increased after LPS treatment ($P < 0.05$) compared with that in untreated cells. However, T β 4 pre-treatment significantly prevented this effect ($P < 0.05$; Fig. 3A and B). These data suggest that T β 4 may confer protection against LPS-induced vascular damage by reducing ICAM1 and VCAM1 gene expression in hBMVECs.

Anti-apoptotic effects of T β 4 on LPS-stimulated hBMVECs. The Caspase-3 mRNA expression level in hBMVECs following LPS treatment was next measured. LPS stimulation significantly increased caspase-3 mRNA expression compared with that in untreated cells. By contrast, T β 4 pre-treatment significantly prevented this LPS-induced increase in caspase-3 mRNA expression ($P < 0.05$; Fig. 4A). The expression of the anti-apoptotic gene, B-cell lymphoma 2 (Bcl-2), was not significantly altered following LPS-stimulation (Fig. 4B). These data suggested that T β 4 pre-treatment protected against LPS-induced apoptosis of hBMVECs by inhibiting caspase-3.

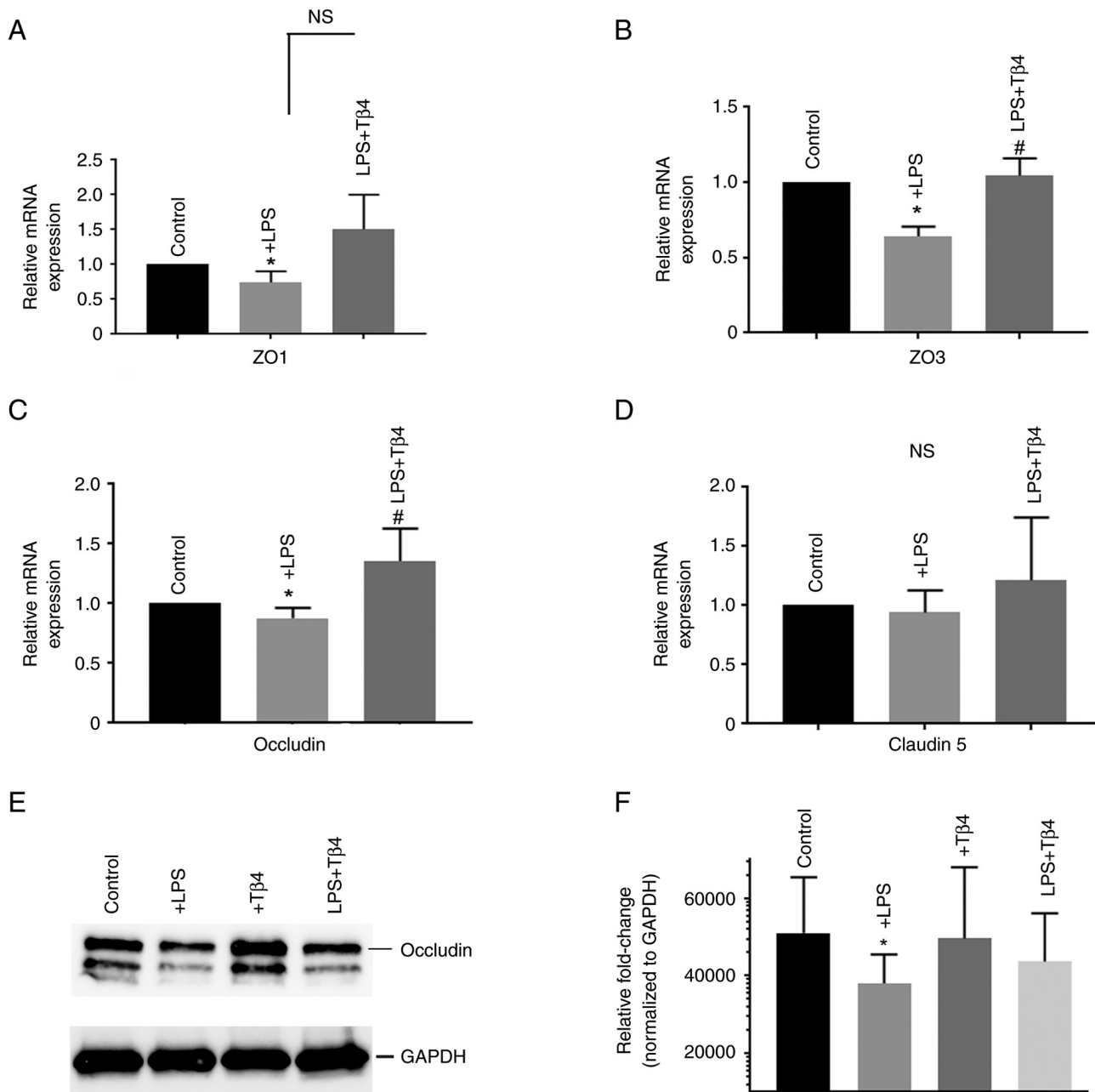


Figure 1. Effect of Tβ4 on LPS-induced relative mRNA expression of tight junction genes in human brain microvascular endothelial cells. Cells were exposed to LPS at 100 ng/ml for a period of 24 h. The mRNA expression of (A) ZO1, (B) ZO3 (C) Occludin and (D) Claudin 5 were measured using reverse transcription-quantitative PCR. (E) Representative image of the western blotting analysis of the Occludin protein expression level. (F) Semi-quantification of the occludin western blot. The experiments were replicated 3-5 times and amplifications were performed and normalized to GAPDH. Results are presented as the mean ± SEM. *P<0.05 vs. untreated cells (control). #P<0.05 vs. LPS treated cells. NS, non-significant; ZO, zonula occludens; LPS, lipopolysaccharide; Tβ4, thymosin β4.

The change in expression of cleaved caspase-3 was further determined by western blotting. The results showed that cleaved caspase-3 was significantly higher after LPS stimulation (P<0.05; Fig. 4C and D). However, Tβ4 pre-treatment significantly reduced levels of cleaved caspase-3 protein expression compared with LPS treated cells.

Effect of Tβ4 on LPS-stimulated Nrg1 expression in hBMVECs. Nrg1 is an endogenous growth factor belonging to the family of epidermal growth factors (22) and has

previously demonstrated neuroprotective effects after ischemic stroke (23-25). The mRNA expression level of Nrg1 in LPS-treated hBMVECs was measured, which showed a significant reduction in Nrg1 mRNA expression (P<0.05; Fig. 5) compared with that in untreated cells. Tβ4 pre-treatment significantly preserved Nrg1 expression (P<0.05; Fig. 5), which was higher compared with that in LPS-treated cells, suggesting that it is a key target molecule. These data suggest that Tβ4 pre-treatment has the potential to prevent LPS-induced injury in hBMVECs by protecting Nrg1 expression.

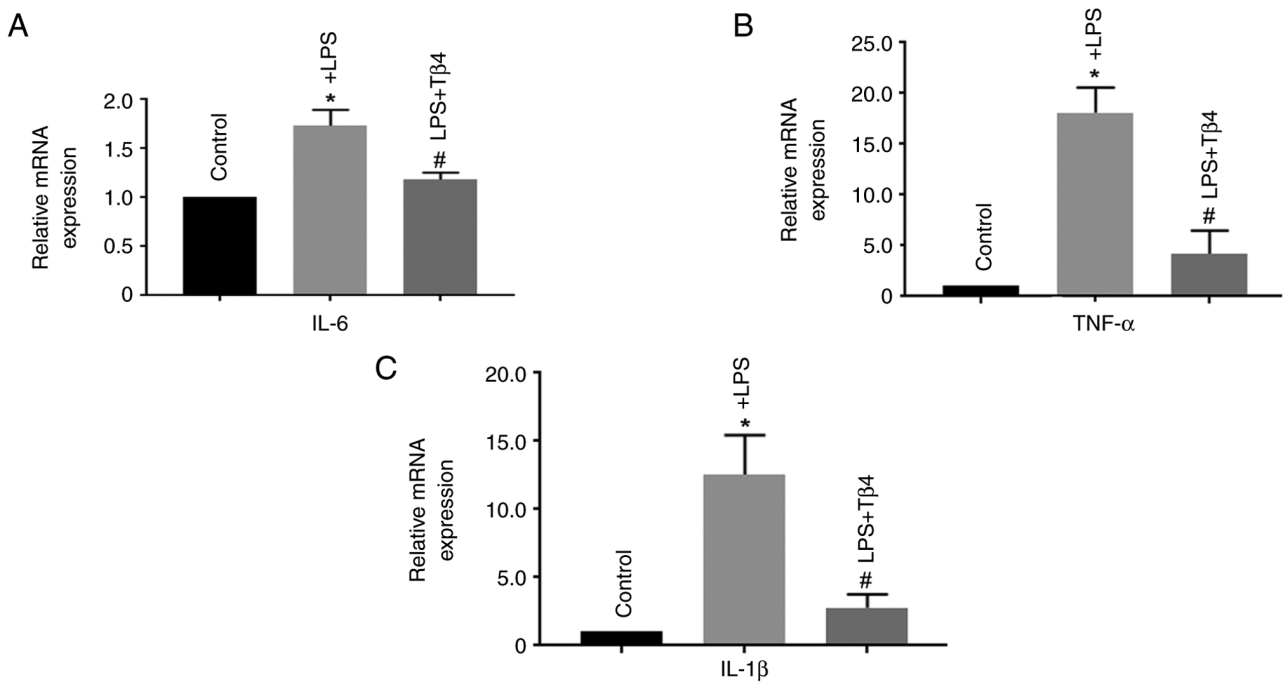


Figure 2. Effect of T β 4 on LPS-stimulated relative mRNA expression of inflammatory genes in human brain microvascular endothelial cells. Cells were exposed to LPS at 100 ng/ml for a period of 24 h. The mRNA expression of (A) IL-6 (B) TNF- α , and (C) IL-1 β were measured using reverse transcription-quantitative PCR. These experiments were replicated 3-5 times and amplifications were performed and normalized to GAPDH. Results are presented as the mean \pm SEM. * P <0.05 vs. untreated cells (control). # P <0.05 vs. LPS treated cells. LPS, lipopolysaccharide; T β 4, thymosin β 4; IL-6, interleukin-6; TNF- α , tumor necrosis factor α ; IL-1 β , interleukin-1 β .

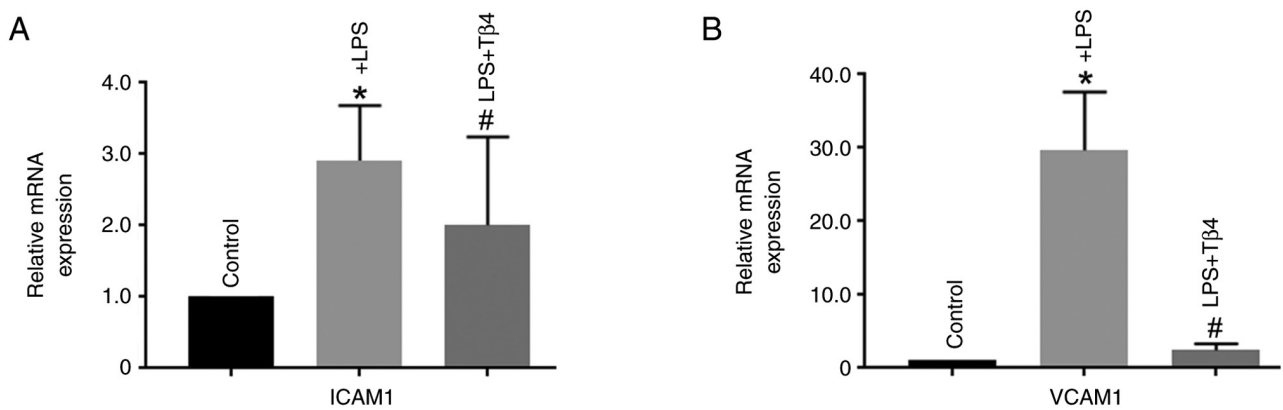


Figure 3. Role of T β 4 in LPS-induced relative mRNA expression of adhesion molecules in human brain microvascular endothelial cells. Cells were exposed to LPS at 100 ng/ml for a period of 24 h. The mRNA expression of (A) ICAM1 and (B) VCAM1 were measured using reverse transcription-quantitative PCR. These experiments were replicated 3-5 times and amplifications were performed and normalized to GAPDH. Results are presented as the mean \pm SEM. * P <0.05 vs. untreated cells (control). # P <0.05 vs. LPS treated cells. LPS, lipopolysaccharide; T β 4, thymosin β 4; ICAM1, intracellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1.

Effects of T β 4 on LPS-stimulated hBMVEC monolayer permeability. Permeability is one of the critical functional parameters of the BBB in maintaining CNS homeostasis (7). Disruption of the BBB due to injury can result in increased BBB permeability, which promotes several neurological diseases (7,11). This suggests that LPS treatment may either disrupt the function of or loosen the TJ proteins, increase inflammation, and promote apoptosis. However, in the present study, T β 4 pre-treatment was shown to preserve TJ protein expression, prevent inflammation and apoptosis. Therefore, the effects of T β 4 on LPS-induced damage of the BBB components was next examined by measuring BBB

permeability. The dose-response of T β 4 in LPS-treated hBMVECs was determined using three doses, 250, 500 and 1,000 ng/ml. The data indicated that 1,000 ng/ml elicits the greatest protection on permeability (Fig. S1). The hBMVECs were then treated with LPS for 24 h in the presence or absence of 1,000 ng/ml T β 4. LPS treatment was found to significantly increase the permeability of the hBMVECs (P <0.05; Fig. 6). By contrast, this LPS-induced increase in hBMVEC permeability was significantly prevented if the cells were pre-treated with T β 4, suggesting that T β 4 may offer protection against LPS-induced dysfunction in endothelial cell permeability (P <0.05; Fig. 6).

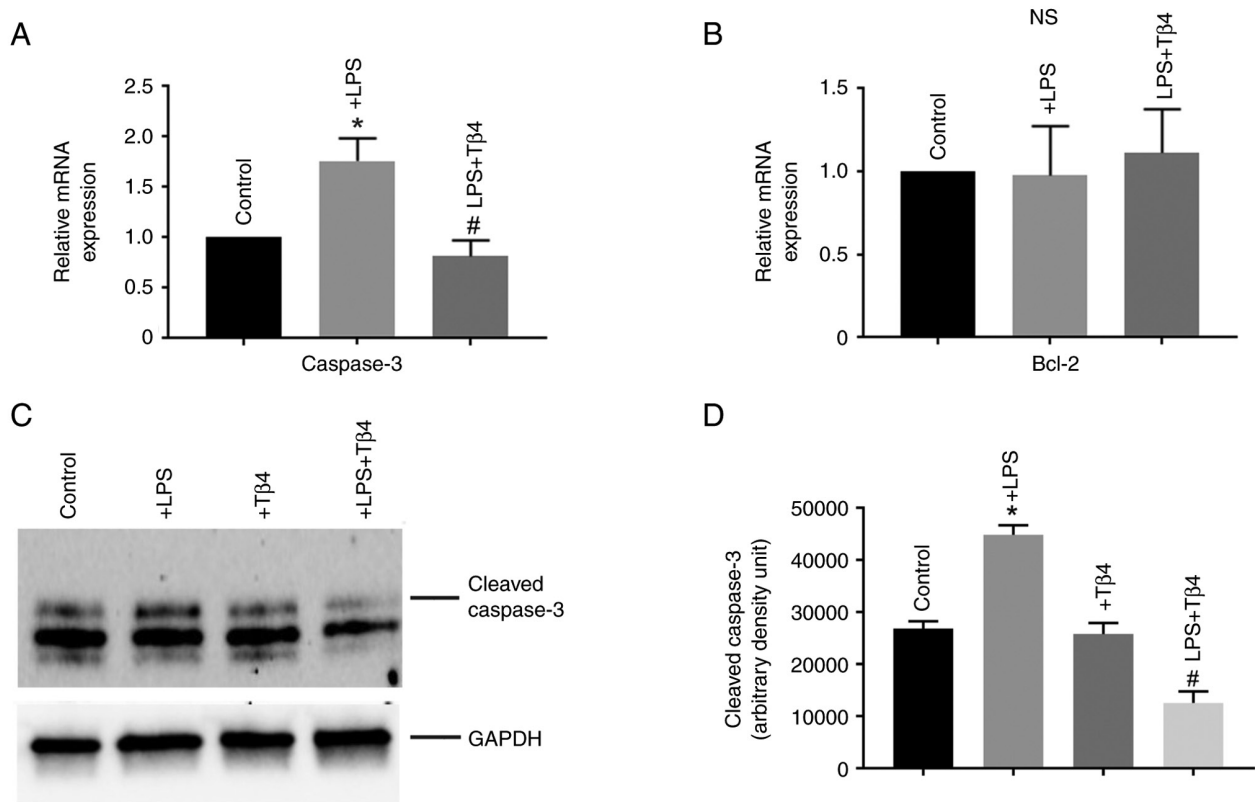


Figure 4. Effect of Tβ4 on LPS-stimulated relative mRNA expression of apoptotic genes and cleaved caspase-3 (arbitrary unit) levels in human brain microvascular endothelial cells. Cells were exposed to LPS at 100 ng/ml for a period of 24 h. The mRNA expression of (A) caspase-3 and (B) Bcl-2 were determined by reverse transcription-quantitative PCR. (C) Representative image of the western blot analysis of cleaved caspase-3 protein level. (D) Semi-quantification of cleaved caspase-3 western blot analysis. These experiments were replicated 3-5 times and amplifications were performed and normalized to GAPDH. Results are presented as the mean ± SEM. *P<0.05 vs. untreated cells (control). #P<0.05 vs. LPS treated cells. NS, Non-significant; LPS, lipopolysaccharide; Tβ4, thymosin β4; Bcl-2, B-cell lymphoma-2.

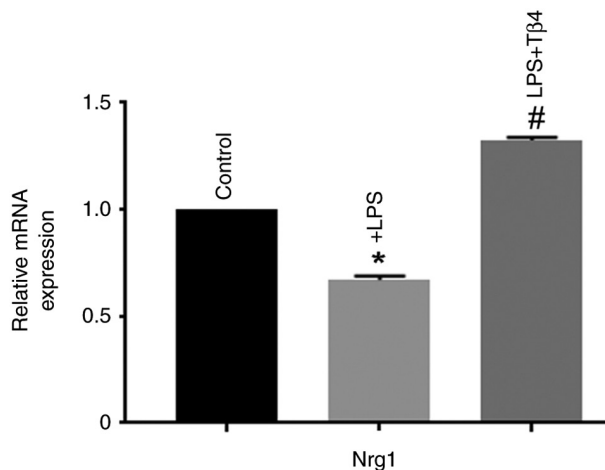


Figure 5. Effect of Tβ4 on LPS-stimulated relative mRNA expression of Nrg1 in human brain microvascular endothelial cells. Cells were exposed to LPS at 100 ng/ml for a period of 24 h. The mRNA expression of Nrg1 was measured using reverse transcription-quantitative PCR. These experiments were replicated three-to-five times and amplifications were performed and normalized to GAPD. Results are presented as the mean ± SEM (n=3). *P<0.05 vs. untreated cells (control). #P<0.05 vs. LPS treated cells. LPS, lipopolysaccharide; Tβ4, thymosin β4; Nrg1, neuregulin-1.

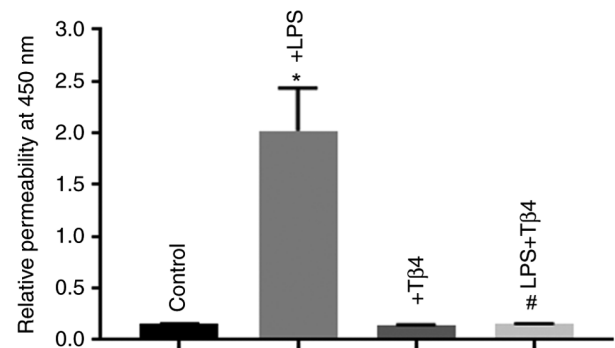
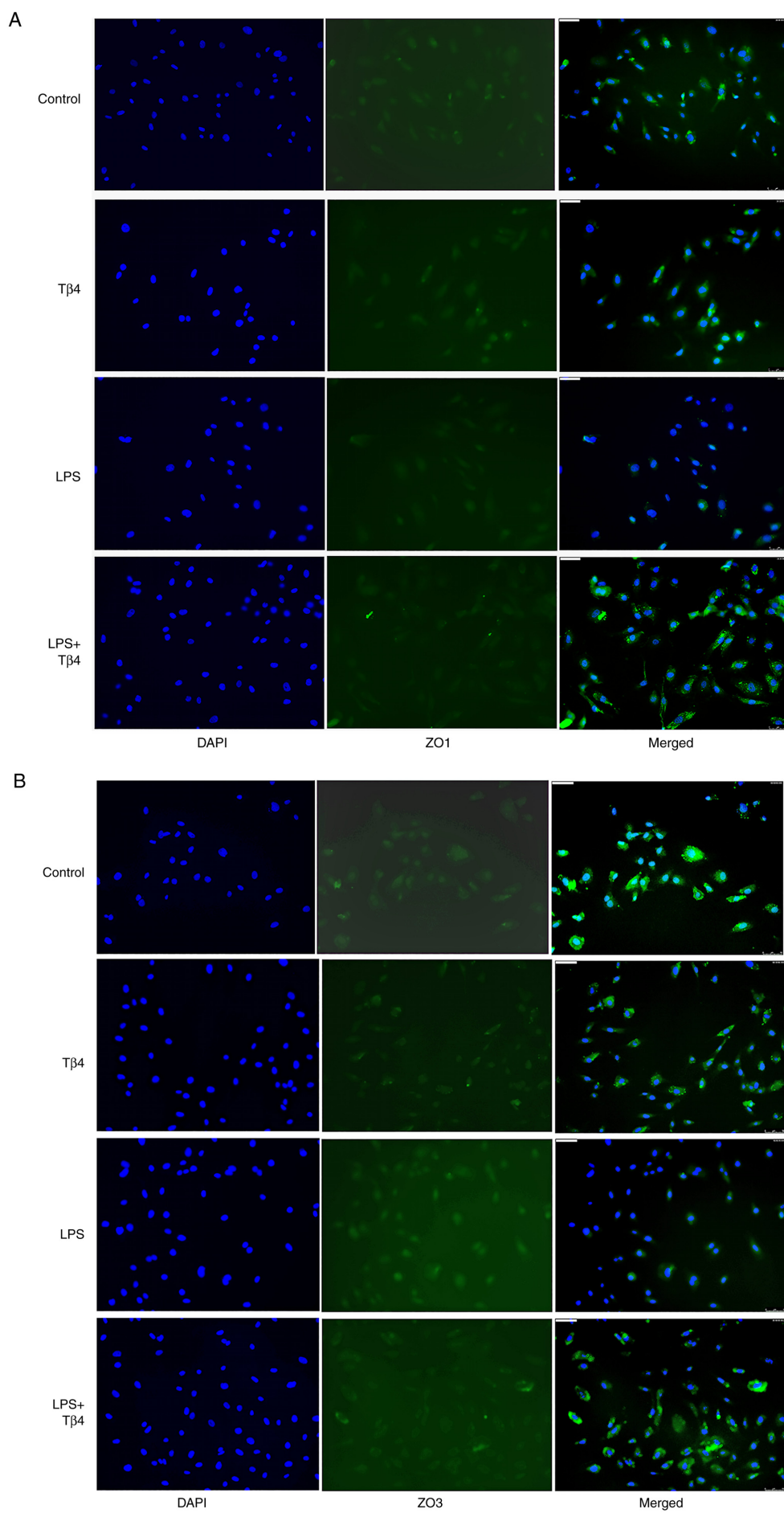


Figure 6. Effect of Tβ4 on LPS-stimulated relative permeability (450 nm) in human brain microvascular endothelial cells. Permeability assays were performed using an Endothelial Trans-well Permeability Assay Kit. Data expressed as A450 absorption readings are considered the relative permeability. Cultured hBMVECs were pre-treated with Tβ4 for 2 h and stimulated with LPS for 24 h. Results are presented as the mean ± SEM (n=3). *P<0.05 vs. untreated cells (control). #P<0.05 vs. LPS treated cell. Tβ4, thymosin β4; hBMVECs, human brain microvascular endothelial cells; LPS, lipopolysaccharide.

Effects of Tβ4 on ZO1, ZO3, occludin and p65 expression in hBMVECs stimulated with LPS. To determine the effect of Tβ4 on LPS-induced degradation of ZO1, ZO3 and occludin

proteins in hBMVECs, immunofluorescence analyses were performed. The data demonstrated that LPS treatment significantly reduced ZO1, ZO3 and occludin protein expression, which was indicated by the low levels of green fluorescence compared with that in untreated cells (Fig. 7A-C). Prior treatment with Tβ4 preserved ZO1, ZO3 and occludin expression,



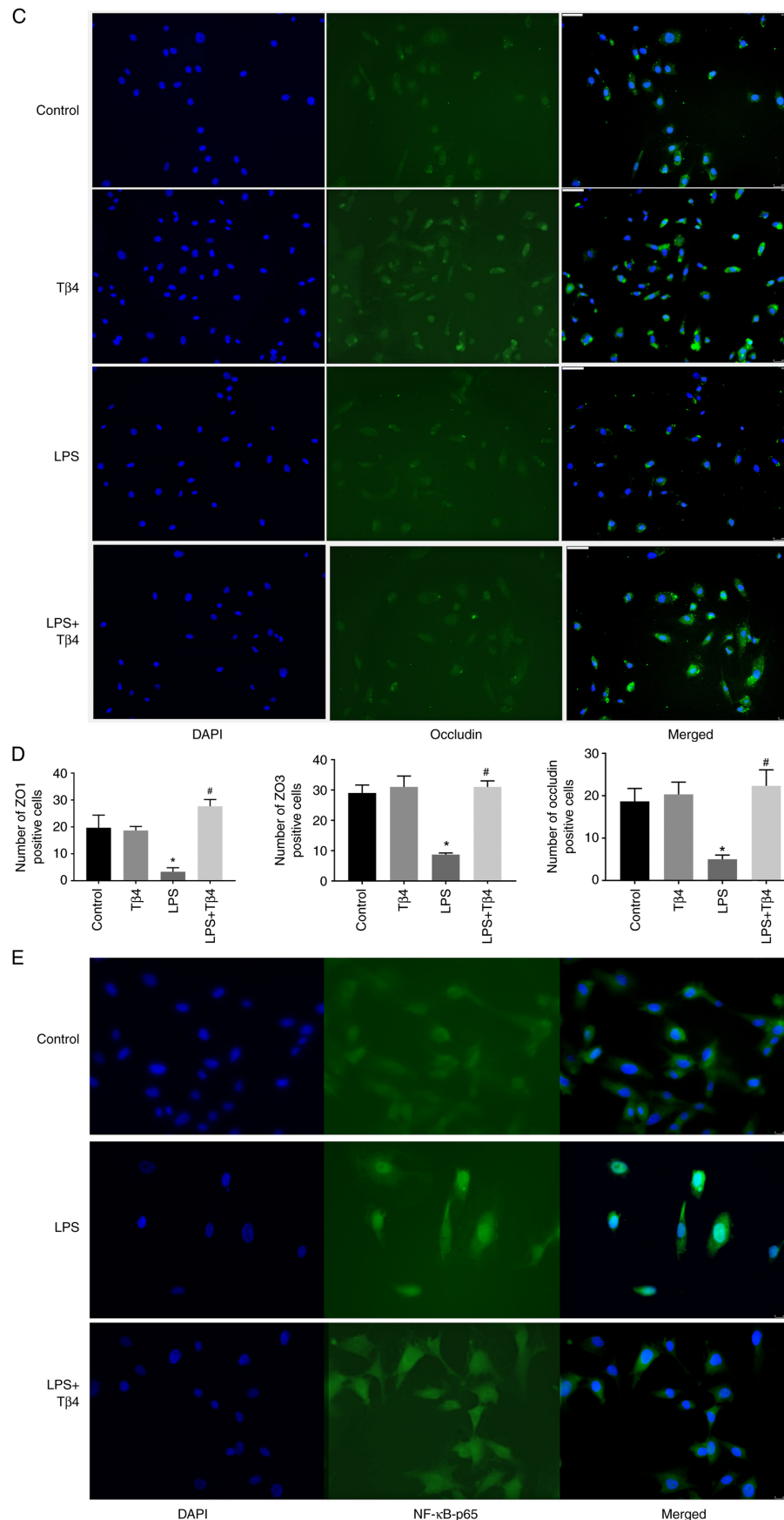


Figure 7. Effect of Tβ4 on ZO1, ZO3, occludin and p65 expression in hBMVECs stimulated with LPS. Cultured hBMVECs were pre-treated with Tβ4 for 2 h and then stimulated with LPS for 24 h. Dual staining immunofluorescence analysis of (A) ZO1, (B) ZO3 and (C) Occludin (green staining; DAPI, blue staining). (D) Semi-quantification of number of green fluorescence-positive cells. (E) Immunofluorescence analysis of p65 (green staining; DAPI, blue staining). Objective magnification, x20. ZO, zonula occludens; Tβ4, thymosin β4; hBMVECs, human brain microvascular endothelial cells; LPS, lipopolysaccharide. *P<0.05 vs. untreated cells (control). #P<0.05 vs. LPS treated cells.

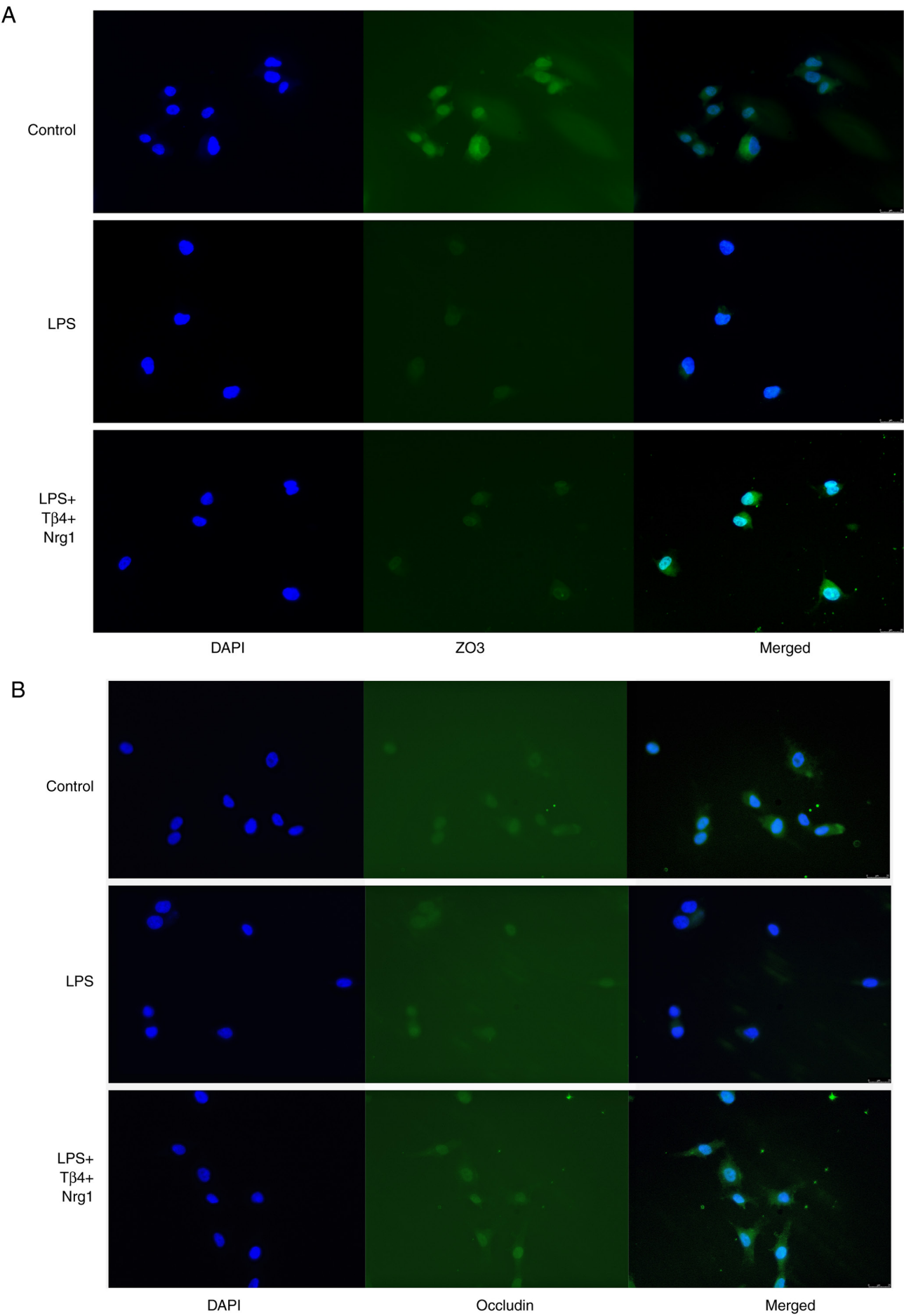


Figure 8. Continued.

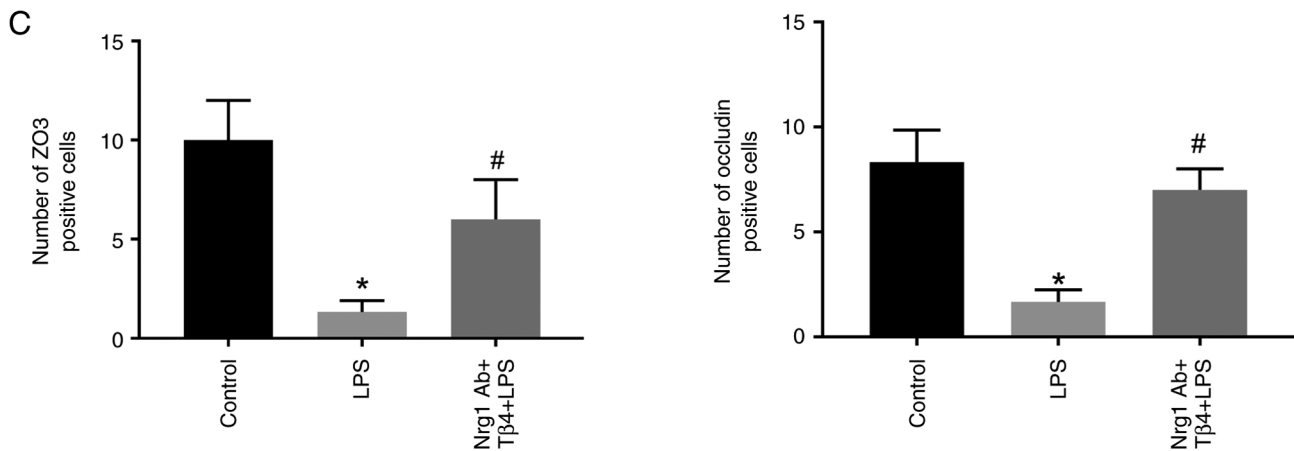


Figure 8. Effect of Nrg1 antibody on LPS-stimulated ZO3 and occludin in hBMVECs. Cultured hBMVECs were pre-treated with Nrg1 antibody for 2 h, Tβ4 for 2 h and stimulated with LPS for 24 h. Dual-staining immunofluorescence analysis of (A) ZO3 and (B) occluding (green staining; DAPI, blue staining). (C) Semi-quantification of number of green fluorescence-positive cells. Objective magnification, x20. hBMVECs, human brain microvascular endothelial cells. ZO, zonula occludens; Nrg1, neuregulin-1; hBMVECs, human brain microvascular endothelial cells; LPS, lipopolysaccharide. Results are presented as the mean \pm SEM. * P <0.05 vs. untreated cells (control). # P <0.05 vs. LPS treated cells.

as indicated by the green fluorescence (Fig. 7A-C). The fluorescence-positive cells were evaluated in 5-7 separate field images in all groups for the determination of statistical significance. The semi-quantification of green fluorescence-positive cells for ZO1, ZO3 and occludin is shown in Fig. 7D (P <0.05).

To assess whether Tβ4 provided protection against inflammation, the expression level of p65, a central regulator for inflammation, was measured. A clear translocation of p65 protein into the nucleus was observed after LPS treatment, whereas Tβ4 pre-treatment significantly prevented this, as indicated by the green fluorescence outside of the nucleus (P <0.05; Fig. 7E). Therefore, the data indicated that the LPS-induced NF- κ B activation is prevented by Tβ4. The hBMVEC-specific marker, CD31, was used as a positive control confirming the correct cell-type was being used in the present study and is shown in Fig. S2.

Together, these data indicate that Tβ4 pre-treatment protects against LPS-induced BBB damage by preserving ZO1, ZO3 and occludin expression, whilst suppressing inflammation in hBMVECs.

Effects of the Nrg1 antibody on LPS-stimulated ZO3 and occludin in hBMVECs. To ascertain the role of Nrg1 in hBMVEC remodeling, cells were pre-treated with the Nrg1 antibody before being treated with LPS and Tβ4. The treatment appears to block the interaction of Tβ4 and Nrg1. The fluorescence-positive cells were evaluated in 5-7 separate field images in all groups for statistical significance determination. Pre-treatment with the Nrg1 antibody significantly prevented the LPS-induced reduction of both ZO3 and occludin protein level compared with that in LPS treatment group (P <0.05; Fig. 8A and B). The semi-quantification of green fluorescence-positive cells for ZO3 and occludin is shown in Fig. 8C (P <0.05). These data suggest that Nrg1 may contribute independently for BBB protection in LPS-stimulated hBMVECs.

Discussion

To the best of our knowledge, the present study is the first to report that Tβ4 has the potential to prevent the BBB damage

induced by LPS treatment. The present study also demonstrated that Tβ4 decreased the inflammatory response during hBMVEC remodeling. In addition, the present study found Nrg1 to be a possible target for Tβ4.

TBI has been previously reported to instigate several secondary injuries, including inflammation and disruption of the BBB (43). The BBB has a pivotal role in the removal of waste materials and provides further protection by preventing entry of pathogenic agents through solute permeability (44). Maintaining the integrity of the BBB is therefore necessary for CNS homeostasis. Permeability of the BBB is typically controlled by several TJ proteins embedded in between hBMVECs, including ZO1, ZO2, occludin and claudin 5 (45-47). The present study demonstrated that the mRNA expression levels of ZO1, ZO2, and occludin were all reduced after LPS stimulation, however, this effect was prevented by Tβ4 pre-treatment, with the exception of ZO1. In addition, the western blotting results using occludin as a representative marker of TJ proteins revealed moderate restoration of occludin protein in response to LPS challenge. This observation was supported by the immunofluorescence analyses of ZO1, ZO3 and occludin protein expression in the LPS-stimulated hBMVECs. It is known that dysfunctional TJ proteins can result in endothelial cell damage, leading to increased BBB permeability (46). The present study also found that LPS stimulation increased hBMVEC permeability, when using an *in vitro* assay. In addition, the Tβ4-pretreated cells demonstrated significantly reduced permeability compared with that in LPS-treated cells, suggesting its potential therapeutic application for BBB damage. However, the mechanism underlying the restoration of TJ protein function by Tβ4 remains poorly understood. Tβ4 can become internalized by cells (48), but the cell surface receptors remain unknown. A hypothesized mechanism is through transcription factor-mediated active transport of positively charged amino acids (48). In addition, nuclear localization of actin and chromatin remodeling have been suggested (49).

BBB disruption allows inflammatory molecules to enter the CNS, thus, triggering the neuroinflammatory response (50).

IL-6 is a major inflammatory factor that can induce the expression of adhesion molecules in activated endothelial cells (51). The present study showed that the LPS-stimulation of IL-6 expression was significantly prevented by T β 4 pre-treatment. Similar observations were found regarding IL-1 β and TNF α expression in LPS-stimulated hBMVECs, suggesting an anti-inflammatory role of T β 4. These findings are consistent with previous studies under various physiological and pathological settings (52-57). Together, these results suggest an anti-inflammatory role for T β 4 in hBMVEC remodeling in the neurovascular unit.

Cellular adhesion was found to be disrupted by LPS in the present study, as demonstrated by the increased ICAM1 and VCAM1 expression observed following LPS treatment of hBMVECs. This increase in ICAM1 and VCAM1 expression was significantly prevented by T β 4 pre-treatment, indicating a potential role of vascular gene regulation during BBB disruption. This observation further suggested that this reduction of vascular gene expression and BBB permeability by T β 4 may attenuate the adhesion and migration of inflammatory cells.

The BBB becomes damaged after ischemic insult or TBI (7,58). Nrg1 is a growth factor with diverse functions in the CNS and has been shown to mediate protective effects in a focal brain ischemic rat model (59). Nrg1 functions by activating ErbB receptor kinases, specifically ErbB4 (60,61). It has been previously demonstrated that Nrg1 expression is reduced in an ischemic stroke model, whereby treatment with Nrg1 prior to brain injury induction provided neuroprotection (62,63). The present study found a reduction of Nrg1 mRNA expression in LPS-stimulated hBMVECs, which was prevented by T β 4 pre-treatment. In addition, cells pre-treated with the Nrg1 antibody prevented the loss of ZO3 and occludin proteins after LPS-stimulation, suggesting Nrg1 is pivotal in hBMVECs remodeling. Together, to the best of our knowledge, these findings demonstrated for the first time that Nrg1 may be a possible target for T β 4. Although T β 4 has been shown to be protective against TBI (36,37), its role in the BBB remains unknown. The present study indicates a possible therapeutic use of Nrg1 for BBB restoration following TBI or ischemic stroke. However, this association between T β 4 and Nrg1 requires further investigation in a BBB model. Furthermore, this observation also requires verification in animal models of TBI or ischemic stroke.

In conclusion, the present study demonstrated that T β 4 can protect against LPS-induced hBMVEC remodeling by reducing inflammation, whilst restoring TJ protein and Nrg1 expression levels. The present study may offer a novel therapeutic platform for treating BBB damage caused by injury or trauma with T β 4 potentially serve as a new therapeutic tool for BBB protection, where inflammation and TJ proteins serve a critical role.

There are many cell lines that can be used in a BBB study. However, hBMVECs were chosen as the model system in the present study as it is one of the pivotal cells in the neurovascular unit. Therefore, the use of only hBMVECs is a limitation of the present study. In addition, the control cell line used is a non-TBI cell line, which is also considered a limitation of the present study.

The present findings indicated that the role of T β 4 in the restoration of TJ proteins and Nrg1 in hBMVECs may be of

clinical relevance. Nrg1 may be an important component in neurovascular remodeling and the use of T β 4 as a therapeutic molecule for neuronal protection may prove to be instrumental.

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Availability of data and materials

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

Authors' contributions

SG conceived the project. SG and RSG designed the experiments. SG wrote the manuscript. RSG critically read the manuscript and provided constructive feedback. WS, CH and SG performed the experiments. SG and RSG analyzed the data. WS and CH cultured cells and performed treatments and maintenance of the cell passages for all experiments. WS performed the western blot and RT-qPCR analyses. CH performed RT-qPCR, immunofluorescence experiments and captured images. SG and WS performed the permeability assay. SG, WS and CH confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The VISN 17 Center of Excellence for Research on Returning War Veterans (Waco, USA) comes under the Central Texas Veterans Health Care System (CTVHCS; Waco, TX, USA), which regulates its research operations. All required approvals to conduct the present study were attained through the various CTVHCS research committees, including ethical approval from the Institutional Review Board (approval no. 00711), which is the human research ethics oversight committee of our institute.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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