HUMAN BRAIN PERICYTE-LIKE CELLS EXHIBIT G PROTEIN COUPLED RECEPTOR-MEDIATED TAB1-P38α INFLAMMATORY SIGNALING

by

MORGAN M. ROOS

(Under the Direction of Neil Grimsey)

ABSTRACT

Brain pericyte cells are essential to vascular development, blood-brain barrier (BBB) stability, cerebral blood flow, cytokine expression, and angiogenesis. During inflammation, pericytes secrete proinflammatory cytokines, migrate, and contribute to BBB breakdown and infiltration of immune cells into the brain. Recent work has focused on pericytes as key targets to treat neuroinflammation. P38 mitogen-activated protein kinase (MAPK) is a critical mediator of proinflammatory signaling. G protein coupled receptors (GPCRs) mediate atypical, direct binding of transforming growth factor-β activated kinase 1 binding protein 1 (TAB1) to p38 to induce p38 autophosphorylation in endothelial cells. Its role in pericytes has not been investigated. Using chemical and cell-penetrating peptide inhibitors, our studies reveal that GPCRs activate atypical p38 signaling in human brain pericyte-like cells. We also show that pericyte migration and morphological change are induced by α-thrombin, PGE2, and PDGF-BB. We predict that GPCR-mediated atypical TAB1-p38α inflammatory signaling in brain pericytes is a critical driver of neuroinflammation.

INDEX WORDS: Pericytes, TAB1-p38α, GPCRs, Atypical p38, Neuroinflammation, Signaling, p38 MAPK

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CHAPTER 1

INTRODUCTION

Neuroinflammation

Neuroinflammation is a defense mechanism that is intended to clear up infection or injury in a beneficial manner so that disease progression or injury is halted and the normal function and structure of the brain is restored [1-3]. While acute inflammatory responses are a protective mechanism, a prolonged, or chronic, inflammatory response leads to cytotoxic effects causing breakdown of normal brain function and severe symptoms of neurodegeneration [4, 5].

The neurovascular unit is made up of neurons, glial cells (microglia, oligodendrocytes, and astrocytes), and vascular cells (mural cells and endothelial cells) [6]. This vast network of cells works together to regulate blood flow, maintain blood-brain barrier (BBB) integrity, and overall is essential for proper central nervous system (CNS) function [6]. Many neuroinflammatory disorders are characterized by a damaged blood-brain barrier, the primary defense mechanism of the brain, making it permeable to any circulating substances, which results in toxic neural effects and perpetuation of the inflammatory state [7, 8]. The blood-brain barrier is made up of endothelial cells, pericytes, astrocytes, and the basement membrane (comprised of extracellular matrix proteins, such as laminins, collagen, and fibronectin), all of which play a critical role in barrier integrity and physiological functioning through their interactions and various signaling mechanisms [5, 9].

Neuroinflammation is a hallmark characteristic of a wide range of CNS disorders, including neurodegenerative diseases such as Alzheimer's and Parkinson's, traumatic brain injury (TBI), stroke, and infection where immune cell infiltration, increased cytokine release, BBB breakdown, and cell migration are a driving force in disease progression [10-13]. Specifically, a proinflammatory environment stimulates an increased production of chemokines, cytokines, and reactive oxygen species, including thrombin, interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), a plethora of interleukins, and many, many more [14-18]. The release of these factors in conjunction with the damaged and now permeable blood-brain barrier results in immune cell recruitment and subsequent cell migration, increased release of inflammatory factors, edema, tissue damage, and cell death if severe enough [5, 11, 19]. The amount of damage that is sustained depends on a number of factors, including the duration, intensity, and characteristics of the inflammatory response [20, 21]. Although the many components of the neurovascular unit serve an important role, pericytes in particular have been shown to be a central driver of vascular development, maintenance, and inflammatory signaling [22, 23].

Pericyte Cells and Their Role in Neuroinflammation

Pericytes are a type of mural cell (which also includes vascular smooth muscle cells) that are responsible for maintaining the blood-brain barrier, clearing toxic substances from the brain, and regulating vascular development, cerebral blood flow, cytokine expression, and angiogenesis [22-28]. Besides the brain, pericytes are found in many organs including the eyes, heart, lungs, kidneys, and liver. However, the ratio of

pericytes to endothelial cells is highest in the brain and retinas [29, 30]. Due to the integral role that pericytes play in brain functioning and dysregulation, brain pericytes have been the most extensively studied, and will be the focus of our discussion unless otherwise stated [22, 24]. As displayed in Figure 1, brain pericytes are embedded in the basement membrane and wrap around endothelial cells to form capillary vessels where the two cell types interact via peg-and-socket contact and gap junctions, which allow for signaling crosstalk [31]. Pericytes are also in close contact and interact with astrocyte end feet, which wrap around the pericytes and endothelial cells and all together, in addition to the basement membrane, form capillary blood vessels [24]. Widespread pericyte loss or migration results in a substantial progressive breakdown of the BBB and has been shown to be a key factor in disorders such as Alzheimer's disease, ischemic stroke, and traumatic brain injury [11, 25, 32-41]. Some of the key characteristics of neuroinflammation, including loss of endothelial cell tight junctions, disruption of the basement membrane, pericyte loss or migration, cytokine release, and infiltration of immune cells into the brain are displayed in Figure 1.

Figure 1.

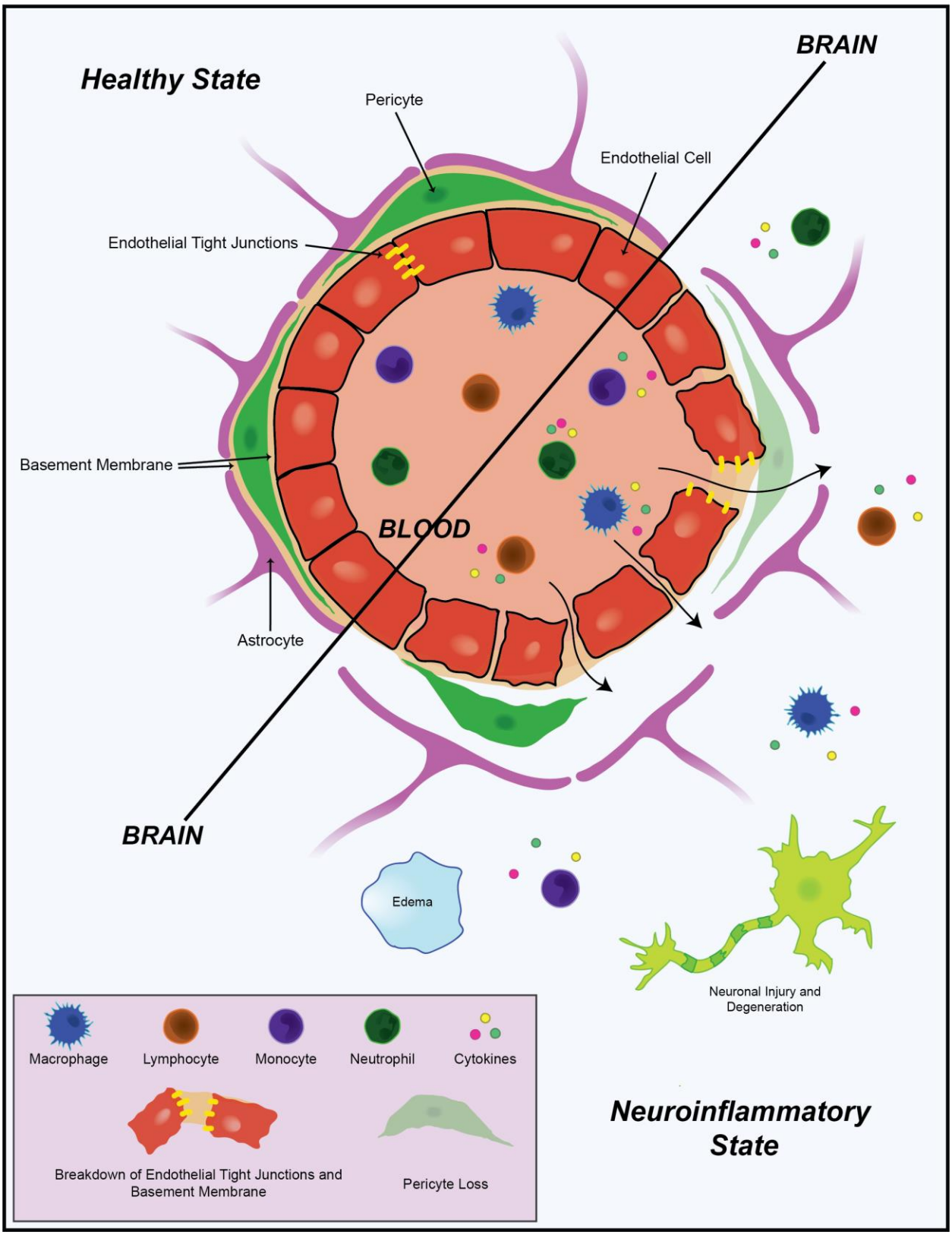
Healthy State
BRAIN
Pericyte
Endothelial Cell
Endothelial Tight Junctions
Basement Membrane
BLOOD
Astrocyte
BRAIN
Edema
Neuronal Injury and Degeneration
Macrophage
Lymphocyte
Monocyte
Neutrophil
Cytokines
Breakdown of Endothelial Tight Junctions and Basement Membrane
Pericyte Loss
Neuroinflammatory State

Figure 1: Blood Brain Barrier in Healthy Versus Neuroinflammatory State

Healthy State. A healthy brain involves the interaction of endothelial cells, pericytes, astrocytes, and the basement membrane, all of which make up the blood-brain barrier and serve to regulate what has the ability to enter the brain. Pericytes in particular play an important role in maintaining the stability of the barrier and homeostatic blood flow through their close interaction with both endothelial cells and astrocytes.

Neuroinflammatory State. In response to and in conjunction with a proinflammatory environment, widespread pericyte loss (through migration, differentiation, or death), disruption of the basement membrane, and loss of endothelial cell tight junctions have all been shown to be key factors in breakdown of the blood-brain barrier. In response to damage, immune cells are recruited, a cytokine storm is released, and increased proinflammatory signaling by pericytes in particular induces edema, neuronal injury and degeneration, and widespread tissue damage. Prolonged stimulation and damage establishes a chronic neuroinflammatory environment, which is characteristic of conditions such as stroke, traumatic brain injury, and Alzheimer's, deteriorating health and physiological functioning in a difficult to target manner.

Since their first identification and preliminary characterization in the late 1870s by Eberth and then Rouget, research of pericyte cells has been perpetually hampered due to the lack of specific markers available to enable pericyte identification from other similar cell types (such as smooth muscle cells) [42]. Pericytes were first named in 1923 by Zimmerman and even today there is still debate regarding the properties of pericyte cells, including characterization of their expression profile (both physiological and inflammatory), where they are located, and by what name they are referred to, particularly in cases where the pericytes migrate away from their position on the blood vessel [43, 44]. Some studies use *mural cell* to refer to both smooth muscle cells and pericytes, while others provide a distinct differentiation between each group and even subtypes of pericytes [41, 45-48]. Pericyte cells themselves have been shown to differ in contractility and expression profile based on their location and environmental stimuli, although there is a large gap in the literature in studying the subtypes of pericyte morphology and how they change in the face of proinflammatory regulation and signaling [41, 48-52]. Furthermore, given the role of pericytes as stem cells and their importance in repair, there are different ideas as to whether mesenchymal stem cells (MSCs) are derived from pericytes, pericytes are derives from MSCs, or if they are both separate populations with similar restorative properties [53-56]. Overtime, as our knowledge on pericyte markers has grown, so has the awareness of just how important pericytes are and the expansive number of roles they perform in vascular development, regulation of the blood-brain barrier, and the inflammatory response [26, 27].

In response to infection or damage, proinflammatory factors such as lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNF-α), and interferon gamma

(IFN-γ) are released [7, 8, 31, 34, 55, 57-59]. Studies have shown that these factors stimulate a further increase in the expression of proinflammatory cytokines, including α-thrombin, TNF-α, IFN-γ, matrix metalloproteinases (MMPs), monocyte chemoattractant protein-1 (MCP-1), nitric oxide, CXCL family chemokines, and various interleukins by brain pericytes [7, 8, 17, 31, 34, 55, 57-60]. Furthermore, *in vivo* studies using BBB models have shown that pericyte loss and subsequent barrier breakdown enables the infiltration and accumulation of toxic molecules, such as immune cells, amyloid beta (Aβ), thrombin, plasmin, hemosiderin, and fibrin, in the brain, which is characteristic of the prolonged inflammatory state observed in progressive neural disorders [7, 23, 36, 61, 62]. In addition to allowing infiltration into the brain, pericyte loss also results in a sustained decrease of capillary blood flow, perpetuating the damage seen in conditions such as cerebral ischemia [33, 63].

In addition to the confusion surrounding pericytes and MSCs, pericyte cells also express some of the same markers as smooth muscle cells, macrophages, and fibroblasts. Furthermore, pericytes have been shown to be able to differentiate into a number of different cells, including microglia, angioblasts, fibroblasts, macrophages, and neural progenitor cells [64-70]. Although this provides further evidence for the potential neurorestorative properties of pericyte cells, it also causes a challenge in proper identification and characterization of pericytes from other similar cell types, especially since there is not one unique pericyte marker. Currently, brain pericytes are classified using markers such as platelet derived growth factor receptor beta (PDGFRβ), neuro-glial 2 (NG2), alpha-smooth muscle actin (α-SMA), neuroepithelial stem cell protein (Nestin),

and cluster of differentiation 146 (CD146) (also known as melanoma cell adhesion molecule (MCAM)) [29].

One of the most widely studied molecular markers for pericytes is PDGFRβ. Multiple researchers over the years have shown through mouse deficiency and knockout experiments that PDGFRβ/PDGF-B signaling between endothelial cells and pericyte cells is crucial to pericyte recruitment and sustained investment onto vasculature [26, 71-73]. During development and in neurodamaging conditions such as spinal cord injury and ischemic stroke, which elicit angiogenesis, PDGFRβ can also be expressed by cells such as perivascular fibroblasts, astrocytes, endothelial cells, and smooth muscle cells in addition to pericytes [72, 74-78]. There is debate, however, regarding expression of PDGFRβ in the healthy adult brain as some studies have found PRGFRβ to be expressed exclusively by pericytes while others have shown PDGFRβ expression by other cell types, such as vascular smooth muscle cells and cultured neurons, as well [23, 27, 72, 75, 79, 80].

Other commonly used identification markers for pericytes include NG2 and CD146. NG2 is a proteoglycan cell surface marker that regulates pericyte proliferation, migration, and interaction with endothelial cells through junction proteins and cellular signaling mechanisms [81]. Although studies have shown the expression of NG2 in cell development and tumor vasculature, more recent research has proven consistent expression of NG2 by neural pericytes, neuroglia, and vascular smooth muscle cells [75, 82, 83]. Similar to NG2, CD146 is a cell adhesion molecule that was originally identified in tumor and endothelial cells responsible for promoting angiogenesis and tumor growth [84, 85]. Since that time, CD146 has also been detected in a number of other cells

including pericytes, mesenchymal stem cells, skeletal fibroblasts, umbilical vein perivascular cells, lymphocytes, smooth muscle cells, and, in addition to tumor metastasis, is primarily a marker of cell-cell cohesion [54, 85-92]. CD146 upregulation is not only associated with development and tumor growth, but also in cellular proliferation and migration [93, 94]. Importantly, this cell adhesion molecule has been shown to control pericyte recruitment and adhesion to endothelial cells in the brain where a knockdown of CD146 in either cell line facilitates breakdown of the blood-brain barrier [91].

Another vastly researched molecular marker that is used for pericyte identification is Nestin. Nestin is a cytoskeletal intermediate filament specific to the CNS that was first shown to be expressed by neural stem cells [95, 96]. Over the years, it has also been found to be expressed by various other stem or progenitor cells during development and tissue repair, including mesenchymal stem cells, neural pericytes, neural stem cell precursors, a subset of pancreatic islet cells, and skeletal muscle satellite stem cells, all located in a variety of tissues, such as the brain, hair follicles, testis, and bone marrow [45, 68, 95, 97-100]. With the growing research surrounding the stem cell-like properties of pericyte cells and their potential role in regeneration and repair, Nestin, in conjunction with other molecular markers, is a more recent tool that is used in many studies to identify brain pericytes [59, 68, 101, 102].

An additional marker, α-SMA, is a specific actin that is responsible for aiding in the formation of microfilaments and is most commonly expressed by mural cells and fibroblasts, although inconsistencies have been observed [103-105]. This marker is particularly interesting as less than 10% of neural pericytes actually express α-SMA *in vivo*, yet many studies have used α-SMA as a pertinent marker specifically for ‘pericyte’

cells [83, 104, 106, 107]. It has been shown that expression of α-SMA, in some instances, is increased in cultured pericytes, possibly due to the use of serum in media, separating *in vitro* pericytes from 'true' *in vivo* pericytes [106, 108].

Since there is not one unique marker for pericytes, negative expression of factors distinct to similar cell types is often assessed. As there is not one clearly defined factor expressed only by mesenchymal stem cells, smooth muscle cells, or fibroblasts to differentiate pericytes from these three populations, detection of a lack of endothelial cell markers is used to confirm identity. Platelet endothelial cell adhesion molecule- 1 (PECAM-1 or EndoCAM), also known as cluster of differentiation 31 (CD31), and hematopoietic progenitor cell antigen, or cluster of differentiation 34 (CD34), are commonly used for this purpose as they are robust indicators of endothelial cells throughout the body in the brain, lungs, heart, liver, and other tissues and have been proven through their use in a plethora of studies to distinguish pericytes from endothelial cells [38, 81, 92, 109-113]. While not expressed in pericytes, CD31 and CD34, in addition to being robust indicators of endothelial cells, have also been shown to display weak expression in fibroblasts and platelets in some proinflammatory and tumor environments [92, 114].

The vast amount of research centered around pericytes has shown the important role that they play in cytokine expression and stimulation of a proinflammatory environment, migration, and blood-brain barrier disruption in response to damage [11, 23, 34, 58]. Although the expression of many different markers makes these cells difficult to characterize, it also provides evidence for the expansive regulation of the neurovascular unit and brain function by pericytes through their stem-cell like, phagocytic, and

neurorestorative abilities [64, 67, 68]. Despite the extensive research surrounding neuroinflammation, pericytes are a complicated cell type that remain underexplored and there are many gaps in our understanding of how they contribute to a proinflammatory environment. One such gap is inflammatory G protein coupled receptor (GPCR) pathways as these play an important role in signal transduction of stimuli and cellular response [115].

G Protein Coupled Receptors and a Family of Ligands

As the most extensive and versatile family of membrane proteins, G protein coupled receptors (GPCRs) are ubiquitously expressed and are host to a vast range of responsibilities in the control of vascular function and inflammation [116-118]. GPCRs mediate many cellular pathways through a seven-transmembrane structure where binding of a signaling molecule activates G protein subunits, which then results in generation of downstream messengers [119, 120]. In endothelial cells, GPCRs have been shown to regulate the gene expression of inflammatory proteins as well as blood vessel permeability [121, 122]. Factors such as thrombin, histamine, and prostaglandin E2 (PGE2) play a substantial role in inflammation, primarily through binding to their respective GPCRs as discussed further below.

During wound healing, thrombin is crucial for blood coagulation and clot formation and functions primarily through the activation of the G protein coupled receptor, protease-activated receptor 1 (PAR1) [123, 124]. In addition, PAR1 activation by thrombin in endothelial cells has been shown to increase the production of proinflammatory cytokines and disrupt tight junctions, in part through actin cytoskeleton rearrangement, which alters the morphology of endothelial cells, both of which serve to promote vascular permeability

in blood vessels [125, 126]. PAR1 is expressed in a broad range of cells, including platelets, endothelial cells, pericytes, fibroblasts, smooth muscle cells, neurons, and many other vascular cells [62, 127-130]. Elevated levels of both thrombin and PAR1 have been demonstrated to be associated with neuronal death and disease progression in CNS trauma, Alzheimer's, and Parkinson's where inhibition of thrombin decreases damage and promotes neuronal survival [12, 14, 57, 62, 131-133]. More specifically, in mouse models, thrombin activation of PAR1 has been shown to promote MMP-2 and MMP-9 expression by pericyte cells, leading to BBB permeability where pericytes have been suggested as the most thrombin-sensitive cell in the blood-brain barrier [8, 62, 133, 134].

The effects of histamine are mediated by four G protein coupled receptors, H1, H2, H3, and H4, located throughout the body on numerous cell populations including neurons, T-cells, endothelial cells, macrophages, mast cells, leukocytes, and smooth muscle cells [135-142]. Histamine plays an important role in cellular proliferation and differentiation, regeneration, and hematopoiesis, and is responsible for vasodilation of vessels and heightened vascular permeability [135, 143-146]. In addition, high levels of histamine are also characteristic of brain trauma, stroke, and Alzheimer's disease and have been shown to disrupt neurotransmission and increase blood-brain barrier permeability in an H1, H2, and H3 dependent manner [147-153]. Although research in this regard is very limited, histamine has been displayed to cause the separation of pericytes from endothelial cells and increase the phagocytic abilities of pericyte cells in a proinflammatory environment [154, 155].

PGE2 functions through the binding to four G protein coupled receptor subtypes, EP1, EP2, EP3, and EP4 [156]. EP receptors have been shown to be expressed on

pericytes, neurons, microglia, endothelial cells, immune cells, and many others where depending on which receptor is activated, PGE2 has the ability to function in a pro- or anti-inflammatory manner [18, 157-163]. PGE2 is a known regulator of vascular permeability and cellular migration, and promotes proinflammatory angiogenesis through upregulation of growth factors [164-167]. High levels of PGE2, which are characteristic of many proinflammatory conditions including ischemic stroke, Parkinson's, and Alzheimer's, have been observed, both *in vitro* and *in vivo*, to disrupt the BBB and attenuate the activation and release of inflammatory signaling factors [18, 160, 162, 168-171]. There is a lack of research surrounding PGE2 and pericyte cells, but a recent study has shown PGE2 mediated disruption of the pericyte-endothelial interaction through breakdown of cell adhesion molecules, leading to an increase in blood-brain barrier permeability [38].

GPCR regulation of BBB permeability and proinflammatory cytokine expression is well documented through thrombin, histamine, and PGE2 as key examples [115, 172]. Despite the extensive abilities of GPCRs and their inflammatory agonists, there is a lack of knowledge surrounding GPCR function specifically in pericyte cells. As both GPCR and pericyte function are so crucial to the progression and mediation of inflammation, inflammatory signaling pathways in pericytes that are activated through G protein coupled receptors provide a potential target to regulate and treat chronic neural conditions.

P38 Inflammatory Signaling Pathway

A key inflammatory target of GPCRs are mitogen-activated protein kinases (MAPKs) [173]. There are three MAPK subfamilies: extracellular signal-regulated kinase

(ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK [174-177]. The MAPK pathways modulate a number of downstream signaling cascades and can be activated by various growth factors, proinflammatory cytokines, oxidative stress, and other environmental stressors [178-182]. Specifically, p38 MAPK is an essential driver in tumorigenesis, inflammation, cell development, cell differentiation, and the overall cell cycle where it is responsible for regulating both homeostasis and acute/chronic inflammation [177, 183]. As p38 plays an essential role in many physiologically important processes, dysregulation of this signaling pathway has been observed in numerous neurological disorders, inflammatory conditions, and cancers [184-188].

P38 has four isoforms, α, β, γ, and δ, all of which are activated through the MAP2K, MKK3/6, in the typical three-tiered kinase cascade [175, 189, 190]. There is a vast amount of research regarding the MKK3/6 dependent p38 activation and this is widely considered to be the primary mechanism for p38 phosphorylation. However, in 2002, an atypical mechanism of action was described where p38α was shown to directly associate with transforming growth factor-beta (TGF-β)- activated kinase 1 (TAK1) binding protein 1 (TAB1), an adaptor protein critical for both TGF-β and TAK1 signaling [191]. While both the MKK3/6 dependent and atypical mechanisms facilitate phosphorylation of Thr180 and Tyr182 residues on p38α, atypical signaling is characterized specifically by autophosphorylation in a cis conformation [192]. In atypical p38 signaling, TAB1 binds directly and selectively to two discrete binding domains on p38α, Thr218 and Ile275 [193]. Both of these domains are essential for the distinctive TAB1-p38α interaction, where one is exclusive to this interface and the other is also expendable for the association of MKK3/6 and p38α [193-195].

Recently, it has been shown that direct binding of TAB1 to p38α and subsequent p38 autophosphorylation can be initiated via a family of proinflammatory GPCRs [122, 196, 197]. This interaction is specifically facilitated by a novel ubiquitin driven signaling cascade mediated by the E3 ligase, neural precursor cell expressed developmentally downregulated protein 4- 2 (NEDD4-2) and takes place on the endosome of cells [122, 197]. Figure 2 details the mechanism of typical MKK3/6 p38 signaling compared to three modes of TAB1 mediated atypical activation (through proinflammatory GPCRs, oxidative stress triggering TGF-β, or ischemia/hypoxia) [175, 190, 198-201].

Figure 2.

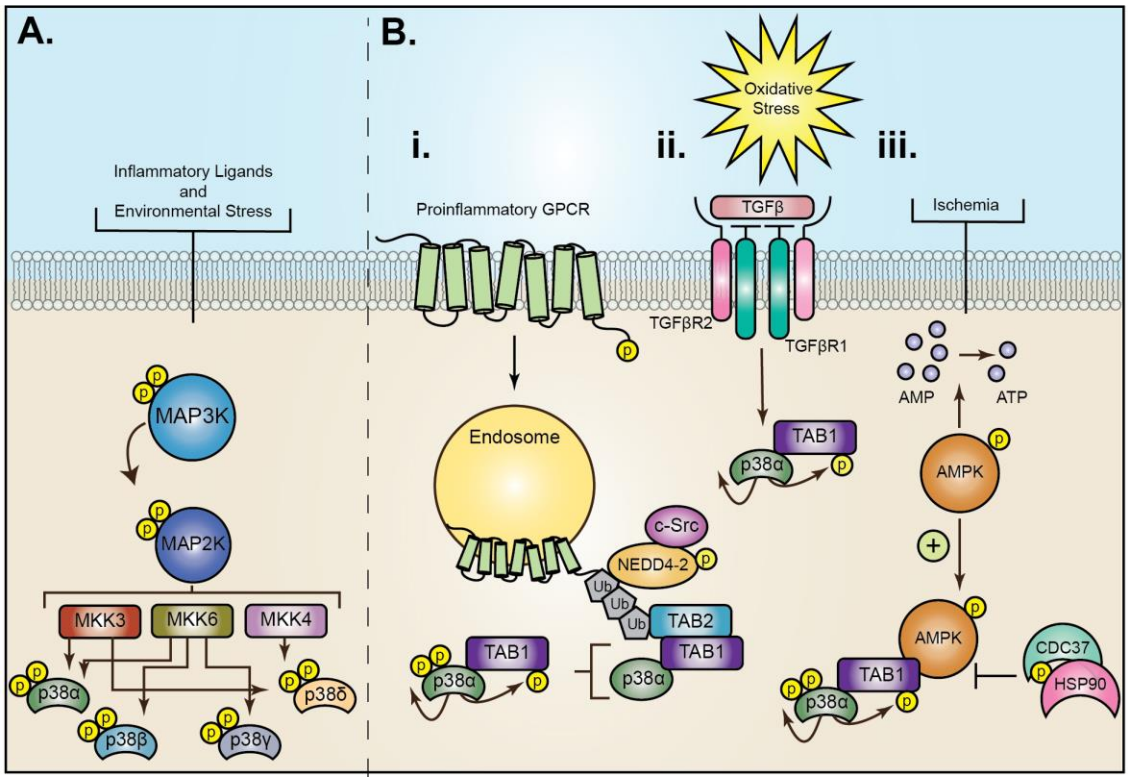

Figure from co-authored review paper: Burton, J.C.; Antoniades, W.; Okalova. J.; **Roos, M.M.**; Grimsey, N.J., *Atypical p38 Signaling, Activation, and Implications for Disease.* International Journal of Molecular Sciences, 2021. **22**(8): p. 4183.

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Figure 2: Mechanisms of p38 MAPK Activation [177]

A. Environmental stress and inflammatory ligands trigger the activation of a three-tiered kinase cascade through a complex array of mechanisms. Inflammatory ligands or environmental factors induce the activation of MAP3Ks which then activate the critical MAP2Ks, MKK3, MKK6, or (less commonly) MKK4. These MAP2Ks are then able to differentially activate the four isoforms of p38 (α, β, γ, δ). **B.** There are three known mechanisms for atypical TAB1-p38α signaling. **i.** GPCR stimulation triggers G protein dependent c-Src phosphoactivation of the E3 ubiquitin ligase, NEDD4-2. GPCRs recruit and are ubiquitinated by NEDD4-2. K63 ubiquitin chains recruit the ubiquitin binding adaptor protein TAB2. TAB2 then recruits TAB1, which binds to p38α and induces a conformational change and autophosphorylation. **ii.** Oxidative stress triggers TGF-β activation, which drives TAB1 and p38 activation, although the exact mechanism is unclear. **iii.** Hypoxia or ischemia events drive the formation of the TAB1-p38α complex and p38α autophosphorylation through activation of AMP-activated protein kinase (AMPK). The heat shock protein 90 (HSP90)-Cdc37 complex negatively regulates this process.

A plethora of inflammatory factors function both upstream and downstream of MAP kinase signaling [183]. Inflammatory ligands such as TNF-α, LPS, and IL-1β have been shown to function primarily through the typical p38 signaling pathway, while others, such as PGE2, α-thrombin, and histamine, are mediated through atypical p38 signal transduction, and specifically in a GPCR-dependent manner [122, 196, 197, 202-204]. For the remainder of the text, atypical p38 signaling will refer to p38 autophosphorylation via the direct binding of TAB1 to p38α and typical p38 signaling will refer to the MKK3/6-driven p38 pathway.

Relative to the extensive amount of research describing MKK3/6 dependent p38 signaling and disease progression, the impact of the TAB1-p38α interaction and p38 autophosphorylation remains largely understudied. That being said, there is a growing body of evidence to support TAB1 induced p38 activation where this atypical signaling pathway has been shown to play a key role in inflammatory conditions such as cardiovascular ischemia, various cancers, diabetes, and infections [122, 201, 205-209]. Table 1 highlights all of the published papers discussing atypical p38 activation, where only three [122, 196, 197] have studied GPCR mediation of this pathway.

***Table 1: TAB1-p38α Inflammatory Signaling in Pathological Conditions* [177]**

Condition	Animal Model	References
Cardiovascular Ischemia and Reperfusion	Murine in-vivo	[210] [201] [211] [212] [194]
	Murine in-vitro	[210] [211] [213] [192] [212] [194]
	Human in-vitro	[211] [212] [194] [214] [215]
	Structural modeling	[195]
Myocardial Infarction, Amyloidosis, and Cardiomyopathy	Murine in-vivo	[216]
	Murine in-vitro	[216] [217] [218]
	Human in-vitro	[218]
	Zebrafish	[219]
General Inflammation and Cancer	Murine in-vivo	[207] [122] [220]
	Murine in-vitro	[207] [122] [220]
	Human in-vitro	[122] [197] [196]
	Structural modeling	[221] [222]
Parasitic Infection	Murine in-vivo	[223] [209]
	Murine in-vitro	[223] [209] [224]
Viral Infection	Murine in-vitro	[225] [226]
	Human in-vitro	[226]
Bacterial Infection	Human in-vitro	[227]
	Shrimp	[228]
Diabetes	Murine in-vitro	[208] [229]
	Human in-vitro	[229]
Leukocyte Dysfunction	Murine in-vivo	[230]
	Murine in-vitro	[230]
	Human in-vitro	[231] [232]
Pregnancy Complications	Murine in-vitro	[233]
	Human in-vitro	[233] [234]
Other	Murine in-vivo	[235]
	Murine in-vitro	[235] [236]
	Human in-vitro	[191] [237] [193] [238]
	Structural Modeling	[239]

Table from co-authored review paper: Burton, J.C.; Antoniades, W.; Okalova. J.; **Roos, M.M.**; Grimsey, N.J., *Atypical p38 Signaling, Activation, and Implications for Disease.* International Journal of Molecular Sciences, 2021. **22**(8): p. 4183.

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Although p38 MAPK signaling has not been as extensively researched in brain pericytes as other cell types or areas of the body, studies have shown that activation of p38 through the MKK3/6 mechanism in pericyte cells leads to inflammatory expression of MMPs, TNF-α, and IL-6 in addition to promoting pericyte migration [240-242]. Regarding p38 activation through the direct binding of TAB1, published research so far has primarily focused on the role that atypical p38 plays in endothelial cells, predominantly in the lungs and heart. Ongoing research by the Grimsey Lab has shown the importance of MKK3/6 independent p38 signaling in endothelial cells and the contribution to vascular leakage moderated through inflammatory GPCRs [122, 196, 197]. The TAB1-p38α mechanism of p38 signaling remains understudied in the brain with no known research on this subject. Furthermore, up until now, there has been no research of TAB1-dependent p38 autophosphorylation (GPCR-mediated or otherwise) in pericyte cells and the role it may play in neuroinflammation.

Current Therapeutics

There are many medications, such as the entire family of non-steroidal anti-inflammatory drugs (NSAIDs), on the market to treat inflammation in the body [16]. A caveat to the blood-brain barrier protecting the neurovascular unit so well is that many drugs which are available to treat inflammation are not able to cross into the brain for local treatment and thus, it is extremely difficult to develop an effective BBB permeably compound [243]. Despite the vast number of drugs available and substantial amount of continued research, due to the complicated nature of neuroinflammatory conditions, there are very limited treatment options available for these diseases. For many conditions,

Alzheimer's for example, there are no effective therapies that are able to halt or reverse disease progression, and for others, such as severe traumatic brain or spinal cord injury, there is oftentimes no way to restore full function and most medications carry risk for harmful adverse reactions [244-247]. For example, tissue plasminogen activator (tPA), the only treatment option that has been approved to treat ischemic stroke, has a high risk of intracerebral hemorrhage [248]. A major factor in being able to treat neurodegenerative disorders lies in understanding the key components that are causing the proinflammatory cytokine stimulation, BBB disruption, and tissue damage in order to be able to effectively target the underlying components of the condition, alleviate symptoms, and halt disease progression.

As detailed above, an increasing growth of knowledge surrounding pericyte cells has substantiated this cell type as a key regulator of the blood-brain barrier and proinflammatory signaling. Importantly, pericytes have recently been shown to exhibit stem cell-like pro-regenerative properties, attracting attention as a promising avenue for regenerative therapeutics in the treatment of neurodegenerative disorders, including traumatic brain injury, Alzheimer's disease, stroke, and Parkinson's disease [59, 64, 68, 109]. The use of stem cell transplantation, and even more recently, mesenchymal stem cell (MSC) derived exosomes, as a promising therapeutic to improve damage sustained by diseases such as spinal cord injury and ischemic stroke is currently a hot topic in research [239, 249-251]. In addition to their neuroprotective abilities, MSCs have been shown to modulate T-cells, which are highly active during neuroinflammation and contribute to long-term cytokine release and blood-brain barrier breakdown [252-254]. *In vivo,* since it was shown that many transplanted MSCs were trapped in the liver and lungs

and very few actually migrated to the injured tissue, alternative avenues involving pericytes have begun to be explored [255]. Due to the stem cell characteristics that pericytes display, their ability to modulate T-cells, and their close relationship with endothelial cells, novel research has shown that pericyte derived exosomes display therapeutic potential in promoting functional recovery after hypoxia, ischemia, and reperfusion, all of which are characteristic of inflammatory conditions such as spinal cord injury and ischemic stroke [256-259]. Not only are pericytes an appealing target for treatment of neuroinflammatory disorders and reparation of the BBB, but the role that these cells play in angiogenesis also makes pericytes a potential target to disrupt the quick and efficient vascularization of cancerous tumors [65, 260, 261]. It is therefore not surprising that pericytes are increasingly becoming a central focus of research to define their wide range of functions and control of many integral processes with regard to blood-brain barrier stability and inflammatory signaling responses. Despite extensive ongoing research, due to the vast amount of roles that pericytes display and many different environments they are involved in, key avenues of function and regulation remain understudied. One such realm is a novel MAPK inflammatory signaling pathway, TAB1-mediated p38 activation.

Cellular stimuli and stress, including cytokines, growth factors, and hormones, as well as environmental stressors such as heat, osmotic shock, UV radiation, ischemia, and reperfusion induce the activation of MAPKs [178-181, 262, 263]. As such, p38 MAPKs have been the subject of intense study in both academia and industry for the generation of novel therapeutics. Despite ongoing clinical trials for many diseases, including cancers, neuropathies, chronic obstructive pulmonary disease (COPD), ischemic cardiac damage,

and acute respiratory distress syndrome (ARDS) / Sars-Cov-2 (COVID-19), only one non-selective p38 inhibitor (pirfenidone) has been approved for clinical use and that is to treat idiopathic pulmonary fibrosis [205, 264-266]. A key contributor to these drugs not making it past clinical trials is that the majority of p38 inhibitors currently being researched compete for ATP binding and block most, if not all, of p38 activity, effectively inhibiting physiological functioning as well as the inflammatory response [267]. Thus, selective inhibition of atypical p38 inflammatory signaling represents a substantially under-investigated avenue and potentially a critical target for treatment of neurodegenerative disorders, cancers, infections, diabetes, and other proinflammatory conditions [122, 201, 205-209]. As discussed above, targeting GPCR-mediated atypical TAB1-p38α signaling is a promising opportunity to effectively inhibit a portion of p38 inflammatory signaling while still allowing for physiologically important cellular signaling and homeostatic functioning.

G protein coupled receptors are currently the largest group of drug targets and make up approximately 35% of approved drugs [268]. Therapeutics targeting GPCRs have long been studied for their potential to treat conditions such as allergies, autoimmune diseases, cancers, mental illnesses, cardiovascular diseases, and many others associated with inflammation in various different parts of the body [135]. In the case of neuroinflammation, where there are many key components at play and various factors have the ability to induce a prolonged inflammatory response, it is very difficult to attempt to block each individual GPCR as some current therapeutics aim to do [268]. Inhibiting atypical p38 signaling is a novel method that has the potential to halt the effects

of multiple different proinflammatory GPCRs, effectively treating a substantial portion of chronic inflammation while physiological signaling remains intact.

Due to the position and function of pericyte cells in the brain and the expansive role of G protein coupled receptors in inflammation, GPCR-mediated atypical p38 signaling in pericytes is a novel target that we hypothesize is critical mediator of neuroinflammation. As this is a subject that has not been studied previously, our research provides insight about this signaling pathway and serves as the beginning of a foundation for atypical p38 as a potential therapeutic target to treat neuroinflammatory conditions.

CHAPTER 2

RESULTS

Characterization of Human Brain Vascular Pericyte-Like Cells

As pericytes fall into the classification of “mural cell” and there are some overlapping characteristics within this group, it is imperative that the pericyte cells being used for our experiments are validated and characterized based on known molecular expression. That is difficult since there is not one unique pericyte marker and there is still a lot of debate surrounding the molecular markers and expression profiles of these cells [26, 106, 269]. However, as described in the introduction section *Pericyte Cells and Their Role in Neuroinflammation*, there are a number of markers that are widely studied and used to classify pericyte-like cells [26, 269]. To validate the characteristics of our primary human brain vascular pericyte cells, we performed analysis of both mRNA and protein expression of platelet derived growth factor receptor beta (PDGFRβ), neuroepithelial stem cell protein (Nestin), neuron-glial antigen 2 (NG2), alpha-smooth muscle actin (α-SMA), cluster of differentiation 146 (CD146) (also known as melanoma cell adhesion molecule (MCAM)), cluster of differentiation 34 (CD34) (also known as hematopoietic progenitor cell antigen CD34), and cluster of differentiation 31 (CD31) (also known as platelet endothelial cell adhesion molecule- 1 (PECAM-1 or EndoCAM)).

To assess pericyte marker expression, we first quantified mRNA levels of CD34 and PDGFRβ for both human brain vascular pericytes (PC) and human pulmonary artery endothelial cells (EC) via real time quantitative PCR (qPCR) (Fig. 3). As discussed

previously, CD34 is a known endothelial cell marker while PDGFRβ is a widely used marker for pericyte cells [75, 270]. Results were normalized to the GAPDH mRNA expression in each respective cell type and represented as fold change values relative to the opposing cell line, i.e. pericyte cells versus endothelial cells or endothelial cells versus pericyte cells (Fig. 3). The pericytes contained 400-fold more PDGFRβ mRNA compared to the endothelial cells (Fig. 3). Conversely, the endothelial cells held 228-fold more CD34 mRNA than the pericytes (Fig. 3). This preliminarily confirms the identity of the human brain vascular pericytes as pericyte-like cells.

Figure 3.

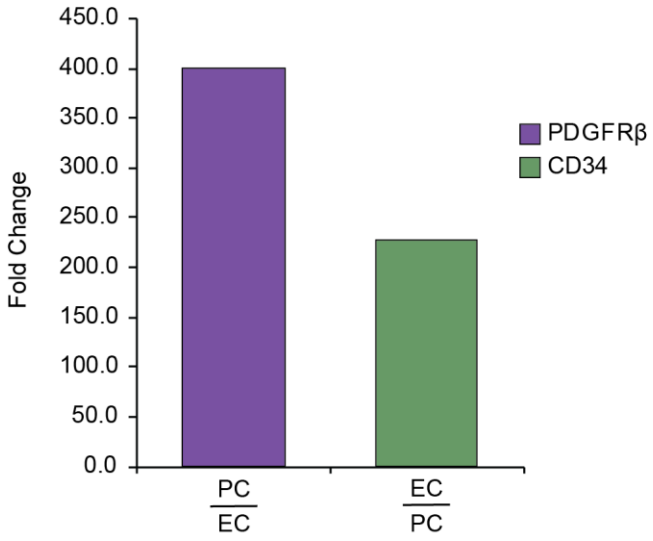

Figure 3: Real-Time Quantitative PCR Characterization of Pericyte-Like Cells

Pericyte cells expressed a high level of PDGFRβ mRNA and no CD34 mRNA compared to endothelial cells.

mRNA expression of PDGFRβ and CD34 was quantified in human brain vascular pericyte cells (PC) and human pulmonary artery endothelial cells (EC) via real-time quantitative PCR (qPCR). Results were normalized to the respective GAPDH levels for each cell type and quantified as the fold expression of PC relative to EC (PDGFRβ) or EC relative to PC (CD34). (n=1)

We next sought to characterize the pericytes further by assessing protein expression of PDGFRβ, Nestin, NG2, α-SMA, and CD31. Pericyte cell (PC) and EA.hy926 cell (EC) lysates were immunoblotted and protein expression was normalized to the GAPDH levels of the respective cell types and represented as fold change values relative to the opposing cell line. Compared to the endothelial cells, the pericyte cells expressed 121-fold more PDGFRβ, 75-fold more Nestin, 393-fold more NG2, and 135-fold more α-SMA (Fig. 4A- B). There was no PDGFRβ, Nestin, NG2, or α-SMA protein expression observed in the endothelial cells (Fig. 4A-B). Conversely, the endothelial cells displayed 110-fold more CD31 than the pericytes, which did not show any expression of this molecular marker (Fig. 4A, C). As many studies mention, lack of a cellular marker unique to only pericytes makes it difficult to classify this cell type [269]. However, using multiple known pericyte expression markers enables us to show the pericyte-like qualities of this cell type *in vitro*. The presence of PDGFRβ, Nestin, NG2, and α-SMA and absence of CD31 in the cultured pericytes via immunoblotting further validates that our subsequent experiments are performed on pericyte-like cells.

Figure 4.

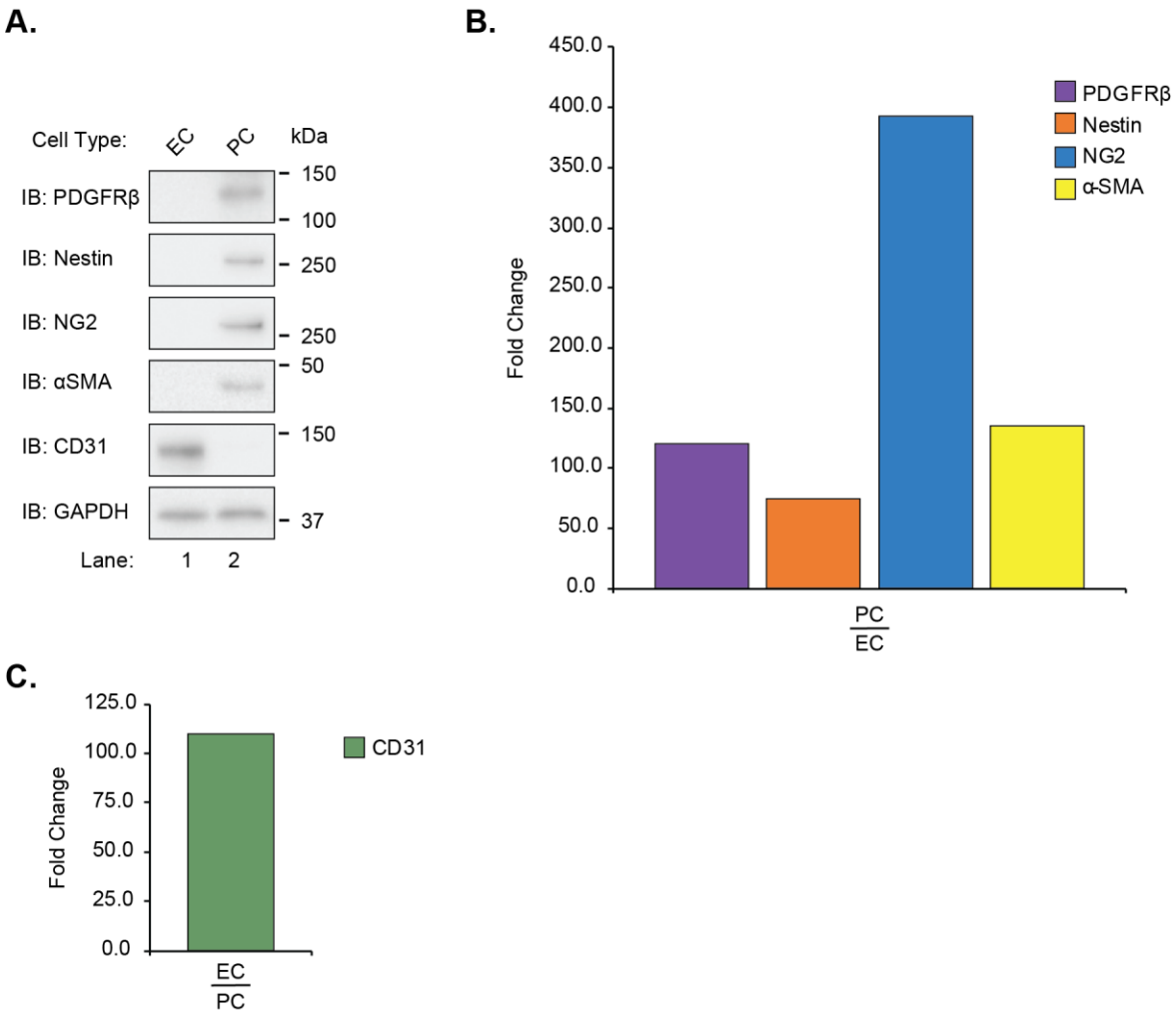

Figure 4: Immunoblot Characterization of Pericyte-Like Cells

Compared to endothelial cells, pericytes expressed PDGFRβ, Nestin, NG2, α-SMA, and no CD31.

A. Equal amounts of lysed pericyte cell (PC) samples from other experiments listed in this paper and EA.hy926 cells (EC) were immunoblotted (IB). Results were normalized to the GAPDH level of each respective cell type and quantified as **B.** the fold change in PC relative to EC or **C.** the fold change in EC relative to PC. (n=1)

To follow our analysis of protein expression using immunoblotting, we then assessed the same marker proteins via immunofluorescence. To achieve this, human brain vascular pericytes (PC) and human pulmonary microvascular endothelial cells (EC) were seeded onto coverslips and fixed and permeabilized using either 4% paraformaldehyde (PFA) and 0.1% Triton-X or 100% methanol, as per the product specifications. The fixed samples were immunolabeled and visualized using a Zeiss confocal laser scanning microscope (LSM800) with widefield Colibri microscopy. We observed PDGFRβ, Nestin, NG2, and α-SMA expression in the pericytes and a limited appearance of these markers in the endothelial cells (Fig. 5A). There was a substantial amount of PDGFRβ staining seen throughout the cell body of all the pericyte cells (Fig. 5A). In addition, a considerable amount of Nestin was also ubiquitously expressed in the pericytes (Fig. 5A). Furthermore, NG2 expression was observed evenly throughout the entire cytoplasm of the pericyte cells (Fig. 5A). α-SMA staining could be seen in a small amount of the pericyte cells, seemingly localized to the cytoplasmic microfilaments, suggesting that when cultured carefully and used at a low passage number, these cells retain some of their expected *in vivo* characteristics (Fig. 5A) [104]. There was limited detection of PDGFRβ, NG2, and α-SMA on the endothelial cells, but staining did reveal a low expression of Nestin in these cells (Fig. 5A). Interestingly, in contrast to many studies that have shown Nestin to be expressed by endothelial cells that are actively proliferating and in tumor angiogenesis, a recent paper has also shown Nestin expression in endothelial cells throughout the body, regardless of proliferative status [96, 271, 272]. CD146 was observed to be highly expressed at endothelial cell junctions and moderately expressed in some pericyte cell junctions as well, but not in all cells (Fig. 5B). CD31

expression was seen in a majority of the endothelial cells and at low levels in the pericytes (Fig. 5B). Heterogeneity of the CD31 molecular marker may be attributed to the endothelial cells being a mixed population of the lung microvasculature, including lymphatic vessels and blood capillaries. Minimal staining was observed in the cells where only secondary antibody was present (Fig. 5C). The combined data displayed in Figure 5 corroborates that these are pericyte-like cells.

Immunofluorescence is a technique that displays the full spectrum of cellular expression where success is dependent on many variables, including the method, reagents, and duration of the fixing and permeabilization steps. Also, the concentration of both the primary and secondary antibodies, the antibody suspension buffer, and antibody incubation times. Each one of these steps must be optimized to achieve optimal results with minimal background. Due to the varying levels of background seen with our one trial, these are preliminary results where the specificity will be improved. The immunoblot and qPCR experiments yielded very clear and specific results with no question of background interference. Further optimization studies are predicted to display a clearer immunofluorescence labeling of these cells. However, based on the specific immunoblot and qPCR, and preliminary immunofluorescence results, we believe we have confirmed that these are pericyte-like cells.

Figure 5.

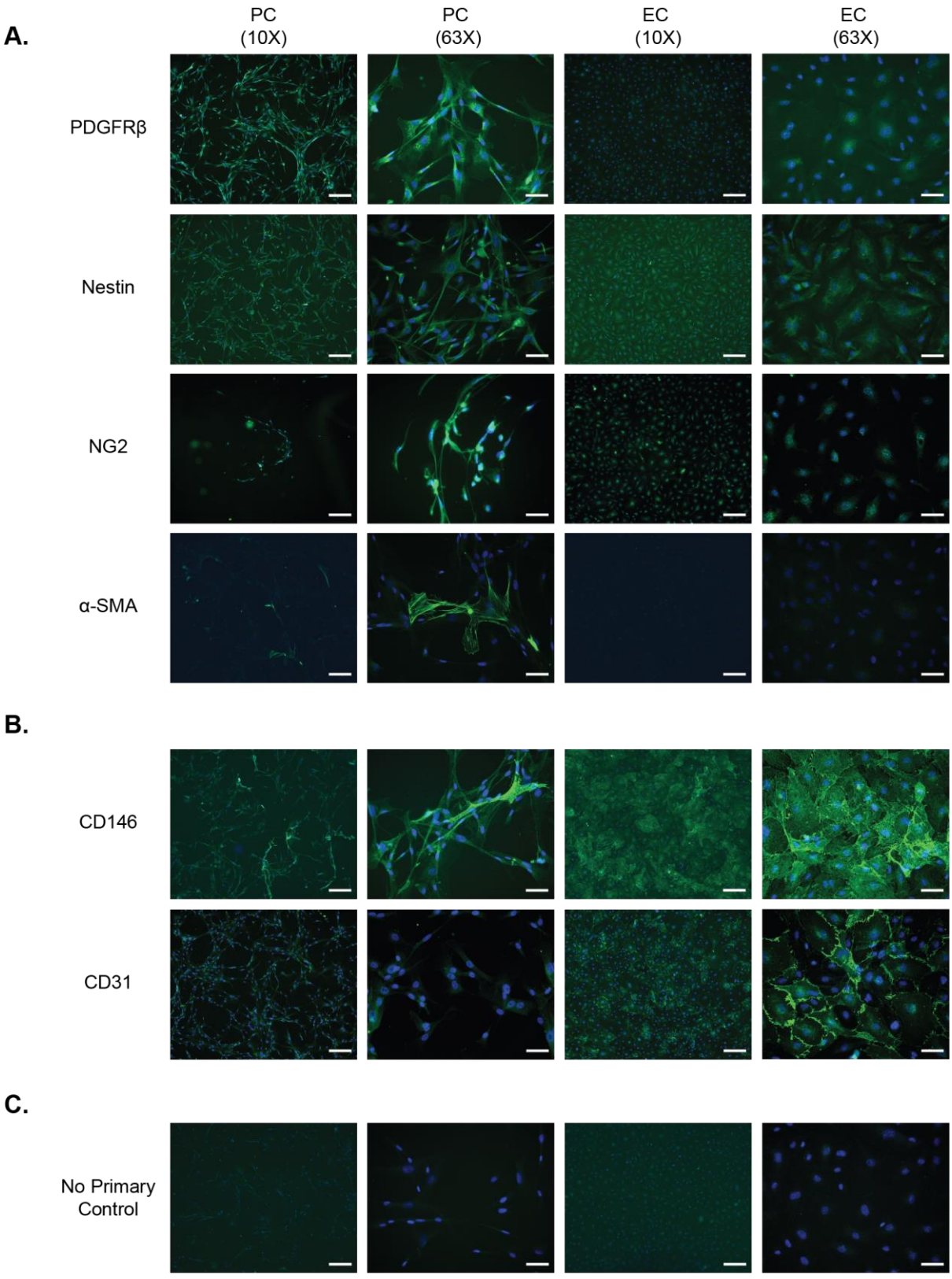
A.
PC (10X)
PC (63X)
EC (10X)
EC (63X)
PDGFRβ
Nestin
NG2
α-SMA
B.
CD146
CD31
C.
No Primary Control

Figure 5: Immunofluorescence Characterization of Pericyte-Like Cells

Immunofluorescence confirmed pericyte expression of PDGFRβ, Nestin, NG2, some α-SMA, CD146, and minimal CD31.

Human brain vascular pericyte cells (PC) and human pulmonary microvascular endothelial cells (EC) adherent to coverslips were fixed and permeabilized with either 4% PFA and 0.1% Triton-X or 100% methanol, stained with primary and secondary antibodies, and mounted onto slides. Results were visualized via Zeiss LSM800 widefield Colibri microscopy. Bar indicates 200μm for 10X (columns one and three) and 50μm for 63X (columns two and four). (n=1)

The use of a single cell marker would be insufficient in identifying these cells as pericytes, but seven different reliable markers (PDGFRβ, Nestin, NG2, α-SMA, CD146, CD31, and CD34) via three methods of identification (quantitative PCR, immunoblotting, and immunofluorescence) shown in Figure 3, Figure 4, and Figure 5 support the conclusion that our cells display multiple characteristic markers of brain pericytes. Our results establish the cultured human brain vascular pericytes as pericyte-like cells, showing that they display similar characteristics to their *in vivo* counterparts. For simplicity, we will refer to these pericyte-like cells as pericytes for the remainder of the text.

Atypical p38 Signaling in Human Brain Pericyte Cells is Activated by Multiple GPCR Agonists

P38 activation can occur via multiple pathways: through the three-tiered kinase cascade mediated by MKK3 and MKK6 or through TAB1 binding to p38, both resulting in phosphorylation dependent activation of p38 [175, 190, 191]. As discussed in the introduction, G protein coupled receptors (GPCRs) have been shown to activate p38 via the atypical, direct TAB1 binding, p38 signaling pathway [122, 196, 197]. However, both GPCR function and p38 activity are vastly under-researched in pericyte cells. Furthermore, there are no published studies showing p38 signaling via TAB1 binding, including GPCR mediated activation of this pathway or otherwise, in pericyte cells from anywhere in the body. There is also a large gap in the literature regarding atypical activation of p38 in neuroinflammation, where, so far, the focus of research on GPCR-mediated atypical activation of p38 has been in the lung vasculature [122, 196, 197]. To

examine the mechanism of p38 phosphorylation and activation, we used SB203580, a specific inhibitor of the alpha and beta isoforms of p38 MAPK. SB203580 functions via binding to the ATP binding pocket on p38, which prevents p38 autophosphorylation and all downstream p38 signaling cascades, both typical and atypical, but upstream MAP2K phosphorylation (via MKK3/6) of p38 remains intact [191, 267, 273, 274] (Fig. 6). As SB203580 blocks all of p38's downstream activity, disrupting both physiological and pathological inflammatory signaling, it is not a viable therapeutic option. However, the fact that this inhibitor specifically prevents p38 phosphorylation of itself while upstream activation remains intact enables the use of SB203580 to observe atypical TAB1-mediated p38 autophosphorylation in a research setting.

Figure 6.

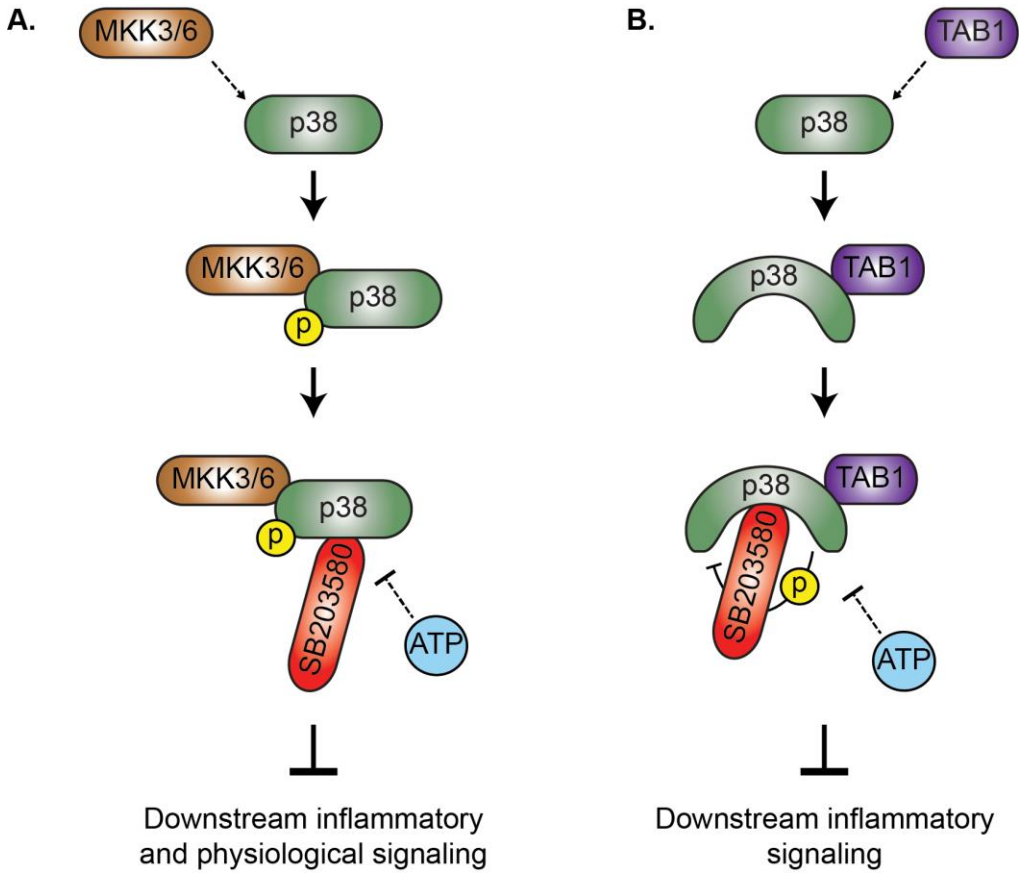

Figure 6: p38α MAPK Inhibitor, SB203580

SB203580 prevents p38 autophosphorylation and all downstream p38α MAPK signaling, but MKK3/6 phosphorylation of p38 remains intact.

A. SB203580 binds to the ATP binding pocket on p38α. MKK3/6 are able to bind to and phosphorylate p38α at the normal Thr180 and Tyr182 residues. As the ATP binding pocket on p38 is occupied, the kinase activity of p38 is prevented and thus it cannot participate in downstream signaling. **B.** In the presence of SB203580, TAB1 is still able to bind to p38 and induce a conformational change, but as the ATP binding pocket is occupied and p38α kinase activity is required for autophosphorylation, p38 is unable to phosphorylate itself. P38α cannot participate in downstream signaling by either mechanism in the presence of SB203580.

To observe whether a family of GPCR agonists function via MKK3/6 phosphorylation or autophosphorylation of p38, human brain vascular pericyte cells were pretreated with 10μM SB203580 for 20 minutes and stimulated with either 10μM α-thrombin, 1μM histamine, or 10μM PGE2. Protein expression of phosphorylated p38 was quantified based on respective p38 levels in each sample and normalized to the DMSO control zero-minute time point.

After treatment with α-thrombin, there was a 1.8-fold increase in p38 activation from zero to five minutes in the DMSO control wells (Fig. 7A; lanes 1-3). This p38 activation, visualized as phosphorylated p38, was significantly inhibited with SB203580 pretreatment at both 5 and 7.5 minutes (Fig. 7A; lanes 4-6). Histamine stimulation of the samples induced a 2.7-fold increase in p38 activation in the DMSO controls from zero to five minutes (Fig. 7B; lanes 1-3). Similar to with α-thrombin treatment, the p38 activation by histamine at 5 and 7.5 minutes was significantly inhibited when cells were pretreated with SB203580 (Fig. 7B; lanes 4-6). Consistent with the results seen with α-thrombin and histamine, PGE2 stimulation yielded a 2.0-fold increase in p38 activation from zero to five minutes in the DMSO control samples (Fig. 7C; lanes 1-3). With SB203580 pre-treatment, a significant inhibition of p38 activation was observed at both 5 and 7.5 minutes of PGE2 stimulation compared to the control wells treated with DMSO (Fig. 7C; lanes 4-6).

Figure. 7.

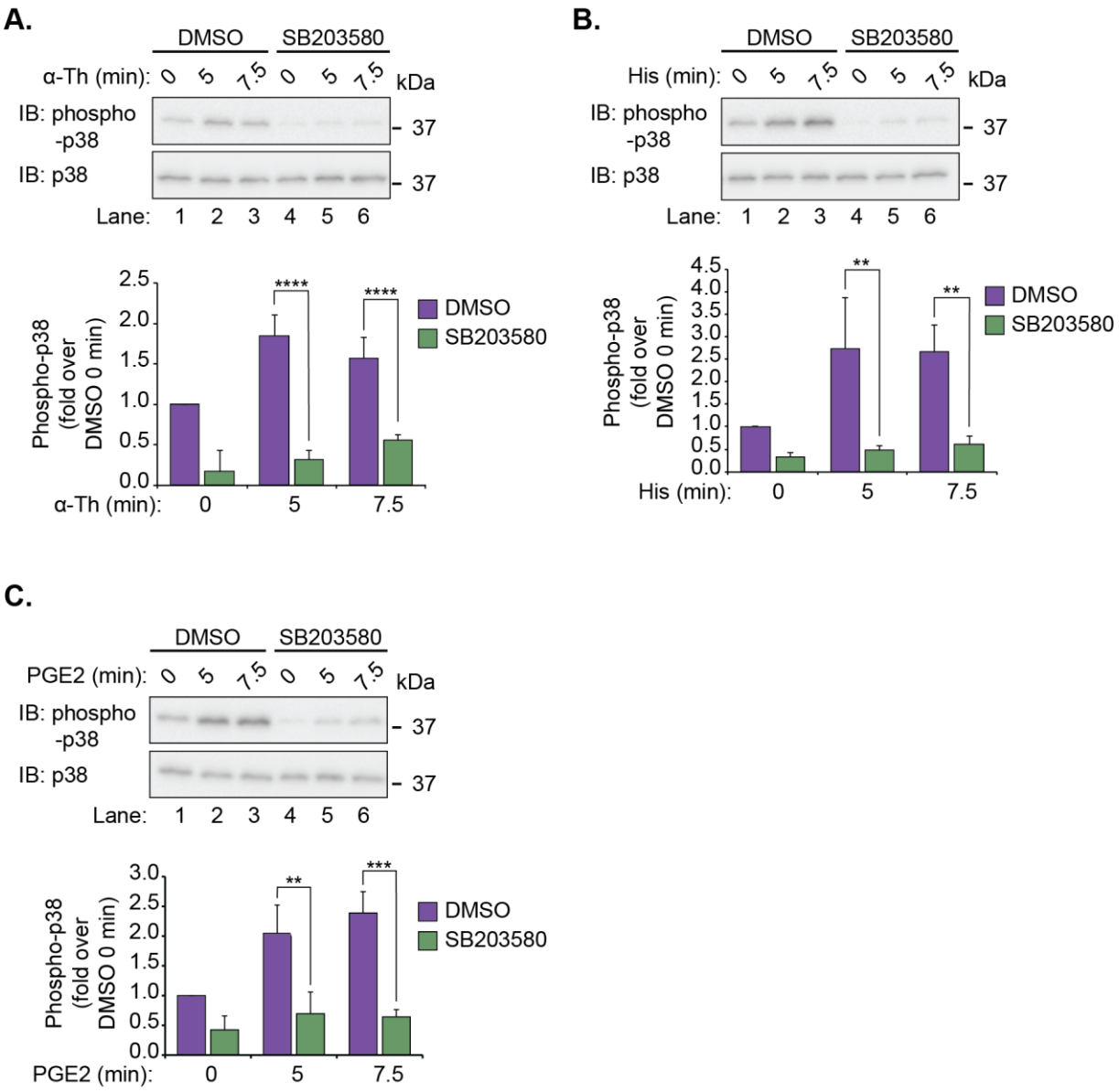

Figure 7: GPCR-Mediated TAB1-p38α Signaling in Pericyte Cells

Multiple GPCR agonists activate atypical p38 signaling in human brain pericyte cells.

Pericytes were stimulated with **A.** 10μM α-thrombin (α-Th), **B.** 1μM histamine (His), or **C.** 10μM PGE2 after pretreatment with 10μM SB203580 or DMSO for 20 minutes. Cells were lysed and equal amounts were immunoblotted (IB). Results were quantified as the fold change over DMSO zero-minute time point and expressed as mean ± SD. Statistical analysis was carried out by ANOVA test (n=3; *p < 0.05, **p < 0.01, ***p < 0.001).

Tumor necrosis factor-alpha (TNF-α) is a well-known inflammatory agonist that activates all four isoforms of p38 MAPK via the typical MKK3/6 dependent signaling pathway [202, 203, 275]. TNF-α was used here to demonstrate that SB203580 is not a broad range inhibitor of upstream MKK3/6 p38 activation in addition to inhibiting the p38 autophosphorylation response, i.e. all p38 MAPK signaling activation. Utilizing the same method discussed in Figure 6, pericytes were pretreated with 10μM SB203580 or DMSO for 20 minutes and then stimulated with either 25ng/mL TNF-α or 40ng/mL PDGF-BB. TNF-α induced a 6.8-fold increase in p38 activation from zero to five minutes in the DMSO control samples (Fig. 8A; lanes 1-3). SB203580 pretreatment, in contrast to what was seen with α-thrombin, histamine, and PGE2 treatment, did not significantly inhibit p38 activation by TNF-α (Fig. 8A; lanes 4-6). With no significant reduction of p38 activation after TNF-α stimulation in the presence of the p38 MAPK inhibitor compared to the control, we can confirm that SB203580 is not functioning as an unselective inhibitor of the upstream MKK3/6 phosphorylation of p38 in addition to p38 autophosphorylation (Fig. 8A).

As an interesting observation, we noted that PDGF-BB, which is not a GPCR agonist or known activator of TAB1-p38α signaling, also increased p38 activation by 1.7-fold from zero to five minutes in the DMSO wells (Fig. 8B; lanes 1-3). Analogous to α-thrombin, histamine, and PGE2, PDGF-BB stimulation, in the presence of SB203580 pretreatment, resulted in a significant decrease in p38 activation at both five and ten minutes compared to the DMSO treated samples at each time point (Fig. 8B; lanes 4-6). PDGF-BB has been shown to activate p38 MAKP, but the explicit mechanism of activation has yet to be elucidated [276-278]. Our data suggests that PDGF-BB can activate p38

through a mechanism that requires p38 autophosphorylation. Whether this autophosphorylation and activation is through a TAB1-TAB2 dependent mechanism similar to what is seen with the GPCR agonists remains to be determined and requires further research.

Figure 8.

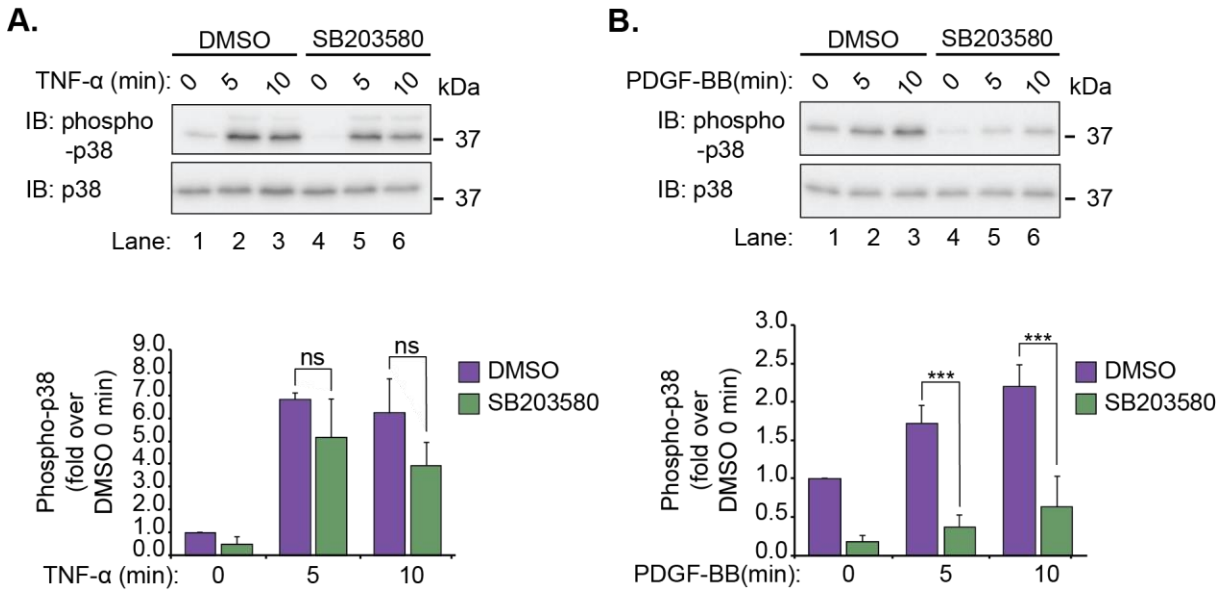

Figure 8: MKK3/6 and Alternate Mechanism of p38 Activation in Pericyte Cells

TNF-α activated p38 via MKK3/6 while PDGF-BB activated p38 through autophosphorylation via an unknown mechanism.

Pericytes were stimulated with **A.** 25ng/mL TNF-α, or **B.** 40ng/mL PDGF-BB after pretreatment with 10μM SB203580 or DMSO for 20min. Cells were lysed and equal amounts were immunoblotted (IB). Results were quantified as the fold change over the DMSO zero-minute time-point and expressed as mean ± SD. Statistical analysis was carried out by ANOVA test (n=3; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

These results suggest that atypical p38 activation via autophosphorylation can be induced by multiple different GPCR agonists (α-thrombin, histamine, and PGE2) as well as PDGF-BB in human brain pericytes. The data displayed here is consistent with the results seen previously in endothelial cells [122, 196, 197]. To the best of our knowledge, this is the first time that TAB1-mediated p38α activation has been shown in human brain vascular pericyte cells.

Confirmation of p38 Atypical Signaling via siRNA Knockdown and TAB1-p38α Peptide Inhibitor

Atypical p38 signaling is activated in a manner where TAB2 associates with TAB1 to form a complex, which then binds to p38 to induce p38 autophosphorylation [191]. Knockdown of TAB1 and TAB2 via siRNA to block atypical signaling is a method that has previously been used in primary endothelial cells to confirm TAB1-mediated p38 signaling [122, 196, 197]. To confirm our hypothesis that GPCR signaling occurs through the TAB1-TAB2 dependent pathway, we attempted to deplete these two critical components to further prove that p38 can be activated through the autophosphorylation pathway in pericyte cells.

To knockdown TAB1 and TAB2 and prevent atypical p38 signaling, cells were transfected with either TAB1 siRNA, TAB1 and TAB2 siRNA, or nonspecific siRNA. However, siRNA transfection proved to be extremely cytotoxic to the cells. To mediate cytotoxicity, we altered the reagent used (TransIT-X2, Lipofectamine 2000, or Oligofectamine 2000), the amount of reagent, the siRNA concentration, the confluency of the cells, and the time of media change after transfection. Various trials yielded a

reduction in TAB1 and TAB2, although scattered across the board and masked by a slew of cell death. A representative trial is indicated in Figure 9. Although there was a decrease in TAB1 and TAB2 protein expression with TransIT-X2 as the transfection reagent, there was also substantial cell death where the vast amount of cytotoxicity can be seen through the decrease of GAPDH (Fig. 9; lanes 4-6). Despite our best efforts, the substantial cell stress and cell death persisted. siRNA transfection is especially difficult because unlike other more robust cells, HeLa cells for example, pericytes are very sensitive and have the potential to differentiate in response to stress, particularly in cultured conditions [64, 279]. In addition, p38 MAPK is a stress induced pathway where if the cells are distressed or have differentiated as a result of the transfection, there will likely be an upregulation of inflammatory signaling by p38 and other proinflammatory signaling pathways, such as extracellular signal-regulated kinase (ERK) or c-Jun N-terminal kinase (JNK), potentially confounding any results that might be found [182]. The unwillingness of the cells to uptake the siRNA and possible differentiation is likely a key contributor to the cytotoxicity and lack of knockdown of TAB1 and/or TAB2, which has proved to be very difficult to fix.

Figure 9.

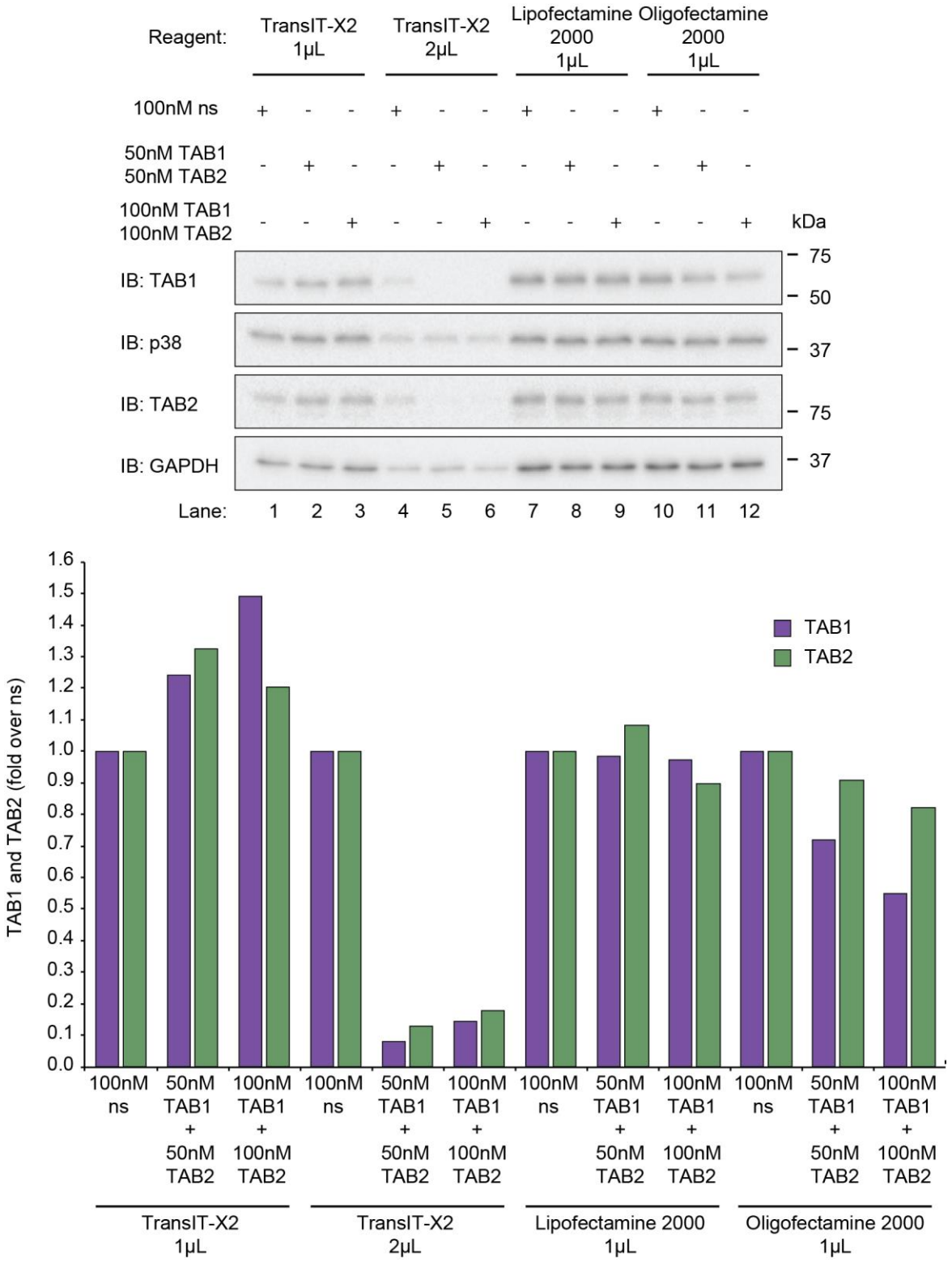
Reagent:
TransIT-X2 1µL
TransIT-X2 2µL
Lipofectamine 2000 1µL
Oligofectamine 2000 1µL
100nM ns
50nM TAB1 50nM TAB2
100nM TAB1 100nM TAB2
kDa
IB: TAB1
IB: p38
IB: TAB2
IB: GAPDH
75
50
37
75
37
Lane: 1 2 3 4 5 6 7 8 9 10 11 12
TAB1
TAB2
TAB1 and TAB2 (fold over ns)
100nM ns
50nM TAB1 + 50nM TAB2
100nM TAB1 + 100nM TAB2
TransIT-X2 1µL
TransIT-X2 2µL
Lipofectamine 2000 1µL
Oligofectamine 2000 1µL

Figure 9: siRNA Transfection is Cytotoxic to Pericytes

Representative data showing TAB1 and TAB2 knockdown cytotoxicity in the pericyte cells.

Pericytes were co-transfected with either 100nM non-specific (ns) siRNA, 50nM TAB1 siRNA + 50nM TAB2 siRNA, or 100nM TAB1 siRNA + 100nM TAB2 siRNA using either 1μL TransIT-X2 (Lanes 1-3), 2μL TransIT-X2 (Lanes 4-6), 1μL Lipofectamine 2000 (Lanes 7-9), or 1μL Oligofectamine 2000 (Lanes 10-12). Cells were lysed and equal amounts were immunoblotted (IB). Results were quantified as the fold change over 100nM ns siRNA for each respective reagent. (n=1)

There are alternate methods that may be attempted to knock down TAB1 and TAB2 in order to prevent p38 autophosphorylation. Possible approaches include siRNA knockdown via electroporation, shRNA delivery via lentiviral vector, or a peptide inhibitor. Lentivirus mediated shRNA transduction has been used in pericyte cells for example to knockdown the protein encoding apelin and observe the effects of hypoxia-induced injury on pericytes [280]. Although this could be a feasible approach, shRNA transduction in pericytes is not very well researched at the moment and there are other more accessible options.

To overcome the challenge posed by the siRNA transfections, we explored the use of a cell-penetrating peptide (CPP) inhibitor. Through molecular modeling, Wang, et al. in 2013 developed a selective CPP inhibitor to block the direct interaction of TAB1 and p38α [212]. They synthesized and provided evidence for cell penetrating peptides PT1, as a control, and PT5, as a peptide with mutations of key TAB1 binding residues at the hydrophobic docking groove and unique surrounding interface of p38α to specifically block the TAB1-p38α interaction [212] (Fig. 10). As such, TAB1 cannot bind to p38, p38 is unable to autophosphorylate, and downstream atypical p38 induced signaling is prevented. The peptide was made permeable to cells through the use of a 10 amino acid HIV TAT transporter sequence linked to the C-terminal [212, 281]. In additionally studies, this CPP inhibitor was successfully used to suppress spontaneous dermal inflammation in the Itch knockout mouse [206]. This research provided evidence that Itch, an E3 ligase that prevents the development of a skin-scratching phenotype, functions through ubiquitinating and degrading TAB1, preventing the TAB1-mediated activation of p38 [206].

Figure 10.

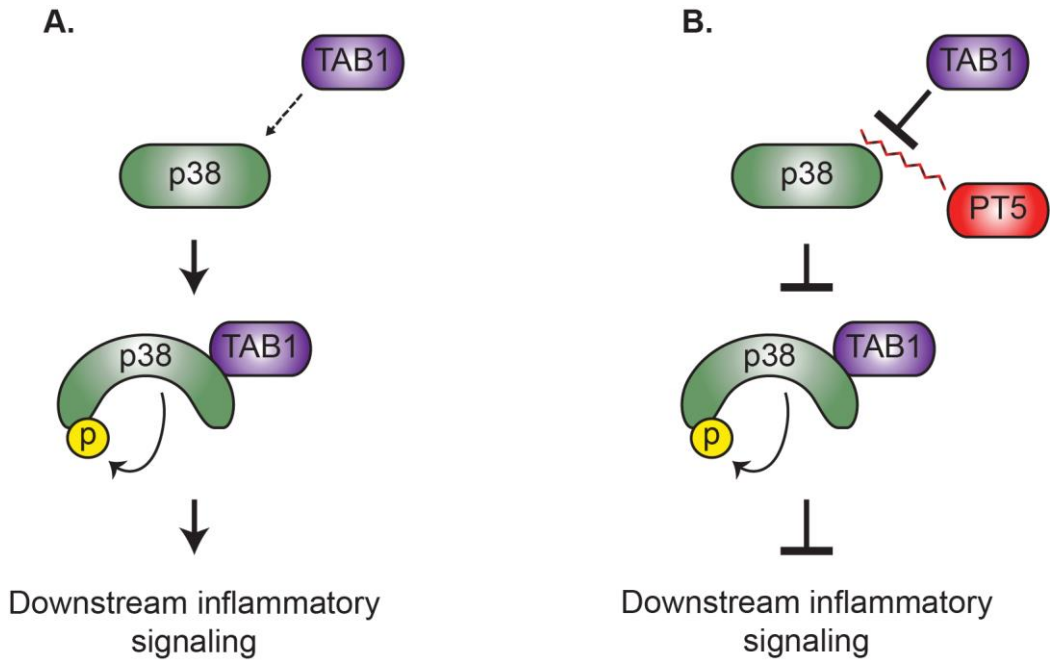

Figure 10: Specific TAB1-p38α Cell-Penetrating Peptide Inhibitor, PT5

A. TAB1 binds to p38α, which induces a conformational change, enabling p38 autophosphorylation and activation to mediate downstream inflammatory signaling. **B.** PT5 binds at and next to the TAB1 binding site on p38α, blocking the direct TAB1-p38α interaction. TAB1 unable to bind to p38 prevents the conformational change and subsequent autophosphorylation of p38. Downstream signaling mediated by the autophosphorylated p38α is thus prevented.

To investigate the potential of the CPP inhibitor to block GPCR signaling, we incubated human brain vascular pericyte cells with either 50μM PT1 or 50μM PT5 for one hour and then stimulated with 10μM PGE2 (Fig. 11). PGE2 induced a 4.6-fold activation of p38 from 0 to 7.5 minutes, as displayed in the control PT1 treated samples (Fig. 11; lanes 1-2). Pretreatment with PT5 produced a substantial 2.0-fold decrease in PGE2 dependent phosphorylation of p38 at 7.5 minutes compared to the 7.5-minute control (Fig. 11; lanes 3-4). To confirm this was selective inhibition of atypical activation and the peptide was not blocking all MAPK signaling, we next assessed extracellular signal-regulated kinase 1 and 2 (ERK1/2) activation and the activation of downstream kinases mitogen- and stress-activated protein kinase 1 (MSK1) and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2 or MK2) (Fig. 11). ERKs are inflammatory signaling kinases that are activated by mitogen-activated protein kinase kinase 1 (MAP2K1 or MEK1) and mitogen-activated protein kinase kinase 2 (MAP2K2 or MEK2), which are activated by upstream mitogen-activated protein kinase kinase kinases (MAP3Ks), the recruitment of which is regulated by Ras GTPases [282-284]. MSK1 is a downstream kinase that can be phosphorylated and activated by both ERK1/2 and p38α [285, 286]. MK2, another downstream kinase, is activated through phosphorylation by p38α and p38β [287-289].

In the presence of PT1, the control peptide, PGE2 produced a 2.0-fold activation of ERK from 0 to 7.5 minutes (Fig. 11; lanes 1-2). Pretreatment with PT5, the TAB1-p38α inhibitor, did not greatly reduce PGE2 stimulated ERK1/2 phosphorylation and activation from 0 to 7.5 minutes (Fig. 11; lanes 3-4). However, in one out of two of the experiments, PT1 was shown to cause a lower baseline of ERK expression than PT5, as indicated by

the SD error bars, perhaps indicative of a non-selective interaction of PT1 as a control, but further investigation is required. Regardless, this provides support that PT5 is a selective inhibitor of p38 MAPK signaling and is not blocking ERK, another MAPK pathway, as well. As MSK1 and MK2 are both downstream targets activated by p38, inhibition of p38 signaling should result in a decrease in their activation as indicated by MSK1 and MK2 phosphorylation in the presence of the inhibitor peptide. PGE2 stimulated a 2.7-fold increase in MK2 activation from 0 to 7.5 minutes in the samples pretreated with PT1 (Fig. 11; lanes 1-2). In the presence of PT5, there is a 1.6-fold lower MK2 activation by PGE2 at 7.5 minutes compared to the 7.5-minute control (Fig. 11; lanes 3-4). With PT1 pretreatment, PGE2 stimulated a 2.5-fold increase in MSK1 (Fig. 11; lanes 1-2). Similar to MK2, PGE2 stimulation, in the presence of PT5, resulted in a 1.1-fold lower MSK1 activation compared to the control at 7.5 minutes (Fig. 11; lanes 3-4). Inhibition of p38 activation in conjunction with inhibition of downstream MSK1 and MK2, compared to lack of inhibition of ERK1/2 activation, provides support that PT5 acts as an inhibitor of p38 activation through the TAB1-mediated pathway (Fig. 11). In addition, the fact that blocking the TAB1-p38α interaction substantially decreased phosphorylated p38 further corroborates the results shown with the SB203580 experiments, that p38, via GPCR mediation, is activated via the atypical signaling pathway in human brain pericyte cells. Significance for these experiments will be performed once an n=3 has been obtained.

Figure 11.

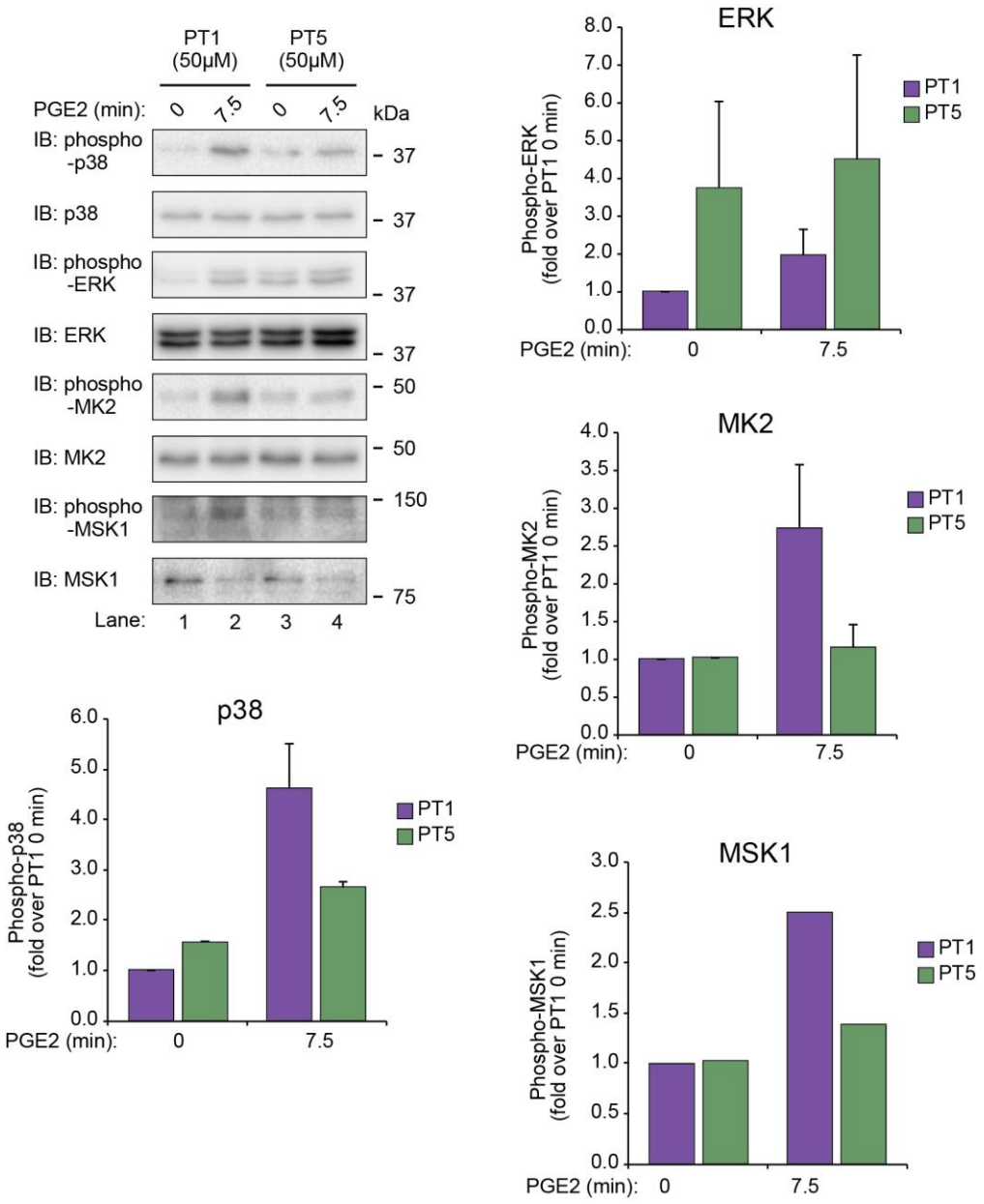

Figure 11: TAB1-p38α Peptide Inhibitor Blocks Atypical p38 Signaling in Pericyte Cells

Inhibition of p38, MK2, and MSK1 activation and no inhibition to ERK signaling with PT5.

Pericytes were stimulated with 10μM PGE2 after pretreatment with 50μM PT1 (control peptide) or 50μM PT5 (cell-penetrating peptide (CPP) inhibitor) for 60 minutes. Cells were lysed and equal amounts were immunoblotted (IB). Results were quantified as the fold change over PT1 zero-minute time-point for each respective protein pair and expressed as mean ± SD. (n=2; significance pending repeats)

Combined, these data support the hypothesis that there is atypical p38 activation in pericyte cells and that this signaling can be blocked through the use of a selective TAB1-p38α cell-penetrating peptide inhibitor. The next step is to look at the effect that blocking this inflammatory signaling pathway may have on pericyte function and regulation in neuroinflammatory conditions.

Inflammatory Morphology of Pericyte Cells

The function of pericytes in response to damage, i.e. a proinflammatory environment, is most commonly characterized through surface markers or cytokine secretion (as discussed in the introduction section *Pericyte Cells and Their Role in Neuroinflammation*). Although this does provide useful information on migration, cytokine release, and other functioning that is characteristic of brain pericyte cells during damage, it is a characterization of pericytes as a whole population and does not take into account differences on a single-cell basis. It is well known that pericyte cells are not a homogenous population and exhibit a wide range of expression patterns and functions [41, 45, 46, 48]. Many studies have characterized a general morphology that is representative of brain pericytes in healthy conditions or as a whole population, but there is a lack of research surrounding the way in which pericyte morphology changes in response to a proinflammatory environment and the possible morphological subpopulations that arise [290]. Recent studies have used a new high-throughput technique, functionally-relevant morphological profiling (FRMP), to analyze the morphological characteristics of mesenchymal stem cells (MSCs) in response to interferon gamma (IFN-y) [291-293]. This method revealed morphological subpopulations of MSCs, which differed in their ability to

suppress T-cells [291, 292]. As pericytes exhibit some of the same pro-regenerative properties as MSCs and have been shown to consist of multiple sub-populations, they are an ideal candidate for FRMP. Morphological profiling has the potential to determine if different pericyte responses to inflammation can be classified as consistent sub-morphologies and how pericyte morphology is influenced by GPCR-mediated atypical p38 signaling. This would enable the prediction of which pericyte roles, such as T-cell modulation, cytokine regulation, and migration, may potentially be therapeutically relevant and targetable.

Preliminary experiments were performed in collaboration with Dr. Ross Marklein, Kanupriya Daga, and Courtney Campagna of the Marklein Lab at the University of Georgia. To observe morphological differences of brain pericytes in response to cytokine stimulation, cells were pretreated with 10μM SB203580 and stimulated with either 10nM α-thrombin, 1μM histamine, 10μM PGE2, or 200ng/mL PDGF-BB. The next day, cells were fixed with 4% PFA, stained for visualization, and imaged using a microscopy-based size analysis software, CellProfiler. Results were analyzed with JMP software. In response to stimulation with α-thrombin, histamine, and PDGF-BB, the size of the cells (displayed as median area) decreased by a fold of 1.14, 1.04, and 1.05 respectively; they became more contracted and smaller (Fig. 12). This could be indicative of a migratory phenotype as pericytes are known to detach from the blood vessels they are regulating in response to inflammation [38]. In the control samples, pericyte size increased by 1.01-fold with SB203580 pretreatment compared to no inhibitor (Fig. 12). Pericyte size of the samples stimulated with α-thrombin, histamine, and PDGF-BB following SB203580 pretreatment was increased compared to the respective sizes without the inhibitor (Fig.

12). The size of these cells remained more similar to the control samples compared to the size change that was seen in their DMSO pretreated counterparts, with size increasing even further in the SB203580 pretreated, PDGF-BB stimulated cells (Fig. 12). In response to PGE2 stimulation, there was a 1.14-fold increase in pericyte size compared to the DMSO control that was increased even further with SB203580 pretreatment (Fig. 12). Although seemingly small, these fold change numbers do represent substantial differences in the size and shape of the cells. As SB203580 prevents all downstream signaling by p38, we cannot make any inferences specifically about the TAB1-p38α pathway, but, speculatively, this may indicate that there is a potential protective mechanism provided to the cells in blocking p38 activation as indicated by their larger area with SB203580 pretreatment. The difference in area between α-thrombin, histamine/PDGF-BB, and PGE2 stimulated pericytes provides preliminary support that there are individual pericyte subpopulations as defined by morphology in response to various cytokines. Future studies are required to fully understand the extent of these morphological changes and the different phenotypes pericyte cells display in the presence of a proinflammatory environment.

Figure 12.

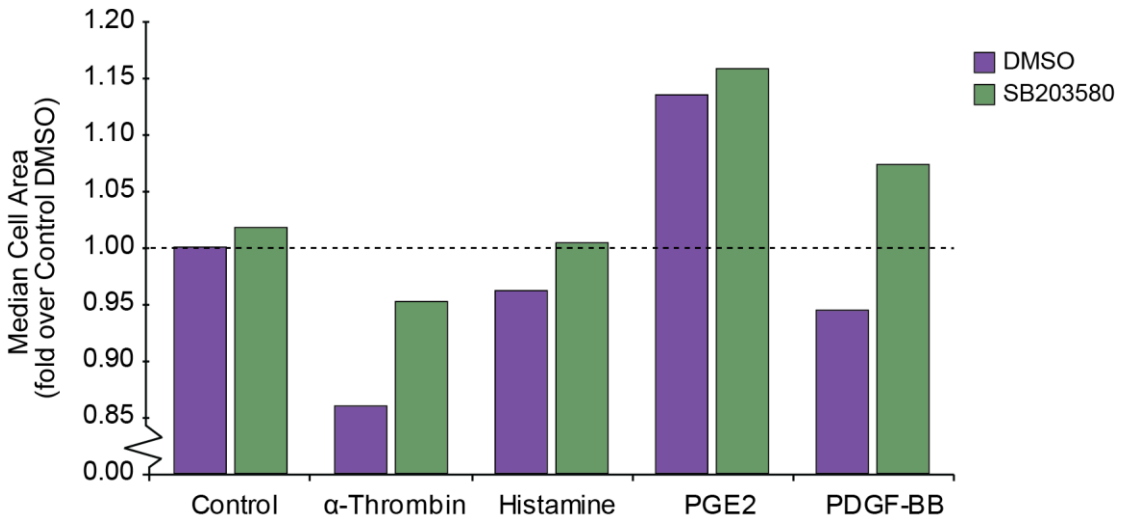

Figure 12: Morphological Analysis of Pericyte Cells in Response to Cytokine Stimulation

Pericyte morphology changed in response to stimulation with α-thrombin, histamine, PGE2, and PDGF-BB.

Pericytes were stimulated with 10nM α-thrombin, 1µM histamine, 10µM PGE2, or 200ng/mL PDGF-BB, after pretreatment with 10µM SB203580 or DMSO for two hours. 24 hours after stimulation, cells were fixed with 4% PFA and stained with cellular Fluorescein-5-Maleimide (FITC-Maleimide) and nuclear Hoechst. Morphological analysis was performed using an inverted Nikon Ti-S microscopy. Cellular and nuclear shape were quantified with CellProfiler, an automated software. Results were visualized as the fold change with respect to the DMSO control. (n=1)

GPCR-Induced Pericyte Migration

Pericyte cells have been shown to migrate away from the vascular wall during states of inflammation, which is both beneficial and detrimental [11, 269]. The pericytes do escape from injury, are able to guide angiogenesis, and help to restore neurological function, but at the same time, their detachment from vessels contributes to blood-brain barrier breakdown and prolonged vascular instability in the brain [26, 294]. More specifically, pericyte migration has been shown to leave blood vessels exposed and weak, vulnerable to the infiltration of unwanted immune cells and cytokines across the BBB and into the brain [23, 36, 242, 295]. The migration of pericyte cells is regulated by PDGFRβ/PDGF-B signaling and has also been shown in inflammatory models in response to factors such as TNFα, IL1β, and PGE2 [38, 73, 296-298]. It is not yet known what role atypical p38 signaling plays in pericyte migration and this is what we aim to investigate using a migration assay model.

The migratory properties of pericytes were observed via a wound healing assay (Fig. 13). A scratch was made in wells of adherent pericytes stained with CellTracker, a fluorescent dye, for visualization. Cells were stimulated with either 10μM α-thrombin, 40ng/mL PDGF-BB, or 10μM PGE2. Wound closure was observed using a Zeiss LSM800 microscope with widefield Colibri imaging and cell migration was quantified with ImageJ software. Over a period of 20 hours, there was a 4% closure of the wound area in the control sample in relation to its zero-hour time-point (Fig. 13). Conversely, α-thrombin induced migration of the pericytes resulting in a 58% wound closure compared to the α-thrombin zero-hour time-point. In addition, PGE2, with respect to its zero-hour time-point, stimulated a substantial 90% closure (Fig. 13). Furthermore, stimulation with PDGF-BB,

a known inducer of pericyte migration, resulted in a 100% closure of the gap area in relation to its zero-hour time-point (Fig. 13). On a very basic level, this 2-D model has preliminarily shown the migratory characteristics of the brain pericyte cells. Further studies will be performed to observe the wound healing role of pericytes in response to α-thrombin, PGE2, and PDGF-BB, and the other GPCR agonist, histamine, which was used in the experiments discussed previously in this text. In addition, we hope to further characterize the role of GPCR-mediated atypical p38 activation in pericytes and assess whether TAB1-p38α signaling can be selectively blocked to mediate pericyte migration. As PT5, the peptide inhibitor discussed above, has been shown to prevent the TAB1-p38α interaction and p38 activation through autophosphorylation in pericyte cells, this will be a useful tool to show the role of the atypical p38 pathway in inflammatory brain pericyte migration.

Figure 13.

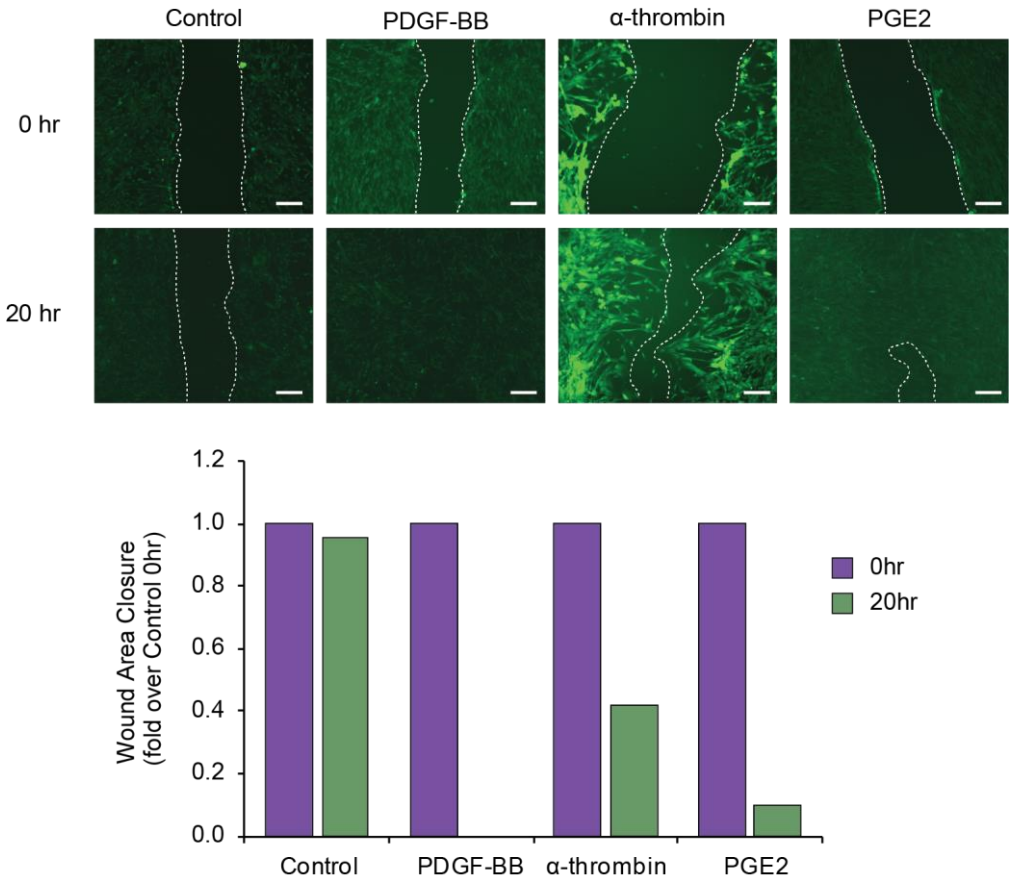

Figure 13: Pericyte Cell Migration in Response to Inflammatory Factors

PDGF-BB, α-thrombin, and PGE2 stimulated pericyte migration into the wound area.

Pericytes were dyed using 1μM CellTracker Green and a scratch wound was made in the cell layer. At the zero-hour time-point, cells were stimulated with 40ng/mL PDGF-BB, 10μM α-thrombin, or 10μM PGE2 and imaged via Zeiss LSM800 widefield Colibri microscopy. Cells were fixed at the 20-hour time-point using 4% PFA and imaged again using the same method. The area of wound closure was measured with ImageJ software and results were visualized as the fold change compared to the zero-hour time-point of each respective agonist. Bar indicates 200μm. (n=1)

Creation of Pericyte and Endothelial Cell Spheroids as a Model of Angiogenesis

Spheroids are a 3-D model that can be utilized to observe capillary sprouting or angiogenesis. This method is useful in that it is able to model a co-culture system of multiple different cell types to visualize the interactions, signaling crosstalk, and capillary investment of cells [299].

Pericytes serve to regulate vascular development through the promotion of angiogenesis [300-302]. This can be beneficial, for example in directing blood flow to promote tissue repair after damage [33]. Or harmful, such as in the case of vessel destabilization causing tumor formation and growth or perpetuation of chronic conditions through tissue damage and scarring [46, 260, 303]. Pericyte coverage of endothelial cells heavily influences the strength of effect seen with proangiogenic factors, such as TNFα, demonstrating the importance of these cells in regulating angiogenesis [304]. As pericytes play a role in inflammatory angiogenesis, the spheroid model is a useful tool that may be used to visualize the investment of pericyte cells onto endothelial cells and their response to GPCR inflammatory factors [112, 300]. These include agonists such as PGE2, α-thrombin, and histamine, all of which are known to act as proangiogenic molecules and which we have shown to function through the atypical p38 signaling pathway [165, 305-307].

Spheroids were formed as hanging drops using human umbilical vein endothelial cells (EC) and pericyte cells (PC) in a ratio of 3:1. After 24 hours, spheroids were imbedded into a collagen matrix and visualized with a Zeiss LSM800 microscope, using laser confocal microscopy. The protocol for formation, embedding, and imaging was designed and required optimization where proper embedding and sprouting have yet to

be achieved. Figure 14 shows a confocal Z-slice of the formed spheroid with the endothelial cells (HUVEC) in the first image, the pericyte cells (HBVP) in the second image, and merged in the third. This model provides the groundwork for future studies to observe sprouting *in vitro*, the interaction of pericytes and endothelial cells, and the pericyte response to proinflammatory factors. This method will also be beneficial to observe a subset of pericytes in which the atypical p38 pathway is inhibited mixed with the uninhibited pericytes and endothelial cells. Possible methods of preventing p38 atypical activation include knockdown of key components, TAB1 and TAB2, or the TAB1-p38α CPP inhibitor. After further repeats to provide more substantial evidence for inhibition of atypical p38 activation by PT5, and timeframe of stable expression, this avenue will be a promising pathway forward for use with the spheroid model. Although speculative, based on the evidence for atypical p38 signaling in brain pericytes that we have shown here and the role of p38 in inflammatory disease progression, it is probable that GPCR mediated TAB1-p38α inflammatory signaling in pericyte cells plays a role in the progression of neuroinflammatory conditions.

Figure 14.

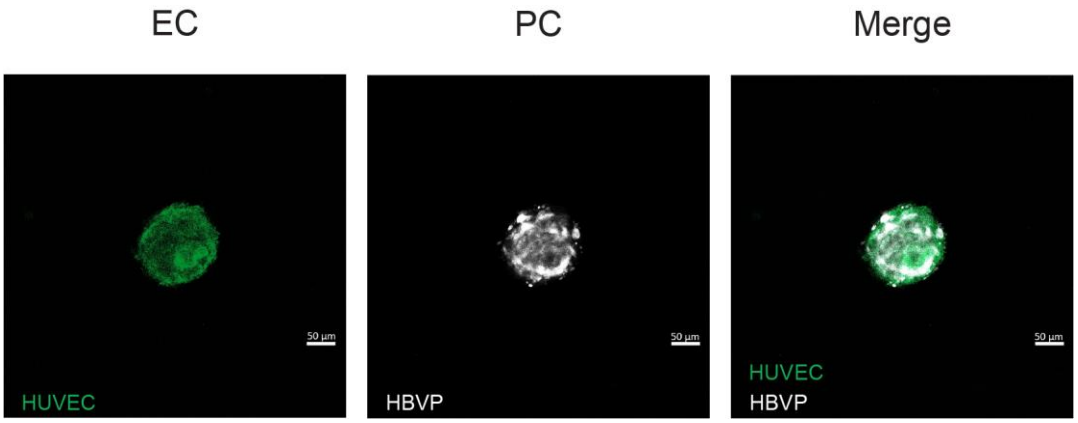

Figure 14: Pericyte and Endothelial Cell Co-Culture Spheroid Model

Human umbilical vein endothelial cells (HUVEC) and human brain vascular pericytes (HBVP) were incubated, at a 3 to 1 ratio, as hanging drops to promote spheroid formation. HUVEC and HBVP were stained with different colors of CellTracker for visualization of the two cell types. Spheroids were embedded into a collagen matrix and imaged with Zeiss LSM800 confocal microscopy. Bar indicates 50µM. (n=1)

CHAPTER 3

METHODS

Cell Culture and Reagents

Details regarding reagents, including the company they were obtained from and the catalog numbers, are available in the *Reagents and Materials Table* proceeding the methods section. Primary human brain vascular pericyte cells (HBVP) purchased from ScienCell were cultured in complete pericyte medium (PM) according to the manufacturer's specifications. Primary human pulmonary artery endothelial cells (HPAEC) were purchased from ATCC and cultured with endothelial cell growth basal medium- 2 (EBM-2) in accordance with the manufacturer's guidelines. EA.hy926 cells, purchased from ATCC, were cultured using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) according to the specifications recommended by the manufacturer. Primary human pulmonary microvascular endothelial cells (HPMEC) were purchased from Sciencell and cultured in supplemented endothelial cell medium (ECM) in accordance with guidelines by the manufacturer. Primary human umbilical vein endothelial cells (HUVEC) were purchased from ScienCell and cultured in supplemented endothelial basal medium (EBM) -plus growth medium according to the manufacturer's instructions. All cells were maintained at 37°C and 5% CO_2 and used at low passage numbers (passage 6 or below). Starvation media for all cells was made of 500µL 50X Low Serum Growth Supplement (LSGS) in 25mL Medium 200. All experimental plates were pre-coated overnight with 2µg/cm^2 Poly-

L-Lysine Hydrobromide in PBS for pericyte cells or 5µg/cm^2 Collagen I in PBS for endothelial cells prior to seeding.

RNA Extraction and Real-Time Quantitative PCR (qPCR)

mRNA was extracted from HBVPs and HPAECs using Qiagen's RNeasy Plus Mini Kit. The quantity and quality of RNA was assessed with a DS-11+ Spectrophotometer. iScript cDNA Synthesis Kit was used to convert RNA to cDNA. cDNA synthesis was performed at 25°C for 5 minutes, 46°C for 20 minutes, and 95°C for 1 minute using a SimpliAmp Thermal Cycler. qPCR was performed on a QuantStudio 3 under standard conditions for the expression of PDGFRβ, CD34, and GAPDH.

Table 2: qPCR Primers

Primer	Sequence	Company
PDGFRβ-F	5'- ACG GAG AGT GTG AAT GAC CA -3'	Integrated DNA Technologies
PDGFRβ-R	5'- GAT GCA GCT CAG CAA ATT GT -3'	Integrated DNA Technologies
CD34-F	5'- ACC ACT AGC ACT AGC CTT GC	Integrated DNA Technologies
CD34-R	5'- CCA GCA GTA GAC ACT GAG GC	Integrated DNA Technologies
GAPDH-F	5'- ACA ACT TTG GTA TCG TGG AAG G -3'	Integrated DNA Technologies
GAPDH-R	5'- GCC ATC ACG CCA CAG TTT C -3'	Integrated DNA Technologies

Immunofluorescence

HBVPs and HPMECs were seeded onto either 2µg/cm^2 Poly-L-Lysine (PC) or 5µg/cm^2 collagen (EC) coated 18mm coverslips in 12-well plates. Cells were grown for three days and fixed with either 4% paraformaldehyde (PFA) in PBS for 10 minutes at

room temperature or 100% methanol for 30 seconds at room temperature. For intracellular staining, PFA fixed cells were permeabilized in the presence of 0.1% Triton-X in PBS. All samples were blocked with 1% Bovine Serum Albumin (BSA) and 22.52mg/mL glycine in 0.1% Tween-20 in PBS (PBST) for 30 minutes at room temperature. They were then incubated with primary antibodies diluted in 1% BSA in PBST overnight at 4°C in a dark humidified chamber. The next day, cells were stained with Alexa Fluor 488 as the secondary antibody diluted in 1% BSA in PBS for one hour in the dark at room temperature. Finally, the cell containing coverslips were mounted onto slides using ProLong Glass Antifade Mountant with NucBlue, left to dry overnight at room temperature protected from light, and stored at 4°C. Cells were visualized with a ZEISS LSM800 confocal microscope, using laser confocal or widefield Colibri imaging. Images for each respective antibody were taken at the same exposure time and magnitude for both cell types and scaled in the same proportion.

Signaling Assay

HBVPs were seeded in 2μg/cm^2 Poly-L-Lysine coated 24-well plates and grown to confluency. Cells were starved for two hours and treated with 10μM SB203580 or dimethyl sulfoxide (DMSO) for 20 min prior to stimulation with 10μM α-thrombin, 1μM histamine, 10μM PGE2, 25ng/mL TNF-α, or 40ng/mL PDGF-BB. Cells were lysed with 1x laemmli sample buffer (LSB) containing 100mM DTT and stored at -80°C.

Cell Transfection

Human brain vascular pericytes were seeded onto 24-well plates and after one day, transfected with 50nM-200nM TAB1, TAB2, or non-specific control siRNA using 1-2μL TransIT-X2 System, Lipofectamine 2000, or Oligofectamine 2000 per each manufacturer's instructions. Media was changed after 8-24 hours. 72 hours after transfection, cells were lysed using 1x LSB containing 100nM DTT and stored at -80°C.

Table 3: siRNA Sequences

siRNA	Sequence	Company
Non-specific (ns)	5'- CUA CGU CCA GGA GCG CAC C -3'	Qiagen
TAB1	5'- CGG CUA UGA UGG CAA CCG ATT -3'	Qiagen
TAB2	5'- GUC AAU AGC CAG ACC UUA ATT -3'	Qiagen

Immunoblot Protein Analysis

Cell lysates were loaded at 10μL per well into a 10% 0.75mm gel, resolved by SDS-PAGE, and transferred to Immobilon-P PVDF membranes. Membranes were blocked using 5% milk in 0.1% Tween-20 in Tris-Buffered Saline (TBST) and incubated overnight at 4°C with primary antibodies in 1% BSA + 0.02% sodium azide (NaN_3) in TBST. Membranes were incubated at room temperature for one hour the subsequent day with secondary antibodies in 5% milk in TBST. Antibodies used and their respective concentrations are listed in Table 4. Membranes were developed with chemiluminescence on a ChemiDoc Imaging System and quantified using ImageJ software.

Morphological Profiling

HBVPs were seeded onto 2µg/cm^2 Poly-D-Lysine coated 96-well plates and grown for 24 hours. Cells were pre-treated with 10µM SB203580 or DMSO for two hours and then stimulated with 10nM α-thrombin, 1µM Histamine, 10µM PGE2, or 200ng/mL PDGF-BB. Following 24 hours of stimulation, cells were fixed with 4% PFA for 15 minutes and stained with cellular Fluorescein-5-Maleimide (FITC-Maleimide) and nuclear Hoechst. Imaging and analysis were performed as described in Klinker et al. [291]. Briefly, single cells were imaged using an inverted Nikon Ti-S microscope and their cellular and nuclear shape were quantified via an automated software, CellProfiler [308].

Wound Healing Assay

Human brain vascular pericytes were seeded onto 2µg/cm$_2$ Poly-L-Lysine coated ibiTreat µ-slide 8-well plates. Cells were grown to confluency, starved for one hour, and incubated with 1µM CellTracker Green for 30 minutes. A scratch was made using a P10 pipette tip and cells were stimulated with either 10µM α-thrombin, 10µM PGE2, or 40ng/mL PDGF-BB. Plates were imaged at time = 0 using ZEISS LSM800 widefield Colibri microscopy and incubated at 37°C and 5% CO_2. After 20 hours, cells were fixed with 4% PFA for 10 minutes and imaged using the same method.

Co-Culture Spheroid Model

Spheroids were formed and embedded based on a method modified from Tetzlaff, F. and Fischer, A. (2018) [309]. HUVECs labeled with 1µM Celltracker Red and HBVPs labeled with 1µM Celltracker Green were mixed 3:1 in 25% methylcellulose and incubated

as hanging drops for 24 hours. Spheroids containing 600 endothelial cells and 200 pericyte cells were formed. Spheroids were embedded in 3mg/mL Rat Tail Collagen I supplemented with Medium 199 and 10% FBS in µ-slide angiogenesis plates and incubated for 30 minutes at 37°C. Embedded spheroids were observed using ZEISS LSM800 widefield Colibri and laser confocal microscopy.

Microscopy, Statistical Analysis, and Figures

Microscope visualization for all experiments except for morphological analysis was performed using widefield Colibri and laser confocal microscopy with a ZEISS LSM800 Confocal Microscope. Morphological profiling was performed using a Nikon Eclipse Ti-Series (Ti-S) Inverted Microscope. All data was analyzed with Microsoft Excel or JMP and results were expressed as the mean ± SD. Statistical analysis was performed via GraphPad Prism using two-way analysis of variance (ANOVA). Figures were made using Adobe Illustrator software.

Table 4: Immunoblot (IB) and Immunofluorescence (IF) Antibodies

Antibody	Dilution for IB	Secondary Dilution for IB	Dilution for IF	Secondary Dilution for IF	Species	Company
PDGFRβ	1:10,000	1:2,000	1:100	1:500	Rabbit	Abcam
Nestin	1:500	1:1,000	1:200	1:500	Rabbit	Abcam
NG2	1:2,000	1:2,000	1:100	1:500	Rabbit	Abcam
α-SMA	1:1,000	1:2,000	1:200	1:500	Rabbit	Abcam
CD31	1:5,000	1:2,000	1:100	1:500	Rabbit	Abcam
CD146	---	---	1:100	1:500	Mouse	Abcam
GAPDH	1:20,000	1:4,000	---	---	Mouse	GenTex
P38	1:20,000	1:4,000	---	---	Rabbit	Cell Signaling Technology
Phospho-p38	1:20,000	1:4,000	---	---	Rabbit	Cell Signaling Technology
TAB1	1:2,000	1:4,000	---	---	Rabbit	Cell Signaling Technology
TAB2	1:2,000	1:4,000	---	---	Rabbit	Cell Signaling Technology
ERK	1:1,000	1:4,000	---	---	Rabbit	Cell Signaling Technology
Phospho-ERK	1:1,000	1:4,000	---	---	Rabbit	Cell Signaling Technology
MK2	1:1,000	1:4,000	---	---	Rabbit	Cell Signaling Technology
Phospho-MK2	1:400	1:1,000	---	---	Rabbit	Cell Signaling Technology
MSK1	1:400	1:1,000	---	---	Rabbit	Cell Signaling Technology
Phospho-MSK1	1:400	1:1,000	---	---	Rabbit	Cell Signaling Technology

Table 5: Reagents and Materials

Product	Company	Catalog Number
Human Brain Vascular Pericytes	Sciencell	1200
Complete Pericyte Medium	Sciencell	1201
Human Pulmonary Artery Endothelial Cells	ATCC	PCS-100-022
Endothelial Basal Medium-2	Lonza	00190860
EA.hy926	ATCC	CRL-2922
Dulbecco's Modified Eagle's Medium	Corning	10-017-CV
Fetal Bovine Serum	Corning	45000-734
Human Pulmonary Microvascular Endothelial Cells	Sciencell	3000
Endothelial Cell Medium	ScienCell	1001
Human Umbilical Vein Endothelial Cells	Sciencell	8000
EBM Plus Medium	Lonza	CC-5036
Low Serum Growth Supplement	Thermo Fisher Scientific	S00310
Medium 200	Thermo Fisher Scientific	M200500
Poly-L-Lysine Hydrobromide	Thermo Fisher Scientific	ICN19454405
Phosphate Buffered Saline	Corning	21-040-CMX12
Rat Tail Collagen I	Enzo Life Sciences	ALX-522-435-0020
RNeasy Plus Mini Kit	Qiagen	74134
DS-11+ Spectrophometer	DeNovix	-----
iScript cDNA Synthesis Kit	Bio-Rad	1708890
SimpliAmp Thermal Cycler	Thermo Fisher Scientific	A24811
QuantStudio 3	Thermo Fisher Scientific	A28567
18mm coverslips	Carolina	633033
12 well plate	Thermo Fisher Scientific	12-556-005
32% Paraformaldehyde	Electron Microscope Sciences	15714
Methanol	Avantor Sciences	9093
Triton-X	Sigma-Aldrich	T9284
Bovine Serum Albumin	Thermo Fisher Scientific	BP1600-100
Glycine	Thermo Fisher Scientific	BP381-5
Tween 20	VWR	97062-332
Alexa Fluor 488 Goat anti-Rabbit	Thermo Fisher Scientific	A-11008

Alexa Fluor 488 Goat anti-Rabbit	Thermo Fisher Scientific	A-11001
ProLong Glass Antifade Mountant with NucBlue	Thermo Fisher Scientific	P36981
24 well plate	Thermo Fisher Scientific	12-556-006
SB203580	Cayman Chemical	13067
Dimethyl sulfoxide	Thermo Fisher Scientific	D128
α-Thrombin	Enzyme Research Laboratories	HT 1002a
Histamine	Tocris Bioscience	3545
Prostaglandin E2	Thermo Fisher Scientific	22-961-0
PDGF-BB	Shenandoah Biotechnology	10787-436
TNF-α	BioVision	1050-50
TransIT-X2 System	Mirus	MIR 6004
Immobilon-P PVDF Membrane	Millipore Sigma	IPVH00010
Non-Fat Milk Powder	Anthony's Goods	-----
NaN_3	Sigma-Aldrich	S2002
ChemiDoc Imaging System	Bio-Rad	-----
µ-slide 8 well plate	Ibidi	80826
CellTracker Green	Thermo Fisher Scientific	C7025
CellTracker Red	Thermo Fisher Scientific	C34552
FITC-maleimde	Thermo Fisher Scientific	-----
Hoechst	Sigma-Aldrich	-----
Methylcellulose	Sigma-Aldrich	M0512
Rat Tail Collagen I	Corning	47743-656
Medium 199	Thermo Fisher Scientific	11043-023
µ-slide angiogenesis plate	Ibidi	81501
LSM800 Microscope	ZEISS	-----
Eclipse Ti-Series Inverted Microscope	Nikon	-----

CHAPTER 4

DISCUSSION

The diverse and critical role of pericytes in vascular homeostasis and their control of inflammatory signaling is very well known [11, 23, 26, 36, 310-312]. However, the intrinsic plasticity of pericytes with their capacity to adapt their morphology, protein expression profile, and function make them challenging to study in an *in vitro* setting [59, 64, 65, 67, 68, 70, 155, 313]. In this research, our central goal was to establish that a TAB1-mediated atypical p38 signaling pathway, which has previously been characterized in endothelial cells, is conserved in human brain pericytes. The central hypothesis being that GPCR-mediated atypical p38 signaling is an understudied critical driver of neurovascular inflammation.

Our initial focus was on defining the expression profile of our pool of human brain pericytes. Using the three different detection methods of qPCR, immunoblotting, and immunofluorescence, one of which requires further optimization, we confirmed the expression of PDGFRβ, Nestin, NG2, α-SMA, and CD146, known pericyte markers, and a lack of endothelial cell markers, CD31 and CD34, in the cultured brain pericyte cells (Figures 3-5). These experiments confirmed the notion that our pericytes display properties similar to their *in vivo* counterparts, effectively classifying them as pericyte-like cells.

The results of our studies confirmed pericyte expression of markers that pertain to other functionally integral cell types in the brain, providing evidence for the wide range of

roles performed by pericytes in the neurovascular unit and why they are a pertinent target for understanding and treating neuroinflammatory disorders. For example, as the majority of neural stem cells express Nestin and expression has been linked to proangiogenic properties in progenitor cells, pericytes staining positive for Nestin could confer pro-regenerative and pro-angiogenic potential to pericyte cells as well [46, 68, 95, 97]. In addition, the fact that some of the pericytes express α-SMA shows the importance of a subset of pericytes in the contractility of blood vessels and control of blood flow [33, 49, 314]. The wide array of markers that pericytes express has been vastly studied, but as research into the functionality of pericytes grows and their expression profiles for each task or physiological location increase, the array of marker proteins will have to be adapted for specific conditions, including the understudied role of pericytes in the regulation of neuroinflammation.

Of particular interest to our group is a subset of GPCR inflammatory signaling that has been shown to activate p38 via an atypical TAB1-p38α signaling transduction pathway. As many GPCRs have the ability to regulate vascular permeability and are pertinent mediators of cytokine expression, their role in p38 signaling is an important, and until recently, understudied, phenomenon [38, 191, 315]. In particular, although GPCR mediated atypical p38 signaling has been characterized in endothelial cells to induce vascular permeability, its implications in neuroinflammation, and specifically in pericyte cells, remain unknown [122, 196, 197]. Through the use of SB203580, an inhibitor of p38 MAPK, we uncovered a novel phenomenon, that p38 can be activated via the atypical signaling pathway in brain pericyte cells (Figures 6-7). This activation is mediated through the GPCR agonists α-thrombin, histamine, and PGE2. TNF-α, a proinflammatory cytokine

that is known to activate p38 via the typical MKK3/6-driven signaling and not the atypical TAB1 mediated pathway, served to substantiate these results [202, 203, 275] (Figure 8). In these studies, we also observed PDGF-BB activation of p38, which we demonstrated is via a mechanism that requires p38 autophosphorylation (Figure 8). However, further research to determine the specific mechanism behind this activation is required. If PDGF-BB activates p38 through TAB1-TAB2 dependent binding, this suggests an avenue for pericyte signaling and migration that has yet to be researched and another implication for TAB1-p38α specific therapeutic development.

The activation of p38 via the atypical signaling mechanism in pericytes shown in the SB203580 experiments was corroborated through the use of a cell-penetrating peptide inhibitor that specifically blocks the direct binding of TAB1 to p38α (Figures 10-11). Substantial reduction in phosphorylated p38 and confirmation that the cell penetrating peptide does not block ERK1/2 signaling provides support for its continued use as a specific inhibitor compound. In addition, the peptide also prevented p38 specific activation of critical downstream substrate kinases, MSK1 and MK2. These data support our hypothesis that pericyte cells exhibit GPCR-mediated proinflammatory atypical p38 signaling (Figure 11). The results shown also support the use of PT5 as an inhibitor of atypical p38 activation in future experiments to fully assess the role of pericyte TAB1-p38α signaling in neuroinflammation.

Our confirmation of TAB1-mediated activation of p38α signaling in pericytes invites the idea that this pathway may be a determinant for the inflammatory signaling modulation, migration, and angiogenesis displayed by pericytes in response to a proinflammatory environment, i.e. the damage that is characteristic of neuroinflammatory

conditions such as ischemic stroke, traumatic brain injury, Parkinson's, and Alzheimer's [11, 23, 34, 290].

Characterization of pericyte morphology and classification of the different subtypes that form in response to inflammatory factors is a novel method to determine the distinct groups of pericytes that are important in cytokine regulation, T-cell modulation, and other potentially therapeutically targetable functions. Preliminary experiments displayed a difference in pericyte size depending on pretreatment with SB205380 and stimulation with α-thrombin, histamine, PGE2, or PDGF-BB (Figure 12). In addition to determining the role of atypical p38 on pericyte morphological change, future inflammatory morphological profiling of individual pericyte cells will also provide a novel mechanism to define functional characteristics depending on what type of damage, cytokine stimulation, and inflammatory environment are present.

In response to inflammatory factors, such as α-thrombin, LPS, PDGF-BB, and TNF-α, brain pericytes have been shown to secrete increased levels of various cytokines, MMPs, interleukins, and chemokine ligands, effectively amplifying a cytokine storm [17, 55, 59, 60]. The continual release of these proinflammatory factors perpetuates a detrimental chronic inflammatory environment in the brain. P38 MAPK in pericytes has been shown to activate downstream pathways and promote the release of factors such as IL-1β, IL-6, IFNγ, and TNF-α, but it remains to be known what cytokines are released by pericytes in response to activation of atypical p38 [316, 317]. Future studies using an *in vitro* cytokine array assay (Ray Biosciences), where the pericytes are stimulated with α-thrombin, histamine, or PGE2 in the presence or absence of the TAB1-p38α inhibitor, will reveal the inflammatory profile of the cells with and without the ability of p38 atypical

signaling to occur. We predict that MKK3/6 independent p38 signaling will be a novel target to prevent critical cytokine release by brain pericytes, potentially helping to restore healthy function to a chronic inflammatory state.

In addition to secretion of inflammatory factors, pericyte migration in states of neuroinflammation also contributes to blood-brain barrier breakdown and a prolonged, or chronic, dysregulation of the neurovascular unit [11, 40, 318]. Preliminary evidence of pericyte migration in response to the GPCR agonists, α-thrombin and PGE2, and growth factor, PDGF-BB, was shown through the use of a wound healing model (Figure 13). As PGE2, and possibly PDGF-BB, activate TAB1-p38α inflammatory signaling, and p38 in pericytes is linked to migration, we hypothesize that brain pericyte migration induced by these agonists is likely to be mediated through the atypical p38 pathway [240, 242]. Further studies, with the use of our PT5 cell-penetrating peptide inhibitor, are necessary to define the specific role and extent of GPCR-induced atypical p38 signaling in pericyte migration.

In conjunction to migration, pericytes also serve to regulate angiogenesis, which is primarily beneficial in acute inflammation and increasingly harmful once inflammation progresses to chronic levels [300, 304]. We have successfully created a co-culture spheroid model of pericytes and endothelial cells (Figure 14). Future studies will observe the investment of pericytes onto endothelial cell tubules and the response of the pericytes to a proinflammatory environment. In addition, the role of GPCR-mediated atypical p38 signaling on neuroinflammatory angiogenesis remains to be determined in further research using the peptide inhibitor or gene depletion studies.

The use of the cytokine array, migration assay, and angiogenesis model will allow us to determine not only how the atypical p38 inflammatory signaling response mediates pericyte function, such as migration, cytokine release, and investment onto endothelial tubules, but also whether blocking the TAB1-p38α interaction restores any facets of healthy functioning. In addition, through future studies, we aim to able to determine whether the TAB1-p38α interaction is a potential therapeutic target to treat neuroinflammation. A path forward to corroborate the *in vitro* results displayed in Figures 3-14, involves *in vivo* murine studies.

Multiple studies have shown evidence for the importance of TAB1-mediated atypical p38 signaling *in vivo* [122, 194, 206, 207, 210]. Of particular prominence, in 2018, De Nicola et al. identified key binding sites required for the TAB1-p38α interaction [194]. Mutation of those binding residues on TAB1 produced a significant lack of atypical activation *in vitro* and a mouse model was subsequently created with the same mutations [194]. These knock-in mutations prevented the interaction of TAB1 and p38, but did not alter the binding properties of TAB1 with other substrates [194]. Our lab is in the initial stages of developing and testing this same mouse model system with the TAB1 knock-in mutation. Intended to function as a disease model for vascular inflammation, the transgenic mouse line may also be utilized to observe pericyte behavior when atypical p38 signaling is prevented. Histological analysis of brain sections and isolation of pericytes from the brain of this TAB1 knock-in mutation model can be used to corroborate the results seen in the SB203580 and CPP-inhibitor experiments. Brain pericytes isolated from the mouse model may also be used for the wound healing and spheroid assays mentioned previously in order to observe the role of atypical p38 signaling in pericyte

migration and angiogenesis. In addition, once a comprehensive profile of the morphological subtypes of pericytes has been created, we can use this assay to observe the morphological classification and changes of pericytes with the TAB1 knock-in mutation. Furthermore, through immunohistochemistry performed on brain slices from the mice, the spatial relationship of pericytes and other cells in the neurovascular unit and their response to cytokine stimulation may also be observed [319].

CHAPTER 5

CONCLUSIONS

Our findings reveal in human brain pericyte cells that p38 inflammatory signaling can be activated through a TAB1-dependent pathway in response to stimulation by the GPCR agonists α-thrombin, histamine, and PGE2. GPCR-mediated atypical p38 signaling has been shown previously in endothelial cells to cause barrier disruption, but this is the first time that activation of p38 through this atypical pathway, rather than the traditional three-tiered kinase cascade, has been shown in pericytes [122, 196, 197]. In addition, preliminary results revealed morphological subpopulations of pericyte cells based on stimulation with α-thrombin, histamine, PGE2, and PDGF-BB.

Furthermore, a pilot study which displayed pericyte migration in response to α-thrombin, PGE2, and PDGF-BB, and the creation of an angiogenesis model, provide a basis for future studies on pericyte cells. This includes their response to proinflammatory factors, interaction with endothelial cells, and role in vessel sprouting. These models, in addition to repeats of the preliminary experiments, will enable us to further research in a more in-depth manner the function of atypical p38 signaling in pericyte cells and role in regulating neuroinflammation.

The TAB1-p38α interaction has been observed *in vitro* and *in vivo* to play a role in conditions such as cardiovascular ischemia, diabetes, cancers, and others associated with chronic vascular inflammation (see Table 1 for a complete list of all the pathological conditions shown to be associated with TAB1-mediated p38 activation) [122, 206-208,

210]. Our novel findings, in corroboration with the known influence of p38 in inflammation, suggests that atypical p38 signaling in pericyte cells may play a role in neuroinflammation as well. More specifically, on immune cell regulation, blood brain-barrier disruption and angiogenesis as pericytes are known for exacerbating these phenomena in neuroinflammatory conditions such as Alzheimer's, brain ischemia, traumatic brain injury, and stroke [11, 241, 318, 320, 321]. In addition, with further studies, the TAB1-p38α interaction may serve as a therapeutic target to selectively block a portion of inflammation in the brain, leaving physiological p38 signaling intact.

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