EXTRACELLULAR VESICLE DELIVERY OF AN INHIBITORY PEPTIDE TO BLOCK VASCULAR INFLAMMATION

by

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(Under the Direction of Neil Grimsey)

ABSTRACT

GPCRs activate atypical p38 signaling to drive vascular inflammation. Recent studies have highlighted atypical p38 signaling as an attractive therapeutic target for a range of pathological signaling. However, current approaches to block atypical p38 signaling are limited to a single cell-penetrating peptide inhibitor (PT5). Our hypothesis is that we can generate a stable cell line to produce extracellular vesicles (EVs) preloaded with monomeric Red fluorescent Protein conjugated PT5 (mRFP-PT5) as an alternative approach for inhibitor delivery to cells. We successfully generated an endothelial cell line that produces mRFP labelled PT5 packaged into EVs. We were able to isolate PT5 loaded EVs, which could block p38 atypical signaling not only in EV producing cells, but in recipient endothelial cells as well. These data provide evidence that EVs are suitable vehicles for delivering engineered biomolecules intracellularly to block atypical p38 signaling.

INDEX WORDS: GPCR, MAPK, atypical p38 signaling, extracellular vesicles, PT5 inhibitor, Inflammation

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CHAPTER 1

INTRODUCTION

Overview of Inflammation

Inflammation is an innate and adaptive response to noxious stimuli to restore homeostasis in the body. Typically, the inflammatory response activates the immune system to eliminate infections agents and repair tissues. Once the issue is resolved, the injured area ceases production of prostaglandins (pro-inflammatory molecules) and activates production of lipoxins (anti-inflammatories). Immune cell infiltration slows, and the tissue begins to heal. If the initial acute inflammatory response is not sufficient to eliminate the pathogen, the situation increases in intensity. Macrophages are signaled to the area of interest and more cytokines are released to act on the nearby endothelial cells. Signaling molecules like IL-6 act on the endothelial cells to induce atherogenesis [1]. Interestingly, IL-6 serves a dual role as an anti-inflammatory signal in immune cells expressing the IL-6 receptor. The purpose of an inflammatory response is to remove the source of homeostatic disturbance, allow the host to adapt in the case of adaptive inflammation, and restore functionality. If abnormal conditions are sustained, the inflammatory insult may enter a chronic state [2].

Pathology of Chronic Inflammation

Chronic inflammatory responses are involved in a variety of disease states such as obesity, type 2 diabetes, asthma, and neurodegenerative diseases. As stated

previously, an enhanced inflammatory state begins when the initial response is insufficient and higher degree of immune response is needed. The situation becomes chronic when homeostatic controls are overridden. Changing the natural homeostasis of the body causes chronic pathological states [2]. Physiologically, homeostatic systems rely on fixed set points for variables that need to be regulated such as blood oxygen concentration [3]. Fixed set points do not yield chronic disease and are not adaptable. Some homeostatic systems in the body employ variable or adjustable set points to efficiently deal with numerous situations. Researchers have referred to this as adaptive homeostasis [4]. One example of this being body temperature [5]. At basal conditions, the body maintains a body temperature of about 37°C but during fever, the body temperature is raised to 38°C or higher because the set point for body temperature is adjusted in some disease states. There is a similar switch point with inflammation. When it is changed to the chronic state, it becomes detrimental to the body. In the case of type II diabetes, an acute inflammatory response causes the initial resistance to insulin [6], but a low grade chronic inflammatory state caused by risk factors such as obesity worsens the insulin resistance. This creates a vicious cycle as insulin resistance leads to inflammation and inflammation weakens insulin's effects. Clearly, the mechanisms for switching the homeostatic set point for inflammation need to be understood to effectively treat and understand diseases with chronic inflammation.

Role of endothelial cells in inflammation

Inflammation affects many types of cells and tissues, but endothelial cells, lining the lumen of blood vessels, are the first point of contact for circulating inflammatory

modulators [7]. In response to inflammatory cytokines like tumor necrosis factor α (TNFα), endothelial cells express adhesion proteins like intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM1) [8]. Through ICAM1 and VCAM-1 [9] and disassembly of the microtubule network, [10] vascular permeability is enhanced [11] to encourage T cell adhesion and infiltration of the inflamed tissue. Endothelial cells play important roles in controlling the inflammatory response by recruiting leukocytes through expression of selectin ligands, ICAM1 and VCAM-1 [12]. Platelet alpha-granule membrane protein (GMP-140) and platelet activating factor (PAF) on endothelium facilitate trans-migration of immune cells in heightened inflammatory states [13, 14]. These proteins allow for leukocyte adhesion to the endothelium, diapedesis and subsequent transendothelial migration. In circumstances where the existing blood vessels are not enough to handle the demand for more nutrients and immune cells, endothelial cells undergo the process of blood vessel growth, angiogenesis [15]. Angiogenesis can crowd tissues with immature blood vessels which contributes to diseases like acute lung injury (ALI) [10, 16]. In the lungs, hypoxic conditions cause vascular remodeling changes that alter the circulation in the lungs [17]. This mechanism of vascular remodeling has a role in a variety of disease states from cancer to ischemia/reperfusion injury [15].

Endothelial cells express many cell-surface receptors that enable rapid responsivity to soluble inflammatory cytokines in the blood plasma. Arguably, one of the largest families is the G protein-coupled receptors (GPCRs). GPCRs are a diverse class of cell surface receptors denoted by seven transmembrane subunits [18]. They are responsible for regulating a wide array of cellular and physiological functions from sensory

information such as sight and taste, to neuronal synaptic transmission. They accomplish this through coupling to heterotrimeric G-proteins and through G-protein independent mechanisms as observed in protease activated receptor 1 (PAR1) activation by activated protein C (APC) [19, 20]. The G-protein independent mechanism is mediated by β-arrestin-2 which controls endothelial barrier protection, contrary to the more rapid thrombin induced heterotrimeric G protein signaling that mediates disruption of the endothelial barrier [21]. Furthermore, prostaglandin E2 receptor 2 (EP2) and 4 (EP4) [22, 23], histamine receptor 1 (H1) [24] and P2Y1 purinergic receptor [25] also display multiple signaling pathways, G-protein dependent and independent, that regulate vascular homeostasis and barrier disruption. The full functional range of GPCRs is still not completely understood, but GPCRs have important signaling roles across all tissues and cell types.

P38 MAPK Signaling in inflammation

GPCR mediated activation of classical and atypical signaling cascades are critical for the amplification and robust inflammatory responses by endothelial cells. Of the key signaling proteins downstream of pro-inflammatory GPCR activation, the p38 mitogen activated protein kinase (p38 MAPK) stands out. MAPK signaling includes a large group of kinases such as c-Jun activated kinase (JNK) and extracellular signal-related kinase 1 and 2 (ERK1/2) [26]. MAP kinases have both physiological and pathophysiological roles which in turn, can create difficulties with selective therapeutic targeting. The differential activation and signal transduction by MAPKs appears to be in part regulated by binding to specific scaffold proteins [26-29]. Where scaffolding proteins residing in different

subcellular locations may assist in the spatiotemporal activation of MAPKs, for example osmotic stress induces the formation of a complex including Rac GTpase, osmosensing scaffold for MEKK3 (OSM), MEKK3, and MKK3 for specific activation of p38 [27]. P38 MAPK is typically activated by a three-tiered kinase cascade resulting in mitogen activated protein kinase kinase 3 and 6 to phosphorylate and activate p38 by phosphorylating threonine residues on the activation loop [28] (figure 1A). This pathway is crucial to acute inflammation however, a research group recently elucidated an atypical p38 activation mechanism that is closely associated with pathological chronic inflammation [29]. GPCR ubiquitination drives p38α activation through an atypical mechanism utilizing transforming growth factor β activated kinase 1 binding protein 1 (TAB1) and 2 (TAB2) to form a signaling complex at endosomal structures to enhance vascular inflammation. The interaction of TAB1 with p38 facilitates a conformational change that allows p38 to autophosphorylate (figure 1B). This unique method of p38 activation and spatial sequestration at the endosome is predicted to selectively modulate the downstream signaling of atypical p38 activation, selectively amplifying pathological chronic inflammatory signaling. This unique p38-TAB1 interaction is a promising, selective therapeutic target for pathological p38 signaling.

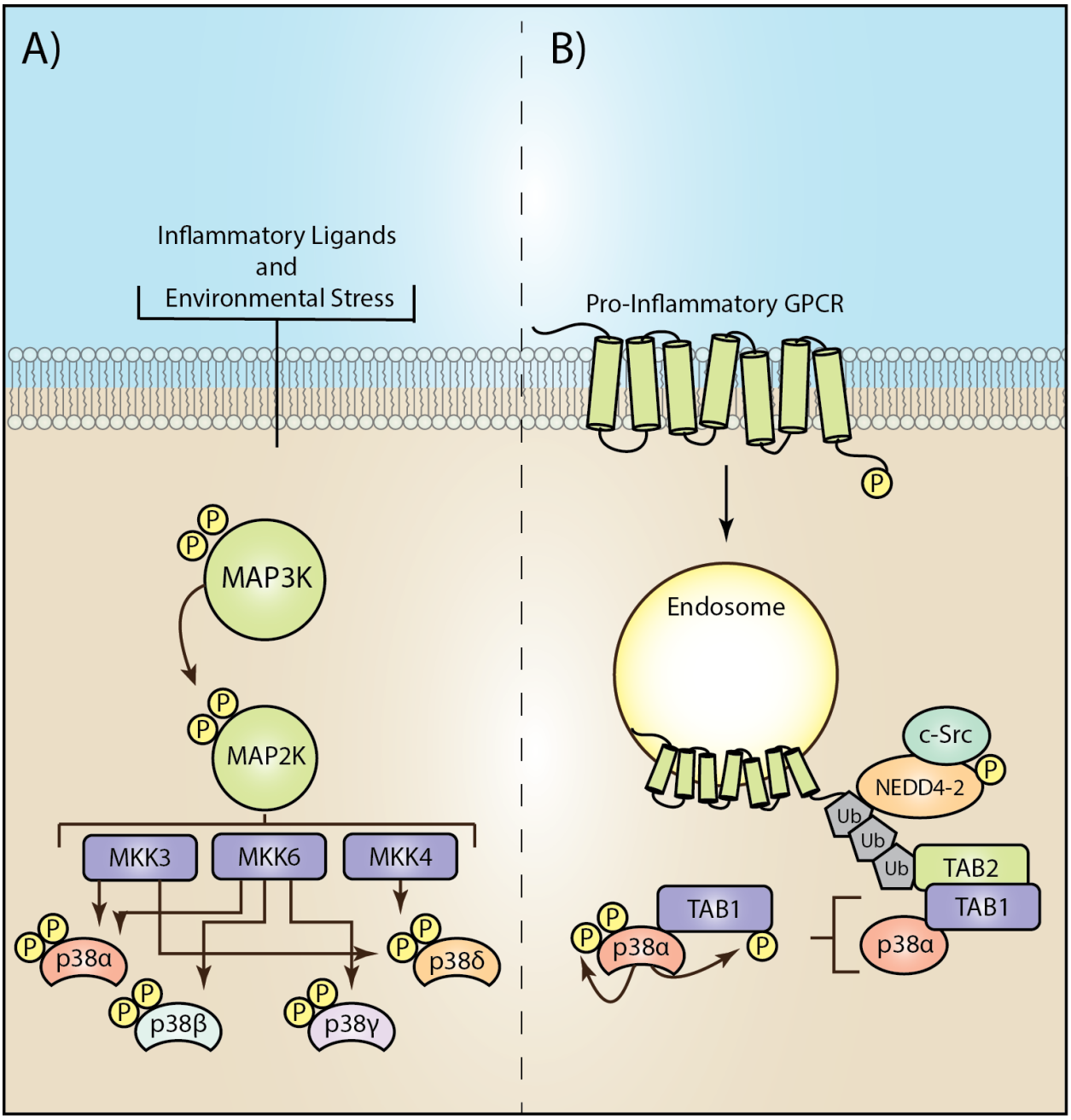


Figure 1: P38 MAPK is activated by **A.** stressors that lead to a three-tiered kinase cascade resulting in phosphorylation of all p38 isoforms. **B.** In an atypical mechanism, GPCR trafficking creates the complex for TAB1 and p38α to interact resulting in p38α autophosphorylation.

Atypical p38 Signaling in Disease

While understudied, there is a growing body of evidence to support atypical p38 signaling in the progression of vascular inflammation and edema [29-31], cardiac ischemic damage [32-34], amyloidosis [35], dermal inflammation [36], and viral infections [37]. Multiple studies have described the role of atypical p38 activation in ischemia/reperfusion injury after myocardial infarction [34, 38, 39]. In mice deficient in MKK3, TAB1 dependent p38 activation is the primary reason for cardiomyocyte necrosis [40]. Additionally, in zebrafish models, amyloid light-chain amyloidosis was found to direct atypical p38 signaling to impair cardiac function and edema [35, 41]. Atypical p38 activation has also been attributed to dermal inflammation. Itch ligase deficient mice showed enhanced expression of TAB1 and subsequent p38 autophosphorylation increasing inflammatory cytokine expression and skin lesions [36]. Atypical p38 signaling resulting in vascular inflammation is thought to be driven by GPCRs. Alpha thrombin is known to activate the PAR1 GPCR found in endothelial cells enhancing the permeability of the endothelial barrier. Interestingly, pretreating endothelial cells with the inhibitor of the ATP binding domain on p38 (SB203580) inhibited the alpha thrombin induced barrier permeability indicating the role of p38 autophosphorylation in inflammatory phenotypes [29].

Activation of p38 MAPK is a major contributor to multiple steps of chronic inflammation such as excessive cytokine expression and immune cell recruitment as shown in diseases like psoriasis and rheumatoid arthritis [42-44]. Furthermore, in the lungs, p38 and chronic inflammation contributes to chronic obstructive pulmonary disease (COPD) [45, 46] and Acute Lung Injury (ALI) [47-49]. While p38 linked neuroinflammation contributes to the progression of neurodegenerative diseases such as Alzheimer’s [50].

Additionally, p38 MAPK signaling in microglia and astrocytes help accelerate tau phosphorylation [51-53]. In each of these disease states GPCR ligands are produced and predicted to contribute to disease progression. It is therefore hypothesized that atypical p38 signaling contributes to a wider array of pathological signaling than currently characterized.

Discovery of an inhibitor peptide blocking the p38-TAB1 interaction

P38 MAPK is an attractive target for therapeutics due to its implication in a multitude of disease states. However, all p38 inhibitors up until now have failed because lack of efficacy and toxicity. One example of these inhibitors, Losmapimod, directly inhibits p38 MAPK and was terminated early in the trial because of low probability of success for effecting COPD outcomes [54]. Interestingly, due to the predicted role of p38 in the regulation of vascular inflammation in the progression of SARS-CoV-2, Losmapimod is in a phase 3 trial for treatment of SARS-CoV-2. Another inhibitor, BMS-582949, was found to not reduce arterial inflammation when compared to statin controls [55]. These inhibitors and others, such as SB203580, target the ATP binding pocket of p38 to prevent kinase activity which is conserved in all p38 isoforms. Therefore, these inhibitors block both physiological and pathological signaling. However, they are useful for inhibiting acute p38 inflammatory signaling and p38 signaling in vitro. Now, small molecules are being investigated to target the specific TAB1-p38 interaction through in silico drug design [34, 56]. As a potential alternative therapeutic strategy, atypical p38 signaling pathways can be selectively targeted. To enable therapeutic targeting, the crystal structure of TAB1 interaction with p38α was characterized to determine the key

interaction domains and residues on both proteins. The two critical residues on p38α are Thr218 which forms interactions with Ser399 and Ser401 on TAB1 and Ile275 which helps stabilize the Thr218 site on p38 [32]. From there, novel peptide inhibitors targeted to sites either at the hydrophobic docking groove for TAB1 on p38, Ile275, or Thr218 were developed. From these studies, the PT5 inhibitor (which targets Thr218) showed the best inhibitory affect and reduced ischemic damage in mice [32]. A separate study with the PT5 inhibitor discovered its capability to suppresses chronic dermal inflammation in the E3 ubiquitin ligase itch deficient mice [36]. In this study the authors discovered that basal TAB1 expression is controlled by Itch mediated ubiquitination of TAB1 which induced TAB1 degradation. In the absence of Itch, TAB1 expression is substantially increased, driving excessive TAB1 dependent atypical p38 signaling. Localized intradermal injections of the PT5 inhibitor blocked TAB1-p38 interaction and suppressed TAB1 dependent p38 activation. PT5 treatment blocked cytokine production and leukocyte recruitment protecting the mice from sever dermal inflammation [36].

Drawbacks to peptide-based drugs

Peptide drugs have recently emerged as promising therapeutics due to their selectivity and safety [57]. Peptides have been previously approved for therapeutic use and still more are in clinical trials. Most of these peptide drugs are designed to mimic natural peptides such as hormones, growth factors, and antibiotics. The approved drug, Giapreza, mimics Angiotensin II to increase blood pressure in patients with vasodilatory shock [58]. Another approved peptide drug, semaglutide, is modified from the glucagon-like peptide 1 (GLP-1) to improve its half-life [59]. One of the largest problems with peptide

therapeutics is their *in vivo* instability which may be combatted through glycosylation that prevents proteolysis [60], cyclization, and PEGylation [61]. Another limitation of peptide drugs is their membrane permeability. Peptides and polypeptide chains, while still relatively small on the biological scale, are much larger than small molecule drugs which further enhances the difficulty of these drugs to cross lipid membranes to reach intracellular targets. Researchers have attempted to combat this by using cell-penetrating peptide motifs from viruses such as HIV [62] and SARS-CoV-2 [63]. Attaching these motifs to peptides facilitates their entry into a cell. However, cell-penetrating peptides struggle to be effective *in vivo* due to their bioavailability, nonspecific cell absorbance and target selectivity [64]

A less expensive, more reproducible system of peptide synthesis uses animals and cells as bioreactors [65]. Bioreactors are used to increase the cost efficiency of commonly used biologics such as insulin [66]. Hijacking the systems of DNA transcription and translation is a well-known technique used for small scale preclinical research and large-scale drug development. It can be accomplished with bacterial plasmids that are transfected into mammalian cells to transiently express a desired protein or viral vectors such as a lentivirus that insert the engineered DNA into the genome of the host cell to create a stable cell line. Viral vectors also have enhanced transfection efficiency compared to bacterial plasmid vectors and are great for smaller DNA fragments which makes them great candidates for stable production of recombinant peptides.

To overcome some of the challenges of peptide drugs, protective carriers were developed synthetically, imitating biological structures, and hijacking biological systems. Nanoparticles encompass a wide field of nanoscale particles from 1-100nm in diameter

and include micelles, dendrimers, viruses, liposomes, polymers, and some extracellular vesicles. These nanostructures are currently being evaluated for their ability to protect volatile or sensitive drugs from early metabolism by enzymes and off target effects. Extracellular vesicles are a promising nanostructure because they are produced from living cells and have common biological markers such as major histocompatibility complex I which aid in T cell recognition [67].

Extracellular Vesicles role in physiology

Extracellular vesicles are phospholipid encapsulated nanoparticles released by cells naturally for paracrine communication. The term extracellular vesicles encompass a range of vesicles including microvesicles or ectosomes formed directly from the plasma membrane and exosomes which are internal vesicles stored within a multivesicular endosomal compartment (MVB). Once the MVB fuses with the plasma membrane, the internal vesicles (exosomes) are released [68]. Recently, exosome populations have been further characterized by large and small exosomes and nonmembraneous exomeres [69, 70].

Extracellular vesicles are natural complexes secreted by animal, plant, and some prokaryotic organisms [71]. They are found in almost all bodily fluids and are known to carry proteins, DNA, RNA, lipids, and other bioactive molecules to cells. Recent studies have shown that these nanoparticles can affect the phenotype of a recipient cell through the delivery of these bioactive payloads. EVs have demonstrated a variety of mechanisms to enter recipient cells such as direct membrane fusion [72], clathrin-mediated endocytosis [73, 74], caveola-mediated endocytosis [75], macropinocytosis [76], and

phagocytosis [73, 77]. In some cases, EVs have enriched protein levels when compared to their parental cell line which can be used as exosomal markers. Proteins like Flotillin-1, Heat shock protein 70, major histocompatibility complex class I and II, and tetraspanins like CD9, CD63, and CD81 are all enriched in EVs [68, 78]. EVs can be used to trigger a response by interacting with antigen presenting cells to activate T cells [79]. T cells also use extracellular vesicles to send genetic material to dendritic cells upon interaction [71]. Preliminary research demonstrates that EVs derived from different cell types have specific therapeutic or pathological qualities due to differential cargo loading [80]. For example, exosomes from mesenchymal stromal cells are known to promote regeneration and decrease inflammation of the endothelium cells in injured areas [81]. Whereas exosomes from dendritic cells (dex) are observed to mirror the functional role of a dendritic cell due to the expression of major histocompatibility complex which can be used to prime T cells against specific antigens. Dex have several advantages over dendritic cells: enrichment of MHC-II, high stability, higher dispersion in the body, and resistant to immunosuppressive molecules [82].

Extracellular vesicles in pathophysiology

Extracellular vesicles are fascinating vehicles for cell-to-cell communication and essential to immune responses through cross-talk with dendritic cells [77, 83], but pathological states can take advantage of the EV biogenesis machinery to advance disease. For example, tumor derived exosomes can carry microRNAs like miR-21-3p can modulate macrophages to become tumor protective [84]. Additionally, exosome production is upregulated in areas of low pH such as the tumor microenvironment [72]

which enhances the spread of tumor derived exosomes. Other studies have shown the role of extracellular vesicles of breast cancer as promoters of brain metastasis [85]. These extracellular vesicles can pass through the blood-brain barrier by delivering payloads that modulate the endocytosis pathway in brain endothelial cells. EVs then transcytose through endothelial cells to breach the blood-brain barrier and set up [85] the pre-metastatic environment potentially through interactions with low-density lipoprotein [86]. In lung inflammatory diseases, epithelial cells in hypoxic conditions secrete EVs that activate macrophages via the ROCK1 pathway to secrete proinflammatory cytokines IL-6 and TNFα [87]. However, EVs can also be used as therapeutic agents.

Therapeutic potential of Extracellular Vesicles

These nanoparticles are promising therapeutic vehicles due to their natural properties that enhance drug delivery. When compared with synthetic nanoparticles, EVs have decreased immunogenicity, enhanced specificity, and accumulate in the lungs, kidneys, and liver [88]. One research group successfully tested dendritic cell derived EVs on patients with non-small cell lung cancer and found that the therapy was tolerated, activated the immune system, and provided some stability to the disease progression [83]. Current research is focused on the therapeutic benefit of unmodified mesenchymal or loaded stem cell-derived extracellular vesicles. It is well known that mesenchymal stem cells have regenerative properties *in vivo* such as bone maintenance [89] and preventing thrombin induced increases in vascular permeability [90]. Through further research on how mesenchymal stem cells modulate their environment, researchers discovered EVs retain the same therapeutic potential of whole cell mesenchymal stem cell treatments.

But with the added benefit of reducing the risks associated with transplantation of stem cells into a patient [91, 92]. MSC EVs have shown to polarize macrophages to a less inflammatory phenotype [92] potentially through microRNAs [93], and are now the focus of MSC based therapeutics. While MSC EVs may have great regenerative and anti-inflammatory properties, less work has been done on genetic engineering of EV content to modulate inflammation with specific inhibitors of pro-inflammatory pathways.

Experimental Design

Clearly, p38 MAPK signaling is involved in a variety of inflammatory responses to external stimuli, but direct inhibition of the kinase's ATP binding pocket is lacking in efficacy, generates toxicities and the blockade of all p38 signaling leads to enhanced compensatory mechanisms. We hypothesize that atypical p38α signaling dependent on TAB1 selectively drives pathological p38 signaling while leaving physiological signaling intact. Based on prior studies, targeting the p38 atypical signaling pathway may be key to development of novel therapeutics for vascular inflammation. The PT5 inhibitor has been shown to selectively inhibit TAB1 interaction with p38 and attenuate phenotypes associated with inflammatory p38 signaling such as myocardial ischemic damage and dermal inflammation. However, peptide therapeutics generated without consideration for their disadvantages are destined to fail *in vivo* due to poor stability and issues crossing the plasma membrane. To overcome the challenges of peptide therapeutics, we hypothesize that the encapsulation of the PT5 inhibitor in extracellular vesicles will protect the peptide from degradation and facilitate its entry into recipient cells. Upon this foundation, a stable cell line producing PT5 in EVs will be generated using a lentiviral

vector. Then, EVs containing the PT5 inhibitor will be isolated and incubated with endothelial cells to block p38 atypical signaling in vitro. Generation of this pilot system opens the door to inexpensive, simple production of peptide therapeutics against chronic inflammatory diseases such as acute lung injury and chronic obstructive pulmonary disease.

CHAPTER 2

RESULTS

Inhibitor Peptide Design and Expression in EA.hy926 cells

Atypical p38 signaling is a major driver of cardiac damage in ischemia/reperfusion injury. To combat this pathological p38 signaling the Wang et. al group generated synthetic peptides designed to inhibit this pathway. Using glutathione-S-transferase pull down assays on *Escherichia coli (E. Coli)* derived TAB1 and p38, co-immunoprecipitations with Fc conjugated peptides in mammalian cells, and a check for inhibition of TAB1 and MKK3/6 mediated activation of p38, they determined which of their synthetic peptides was best for blocking the interaction of TAB1 and p38. After discovering PT5 as the most effective peptide sequence (QGQVVSNGKSTDEQS) it was ligated to a short sequence from the HIV Tat protein (48-58) [62] to allow the peptide to penetrate the cell membrane [32] . Furthermore, Wang et. al found that the cell-penetrating form of PT5 was able to decrease apoptosis of cardiomyocytes after ischemia/reperfusion injury. Unfortunately, it is expensive to generate synthetic cell-penetrating peptides and often difficult to reproduce. Additionally, peptides suffer from poor oral bioavailability due to difficulty crossing the epithelial barrier into the bloodstream [94]. Even peptides delivered intravenously are unstable in blood serum [95]. Peptide based therapeutics have huge potential but require future innovation to overcome their current setbacks.

As mentioned previously, extracellular vesicles (EVs) are promising vehicles for protection of sensitive biomolecules such as RNA and facilitate their delivery through the plasma membrane through a variety of different mechanisms as discussed in the in the introduction (extracellular vesicles role in physiology section). Recombinant proteins and peptides can be targeted to newly formed extracellular vesicles with a proprietary N-terminal Xpack targeting sequence (System Biosciences, LLC (SBI)), using a lentiviral vector to enable translation of a recombinant protein and packaged it into an EV. Keeping in mind that the EV origin cell can modulate cargo and surface markers it is important to choose a cell relevant to the disease model. The EA.hy926 cell line is derived from human umbilical veins and fused with A549 cells. As discussed in the role of endothelial cells in inflammations section of the introduction, endothelial cells are key regulators of the inflammatory response and dysregulation of endothelial cell inflammatory mechanisms leads to the chronic inflammatory response. For diseases such as ALI and COPD, the inflammatory response of endothelial cells is critical to disease progression, so PT5 inhibitor mediated attenuation of atypical p38 could be a potentially important therapeutic approach for these diseases.

To overcome the hurdles faced by CPP-PTs we set about developing an EV packaging approach. Using the Xpack lentiviral system, monomeric red fluorescent protein was ligated to the PT5 inhibitor peptide sequence (Figure 2A). The mRFP allows for visualization of peptide translation in PT5 EV producing cells and tracking EV uptake into recipient cells. The lentiviral particles were generated by transfecting HEK.293 cells with pPACKH1 for packaging and the XPack lentivector. We then isolated viral particles from the HEK.293 cell media using PEG-it™ Virus Precipitation Solution (SBI). We then

transduced EA.hy926 cells with the engineered lentivirus to stably incorporate X-pack-mRFP-PT5 into the genome. A puromycin resistance gene incorporated into the lentiviral vector ensures proper selection of transduced cells and PT5 producing EA.hy926 cells were cultured with 0.01 mg/mL puromycin to apply pressure to produce the lentiviral DNA. When expressed the X-pack encoded mRFP-PT5 will be tethered to the interior lipid bilayer of the extracellular vesicles (EVs) via conjugation to the X-pack peptide (Figure AB). Upon EV delivery to cells, we predict that the mRFP inhibitor peptide will bind to p38 around the Thr218 residue, preventing TAB1 from interacting with p38. (Figure 2B). EA.hy926 cells with and without addition of engineered lentivirus were seeded on coverslips, fixed, and imaged with fluorescent microscopy. EA.hy926 cells showed no red fluorescence (Figure 2C) when compared to EA.hy926 transduced with the engineered lentivirus (Figure 2D). This proves that endothelial cells transduced with the engineered lentivirus are stably producing the mRFP-PT5 Xpack constructs.

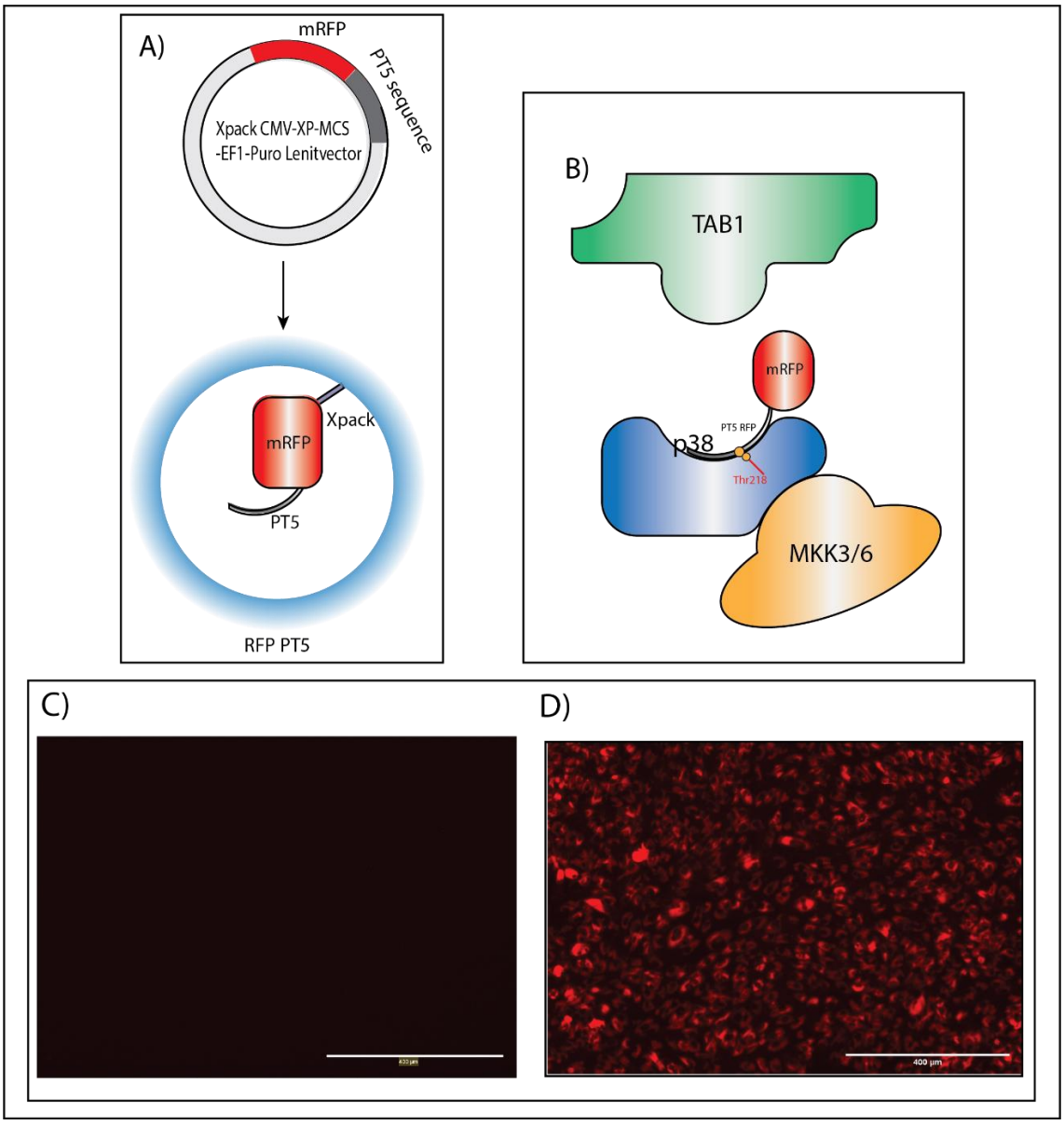


Figure 2. Lentivector Design and Transduction A. A schematic for the Xpack CMV-XP-MCS-EF1-Puro Lentivector from system biosciences, mRFP and PT5 were ligated at the MCS. Upon transcription and translation of this construct, RFP-PT5 will be tethered to the interior wall of extracellular vesicles via Xpack. **B.** A Schematic model showing that mRFP-PT5 will bind to p38 centered around Thr218 to block TAB1 interaction with p38. **C**. EA.hy926 wt and EA.hy926 cells expressing mRFP-PT5 were grown on coverslips, fixed and permeabilized with 4% PFA and mounted onto slides. Results were visualized via Zeiss LSM800 Colibri widefield microscopy. Bar indicates 400 µm for 10x (n=1)**.**

PT5 Inhibitor Peptide attenuates p38 atypical signaling in transformed EA.hy926 Cells

The PT5 inhibitor has previously been shown to block the TAB1 p38 interaction by binding proximal to the TAB1 binding site on p38 and suppressing inflammatory signaling [32, 36, 96]. In preliminary studies our group has also shown that a cell penetrating PT5 inhibitor can suppress GPCR induced p38 activation in primary human endothelial cells and pericyte cells (unpublished data). However, the PT5 inhibitor has never been ligated to RFP or encapsulated in an EV. Therefore, it is important to confirm the efficacy of the PT5 inhibitor with these new modifications. To do this, EA.hy926 cells and EA.hy926 cells transformed with the RFP PT5 engineered lentivirus were stimulated with prostaglandin E2 (PGE2) or adenosine diphosphate (ADP) to activate atypical p38 signaling. PGE2 activation of the prostaglandin E2 receptor 2 (EP2) and 4 (EP4) contributes to inflammatory phenotypes such as production of pathogenic helper T 17 cells [97] and advancing the tumor microenvironment in colorectal cancer [98], both receptors have been implicated p38 activation [23]. ADP activates the P2Y1 receptor on endothelial cells to promote migration [99].

Prior studies from our group have shown that both PGE2 and ADP utilize the TAB1-dependent atypical p38 activation pathway [100]. To examine whether atypical signaling is retained in our stable cell line, EA.hy926 wt and mRFP-PT5 cell lines were seeded at 140,000 cells per well in a 24 well plate and stimulated with 10 μm PGE2 or 10μM ADP. Both cell lines serum starved and stimulated for five, ten, and fifteen minutes in line with our previously published p38 activation profiles [29] and lysed for analysis by

immunoblotting. The immunoblots were probed with antibodies specific for total p38, phosphorylated p38, TAB1, and mRFP.

In the control cells, PGE2 stimulation induced p38 activation indicated by an increase in phosphorylated p38 3.5 fold (Figure 3A comparing lanes 1 and 3). Conversely, in the cells expressing PT5, p38 activated 2.8 fold lower than the control cells (Figure 3A comparing lanes 3 and 7). ADP stimulation produced similar results with control cells activating p38 1.5 fold above baseline and PT5 producing cells activating p38 1.4 fold less than the control (Figure 3C comparing lanes 3 and 7). Shown graphically in figure 2B and 2D and analyzed with 2-way ANOVA, PT5 producing cells significantly reduced p38 activation at 10 minutes of PGE2 stimulation and 5 minutes of ADP stimulation. TAB1 and total p38 are mostly unchanged confirming that only p38 phosphorylation is changing upon stimulation. We observe a clear reduction in PGE2 and ADP dependent signaling, however it is not known whether the mRFP-PT5 inhibitor is blocking p38 activity because of free PT5 that is in the cell during translation or whether our EA-PT5 cells are absorbing secreted mRFP-PT5-EVs. An additional concern is the generation of the stable cell line with the selection process may have made the cells less responsive to PGE2 and ADP compared to control cells. Further studies will be needed to assess this.

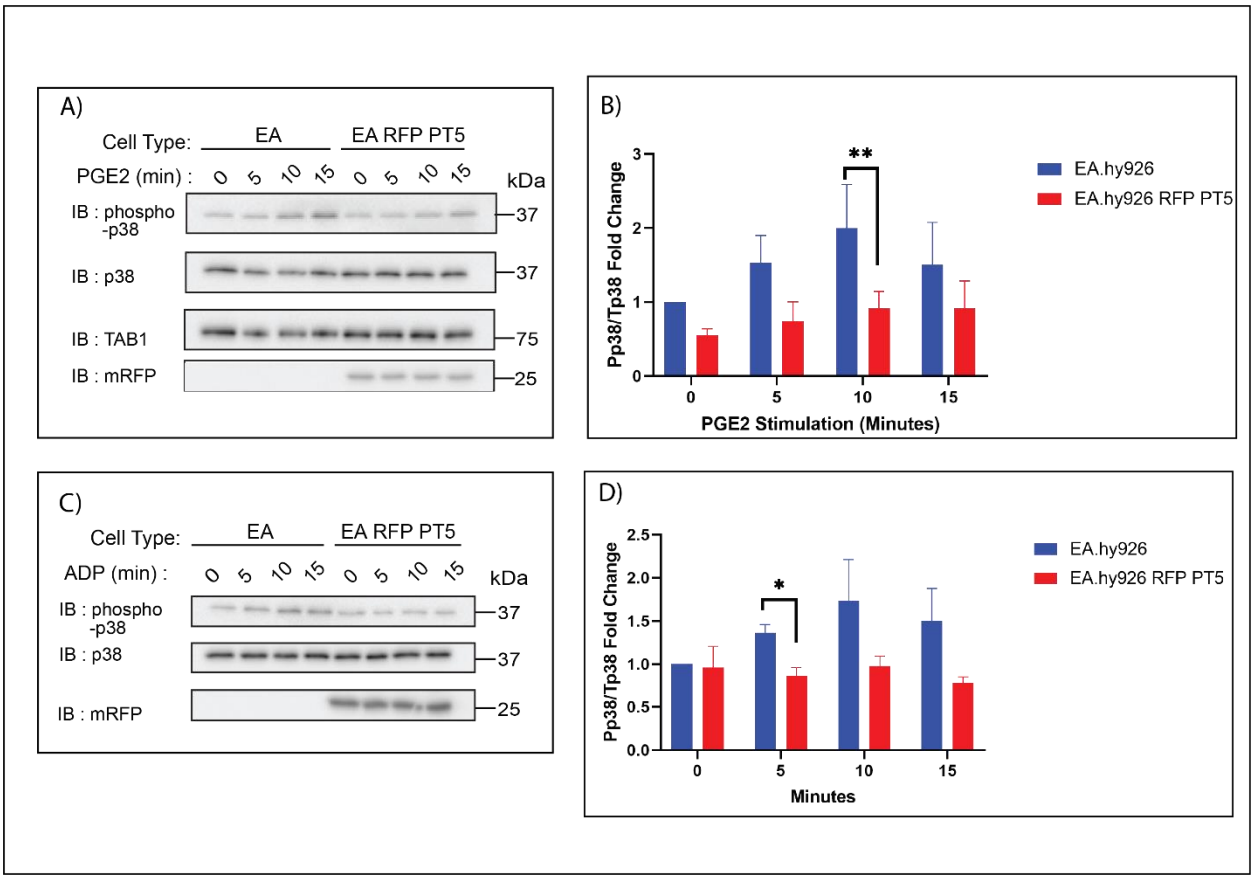


Figure 3. Impact of PT5 in EA.hy926 RFP-PT5 transduced cell line. EA.hy926 and EA.hy926 RFP PT5 producing cells were stimulated with **A.** 10 µM PGE2 or 10 µm ADP **B.** for 5, 10 and 15 minutes. Cells were lysed and equal amounts of lysate were immunoblotted (IB) with antibodies as indicated. **C. D.** Results were quantified as fold change of pp38/tp38 compared to EA.hy926 cells at the zero 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).

Extracted EVs fall within the nanometer scale and contain EV enriched proteins

Somatic mammalian cells *in vivo,* release many molecules into the extracellular space such as small neurotransmitters [101] and large oncosomes [102]. Cultured cells similarly secrete a variety of substances into the media to keep themselves healthy and living. EVs are a designation of these secreted structures than contain useful bioactive molecules and have a large size range. Small EVs, sometimes known as exosomes, are generally considered <200nm in diameter, while oncosomes can be as large as 10 μm in diameter [78]. Due to the large variety of secreted structures by living cells, it can be difficult to determine if an extracted product contains extracellular vesicles. Therefore, it is critical to examine isolated EVs to confirm.

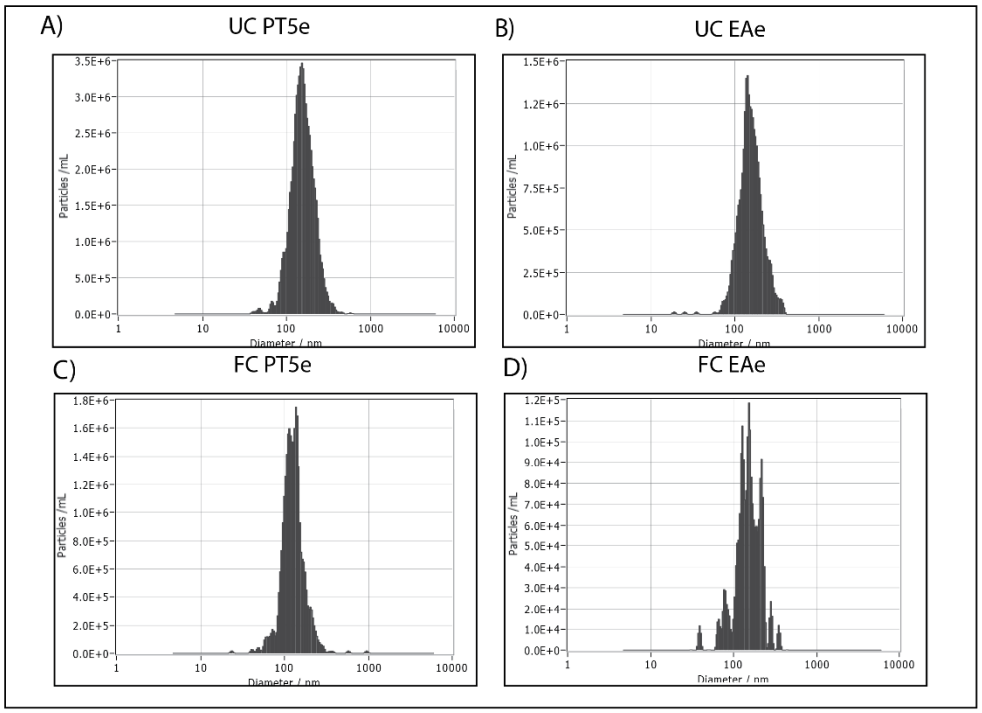
EVs were analyzed with dynamic light scattering and immunoblotting to determine size and composition. Size distribution of EVs was unimodal for ultracentrifugation isolated EVs (figure 4A and B) and multimodal for filter centrifugation isolated EVs (figure 4C and D). There is no difference between the size of PT5 containing EVs and control EA.hy926 EVs.

One preliminary way to check for extracellular vesicles is a size analysis. As previously mentioned, the small EVs fall within the 30-200nm diameter range. Therefore, if most of the isolated product falls within this range, it indicates the presence of EVs. To isolate EVs, cells were plated on 15cm dishes and allowed to grow until 60-70% confluent. The growth media was changed to serum free FBS DMEM for 48 hours. The media was then removed from the cells and either centrifuged at increasing speeds up to 25,000 RPM for ultracentrifugation [103] or up to 3280 RPM in Amicon® Ultra-15 Centrifugal

Filter Units for filter centrifugation. Purified EVs were then analyzed by dynamic light scattering (DLS) [104]. DLS analyzes the scattering of a laser beam to measure the Brownian motion of particles and the distance between scattered light to calculate a particle size range. DLS of our EVs found that the median size of isolated EVs was 150nm (Figure 4A-D) which is the upper end of the small EV scale. EVs isolated with both methods had similar median particle size and range (figure 4E), but ultracentrifugation isolated a higher yield of EVs per mL than filter centrifugation (figure 4E).

Size range alone is not enough to confirm or deny the presence of EVs. According to the minimal information for studies of extracellular vesicles 2018 (MISEV2018) [78], EVs need to be confirmed by the presence of at least 3 EV enriched proteins. As per the guidelines in MISEV2018, protein content cannot be used to differentiate classes of EVs such as exosomes. EV biogenesis results in the enrichment of many different types of membrane proteins and intracellular proteins such as tetraspanins [105] heat-shock proteins [106], and Alix [107]. EVs were probed for CD9, HSP70, Alix, and RFP with immunoblotting. Using the whole cell lysates as positive controls, all markers except RFP and HSP70 were found in the EA.hy926 EVs (Figure 5A) isolated with ultracentrifugation and all markers including RFP were found in EA.hy926 RFP-PT5 EVs (Figure 5B) isolated with ultracentrifugation.

To further assess the purified EVs, we labelled our EVs with Calcien AM. Calcein AM is metabolized to fluorescent Calcein by esterases in the plasma membrane. Labeled EVs were imaged using our Zeiss LSZ800 microscope and RFP and the green Calcien were detected in small aggregates. Suggesting that RFP is indeed present in the EVs (Figure 5C).



E)

EV Isolation Method and Cell Derivative	EV Concentration (particles/mL)	Median Size (nm)	Span (nm)	Mean Size (nm)
FC EA	1.5E10^8	149.5	55.0	157.8
UC EA	5.2E10^9	150.7	55.1	163.4
FC PT5	2.0E10^9	123.3	54.1	133.3
UC PT5	1.4E10^10	151.8	54.4	163.1

Figure 4. EV Size Characterization. Dynamic Light scattering of **A**. & **B.** ultracentrifugation (UC) isolated PT5 EVs and wt EA.hy926 EVs, respectively. **C. & D.** filter centrifugation (FC) isolated PT5 EVs, and wt EA.hy926 EVs. **E.** Particle concentration, median size, span, and mean size are displayed for each cell type and isolation method.

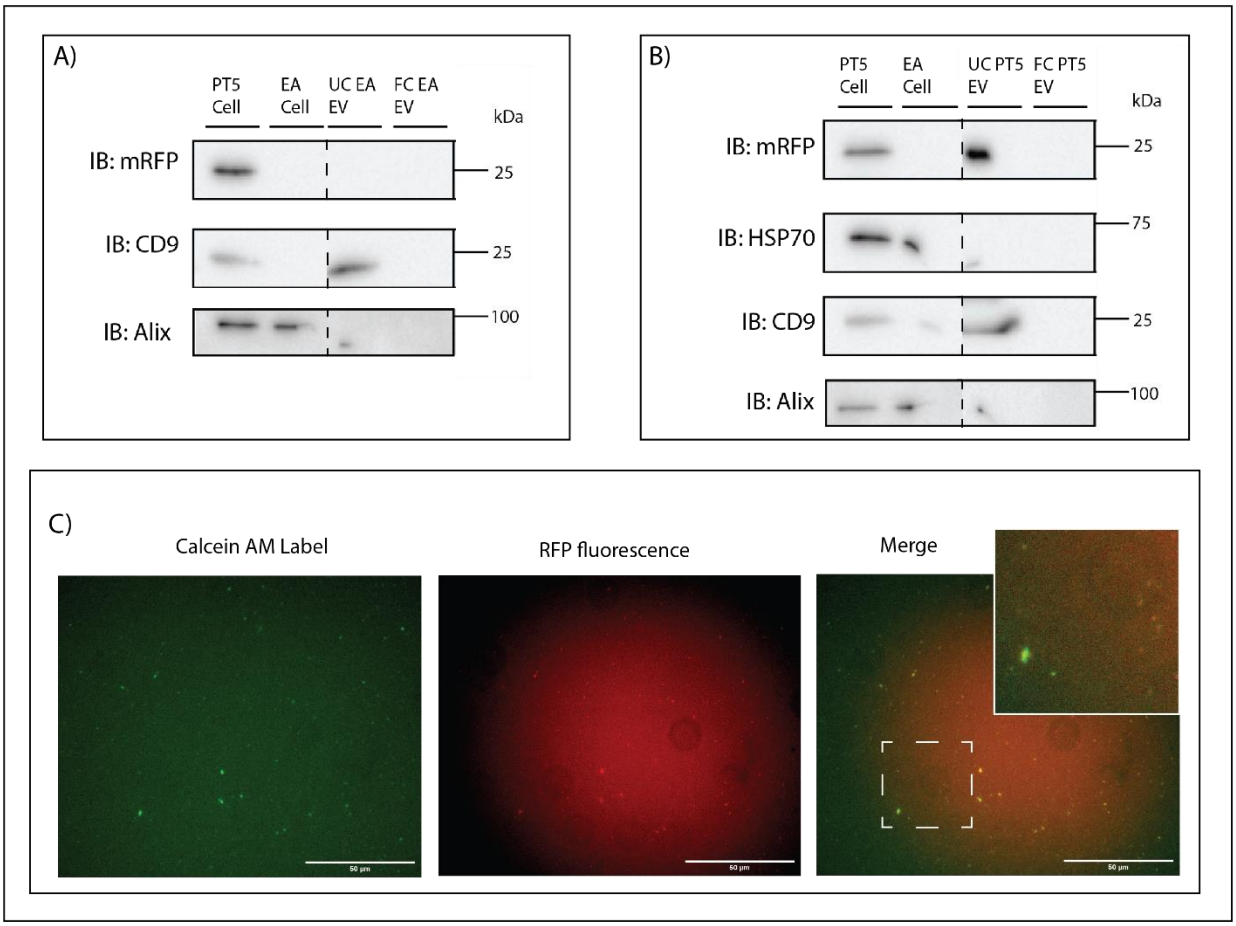


Figure 5. EV Composition and RFP Fluorescence. Immunoblotting of EA.hy926 RFP PT5 cells and wt EA.hy926 cells **A.** EA.hy926 EVs isolated with ultracentrifugation (UC) and filter centrifugation (FC) **B.** PT5-RFP Ea.hy926 EVs isolated with UC and FC. Approximately 500 µg of protein for FC samples and 1 µg of protein for UC samples were loaded. Blots were probed as indicated for EV markers CD9, HSP70, Alix, and mRFP. **C.** EVs stained with Calcein AM, mounted on coverslips, and visualized via Zeiss LSM800 Colibri widefield microscopy (Calcien AM, green, mRFP, Red). Bar indicates 50 µm for 63X (n=1).

EA.hy926 cells uptake RFP PT5 containing EVs

As we have just shown, EVs isolated from EA.hy926 cells and PT5 expressing EA.hy926 cells contain typical EV markers and PT5 containing EVs express RFP. However, for PT5 EVs to be an efficient delivery system they need to traverse the plasma membrane. There are a variety of mechanisms EVs deposit cargo into cells such as membrane fusion, clathrin-mediated endocytosis, phagocytosis, and macropinocytosis. The mechanisms EVs use to enter cells is almost as variable as EV heterogeneity [108]. In this study, the exact EV internalization mechanism was not explored but fluorescence microscopy was used to confirm the uptake of RFP containing EVs. EA.hy926 cells were plated into a μ-8 chamber slide. After reaching confluency, EVs were added and allowed to incubate for 24 hours. Cells were fixed with 4% Para-formaldehyde and mounted on slides using NucBlue containing ProLong Gold. EA.hy926 cells incubated with RFP PT5 containing EVs (Figure 6A) more showed disperse and punctate RFP in the cytosol compared to cells incubated with EVs without RFP (Figure 6B). Regions of interest (ROIs) were drawn around 15 cells and the average fluorescence intensity of RFP and DAPI was measured. EVs from mRFP-PT5 producing EA.hy926 cells isolated with ultracentrifugation had 1.5 fold more RFP/DAPI fluorescence when compared with EVs from EA.hy926 cells isolated with ultracentrifugation (Figure 6C). Filter centrifugation isolated EVs from mRFP-PT5 producing EA.hy926 cells had 1.8 fold higher fluorescence of RFP/DAPI compared to filter centrifugation isolated EA.hy926 EVs. This demonstrates that RFP containing EVs can transmit the RFP to recipient cells, but it is unknown what mechanism the EVs use to accomplish this. Additionally, filter centrifugation isolated EVs demonstrate RFP transmittance but is undetectable in the immunoblot of these EVs.

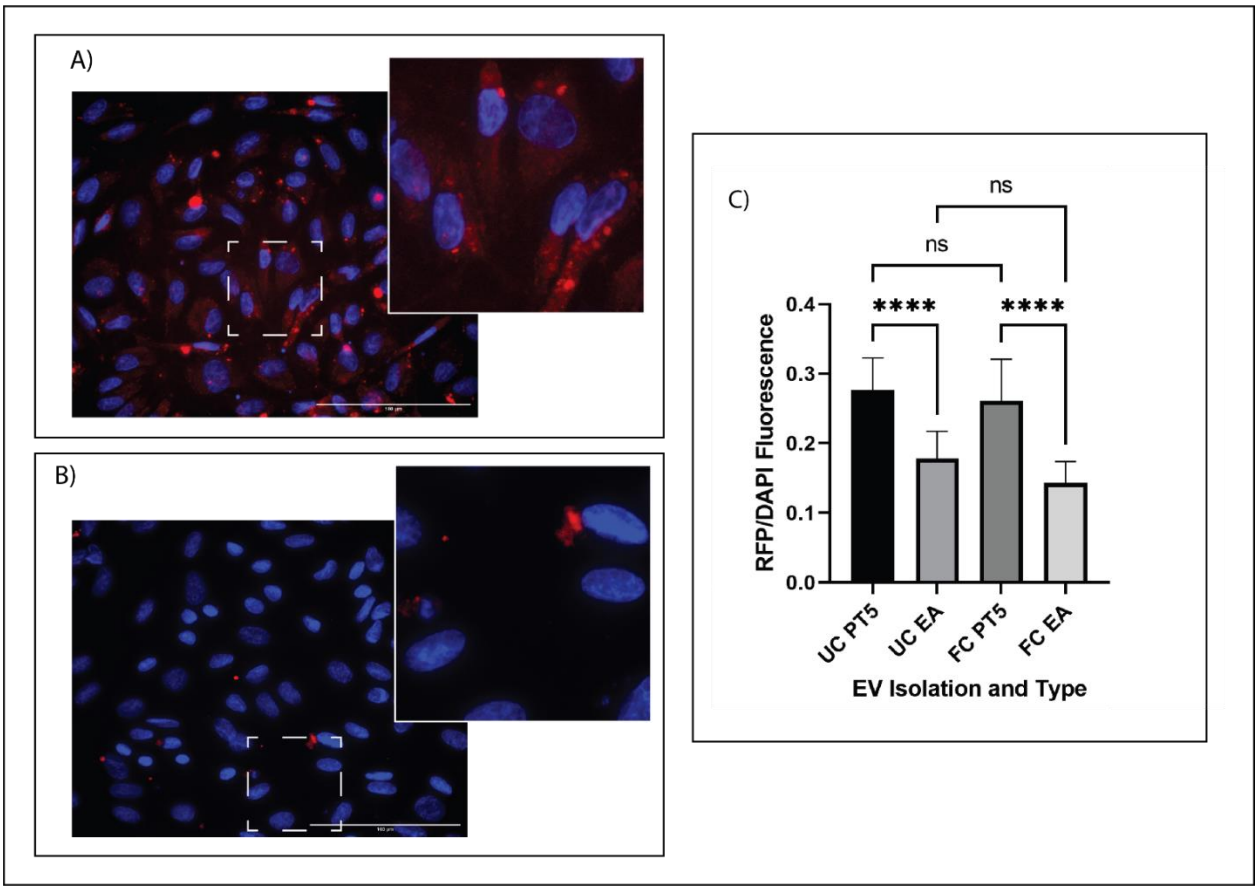


Figure 6. RFP Containing EVs Deposit Cargo in Recipient Cells. Fluorescence analysis of EA.hy926 cells incubated for 24 hours with UC RFP PT5 EVs reveals disperse RFP and punctate RFP within the cell. **A.** EA.hy926 cells were grown in an µ-chamber slide, incubated with RFP PT5 EVs or **B.** EA.hy926 EVs. Cells were fixed, permeabilized with 4% PFA and mounted with glass slips with DAPI stained nuclei (blue) visualized via Zeiss LSM800 Colibri widefield microscopy **C.** Results were quantified as RFP/DAPI fluorescence and expressed as mean ± SD. Statistical analysis was carried out by ANOVA test (n=15, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

EV packaged PT5 inhibitor peptide modulates p38 atypical signaling in recipient cells

Knowing that EVs containing RFP can transfer that RFP to recipient cells, it can be inferred that the PT5 inhibitor peptide is also transmitted to recipient cells and can affect p38 atypical signaling. Confirmation of attenuation of atypical p38 signaling after incubation with RFP PT5 containing EVs allows for further study into the effectiveness of this system in more complex *in vitro* models. Additionally, these tests can indicate the potential of this system to be developed into a therapeutic against atypical p38 associated diseases such as COPD and ARDS.

To study the impact of RFP PT5 containing EVs isolated by filter centrifugation on atypical p38 signaling, EA.hy926 cells were seeded in a 24 well plate and grown to confluency. Cells were treated with either 1000µg FC or 1 µg UC isolated EA.hy926 EVs or RFP PT5 EA.hy926 EVs for 24 hours and stimulated with prostaglandin E2. Signaling responses were analyzed by immunoblot and densitometry with ImageJ. EVs were initially tested with and without a media change before stimulating with PGE2. EA.hy926 EVs with and without media change demonstrate no change in phosphorylated p38 fold change expect for the 10 minute time point when the EVs with no media change had around a 0.8 fold higher activation than the EVs with the media change (figure 7A and B comparing lane 3 with lane 9). PT5 containing EVs showed a significant decrease in phosphorylated p38 at the 10 minute time point without a media change (figure 7A and B comparing lane 9 to 12). Recognizing that EVs isolated by ultracentrifugation had higher particle concentrations and confirmed protein content, we wanted to compare p38

activation in the presence of ultracentrifugation isolated EVs. In these preliminary experiments, filter centrifugation isolated PT5 containing EVs showed the highest blockade of p38 activation (figure 7C), but this data is only n=2 and needs further testing to confirm.

In combination, this data suggests that EVs can deliver functional PT5 to block PGE2 mediated p38 atypical signaling in endothelial cells. However additional tests are required to determine whether the PT5 EVs disrupt all MAPK signaling or only the p38 pathway in recipient cells.

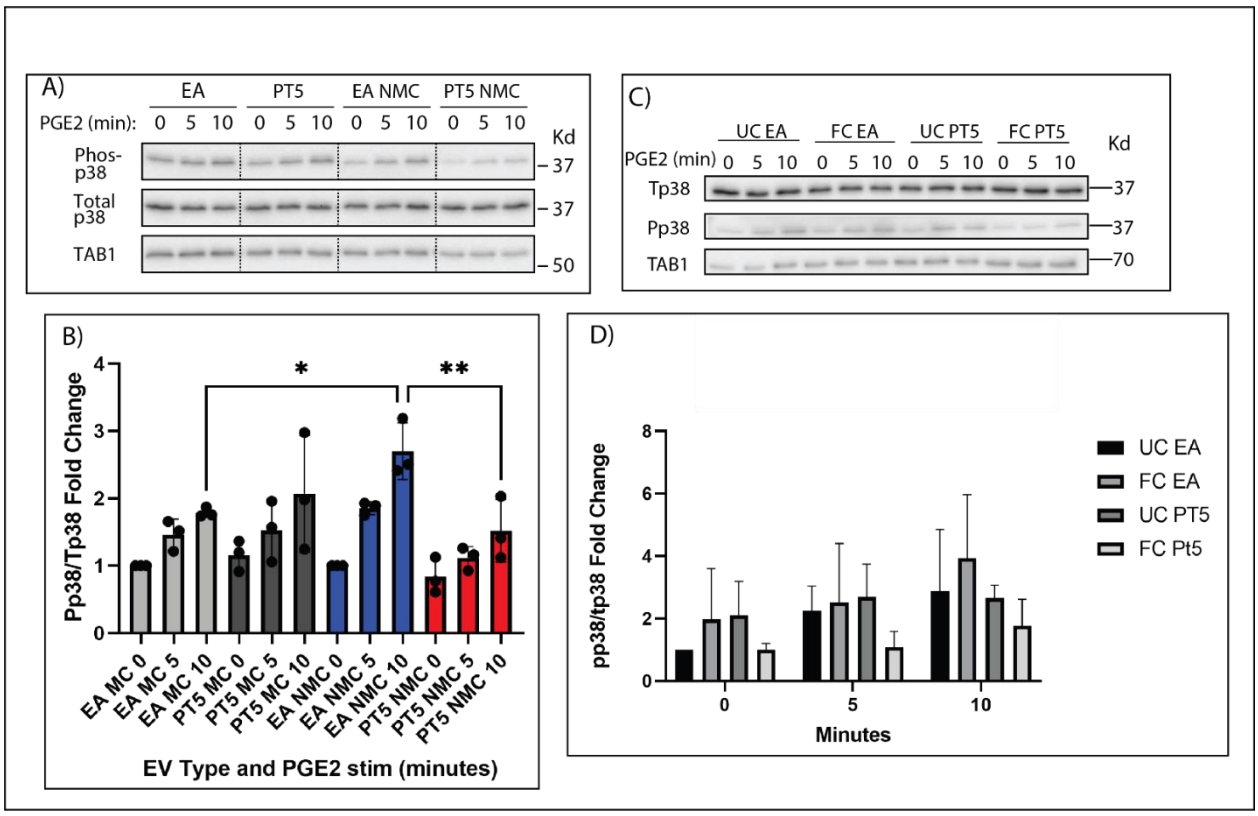


Figure 7. RFP PT5 Loaded EVs Affect p38 Atypical Signaling in Recipient Cells. EA.hy926 cells were incubated with FC EA or PT5 EVs with (MC) and without a media change (NMC) for 24 hours. **A.** Cells were stimulated with 10 μm PGE2 and lysed. Equal amounts were immunoblotted with antibodies as indicated. **B.** Results were quantified as fold change of pp38/tp38 compared to EA.hy926 cells at the zero 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). **C.** EA.hy926 cells were incubated with UC and FC EVs for 24 hours and stimulated with 10 μm PGE2. Cells were lysed, immunoblotted with antibodies as indicated **D.** quantified as described in **B.** (n=2).

CHAPTER 3

METHODS

Isolation of Extracellular Vesicles (EVs)

$1.4x10^6$ EA.hy926 and EA.hy926 stably expressing mRFP-PT5 cells were seeded in 100 mm dishes and grown to approximately 60% confluence ~48 hours. Cell growth media was harvested after 24 hr treatment of 20% Serum-free FBS DMEM. Culture media was passed through a 0.2 μm filter and isolated by filter centrifugation using Amicon® Ultra-15 Centrifugal Filter Units. Filters were rinsed with PBS before adding up to 15 mL of supernatant and centrifuging for 15 minutes at 4000g. The retentate was collected after washing the filter with PBS and centrifuging for 40 minutes at 2000g. EVs were quantified using a nanodrop on A280 and Fluorocet (SBI, LLC), aliquoted, and stored at -80℃.

For the Ultracentrifugation method of EV extraction, 3 million cells were seeded into 150mm culture dishes and grown to ~60% confluency. The media was replaced with 20% FBS (serum-free) DMEM and allowed to grow for 48 hours. The media was collected and stored at 4℃ overnight while the cells were given fresh 20% FBS (serum-free) DMEM for 24 hours. The collected media was centrifuged at 2000g for 20 minutes to remove pellet cells. The supernatant was centrifuged at 10,000g at 4℃ for 30 minutes to pellet cell debris. The supernatant was centrifuged at 100,000g at 4℃ for 70 minutes to pellet EVs. After a wash step, the EVs were resuspended in PBS, quantified using a nanodrop on A280 and Fluorocet (SBI, LLC), aliquoted, and stored at -80℃.

Particle Size Analysis

EVs were analyzed for size distribution and particle concentration with dynamic light scattering at 520 nm wavelength and Transmission-Election Microscopy in collaboration with Dr. Yutao Liu at Augusta University.

EVs were quantified by the enzymatic activity of acetylcholinesterase according to the SBI FluoroCet kit.

Cell Cultures

The immortalized human endothelial cell line (human umbilical vein endothelial cells fused with A549 cells) EA.hy926 (ATCC® CRL-2922™), were maintained in Dulbecco's Modified Eagle Media supplemented with 10% Fetal Bovine Serum. Embryonic kidney cells, HEK293 (ATCC® CRL-1573™), were also grown in Dulbecco's Modified Eagle Media supplemented with 10% FBS and used to produce viral particles.

The PT5 inhibitor producing cell line was created through transduction of EA.hy926 cells with engineered lentiviral particles. The EA.hy926 PT5 inhibitor-producing cells were initially grown with Dulbecco's Modified Eagle Media supplemented with 10% FBS and 0.1 mg/mL puromycin for selection. After selection, puromycin concentration was decreased to 0.01mg/mL. All cell lines were cultured at 37℃ in a humidified incubator of 5% CO2.

Immunoblotting

Cells were grown as described above and pretreated (as indicated) with 1µg-1 mg EA derived EVs or 1µg-1mg PT5 derived EVs for 24 hr, prior to stimulation with 10 µM

PGE2 or 10nM ADP. Cells were lysed in 1X Laemmli sample buffer with 100 mM DTT, sonicated and resolved by SDS-PAGE, transferred to PVDF membranes and incubated with specific antibodies as indicated. Membranes were developed by chemiluminescence and quantified by densitometry using NIH ImageJ software.

Immunofluorescence

Cells were grown as described above and seeded into an ibidi µ-slide 8 well chamber at a density of 79,100 cells per well. When cells reached confluency, the media was switched to 0.4% FBS DMEM for ~12 hours. Approximately 1 mg of EVs from the filter centrifugation method and 2 µg from the ultracentrifugation method of EV isolation were added to separate wells for 24 hours. The slide was fixed with 4% PFA in the dark and blocked with 1% BSA and 0.3% TX100 in PBS. Mouse-anti RFP antibody was diluted 1:500 and added to select wells overnight at 4℃. The goat-anti-mouse AlexaFluor 488, secondary antibody was added at a 1:500 dilution at 4℃ for an hour before fixed cells were washed with PBS and mounted with Nucblue containing ProLong Gold mounting media (ThermoFisher) onto glass slips. The cells were imaged using Zeiss LSM800, 2 channel GaAsP, confocal microscope with Axiocam 506, Definite focus.2, and a solid-state Light Source Colibri 7 fluorescence and quantified using Zen Blue software.

Statistical Analysis

Data from signaling assays was analyzed with two-way ANOVA at a 95% confidence interval using Prism software. Multiple comparisons were done between different conditions at the same time points.

Data from fluorescence microscopy was analyzed with one way ANOVA at the 95% confidence interval using Prism software.

KEY RESOURCES TABLE

Reagent or Resource	*Source*	*Identifier*
Antibodies		
Rabbit anti-TAB1	Cell Signaling Technology	Cat#3226S; RRID:AB_2140247
Rabbit Anti-p38 MAPK, phospho (Thr180 / Tyr182) Monoclonal Antibody, Unconjugated Clone D3F9	Cell Signaling Technology	Cat#4511L; RRID:AB_2139689
Rabbit Anti-pan p38, monoclonal	Cell Signaling Technology	Product# 9212S
HRP-conjugated goat-anti-rabbit	Bio-Rad Laboratories	Cat#170-6515; RRID:AB_11125142
Alexa fluor 488 anti-mouse	Life Technologies	Cat# A-11029; RRID:AB_2534088

Alexa flour 594 anti-rabbit	Life Technologies	Cat# A-11037; RRID:AB_2534095
Alix (3A9) Mouse mAb 2171	Cell Signaling Technology	Product# 74220T
HSP70 (D60) Rabbit 4876	Cell Signaling Technology	Product# 74220T
CD9 (D801A) Rabbit mAb 13174	Cell Signaling Technology	Product# 74220T
CD81	System Biosciences	EXOAB-CD81A-1
Anti-RFP rabbit	Rockland	42896
Chemicals, Peptides, and Recombinant Proteins		
Prostaglandin E2	Fisher Scientific	22-961-0
ADP	Fisher Scientific	20398-34-9
Calcein AM	Thermofisher	C1430
FluoroCet EV Quantification Kit	System Biosciences	FCET96A-1
Puromycin dihydrochloride	Sigma Aldrich	P7255
Lentistarter 3.0 Kit (includes	System Biosciences	LV060A-1

pPACKH1, PureFection, PEG-it, and TransDux MAX)		
Cell Lines		
EA.hy926 Endothelial cells	ATCC	Cat# CC-2519
Hek.293T	ATCC	CRL-1573
Recombinant DNA		
XPack MSCV-XP-MCS-EF1α-Puro Cloning Lentivector	System Biosciences	Cat# XPAK710PA-1
Software and Algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/
Prism 9.0, Statistical Analysis Software	Graphpad	https://www.graphpad.com/scientific-software/prism/
Instruments		
Amicon Ultra 15mL Centrifugal Tubes	Millipore Sigma	Cat# C7715
LSM800	Zeiss	

Ultracentrifuge. Optima L-100XP	Beckman Coulter	392052
Zetaview	Particle Metrix	PMX-120

CHAPTER 4

DISCUSSION

Large compounds, peptides, and proteins are historically difficult to pass through the plasma membrane of cells. This barrier to entry has limited the potential of these highly specific and promising therapeutics *in vivo*, but researchers are trying to better understand how cells receive large, bioactive molecules such as proteins, RNA, and lipids. Upon the discovery of phospholipid capsules emitted from cells, termed extracellular vesicles, EVs emerged as a wide field of study into cell-to-cell communication, disease pathology, and therapeutic development.

EVs are naturally produced nanoparticle carriers similar to liposomes which are often used as empty vehicle controls when compared to EVs [109]. Liposomes are synthetically produced lipid nanoparticles that can encapsulate drugs. While the production efficiency of liposomes is higher than extracellular vesicles, they have issues with delivering cargo to intracellular targets, decreased capacity, encapsulating high molecular weight drugs, and decreased circulation [110-112]. EVs bypass the problems of synthetic nanocarriers because they can hold large molecular weight molecules [69], have enhanced cellular uptake [111], and have a long circulation time [113, 114]. Additionally, EVs have demonstrated the ability to target certain tissues depending on their origin cell types [115]. On the contrary, EVs are more difficult to generate and reproduce because they are derived from living systems.

Targeting recombinant proteins and peptides to EVs requires specific modifications. As discussed in the introduction, EVs have enriched populations of only a handful of proteins. Researchers can accomplish EV targeting with a couple of methods including myristoylation [116], conjugation to EV enriched proteins such as lactadherin [117] and the Xpack sequence mentioned previously (System Biosciences LLC). Alternatively, physical methods can be used to drive molecules into the EV, such as sonication [118]. Myristoylation is a large post-translation modification that adds fatty acid chains to a translated protein meanwhile the Xpack sequence is a short amino acid sequence that targets translated protein to the interior wall of vesicles in the MVB.

In this study, the Xpack peptide was used to target PT5 inhibitor linked to mRFP to EVs. We chose to use a lentiviral vector to generate a stable cell line producing this construct for continuous production of EVs with the desired content. As shown in previous studies by Wang et al. [32] and Levine et al. [36], the PT5 peptide with little to no modifications can block the p38-TAB1 interaction, but we did not know if fusion to a fluorescent protein would hinder its ability to bind p38. RFP tagging of the peptide allowed for tracking and confirmation of expression, but it was unknown if this would impact the peptide's ability to inhibit TAB1-dependent p38 signaling. To do this, we checked for attenuated p38 signaling in the stable cell line expressing RFP-PT5 and found that the RFP-PT5 was still functional when the cells were stimulated with GPCR ligands, resulting in a suppression of atypical p38 signaling.

Another challenge of using EVs as therapeutics or carriers is their limited yield. The standard method of isolation uses ultracentrifugation to isolate the small EV pellet, but the yield is low. Other methods of isolation such as filter centrifugation have increased

total yield, but lower specificity for small EVs. In this study, ultracentrifugation and filter centrifugation were both used and extracted EVs showed similarities in size distribution, but only ultracentrifugation isolated EVs showed visible EV markers and RFP through immunoblot.

As discussed in the introduction section on EVs role in physiology, there are a variety of mechanism EVs use to enter the cell and/or deposit their cargo such as clathrin-mediated endocytosis, phagocytosis, direct membrane fusion, and macropinocytosis [108]. While we did not study the exact uptake mechanisms, we found evidence of RFP inside EA.hy926 after incubation with RFP containing EVs. This might be dispersed RFP from direct fusion of the EV to the plasma membrane or punctate RFP from EVs that have been internalized into endosomes. Future investigations should be directed towards identifying the main mechanism of entry PT5 loaded EVs use to enter endothelial cells.

EVs in physiology and pathology have shown their ability to modulate recipient cell phenotypes by delivery of bioactive molecules. We hypothesized that encapsulating the PT5 inhibitor in endothelial cell derived EVs would facilitate PT5 delivery into the cytoplasm of receiving cells and suppress GPCR induced inflammatory signaling. From our studies, we determined that PT5 loaded EVs attenuated p38 signaling both in the EV producing cells and recipient endothelial cells indicating a potential simple therapeutic for vascular inflammation. It will be important for future studies to confirm that the EV producing cells and recipient cells display “normal” GPCR cell surface expression and “normal” activation of other members of the MAPK family. Surface expression is particularly important, as a reduce level of surface GPCR expression would likely result in a reduced MAPK signaling response. Extracellular stimuli are critical for cell survival,

so it is important for PT5 to have specificity for p38 MAPK over JNK and ERK and indeed also selectivity for p38 over NFκB signaling. This is particularly important as TAB1 can also play a critical role in NFκB signaling [119].

Further testing of PT5 loaded EVs will need to be completed to understand their strengths in preventing inflammatory phenotypes. Since, as a proof of concept, this approach was only tested in EA.hy926 cells , further experimentation with a more physiologically relevant cell line such as human pulmonary microvascular endothelial cells (HPMEC) would increase our confidence in the viability of this design in living systems. In addition, we want to explore the capacity of PT5 EVs to suppress p38 atypical signaling after stimulation with other GPCR agonists such as histamine and α-thrombin. Furthermore, it would be essential to assess whether PT5 EVs can suppress other GPCR induced cellular phenotypes. This could include assessing angiogenesis using endothelial cell tubulation and spheroids to observe PT5's impact on blood vessel branching. Alternatively, we could use RT-qPCR of endothelial RNA after incubation with PT5 loaded EVs to determine changes to pro-inflammatory and pro-angiogenic signaling by measuring cytokine production. Outside of endothelial cells, it would also be interesting to explore the impact of PT5 EVs on other cells involved in inflammation such as T-cells and vascular mural/pericyte cells.

Due to issues with peptide drugs, we are also examining the capability of a nanobodies for blocking the TAB1 p38 interaction. Humanized nanobodies are llama variable heavy domain and are known for their ability to recognize antigens. We believe an intracellular nanobody (intrabody) targeted to block TAB1-mediated p38 signaling may have higher affinity and specificity than a peptide inhibitor and can still be packaged into

extracellular vesicles. Our group has recently identified 40 intrabodies that can bind to TAB1 at the p38 docking domain. These antibodies went through a two-phase screening approach, using a phage display library and a yeast two hybrid screen. As the nanobodies still detected TAB1 inside the cytosol of yeast cells, they can be called intrabodies and are predicted to bind to TAB1 in the cytosol of mammalian cells. Research groups have used nanobody technology to target intracellular pathological events such as tauopathy in neurons [120] and HIV replication [121]. We believe intrabodies alone and/or intrabodies encapsulated in EVs may enhance blockade of TAB1-dependent p38 signaling when compared to the PT5 inhibitor to treat chronic inflammatory diseases.

In summary, our data is the first to demonstrate that RFP labeled PT5 loaded into extracellular vesicles is able block p38 atypical signaling in EV producing cells and EV recipient cells. If confirmed, this approach has the potential to block p38 atypical signaling in other inflammatory regulating cells and prevent pathological p38 associated phenotypes. This work provides a basis for a novel, easily generated therapeutic against chronic inflammatory diseases.

REFERENCES

1. Ohta, M., et al., *IL-6 promotes cell adhesion in human endothelial cells via microRNA-126-3p suppression.* Exp Cell Res, 2020. **393**(2): p. 112094.
2. Medzhitov, R., *Origin and physiological roles of inflammation.* Nature, 2008. **454**(7203): p. 428-35.
3. Acker, H., et al., *Indications to an NADPH oxidase as a possible pO2 sensor in the rat carotid body.* FEBS Lett, 1989. **256**(1-2): p. 75-8.
4. Davies, K.J., *Adaptive homeostasis.* Mol Aspects Med, 2016. **49**: p. 1-7.
5. Hammel, H.T., et al., *Temperature Regulation by Hypothalamic Proportional Control with an Adjustable Set Point.* J Appl Physiol, 1963. **18**: p. 1146-54.
6. Choi, K.M., et al., *Inflammation, insulin resistance, and glucose intolerance in acute myocardial infarction patients without a previous diagnosis of diabetes mellitus.* J Clin Endocrinol Metab, 2005. **90**(1): p. 175-80.
7. Gimbrone, M.A., Jr. and G. Garcia-Cardena, *Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis.* Circ Res, 2016. **118**(4): p. 620-36.
8. Clark, P.R., et al., *Increased ICAM-1 expression causes endothelial cell leakiness, cytoskeletal reorganization and junctional alterations.* J Invest Dermatol, 2007. **127**(4): p. 762-74.
9. Briscoe, D.M., R.S. Cotran, and J.S. Pober, *Effects of tumor necrosis factor, lipopolysaccharide, and IL-4 on the expression of vascular cell adhesion molecule-1 in vivo. Correlation with CD3+ T cell infiltration.* J Immunol, 1992. **149**(9): p. 2954-60.
10. Petrache, I., et al., *The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability.* Am J Respir Cell Mol Biol, 2003. **28**(5): p. 574-81.
11. Pober, J.S. and W.C. Sessa, *Evolving functions of endothelial cells in inflammation.* Nat Rev Immunol, 2007. **7**(10): p. 803-15.
12. Shapiro, N.I., et al., *The association of endothelial cell signaling, severity of illness, and organ dysfunction in sepsis.* Crit Care, 2010. **14**(5): p. R182.
13. Lorant, D.E., et al., *Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils.* J Cell Biol, 1991. **115**(1): p. 223-34.
14. Feng, D., et al., *Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP.* J Exp Med, 1998. **187**(6): p. 903-15.
15. Kim, Y.W. and T.V. Byzova, *Oxidative stress in angiogenesis and vascular disease.* Blood, 2014. **123**(5): p. 625-31.
16. Hoeben, A., et al., *Vascular endothelial growth factor and angiogenesis.* Pharmacol Rev, 2004. **56**(4): p. 549-80.
17. Matarese, A. and G. Santulli, *Angiogenesis in chronic obstructive pulmonary disease: a translational appraisal.* Transl Med UniSa, 2012. **3**: p. 49-56.

18. Weis, W.I. and B.K. Kobilka, *The Molecular Basis of G Protein-Coupled Receptor Activation.* Annu Rev Biochem, 2018. **87**: p. 897-919.
19. Billi, A.C., et al., *KLK6 expression in skin induces PAR1-mediated psoriasiform dermatitis and inflammatory joint disease.* J Clin Invest, 2020. **130**(6): p. 3151-3157.
20. Lin, Y., et al., *Phosphoproteomic analysis of protease-activated receptor-1 biased signaling reveals unique modulators of endothelial barrier function.* Proc Natl Acad Sci U S A, 2020. **117**(9): p. 5039-5048.
21. Soh, U.J.K. and J. Trejo, *Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through β-arrestin and dishevelled-2 scaffolds.* Proceedings of the National Academy of Sciences, 2011. **108**(50): p. E1372-E1380.
22. Omori, K., et al., *Multiple roles of the PGE2 -EP receptor signal in vascular permeability.* Br J Pharmacol, 2014. **171**(21): p. 4879-89.
23. Minamizaki, T., et al., *EP2 and EP4 receptors differentially mediate MAPK pathways underlying anabolic actions of prostaglandin E2 on bone formation in rat calvaria cell cultures.* Bone, 2009. **44**(6): p. 1177-85.
24. Powell, J.R. and M.J. Brody, *Participation of H1 and H2 histamine receptors in physiological vasodilator responses.* Am J Physiol, 1976. **231**(4): p. 1002-9.
25. Woehrle, T., et al., *Autocrine stimulation of P2Y1 receptors is part of the purinergic signaling mechanism that regulates T cell activation.* Purinergic Signal, 2019. **15**(2): p. 127-137.
26. Burton, J.C.A., W.; Okalova, J.; Roos, M.; Grimsey, N.J. , *Atypical p38 Signaling, Activation, and Implications for Disease.* Int. J. Mol. Sci., 2021(22).
27. Uhlik, M.T., et al., *Rac-MEKK3-MKK3 scaffolding for p38 MAPK activation during hyperosmotic shock.* Nat Cell Biol, 2003. **5**(12): p. 1104-10.
28. Min, X., et al., *The structure of the MAP2K MEK6 reveals an autoinhibitory dimer.* Structure, 2009. **17**(1): p. 96-104.
29. Grimsey, N.J., et al., *Ubiquitin plays an atypical role in GPCR-induced p38 MAP kinase activation on endosomes.* J Cell Biol, 2015. **210**(7): p. 1117-31.
30. Grimsey, N.J., et al., *A Tyrosine Switch on NEDD4-2 E3 Ligase Transmits GPCR Inflammatory Signaling.* Cell Rep, 2018. **24**(12): p. 3312-3323 e5.
31. Grimsey, N.J., et al., *G protein-coupled receptors activate p38 MAPK via a non-canonical TAB1-TAB2 and TAB1-TAB3 dependent pathway in endothelial cells.* 2019.
32. Wang, Q., et al., *Disruption of TAB1/p38alpha interaction using a cell-permeable peptide limits myocardial ischemia/reperfusion injury.* Mol Ther, 2013. **21**(9): p. 1668-77.
33. De Nicola, G.F., et al., *Mechanism and consequence of the autoactivation of p38alpha mitogen-activated protein kinase promoted by TAB1.* Nat Struct Mol Biol, 2013. **20**(10): p. 1182-90.
34. De Nicola, G.F., et al., *The TAB1-p38alpha complex aggravates myocardial injury and can be targeted by small molecules.* JCI Insight, 2018. **3**(16).
35. Shi, J., et al., *Amyloidogenic light chains induce cardiomyocyte contractile dysfunction and apoptosis via a non-canonical p38alpha MAPK pathway.* Proc Natl Acad Sci U S A, 2010. **107**(9): p. 4188-93.

36. Theivanthiran, B., et al., *The E3 ubiquitin ligase Itch inhibits p38alpha signaling and skin inflammation through the ubiquitylation of Tab1.* Sci Signal, 2015. **8**(365): p. ra22.
37. Cheng, Y., et al., *Virus-induced p38 MAPK activation facilitates viral infection.* Theranostics, 2020. **10**(26): p. 12223-12240.
38. De Nicola, G.F., et al., *Mechanism and consequence of the autoactivation of p38α mitogen-activated protein kinase promoted by TAB1.* Nature Structural & Molecular Biology, 2013. **20**(10): p. 1182-1190.
39. Thapa, D., et al., *TAB1-Induced Autoactivation of p38α Mitogen-Activated Protein Kinase Is Crucially Dependent on Threonine 185.* Mol Cell Biol, 2018. **38**(5).
40. Tanno, M., et al., *Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: evidence for MKK-independent activation by a TAB1-associated mechanism contributing to injury during myocardial ischemia.* Circ Res, 2003. **93**(3): p. 254-61.
41. Mishra, S., et al., *Human amyloidogenic light chain proteins result in cardiac dysfunction, cell death, and early mortality in zebrafish.* Am J Physiol Heart Circ Physiol, 2013. **305**(1): p. H95-103.
42. Choy, E.H. and G.S. Panayi, *Cytokine pathways and joint inflammation in rheumatoid arthritis.* N Engl J Med, 2001. **344**(12): p. 907-16.
43. Stokes, D.G. and J.M. Kremer, *Potential of tumor necrosis factor neutralization strategies in rheumatologic disorders other than rheumatoid arthritis.* Semin Arthritis Rheum, 2003. **33**(1): p. 1-18.
44. Canovas, B. and A.R. Nebreda, *Diversity and versatility of p38 kinase signalling in health and disease.* Nat Rev Mol Cell Biol, 2021.
45. Renda, T., et al., *Increased activation of p38 MAPK in COPD.* Eur Respir J, 2008. **31**(1): p. 62-9.
46. Domscheit, H., et al., *Molecular Dynamics of Lipopolysaccharide-Induced Lung Injury in Rodents.* Front Physiol, 2020. **11**: p. 36.
47. Singh, R.K. and A.K. Najmi, *Novel Therapeutic Potential of Mitogen-Activated Protein Kinase Activated Protein Kinase 2 (MK2) in Chronic Airway Inflammatory Disorders.* Curr Drug Targets, 2019. **20**(4): p. 367-379.
48. Feng, Y., et al., *p38MAPK plays a pivotal role in the development of acute respiratory distress syndrome.* Clinics (Sao Paulo), 2019. **74**: p. e509.
49. Grimes, J.M. and K.V. Grimes, *p38 MAPK inhibition: A promising therapeutic approach for COVID-19.* J Mol Cell Cardiol, 2020. **144**: p. 63-65.
50. Lee, J.K. and N.J. Kim, *Recent Advances in the Inhibition of p38 MAPK as a Potential Strategy for the Treatment of Alzheimer's Disease.* Molecules, 2017. **22**(8).
51. Correa, S.A. and K.L. Eales, *The Role of p38 MAPK and Its Substrates in Neuronal Plasticity and Neurodegenerative Disease.* J Signal Transduct, 2012. **2012**: p. 649079.
52. He, J., et al., *P38 Mitogen-activated Protein Kinase and Parkinson's Disease.* Transl Neurosci, 2018. **9**: p. 147-153.
53. Germann, U.A. and J.J. Alam, *P38alpha MAPK Signaling-A Robust Therapeutic Target for Rab5-Mediated Neurodegenerative Disease.* Int J Mol Sci, 2020. **21**(15).

54. Pascoe, S., et al., *Biological effects of p38 MAPK inhibitor losmapimod does not translate to clinical benefits in COPD.* Respir Med, 2017. **130**: p. 20-26.
55. Emami, H., et al., *The effect of BMS-582949, a P38 mitogen-activated protein kinase (P38 MAPK) inhibitor on arterial inflammation: a multicenter FDG-PET trial.* Atherosclerosis, 2015. **240**(2): p. 490-6.
56. Nichols, C., et al., *Mining the PDB for Tractable Cases Where X-ray Crystallography Combined with Fragment Screens Can Be Used to Systematically Design Protein-Protein Inhibitors: Two Test Cases Illustrated by IL1beta-IL1R and p38alpha-TAB1 Complexes.* J Med Chem, 2020. **63**(14): p. 7559-7568.
57. Fosgerau, K. and T. Hoffmann, *Peptide therapeutics: current status and future directions.* Drug Discov Today, 2015. **20**(1): p. 122-8.
58. Khanna, A., M. Ostermann, and R. Bellomo, *Angiotensin II for the Treatment of Vasodilatory Shock.* N Engl J Med, 2017. **377**(26): p. 2604.
59. Marso, S.P., A.G. Holst, and T. Vilsboll, *Semaglutide and Cardiovascular Outcomes in Patients with Type 2 Diabetes.* N Engl J Med, 2017. **376**(9): p. 891-2.
60. Levine, P.M., et al., *O-GlcNAc Engineering of GPCR Peptide-Agonists Improves Their Stability and in Vivo Activity.* J Am Chem Soc, 2019. **141**(36): p. 14210-14219.
61. Miranda, L.P., et al., *Design and synthesis of conformationally constrained glucagon-like peptide-1 derivatives with increased plasma stability and prolonged in vivo activity.* J Med Chem, 2008. **51**(9): p. 2758-65.
62. Vives, E., P. Brodin, and B. Lebleu, *A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus.* J Biol Chem, 1997. **272**(25): p. 16010-7.
63. Kardani, K. and A. Bolhassani, *Exploring novel and potent cell penetrating peptides in the proteome of SARS-COV-2 using bioinformatics approaches.* PLoS One, 2021. **16**(2): p. e0247396.
64. Skotland, T., et al., *Cell-penetrating peptides: possibilities and challenges for drug delivery in vitro and in vivo.* Molecules, 2015. **20**(7): p. 13313-23.
65. Dyck, M.K., et al., *Making recombinant proteins in animals--different systems, different applications.* Trends Biotechnol, 2003. **21**(9): p. 394-9.
66. Amini, N., et al., *Insulin production from hiPSC-derived pancreatic cells in a novel wicking matrix bioreactor.* Biotechnol Bioeng, 2020. **117**(7): p. 2247-2261.
67. Lynch, S., et al., *Novel MHC class I structures on exosomes.* J Immunol, 2009. **183**(3): p. 1884-91.
68. Kowal, J., et al., *Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes.* Proc Natl Acad Sci U S A, 2016. **113**(8): p. E968-77.
69. Raposo, G. and W. Stoorvogel, *Extracellular vesicles: exosomes, microvesicles, and friends.* J Cell Biol, 2013. **200**(4): p. 373-83.
70. Zhang, H., et al., *Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation.* Nat Cell Biol, 2018. **20**(3): p. 332-343.

71. Zhou, X., et al., *The function and clinical application of extracellular vesicles in innate immune regulation.* Cell Mol Immunol, 2020. **17**(4): p. 323-334.
72. Parolini, I., et al., *Microenvironmental pH is a key factor for exosome traffic in tumor cells.* J Biol Chem, 2009. **284**(49): p. 34211-22.
73. Feng, D., et al., *Cellular internalization of exosomes occurs through phagocytosis.* Traffic, 2010. **11**(5): p. 675-87.
74. Vallee, R.B., et al., *Dynamin, a GTPase involved in the initial stages of endocytosis.* Ciba Found Symp, 1993. **176**: p. 185-93; discussion 193-7.
75. Nanbo, A., et al., *Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells.* J Virol, 2013. **87**(18): p. 10334-47.
76. Fitzner, D., et al., *Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis.* J Cell Sci, 2011. **124**(Pt 3): p. 447-58.
77. Montecalvo, A., et al., *Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes.* Blood, 2012. **119**(3): p. 756-66.
78. Thery, C., et al., *Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines.* J Extracell Vesicles, 2018. **7**(1): p. 1535750.
79. Danesh, A., et al., *Exosomes from red blood cell units bind to monocytes and induce proinflammatory cytokines, boosting T-cell responses in vitro.* Blood, 2014. **123**(5): p. 687-96.
80. Colombo, M., G. Raposo, and C. Thery, *Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles.* Annu Rev Cell Dev Biol, 2014. **30**: p. 255-89.
81. Bernardo, M.E. and W.E. Fibbe, *Mesenchymal stromal cells: sensors and switchers of inflammation.* Cell Stem Cell, 2013. **13**(4): p. 392-402.
82. Pitt, J.M., et al., *Dendritic cell-derived exosomes for cancer therapy.* J Clin Invest, 2016. **126**(4): p. 1224-32.
83. Morse, M.A., et al., *A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer.* J Transl Med, 2005. **3**(1): p. 9.
84. Chen, X., et al., *Exosomes derived from hypoxic epithelial ovarian cancer cells deliver microRNAs to macrophages and elicit a tumor-promoted phenotype.* Cancer Lett, 2018. **435**: p. 80-91.
85. Morad, G., et al., *Tumor-Derived Extracellular Vesicles Breach the Intact Blood-Brain Barrier via Transcytosis.* ACS Nano, 2019. **13**(12): p. 13853-13865.
86. Busatto, S., et al., *Brain metastases-derived extracellular vesicles induce binding and aggregation of low-density lipoprotein.* J Nanobiotechnology, 2020. **18**(1): p. 162.
87. Moon, H.G., et al., *Lung epithelial cell-derived extracellular vesicles activate macrophage-mediated inflammatory responses via ROCK1 pathway.* Cell Death Dis, 2015. **6**: p. e2016.
88. Wang, J., Y. Zheng, and M. Zhao, *Exosome-Based Cancer Therapy: Implication for Targeting Cancer Stem Cells.* Front Pharmacol, 2016. **7**: p. 533.
89. Tang, Y., et al., *TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation.* Nat Med, 2009. **15**(7): p. 757-65.

90. Zhao, Y.D., et al., *Bone marrow-derived progenitor cells prevent thrombin-induced increase in lung vascular permeability.* Am J Physiol Lung Cell Mol Physiol, 2010. **298**(1): p. L36-44.
91. Lai, R.C., et al., *Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury.* Stem Cell Res, 2010. **4**(3): p. 214-22.
92. Willis, G.R., et al., *Mesenchymal Stromal Cell Exosomes Ameliorate Experimental Bronchopulmonary Dysplasia and Restore Lung Function through Macrophage Immunomodulation.* Am J Respir Crit Care Med, 2018. **197**(1): p. 104-116.
93. Chen, Q., et al., *Bone marrow mesenchymal stem cell-secreted exosomes carrying microRNA-125b protect against myocardial ischemia reperfusion injury via targeting SIRT7.* Mol Cell Biochem, 2020. **465**(1-2): p. 103-114.
94. Wang, B. and B. Li, *Effect of molecular weight on the transepithelial transport and peptidase degradation of casein-derived peptides by using Caco-2 cell model.* Food Chem, 2017. **218**: p. 1-8.
95. Bottger, R., R. Hoffmann, and D. Knappe, *Differential stability of therapeutic peptides with different proteolytic cleavage sites in blood, plasma and serum.* PLoS One, 2017. **12**(6): p. e0178943.
96. Pei, Y., Wang, Q., Zhang, J. et al., *Characterization and Evaluation of Key Sites in the Peptide Inhibitor of TAB1/p38α Interaction.* Int J Pept Res Ther, 2018. **24**: p. 225-233.
97. Lee, J., et al., *T cell-intrinsic prostaglandin E2-EP2/EP4 signaling is critical in pathogenic TH17 cell-driven inflammation.* J Allergy Clin Immunol, 2019. **143**(2): p. 631-643.
98. Aoki, T. and S. Narumiya, *Prostaglandin E2-EP2 signaling as a node of chronic inflammation in the colon tumor microenvironment.* Inflamm Regen, 2017. **37**: p. 4.
99. Shen, J. and P.E. DiCorleto, *ADP stimulates human endothelial cell migration via P2Y1 nucleotide receptor-mediated mitogen-activated protein kinase pathways.* Circ Res, 2008. **102**(4): p. 448-56.
100. Grimsey, N.J., et al., *G protein-coupled receptors activate p38 MAPK via a non-canonical TAB1-TAB2 and TAB1-TAB3 dependent pathway in endothelial cells.* J Biol Chem, 2019.
101. Levi, G. and M. Raiteri, *Carrier-mediated release of neurotransmitters.* Trends Neurosci, 1993. **16**(10): p. 415-9.
102. Di Vizio, D., et al., *Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease.* Cancer Res, 2009. **69**(13): p. 5601-9.
103. Thery, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3 22.
104. Szatanek, R., et al., *The Methods of Choice for Extracellular Vesicles (EVs) Characterization.* Int J Mol Sci, 2017. **18**(6).
105. Hemler, M.E., *Tetraspanin functions and associated microdomains.* Nat Rev Mol Cell Biol, 2005. **6**(10): p. 801-11.

106. Kalluri, R. and V.S. LeBleu, *The biology, function, and biomedical applications of exosomes.* Science, 2020. **367**(6478).
107. Iavello, A., et al., *Role of Alix in miRNA packaging during extracellular vesicle biogenesis.* Int J Mol Med, 2016. **37**(4): p. 958-66.
108. Mulcahy, L.A., R.C. Pink, and D.R. Carter, *Routes and mechanisms of extracellular vesicle uptake.* J Extracell Vesicles, 2014. **3**.
109. Venkat, P., et al., *MiR-126 Mediates Brain Endothelial Cell Exosome Treatment-Induced Neurorestorative Effects After Stroke in Type 2 Diabetes Mellitus Mice.* Stroke, 2019. **50**(10): p. 2865-2874.
110. Vyas, S.P. and T. Sakthivel, *Pressurized pack-based liposomes for pulmonary targeting of isoprenaline--development and characterization.* J Microencapsul, 1994. **11**(4): p. 373-80.
111. Lu, M., et al., *Comparison of exosome-mimicking liposomes with conventional liposomes for intracellular delivery of siRNA.* Int J Pharm, 2018. **550**(1-2): p. 100-113.
112. Zucker, D., et al., *Liposome drugs' loading efficiency: a working model based on loading conditions and drug's physicochemical properties.* J Control Release, 2009. **139**(1): p. 73-80.
113. Aqil, F., et al., *Milk exosomes - Natural nanoparticles for siRNA delivery.* Cancer Lett, 2019. **449**: p. 186-195.
114. Yong, T., et al., *Tumor exosome-based nanoparticles are efficient drug carriers for chemotherapy.* Nat Commun, 2019. **10**(1): p. 3838.
115. Park, E.J., et al., *Exosomal regulation of lymphocyte homing to the gut.* Blood Adv, 2019. **3**(1): p. 1-11.
116. Maguire, J.E., et al., *Myristoylated CIL-7 regulates ciliary extracellular vesicle biogenesis.* Mol Biol Cell, 2015. **26**(15): p. 2823-32.
117. Bliss, C.M., et al., *Targeting Antigen to the Surface of EVs Improves the In Vivo Immunogenicity of Human and Non-human Adenoviral Vaccines in Mice.* Mol Ther Methods Clin Dev, 2020. **16**: p. 108-125.
118. Kim, M.S., et al., *Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells.* Nanomedicine, 2016. **12**(3): p. 655-664.
119. Mihaly, S.R., J. Ninomiya-Tsuji, and S. Morioka, *TAK1 control of cell death.* Cell Death Differ, 2014. **21**(11): p. 1667-76.
120. Gallardo, G., et al., *Targeting tauopathy with engineered tau-degrading intrabodies.* Mol Neurodegener, 2019. **14**(1): p. 38.
121. Vercruysse, T., et al., *An intrabody based on a llama single-domain antibody targeting the N-terminal alpha-helical multimerization domain of HIV-1 rev prevents viral production.* J Biol Chem, 2010. **285**(28): p. 21768-80.