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UNIVERSITY OF CALIFORNIA SAN DIEGO

Regulation of Blood–Brain Barrier Stra6 Expression Under Pro-Inflammatory Cytokine Exposure

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Bioengineering

By

Pavithra Ramesh

Committee in charge:

Professor Richard Daneman, Chair
Professor Erika Cyphert, Co-Chair
Professor Adam Engler

2026

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The Thesis of Pavithra Ramesh is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2026

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List of Abbreviations

BBB – Blood-Brain Barrier

CNS – Central Nervous System

Stra6 – Stimulated by Retinoic Acid 6

EAE – Experimental Autoimmune Encephalomyelitis

LPS – Lipopolysaccharide

MS – Multiple Sclerosis

IL-1 β – Interleukin-1 Beta

TNF- α – Tumor Necrosis Factor Alpha

IFN- γ – Interferon Gamma

ICAM-1 – Intercellular Adhesion Molecule 1

VCAM-1 – Vascular Cell Adhesion Molecule 1

RBP – Retinol-Binding Protein

RBP4 – Retinol-Binding Protein 4

CRBP-I – Cellular Retinol-Binding Protein I

ATRA – All-Trans Retinoic Acid

LRAT – Lecithin Retinol Acyltransferase

JAK2 – Janus Kinase 2

STAT5 – Signal Transducer and Activator of Transcription 5

NF- κ B – Nuclear Factor Kappa B

JAK/STAT – Janus Kinase / Signal Transducer and Activator of Transcription

GPCR – G Protein-Coupled Receptor

TLR4 – Toll-Like Receptor 4

GLUT1 – Glucose Transporter 1

ZO-1 – Zonula Occludens-1

ZO-2 – Zonula Occludens-2

ROS – Reactive Oxygen Species

OCT – Optimal Cutting Temperature

PBS – Phosphate-Buffered Saline

PFA – Paraformaldehyde

ROI – Region of Interest

IACUC – Institutional Animal Care and Use Committee

i.p. – Intraperitoneal

AF488 – Alexa Fluor 488

AF568 – Alexa Fluor 568

AF647 – Alexa Fluor 647

DAPI – 4',6-Diamidino-2-Phenylindole

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Finally, I would like to thank my family for their unconditional love, encouragement, and belief in me throughout my academic journey. Their support has been my foundation during both the challenges and successes of graduate school, and I would not be where I am today without them.

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Publications

Saminathan P, Corey M, Gibbons A, Rayadurgam M, Reddy N, Ramesh P, Sharma S. "Sex Differences in Metabolite–Immune Circuits of Neuroinflammation." Immunological Reviews. March 2026. <https://doi.org/10.1111/imr.70114>

Field of Study

Major Field: Bioengineering
Studies in Neuroinflammation
Professor Richard Daneman

Abstract of the Thesis

Regulation of Blood–Brain Barrier Stra6 Expression Under Pro-Inflammatory Cytokine
Exposure

by

Pavithra Ramesh

Master of Science in Bioengineering

University of California San Diego, 2026

Professor Richard Daneman, Chair
Professor Erika Cyphert, Co-Chair

The blood-brain barrier (BBB) regulates the exchange of molecules between the circulation and the central nervous system through specialized endothelial transport systems. One such transporter is Stra6 which mediates retinol uptake and displays regional enrichment at the BBB. It has high expression in the nucleus accumbens shell and ventral cochlear nucleus. While this

region-specific expression pattern is well established, little is known about how Stra6 is regulated during neuroinflammatory conditions.

Previous work from our laboratory demonstrated that Stra6 expression is increased at the BBB in both Experimental Autoimmune Encephalomyelitis (EAE), a model of multiple sclerosis, and lipopolysaccharide (LPS)-induced inflammation - an endotoxin that triggers the innate immune system. Endothelial-specific deletion of Stra6 also reduced EAE disease severity, suggesting that Stra6 may play an active role in neuroinflammatory processes rather than simply being a marker of inflammation. Because pro-inflammatory cytokines are central mediators of both EAE and LPS-induced inflammation, we hypothesized that cytokine signaling may drive Stra6 upregulation at the BBB.

To test this hypothesis, mice were treated with IL-1 β , TNF- α , IFN- γ , and a combination of all three cytokines (cytokine cocktail), and Stra6 expression was assessed in the nucleus accumbens shell, hippocampus, and cortex. Cytokine treatment successfully induced endothelial activation, as observed by increased ICAM1 expression. Despite this robust inflammatory response, Stra6 expression remained largely unchanged across all treatment conditions. The established regional pattern of Stra6 expression was also preserved, with the nucleus accumbens shell consistently exhibiting the highest expression levels.

These findings indicate that acute cytokine exposure alone is insufficient to induce Stra6 expression at the BBB. Although Stra6 is upregulated in neuroinflammatory disease models, the present results suggest that this response is not driven by direct acute cytokine signaling and may

instead require sustained inflammation or other additional disease-associated signals. Together, these findings provide insight into the regulation of Stra6 during neuroinflammation and highlight the stability of regional BBB specialization under acute inflammatory challenge.

Introduction

The blood-brain barrier (BBB) is a highly selective interface between peripheral circulation and the central nervous system that regulates the movement of ions, molecules, and cells, protecting neural tissue from fluctuations in the systemic environment. Structurally, it is formed by endothelial cells lining cerebral vessels, connected by continuous tight junctions that restrict paracellular diffusion and limit passive permeability. These endothelial cells exhibit low rates of transcytosis and express selective transport systems that enable controlled exchange between the blood and the brain [1, 2].

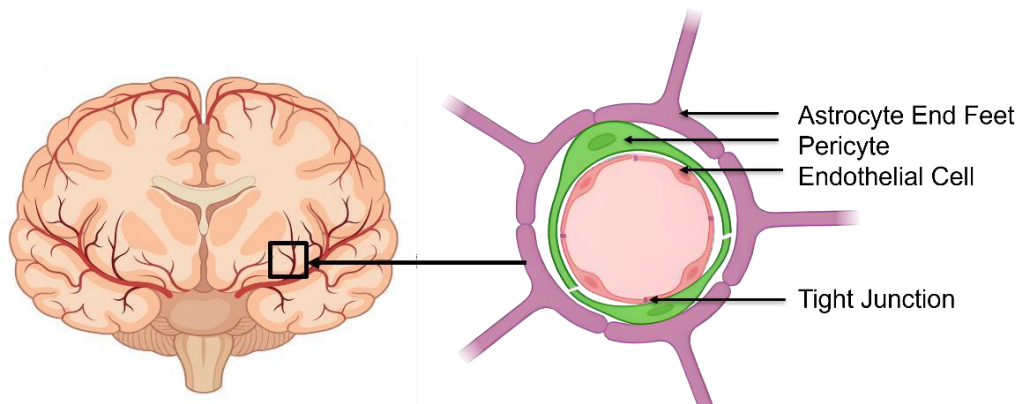


Figure 1: Schematic representation of the blood–brain barrier (BBB) and neurovascular unit.

On the luminal side, a dense glycocalyx layer composed of glycoproteins and glycosaminoglycans forms the first interface with circulating blood, contributing to the regulation of molecular and cellular access to the endothelial surface [3]. Beyond the endothelium, pericytes, astrocytes, and the basement membrane contribute to BBB function by regulating vascular stability, permeability, and signaling. Together, these cellular and structural features allow the BBB to tightly control nutrient delivery, ion balance, and immune cell entry into the brain.

BBB disruption is a common feature of neuroinflammatory conditions including multiple sclerosis, stroke, sepsis, and neurodegenerative disease, and contributes to disease progression and

neural injury. Understanding the mechanisms driving these changes remains an important unanswered question. Pro-inflammatory cytokines such as IL-1 β , TNF- α , and IFN- γ are released during neuroinflammation and are known to modulate BBB function by altering endothelial behavior, disrupting barrier integrity, and influencing immune cell trafficking within the central nervous system [4]. Whether inflammatory cytokines regulate endothelial transport systems in the same way they regulate classical inflammatory markers, however, remains unclear.

One endothelial transport protein whose regulation under inflammatory conditions remains poorly understood is Stra6, a transmembrane receptor that mediates cellular uptake of retinol through its interaction with retinol-binding protein. Stra6 is expressed in brain endothelial cells where it contributes to retinoid transport into the central nervous system [5, 6, 7]. Beyond its role as a retinol transporter, Stra6 can also function as a signaling receptor capable of activating JAK2/STAT5 signaling [8] in response to retinol-bound retinol-binding protein, linking vitamin A uptake to intracellular signaling and transcriptional regulation. This signaling activity depends on coordinated interactions with intracellular retinol-binding proteins such as CRBP-I and retinoid metabolism pathways involving LRAT, suggesting that Stra6 operates within an integrated retinoid-sensitive signaling and transport network rather than as a passive transporter alone.

Stra6 expression is not uniform across the BBB. Strong regional enrichment is observed in areas such as the nucleus accumbens shell, while comparatively lower expression is found in the cortex and hippocampus [9]. This spatial heterogeneity raises the question of whether inflammatory regulation of Stra6 occurs uniformly across brain regions or instead disrupts the normally restricted endothelial expression pattern associated with BBB regional specialization. Prior work from our laboratory provided the key observations motivating this project. RNA sequencing of brain endothelial cells from EAE models of multiple sclerosis revealed upregulation

of Stra6, and a similar increase was observed following LPS administration in a model of sepsis. These findings suggest that neuroinflammation consistently drives Stra6 upregulation at the BBB. Endothelial-specific Stra6 knockout mice additionally displayed reduced EAE severity, indicating that this upregulation is not merely a passive inflammatory response but may actively contribute to neuroinflammatory pathology. The mechanism by which inflammation drives Stra6 upregulation, however, remains unknown. Given that pro-inflammatory cytokines are central mediators of both EAE and LPS-induced inflammation, we hypothesized that cytokine signaling may represent an upstream regulatory mechanism controlling Stra6 expression.

This study therefore investigates how IL-1 β , TNF- α , and IFN- γ , individually and in combination, influence Stra6 expression across distinct brain regions in a mouse model of acute cytokine-induced neuroinflammation. The aim was to determine whether inflammatory signaling intersects with endothelial retinoid transport mechanisms at the BBB, and whether cytokine exposure disrupts the normal regional patterning of Stra6 expression.

Blood–Brain Barrier Structure and Function

The blood-brain barrier (BBB) is a specialized vascular interface that separates circulating blood from the brain and plays a central role in maintaining a stable environment for neural function. Unlike blood vessels in most peripheral tissues, the BBB is highly restrictive, allowing only selected molecules to pass while excluding potentially harmful substances. This selective permeability is essential for protecting the brain from systemic fluctuations and ensuring proper neuronal activity.

At the structural level, the BBB is formed by endothelial cells lining cerebral capillaries. On the luminal side, a dense glycocalyx composed of glycoproteins and glycosaminoglycans forms

the primary interface with circulating blood, regulating molecular and cellular access to the endothelial surface. Beneath this interface, endothelial cells are connected by junctional complexes that restrict paracellular movement. Tight junctions, composed of transmembrane proteins such as claudin-5, occludin, and junctional adhesion molecules, are anchored to intracellular scaffold proteins including zonula occludens-1 (ZO-1) and zonula occludens-2 (ZO-2), forming a continuous seal that limits passive diffusion. These complexes are not static; they can be reorganized in response to physiological or pathological signals, reflecting the barrier's capacity to adapt dynamically to changing conditions [1, 2, 10].

Beyond restricting passive diffusion, the BBB regulates molecular exchange through specialized transport systems. Essential nutrients including glucose and amino acids cross the endothelium via carrier-mediated mechanisms such as glucose transporter 1 (GLUT1), while efflux transporters actively remove xenobiotics and metabolic waste. Together, these mechanisms ensure that the brain receives necessary substrates while limiting exposure to harmful compounds.

The BBB functions not in isolation but as part of the neurovascular unit, which includes pericytes, astrocytes, neurons, and extracellular matrix components. Pericytes contribute to vascular stability and help regulate endothelial permeability, while astrocytic endfeet surround the vasculature and provide structural and signaling support. The basement membrane adds further structural integrity and contributes to the regulation of cellular and molecular exchange. These coordinated interactions maintain the tightly controlled microenvironment required for normal brain function [2].

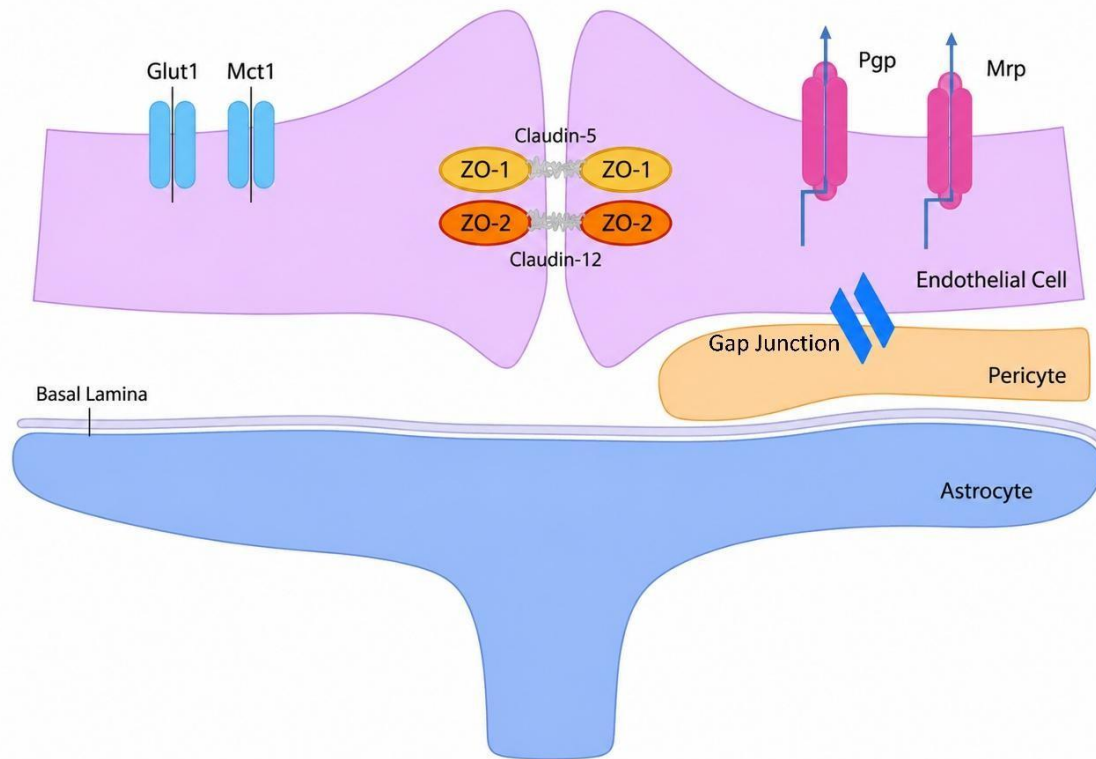


Figure 2: Schematic of the blood–brain barrier (BBB) illustrating key structural components and transport systems of the neurovascular unit.

Effects of Pro-inflammatory Cytokines on BBB Function

Under inflammatory conditions, the integrity of the BBB can be significantly altered by pro-inflammatory cytokines such as IL-1 β , TNF- α , and IFN- γ . These cytokines act directly on brain endothelial cells to disrupt tightly regulated barrier properties, and can also signal indirectly to pericytes and astrocytes within the neurovascular unit, which in turn influence endothelial behavior and barrier integrity.

A primary consequence of cytokine exposure is disruption of the inter-endothelial junctions that regulate paracellular transport. Tight junction proteins including claudin-5, occludin, and ZO-1, together with adherens junction components, are essential for maintaining barrier integrity. Pro-

inflammatory cytokines alter both the expression and spatial organization of these junctional proteins, leading to increased permeability and loss of barrier tightness. Cytokine-induced changes in cytoskeletal organization further weaken endothelial cohesion and contribute to increased paracellular flux [11].

In parallel with these structural changes, cytokines promote an inflammatory endothelial phenotype characterized by upregulation of adhesion molecules such as ICAM-1 and VCAM-1 [12], enhancing the ability of circulating immune cells to adhere to and migrate across the endothelium. IL-1 β , in particular, has been shown to reduce barrier tightness while increasing adhesion molecule expression, facilitating leukocyte transmigration across the endothelial layer [13].

Cytokine exposure also induces oxidative stress within endothelial cells. Activation of NADPH oxidase and related pathways generates reactive oxygen species (ROS), which further destabilize junctional proteins and amplify permeability [11, 14]. These combined structural and molecular changes underscore the multifactorial nature of BBB disruption under inflammatory conditions, collectively driving endothelial cells toward a more permeable and immunologically active state [4].

Intracellular Signaling Pathways in Endothelial Activation

The effects of pro-inflammatory cytokines on endothelial cells are mediated through intracellular signaling pathways that translate extracellular inflammatory cues into coordinated changes in gene expression. Among these, nuclear factor kappa B (NF- κ B) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways play central roles in regulating endothelial activation at the BBB.

NF- κ B signaling is a key pathway activated in response to cytokines such as IL-1 β and TNF- α [13, 14]. Upon stimulation, NF- κ B translocates to the nucleus and drives expression of pro-inflammatory genes, including adhesion molecules such as ICAM-1 and VCAM-1, as well as mediators that contribute to junctional disruption and increased permeability. Through these transcriptional changes, NF- κ B promotes a shift in endothelial cells toward a pro-inflammatory and barrier-disruptive state.

The JAK/STAT pathway, by contrast, is prominently activated by IFN- γ [15]. Activation results in phosphorylation and nuclear translocation of STAT proteins, which regulate genes associated with immune responses, antigen presentation, and cellular activation. These changes reinforce the inflammatory endothelial phenotype and enhance interactions with circulating immune cells.

Beyond direct transcriptional regulation, cytokine-induced signaling also stimulates chemokine production that coordinates immune cell recruitment to the BBB. These chemokines engage G protein-coupled receptor (GPCR) signaling in circulating immune cells, leading to integrin activation and strengthened endothelial adhesion, supporting a coordinated multi-step process of immune cell transmigration across the barrier.

Although NF- κ B and JAK/STAT pathways can each be activated by multiple cytokines, their downstream effects are not identical. IL-1 β and TNF- α are more commonly associated with structural changes including junctional protein remodeling and cytoskeletal reorganization, whereas IFN- γ primarily drives transcriptional programs related to immune activation. These differences highlight that individual cytokines can produce distinct functional outcomes at the BBB, and that the net effect of inflammatory signaling depends on the specific cytokine milieu present.

Importantly, not all endothelial proteins respond uniformly to these inflammatory pathways. While adhesion molecules such as ICAM-1 and VCAM-1 are direct transcriptional targets of NF- κ B and JAK/STAT signaling, other endothelial proteins governed by metabolic or ligand-dependent regulatory programs may not respond to inflammatory cues in the same way. This raises the possibility that certain transport systems at the BBB, particularly those involved in nutrient delivery, operate independently of canonical inflammatory endothelial activation.

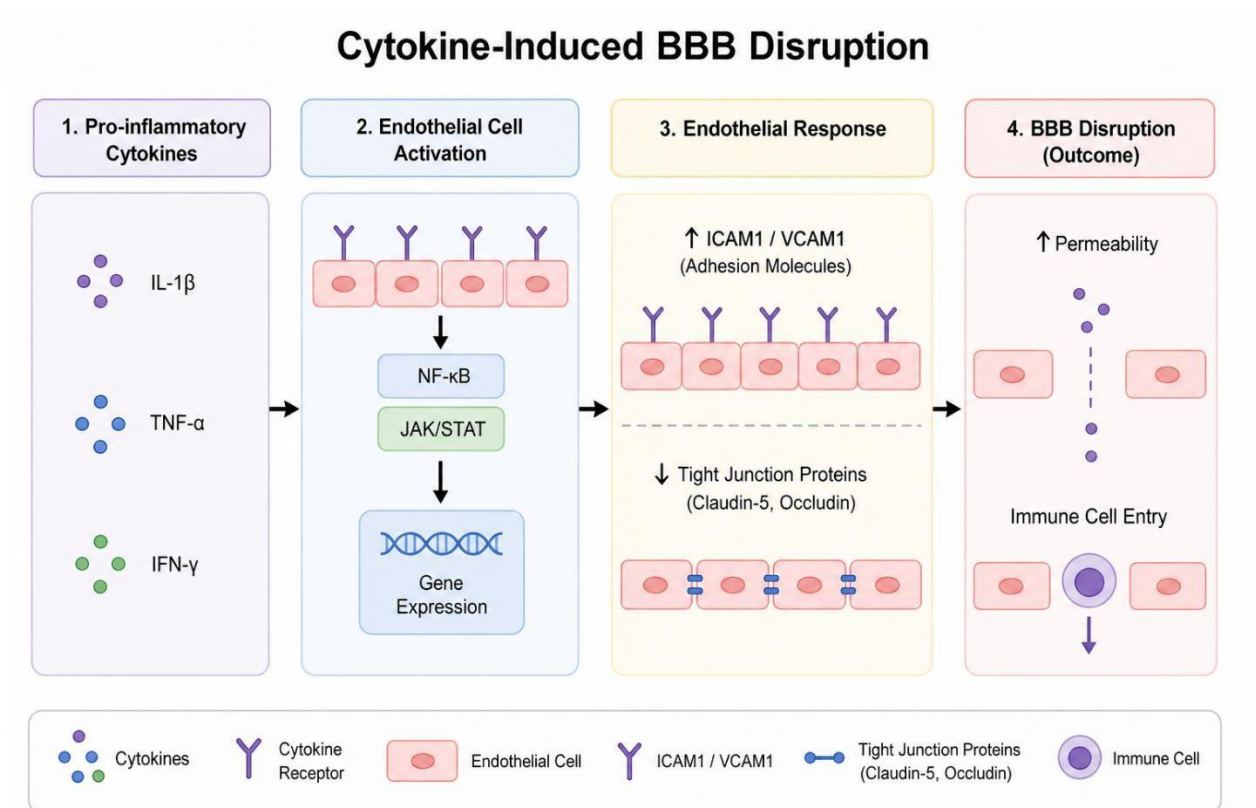


Figure 3: Overview of cytokine-induced blood–brain barrier (BBB) disruption. Created in BioRender by Doe, J. (2025)

Stra6 Retinoid Transport at the BBB and Regional Heterogeneity of the Blood–Brain Barrier

Stra6 (stimulated by retinoic acid 6) is a multispinning transmembrane receptor that mediates cellular uptake of retinol through its interaction with retinol-binding protein (RBP).

Vitamin A and its derivatives are essential for cellular differentiation, immune regulation, and tissue homeostasis, making controlled retinol transport critical for normal physiological function. At the BBB, Stra6 is expressed in endothelial cells where it facilitates the transfer of circulating retinol into the central nervous system.

Beyond its transport function, Stra6 can act as a signaling receptor. Binding of retinol-loaded RBP activates intracellular signaling pathways and influences downstream gene expression, linking vitamin A uptake to broader cellular responses [5-8].

Recent transcriptomic studies have established that the BBB is not a uniform structure but exhibits substantial regional heterogeneity [9]. While many barrier-defining genes are expressed throughout the cerebrovasculature, subsets of endothelial genes show region-specific expression. Stra6 is a prominent example, with expression highly enriched in the nucleus accumbens shell and ventral cochlear nucleus and comparatively low in the cortex and hippocampus [9].

This region-specific pattern suggests that Stra6-mediated retinoid transport is tuned to local physiological requirements. Whether this spatial pattern is preserved during inflammation remains unknown. Because pro-inflammatory cytokines broadly alter endothelial gene expression and BBB function, they could plausibly influence not only overall Stra6 levels but also its regional distribution, a possibility that has not previously been examined.

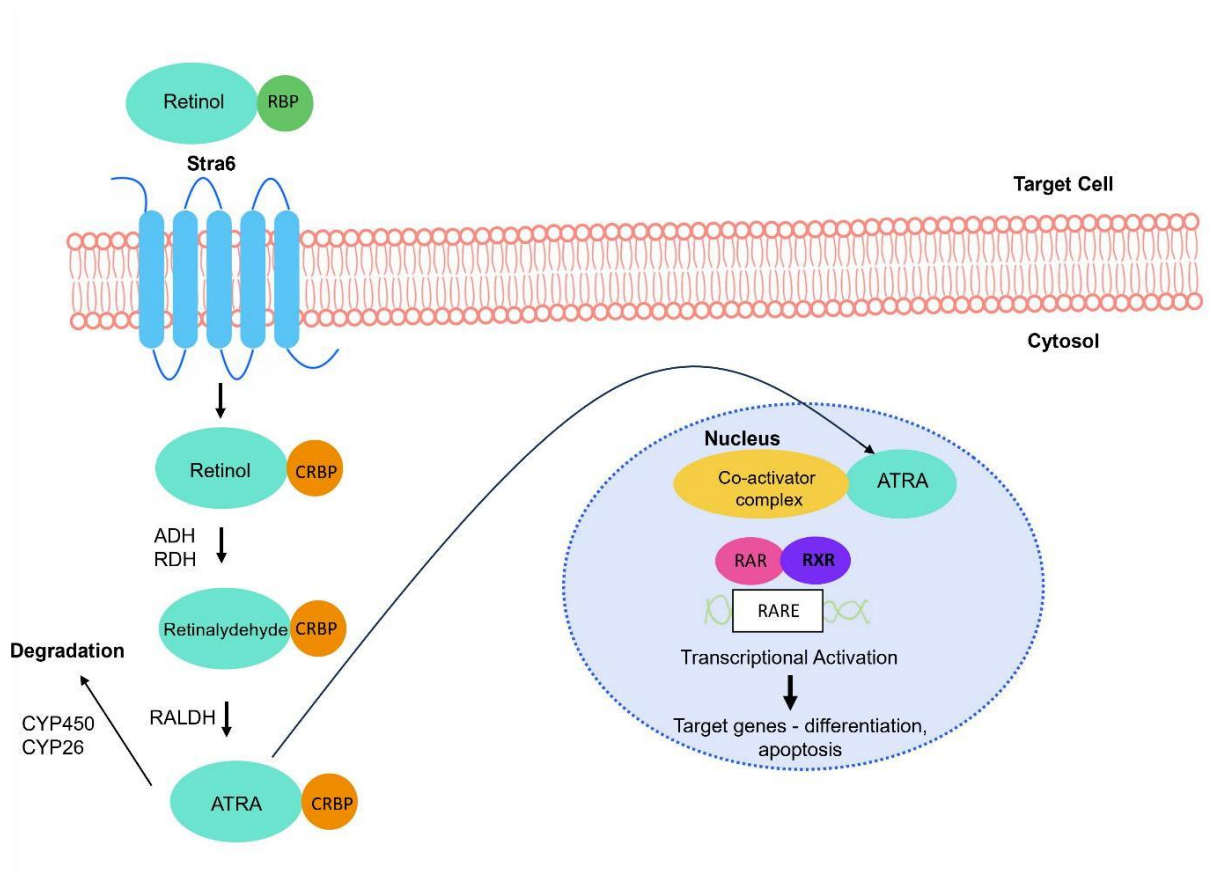


Figure 4: Schematic of Stra6-mediated retinol transport and retinoic acid signaling at the BBB. Adapted from Dhokia, V. et al., 2021

Stra6 in Neuroinflammatory Disease Models

Experimental Autoimmune Encephalomyelitis (EAE)

Experimental Autoimmune Encephalomyelitis (EAE) is the most widely used animal model for studying multiple sclerosis (MS) and the mechanisms underlying neuroinflammation. EAE is induced by immunization with myelin-derived antigens, which activates autoreactive immune cells that subsequently infiltrate the central nervous system. This triggers a cascade of pathological events including BBB disruption, pro-inflammatory cytokine production, immune cell infiltration, demyelination, and neurological deficits that closely resemble key features of MS.

Because EAE reproduces many of the inflammatory and vascular changes associated with neuroinflammatory disease, it provides a useful platform for investigating endothelial responses, BBB dysfunction, and molecular pathways that may contribute to disease progression [16].

Previous work from the Daneman laboratory demonstrated that Stra6 expression is increased at the BBB during EAE progression. Endothelial-specific deletion of Stra6 additionally reduced disease severity, suggesting that Stra6 is not merely a marker of inflammation but may actively contribute to disease-associated processes at the BBB. These findings established Stra6 as a potentially important mediator of neuroinflammatory responses and motivated further investigation into the mechanisms regulating its expression.

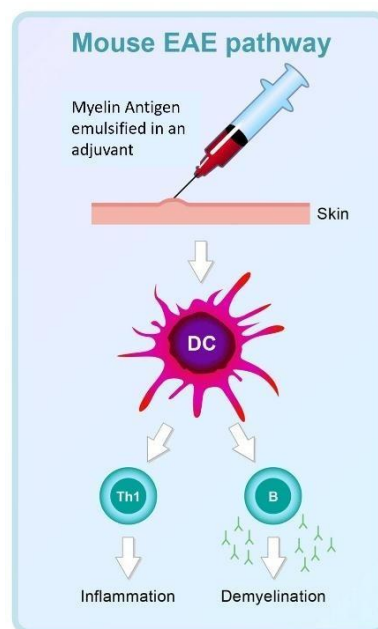


Figure 5: Overview of Experimental Autoimmune Encephalomyelitis (EAE) immunization in mice. Reproduced from 't Hart et al., 2019, licensed under CC BY 4.0.

Lipopolysaccharide (LPS) Model of Neuroinflammation

To determine whether Stra6 regulation was specific to autoimmune neuroinflammation or reflected a broader inflammatory response, Stra6 expression was also examined in a

lipopolysaccharide (LPS) model. Lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria, activates innate immune signaling through Toll-like receptor 4 (TLR4). Systemic LPS administration induces rapid pro-inflammatory cytokine production, endothelial activation, and BBB dysfunction, making it a widely used model of acute systemic inflammation and neuroinflammation [17].

Similar to observations in EAE, Stra6 expression increased following LPS administration. The fact that Stra6 is upregulated in both models suggests that its regulation may represent a common response to inflammatory conditions rather than a phenomenon restricted to any single disease context.

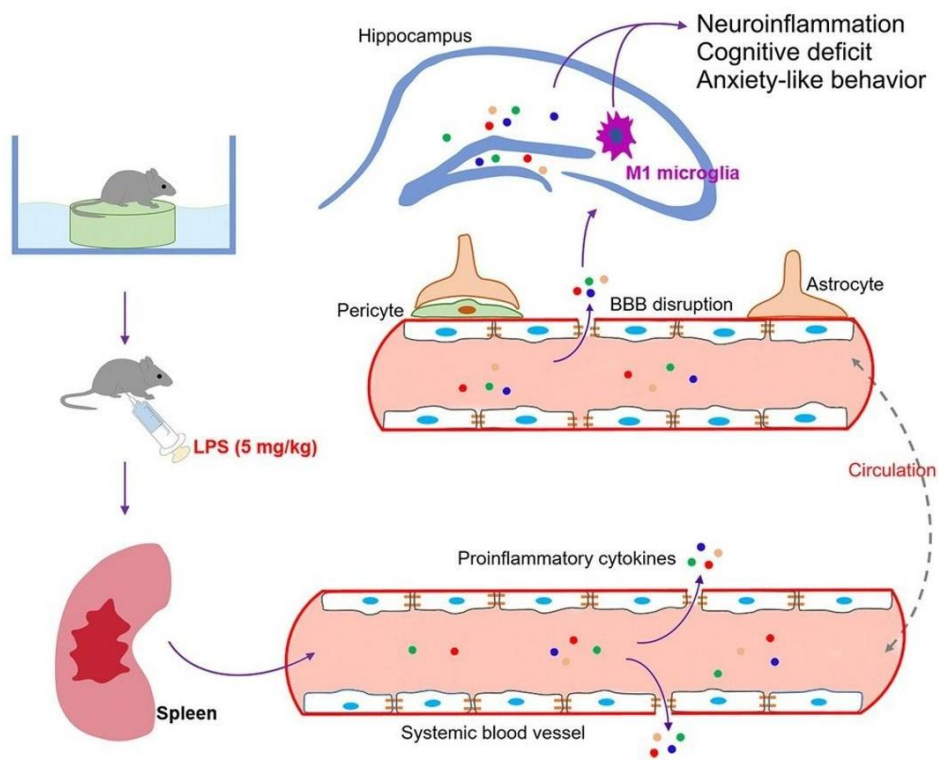


Figure 6: Overview of LPS-induced neuroinflammation. Adapted from Xu et al., 2020, licensed under CC BY 3.0.

Research Gap and Study Objective

Previous work from the Daneman laboratory demonstrated that Stra6 is upregulated at the BBB in both EAE and LPS models of neuroinflammation, and that endothelial-specific deletion of Stra6 reduces EAE severity. Together, these findings suggest that Stra6 is functionally involved in neuroinflammatory processes and may contribute to disease-associated changes at the BBB.

Both EAE and LPS are characterized by elevated levels of pro-inflammatory cytokines and endothelial activation. Because cytokines broadly alter endothelial gene expression and BBB function, they represent a plausible upstream mechanism responsible for the observed Stra6 increase. Whether cytokine signaling directly regulates Stra6 expression, however, has not been experimentally tested. Addressing this question is important for understanding whether Stra6 upregulation during neuroinflammation is a direct consequence of cytokine signaling or instead requires additional disease-associated mechanisms.

The aim of this study was therefore to determine whether acute exposure to IL-1 β , TNF- α , and IFN- γ , individually and in combination, is sufficient to alter Stra6 expression at the BBB. Given the previously reported regional heterogeneity of Stra6 expression [9], the nucleus accumbens shell, hippocampus, and cortex were examined to assess whether cytokine exposure influences Stra6 across regions with distinct baseline expression levels.

Materials and Methods

Animal Model

Eight- to twelve-week-old male and female C57BL/6J wild-type (+/+) mice (Jackson Laboratory) were housed at the University of California, San Diego under standard conditions on a 12-hour light/dark cycle with ad libitum access to food and water. All experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at UCSD.

Cytokine Treatment

Mice received interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), or a combination of all three cytokines (cocktail). Phosphate-buffered saline (PBS) served as the vehicle control.

Cytokine solutions were prepared by diluting stock solutions in PBS to a total injection volume of 100 μ L per mouse. IL-1 β was administered at 0.5 μ g per mouse (20 μ g/kg), TNF- α at 0.5 μ g per mouse, and IFN- γ at 0.5 μ g per mouse, consistent with published low-end effective doses [18, 19, 20]. Individual cytokine treatments consisted of 5 μ L IL-1 β stock, 2.5 μ L TNF- α stock, or 1 μ L IFN- γ stock diluted in vehicle to a final volume of 100 μ L. The combination treatment contained 5 μ L IL-1 β , 2.5 μ L TNF- α , and 1 μ L IFN- γ combined with vehicle to reach the same final volume. Following injection, mice were maintained for 18 hours to allow endothelial responses to develop prior to perfusion and tissue collection.

Tissue Collection and Perfusion

Mice were anesthetized via intraperitoneal (i.p.) injection of ketamine (12 mg/kg) and xylazine (3.6 mg/kg) in PBS. Once fully anesthetized, mice were transcardially perfused with dPBS (Gibco) for 4 minutes to clear vascular blood, followed by 20 mL of 4% paraformaldehyde (PFA) in 1× PBS for fixation. Brains were extracted and immersed in 30% sucrose solution at 4°C overnight for cryoprotection.

Tissue Embedding

Following fixation, brains were prepared for cryo-embedding. To ensure consistent anatomical orientation and capture of all regions of interest, brains were trimmed using a brain matrix with coronal cuts made to retain tissue spanning from anterior regions through the hippocampus. Trimmed brains were embedded in optimal cutting temperature (OCT) compound (Thermo Fisher Scientific), oriented for coronal sectioning, rapidly frozen on dry ice, and stored at −80°C until sectioning.

Cryosectioning

Frozen brain samples were equilibrated to cryostat conditions and sectioned at −21 to −22°C. Coronal sections were collected at a thickness of 7 µm. Sections corresponding to the nucleus accumbens shell, cortex, and hippocampus were mounted onto glass slides and stored at 4°C until staining.

Immunofluorescence Staining

Sections were brought to room temperature and washed in PBS. Blocking was performed with 10% normal goat serum and 2.5% Triton X-100 in PBS for 1 hour at room temperature. Sections were then incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: rabbit anti-Stra6 (Proteintech, cat# 22001-1-AP) at approximately 14.3 µg/mL, cross-absorbed against mouse knockout brain tissue; rat anti-CD31 (BD Biosciences, cat# 553370) at 1 µg/mL (1:500); and Armenian hamster anti-ICAM-1 (Invitrogen, cat# MA5-405) at 20 µg/mL (1:50).

After washing (3×10 minutes in PBS), sections were incubated with fluorescent secondary antibodies for 1.5 hours at room temperature: goat anti-Armenian hamster AF488 (ICAM-1), goat anti-rabbit AF568 (Stra6), and goat anti-rat AF647 (CD31), each diluted 1:1000 in antibody buffer (1:10 blocking buffer in PBS). Sections were washed again (3×5 minutes) and mounted with DAPI-Fluoromount-G.

Imaging

Images were acquired using an epifluorescence microscope (Axio Imager D2; Carl Zeiss) equipped with a digital camera (AxioCam HRm; Carl Zeiss). Imaging was performed across multiple fluorescence channels, with acquisition parameters held consistent across all samples. Images were collected using AxioVision software.

Image Analysis and Quantification

Quantification of Stra6 and ICAM1 expression was performed in ImageJ. Blood vessels were identified using the CD31 channel, and individual vessels were manually traced using the

freehand line tool. Regions of interest (ROIs) corresponding to CD31-positive vessels were recorded using the ROI Manager and applied consistently to all subsequent analyses.

For Stra6 quantification, CD31-defined ROIs were overlaid onto the corresponding Stra6 channel to assess vessel-associated signal. Vessels with detectable Stra6 staining were classified as Stra6-positive and further categorized as high or low Stra6-expressing based on relative signal intensity, with the low-expression reference established from the mean intensity of the dimmest Stra6-positive vessels within each image.

For ICAM1 quantification, the same ROIs were overlaid onto the ICAM1 channel, and vascular segments with detectable ICAM1 staining were classified as ICAM1-positive. For each image, total CD31-positive vascular length, Stra6-positive vascular length, high Stra6-expressing vascular length, and ICAM1-positive vascular length were recorded. Stra6 and ICAM1 expression were each reported as the percentage of positive vascular length relative to total CD31-positive vascular length.

Threshold criteria for vessel classification were defined prior to analysis and applied consistently across all samples. All classification and quantification were performed by a single observer blinded to treatment conditions.

Data Visualization

Quantified data were analyzed and visualized using GraphPad Prism and Python. Results are presented as mean $\pm$ standard deviation with individual data points overlaid. Comparisons across cytokine treatments and brain regions were made using consistent color coding.

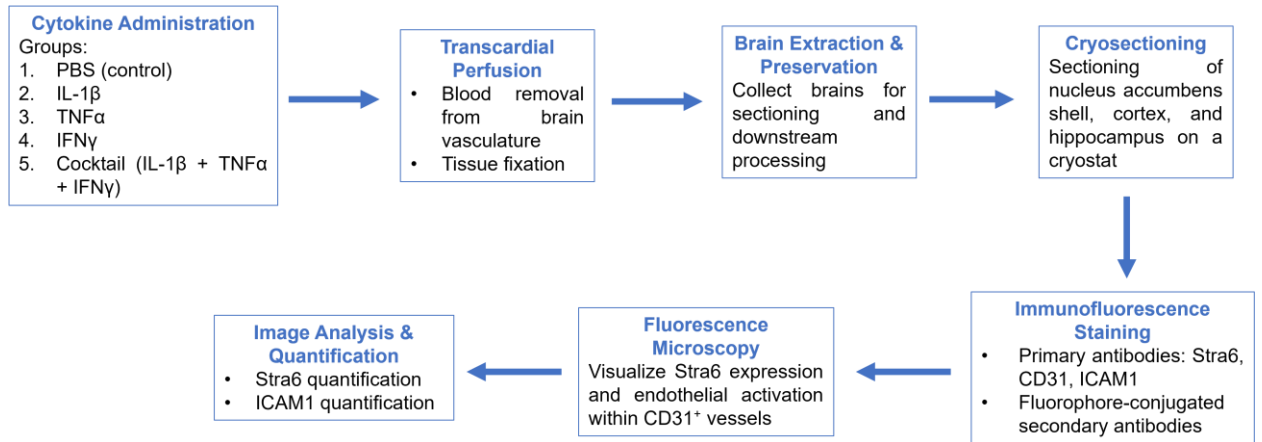


Figure 7: Overview of the experimental workflow used to assess Stra6 expression at the BBB.

Results

Visualization and classification of Stra6 expression in endothelial vessels

Brain sections were stained for CD31 to identify endothelial vessels and for Stra6 to evaluate vascular Stra6 expression. Representative immunofluorescence images across treatment conditions demonstrated Stra6 signal together with CD31 and ICAM1 staining (Figure 8). To quantify endothelial Stra6 expression, CD31-positive vessels were manually traced and overlaid onto the corresponding Stra6 channel. Vessels containing detectable Stra6 signal were classified as Stra6-positive and subsequently categorized as either high or low Stra6-expressing based on staining intensity.

Stra6 signal was localized along CD31-positive endothelial structures and closely followed vascular morphology. Within individual brain regions, Stra6 expression varied between vessels, supporting the use of high and low expression categories for subsequent analyses. Qualitatively, Stra6 staining appeared strongest in the nucleus accumbens shell, while the hippocampus and cortex exhibited lower overall signal intensity.

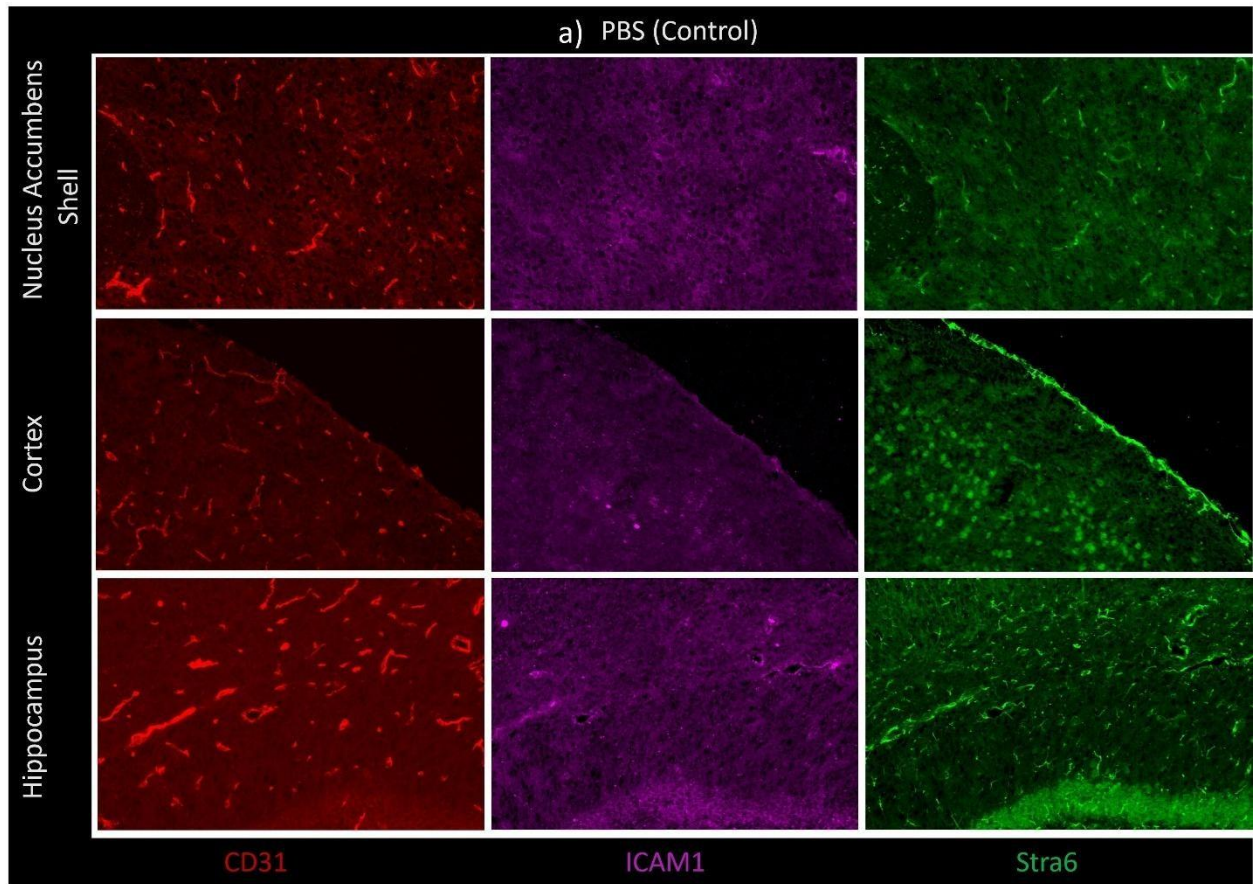


Figure 8: Representative immunofluorescence images of CD31, ICAM1, and Stra6 in brain sections treated with (a) PBS, (b) IL-1 β , (c) TNF- α , (d) IFN- γ , and (e) cytokine cocktail across the nucleus accumbens shell, hippocampus, and cortex.

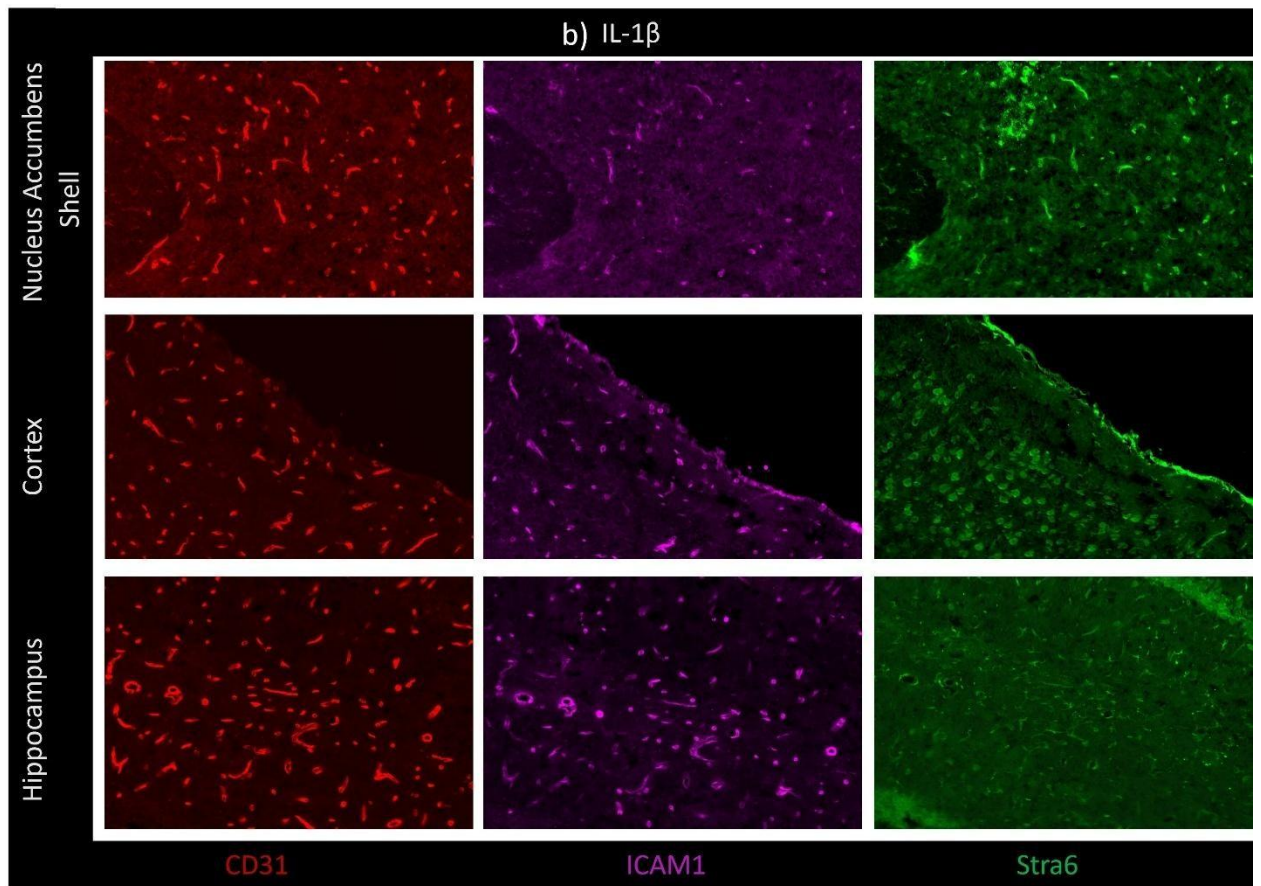


Figure 8 (continued)

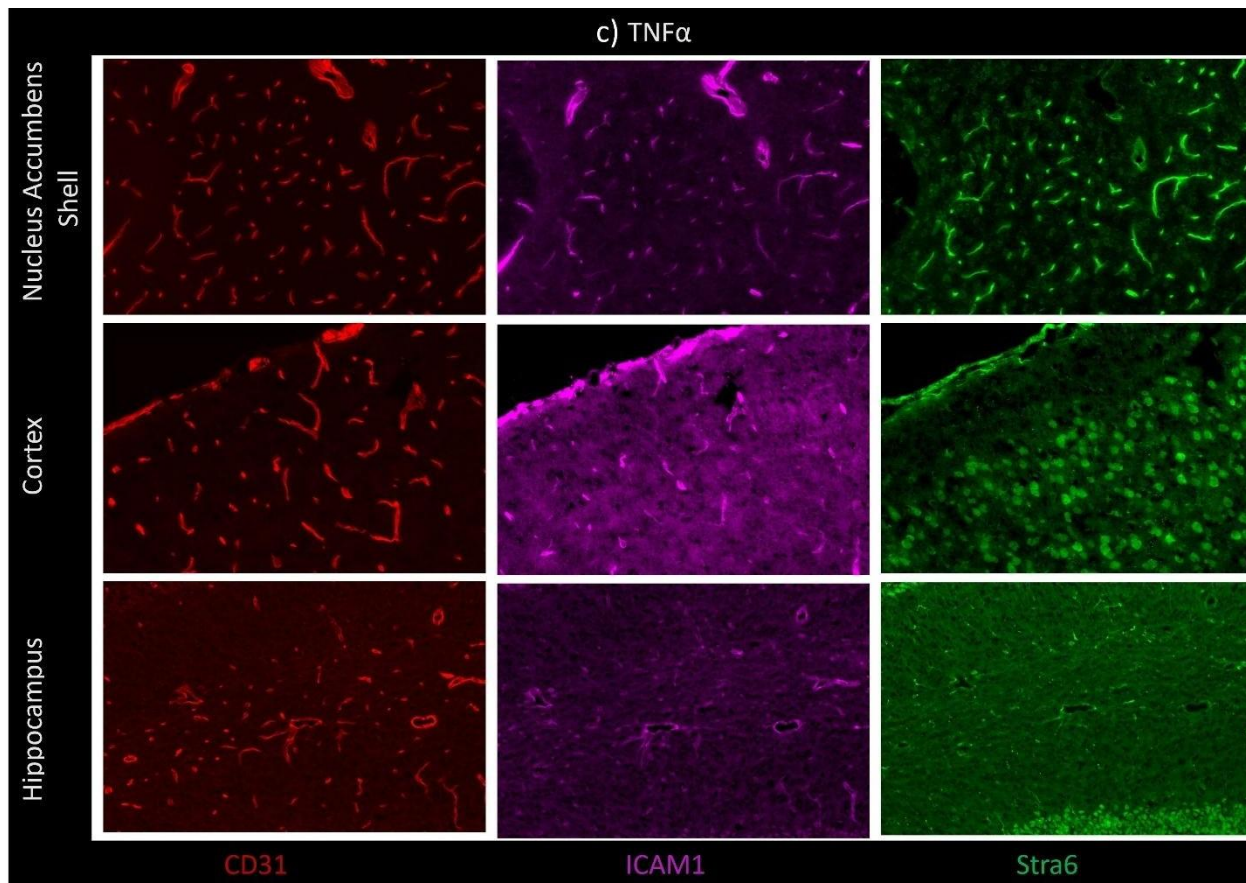


Figure 8 (continued)

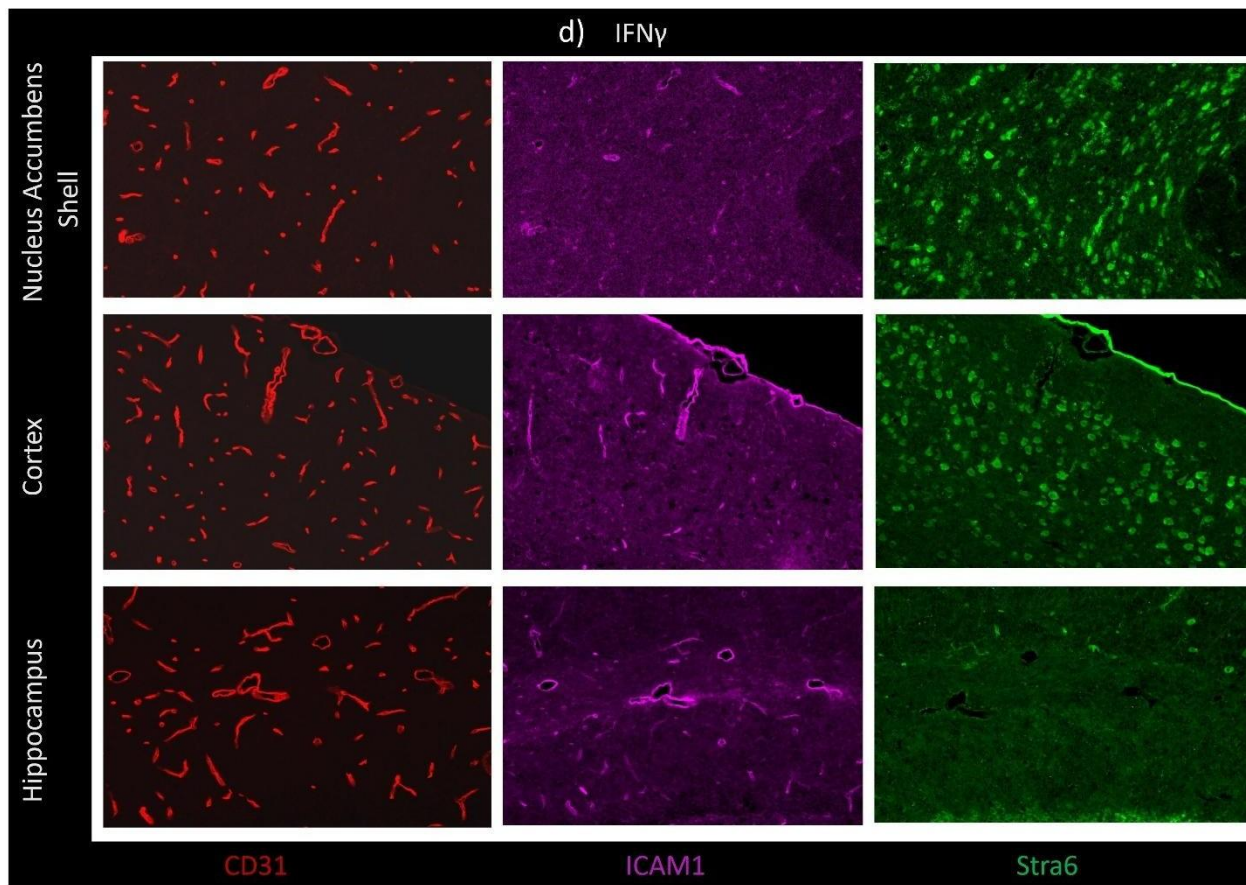


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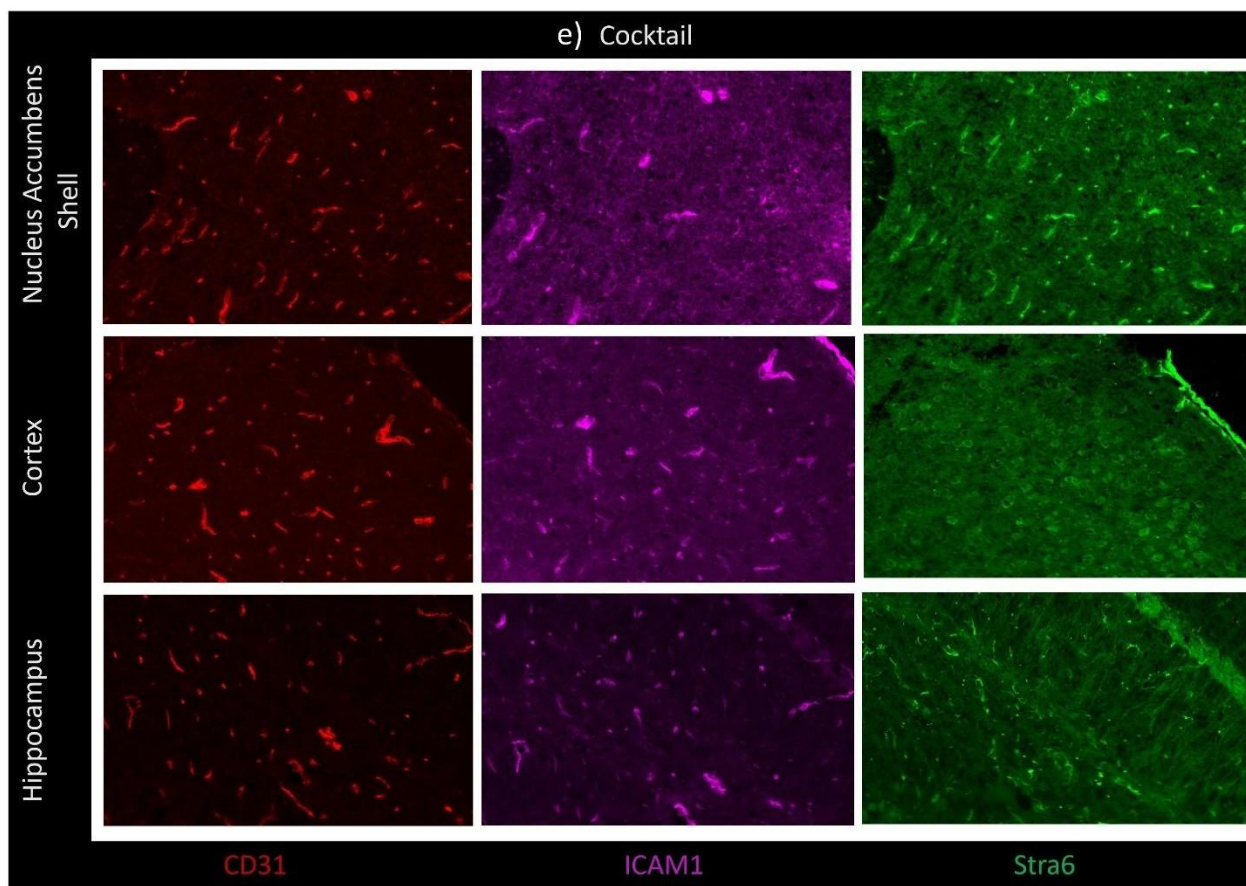


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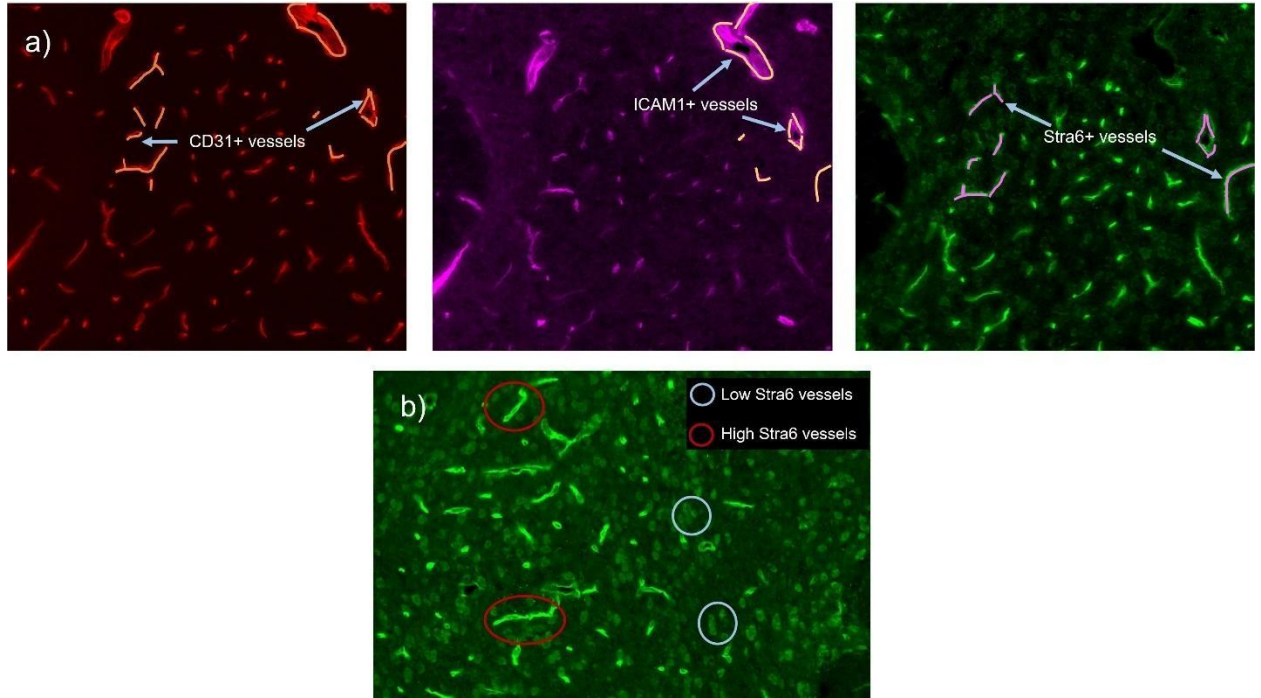


Figure 9: Quantification workflow. (a) CD31-positive vessels were traced and used as ROIs to quantify vessel-associated Stra6 and ICAM1 expression. (b) Stra6-positive vessels were categorized as High (red) or Low (blue) based on relative staining intensity.

Cytokine treatment induces endothelial activation

To verify that cytokine treatment successfully activated inflammatory signaling within brain endothelial cells, ICAM1-positive vascular length was quantified and normalized to total CD31-positive vascular length. ICAM1 is a well-established marker of endothelial activation and is rapidly induced by pro-inflammatory cytokines.

Representative immunofluorescence images revealed increased ICAM1 staining in cytokine-treated tissues compared with PBS controls (Figure 8). Quantitative analysis confirmed this observation, with all cytokine-treated conditions exhibiting greater ICAM1-positive vascular coverage than PBS controls across the nucleus accumbens shell, hippocampus, and cortex (Figure 10). Elevated ICAM1 expression was observed following treatment with $\text{TNF-}\alpha$, $\text{IL-1}\beta$, $\text{IFN-}\gamma$,

and the cytokine cocktail, although the magnitude of induction varied between individual animals and treatment groups. The cytokine cocktail generally produced the highest levels of ICAM1 expression across the examined regions.

These findings confirm that the cytokine treatment paradigm successfully engaged endothelial inflammatory signaling, and that subsequent analyses of Stra6 expression were therefore conducted under conditions of verified endothelial activation.

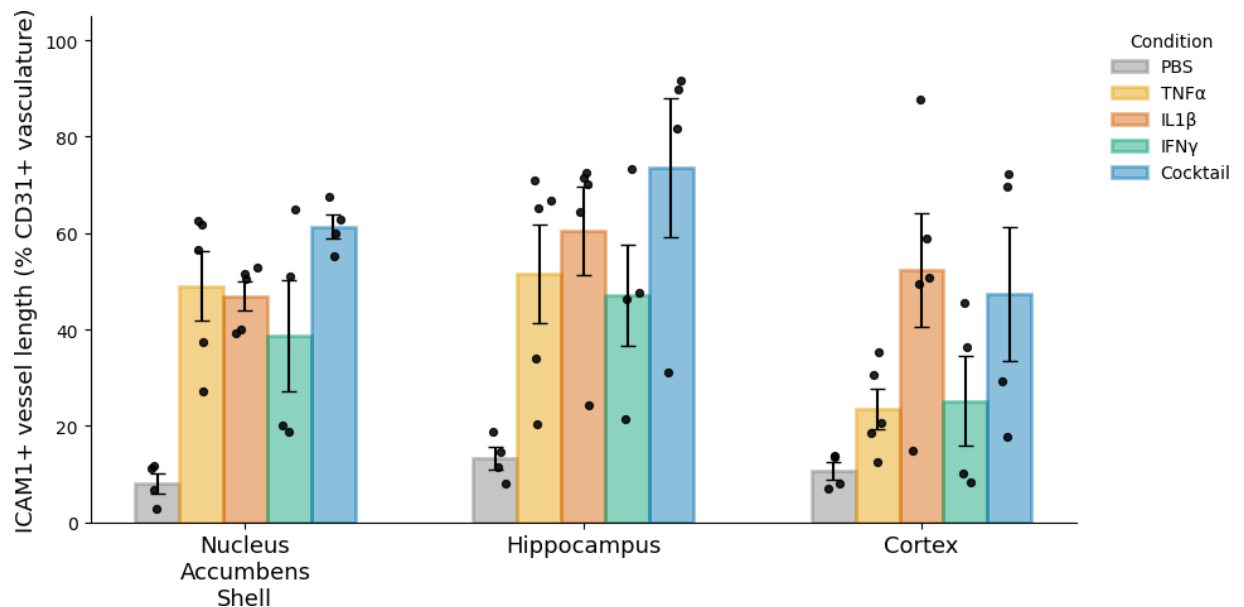


Figure 10: Quantification of ICAM1-positive vascular length as a percentage of CD31-positive length across cytokine conditions and brain regions. Bars indicate mean $\pm$ SD.

Acute cytokine exposure does not alter Stra6 expression

The percentage of Stra6-positive vascular length was quantified across PBS, TNF- α , IL-1 β , IFN- γ , and cytokine cocktail treatment conditions. Despite robust endothelial activation confirmed by increased ICAM1 expression, Stra6 expression remained broadly consistent across all treatment groups.

No treatment-dependent increase in Stra6-positive vascular length was observed following exposure to any individual cytokine or the cytokine cocktail. When Stra6-positive vessels were further separated into high and low expression categories, the proportion of high Stra6-expressing vessels similarly showed no reproducible association with cytokine treatment. Variability was present between biological replicates, but did not follow a treatment-specific pattern.

These findings indicate that acute cytokine exposure alone is insufficient to induce Stra6 expression at the blood-brain barrier. Despite confirmed endothelial activation, cytokine treatment failed to reproduce the Stra6 upregulation previously observed in EAE and LPS models, suggesting that additional inflammatory, metabolic, or disease-associated signals are required to drive Stra6 expression during neuroinflammation.

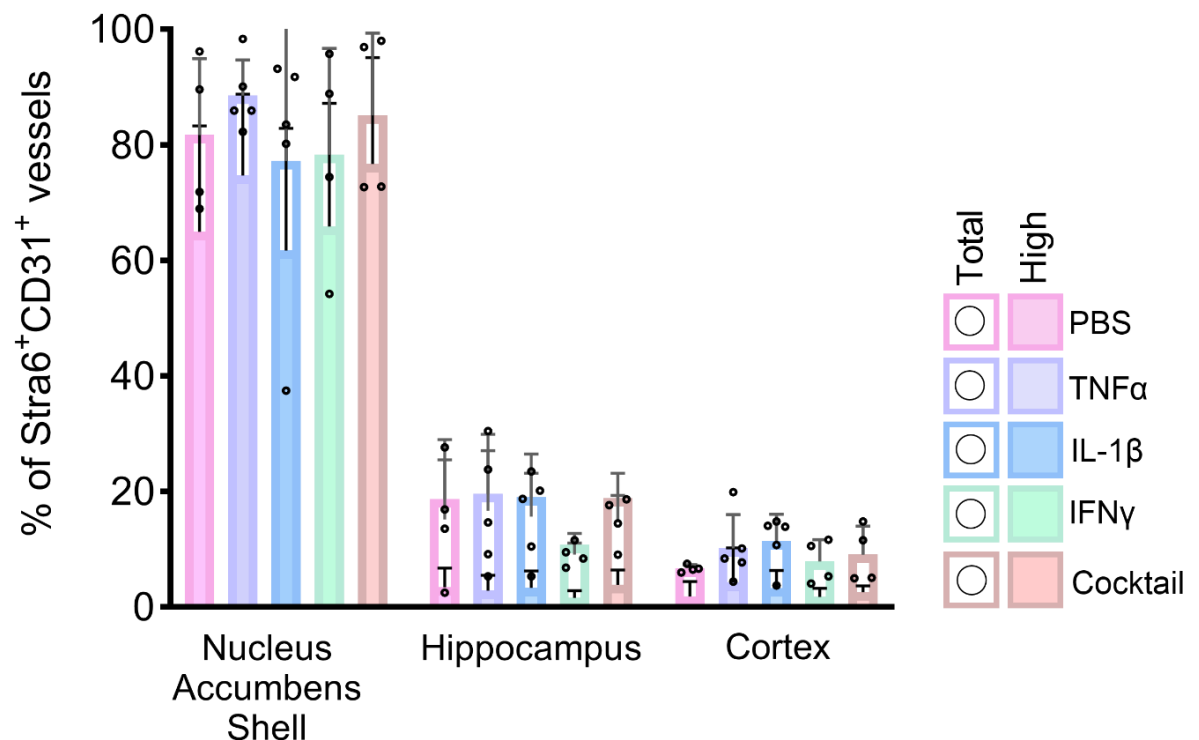


Figure 11: Quantification of Stra6-positive and high Stra6-expressing vascular length as a percentage of CD31-positive length across cytokine conditions and brain regions. Bars indicate mean $\pm$ SD.

Discussion

The present study investigated whether pro-inflammatory cytokines regulate Stra6 expression at the blood-brain barrier. Cytokine treatment successfully activated endothelial inflammatory signaling, as demonstrated by ICAM1 upregulation, yet no consistent changes in Stra6 expression were observed across any individual cytokine or cocktail condition. Given that cytokines are well-established drivers of endothelial activation and BBB dysfunction [4], the confirmed increase in ICAM1 indicates that the experimental paradigm effectively engaged inflammatory signaling. The absence of a corresponding Stra6 response therefore suggests that acute cytokine exposure alone is insufficient to induce its expression at the BBB.

These findings must be interpreted alongside previous work from the Daneman laboratory showing that Stra6 is upregulated at the BBB in both EAE and LPS models of neuroinflammation, and that endothelial-specific Stra6 deletion reduces EAE disease severity. Those observations place Stra6 not merely as a passive marker of inflammation, but as a potential functional contributor to disease-associated neurovascular changes. The present study was designed to test whether pro-inflammatory cytokines represent the upstream mechanism driving this upregulation. Despite robust endothelial activation, however, acute cytokine exposure failed to reproduce the Stra6 induction seen in either disease model.

One straightforward explanation is that Stra6 induction requires a more sustained inflammatory environment than the one modeled here. EAE and LPS-induced neuroinflammation develop over several days and involve prolonged, evolving exposure to inflammatory mediators, whereas this study captured only an acute cytokine response. Stra6 regulation may therefore depend on repeated or cumulative inflammatory signaling rather than a single exposure, and the temporal dynamics governing its expression may be more complex than initially anticipated.

Importantly, the absence of a Stra6 response under acute conditions does not rule out a role for cytokine signaling; it simply suggests that the threshold for induction has not been met within this experimental window.

An equally plausible explanation is that Stra6 upregulation depends on disease-associated signals that systemic cytokine administration cannot replicate. LPS engages Toll-like receptor 4 (TLR4) directly on endothelial cells, potentially activating transcriptional programs distinct from those triggered by downstream cytokines alone. EAE presents an even more complex inflammatory milieu, with immune cell infiltration, microglial activation, astrocyte reactivity, demyelination, and progressive BBB disruption all unfolding in concert. Any of these processes, or their interaction, could contribute to Stra6 regulation in ways that a cytokine-only paradigm cannot capture.

Stra6 protein abundance does not necessarily reflect the functional state of retinoid transport at the BBB. Retinol uptake is governed by a coordinated network of circulating retinol-binding protein (RBP4), transthyretin, intracellular binding proteins, and downstream metabolic enzymes [5, 6], and Stra6 itself can function as a signaling receptor that couples RBP4 binding to intracellular JAK2/STAT5 activation [7, 8]. Changes in ligand availability, intracellular retinoid handling, or downstream signaling could therefore alter retinoid biology at the BBB independently of any detectable shift in Stra6 protein levels. The present findings do not exclude this possibility, and functional transport assays will be needed to address it directly.

A particularly notable finding was the preservation of regional Stra6 expression patterns across all treatment conditions. Consistent with prior observations from the Daneman laboratory [9], the nucleus accumbens shell exhibited the highest proportion of Stra6-positive vessels, the cortex the lowest, and the hippocampus intermediate levels, and these regional differences

persisted despite robust cytokine-induced endothelial activation. This suggests that the mechanisms governing regional Stra6 patterning are relatively resistant to acute inflammatory perturbation. Rather than overriding the endothelial regional identity established under homeostatic conditions, acute cytokine exposure appears to act upon a vascular landscape in which Stra6 expression is already strongly and stably patterned.

Taken together, these findings indicate that acute cytokine signaling alone cannot account for the Stra6 upregulation observed in EAE and LPS models. Inflammatory signaling clearly activates brain endothelial cells, as ICAM1 induction confirms, but driving a change in Stra6 expression likely requires prolonged inflammation, additional disease-associated cellular signals, or more complex neurovascular interactions than were present in this model. These results refine our understanding of Stra6 regulation during neuroinflammation and argue against a simple, cytokine-driven mechanism as the sole upstream regulator of its expression at the BBB.

Limitations

The most consequential limitation of this study is the acute nature of the cytokine exposure paradigm. Tissues were collected following a single cytokine administration, capturing only an early inflammatory response, whereas EAE and LPS models involve sustained inflammation unfolding over several days. Stra6 may simply require more prolonged or repeated inflammatory stimulation before its expression changes detectably, and the absence of a response here should not be read as evidence that inflammatory signaling is irrelevant to Stra6 regulation.

A related limitation is that systemic cytokine administration via retro-orbital injection does not replicate the full complexity of the neuroinflammatory environment. In EAE, brain endothelial cells are simultaneously exposed to signals from activated microglia, reactive astrocytes, infiltrating immune cells, and damaged neural tissue, a cellular crosstalk that cytokine injection

alone cannot reproduce. The mechanisms responsible for disease-associated Stra6 upregulation may therefore depend on components of the inflammatory milieu that were entirely absent from the current model.

Third, this study assessed Stra6 at the protein level without directly measuring retinoid transport or downstream retinoid signaling activity. Because retinol transport across the BBB is regulated by multiple interacting proteins and signaling cascades, stable Stra6 expression does not necessarily indicate unchanged retinoid transport function [12, 13]. Whether inflammatory conditions alter BBB retinoid biology through mechanisms independent of Stra6 protein abundance remains an open question that will require functional assays to resolve.

Finally, the study was constrained by sample size and methodological considerations. With four biological replicates per condition, the dataset was not powered for formal statistical analysis. Immunofluorescence-based classification of Stra6-expressing vessels also carries some degree of observer-dependent variability, despite the use of predefined thresholds and blinded analysis. These approaches were applied consistently across all conditions, but larger cohorts and more quantitative imaging methods would substantially strengthen the conclusions.

Future Directions

The most immediate priority for future work is to determine whether Stra6 responds to prolonged or repeated inflammatory stimulation. The current study used a single cytokine administration with an 18-hour collection window; future experiments should explore repeated dosing and extended timepoints that better approximate the sustained inflammatory environment of EAE and LPS models. If Stra6 induction is temporally gated, a chronic paradigm may be sufficient to reveal it.

Beyond timing, it will be important to identify which disease-associated signals, beyond circulating cytokines, contribute to Stra6 upregulation during neuroinflammation. EAE and LPS models engage many processes simultaneously, including immune cell infiltration, microglial activation, and progressive BBB dysfunction. Dissecting these individual components in isolation or in combination could clarify whether Stra6 regulation is driven by endothelial-intrinsic cytokine responses, signals from neighboring cell types, or a convergence of multiple inflammatory inputs.

Future studies should also directly assess retinoid transport and downstream signaling under inflammatory conditions, independent of Stra6 protein abundance. Since Stra6 functions within a broader retinoid transport and signaling network, measuring retinol flux or the expression of retinoid-responsive target genes would reveal whether neuroinflammation alters BBB retinoid biology through mechanisms not captured by protein-level staining alone.

Finally, expanding biological replicates and integrating more quantitative approaches, such as single-molecule imaging, flow cytometry of isolated endothelial cells, or bulk RNA sequencing, would improve sensitivity and reduce observer-dependent variability. This would be particularly valuable in the cortex and hippocampus, where baseline Stra6 expression is low and subtle treatment effects are most difficult to detect reliably.

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