The role of TRPV4 in flow-mediated inhibition of rat mesenteric collecting lymphatic vessel pumping

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The role of TRPV4 in flow-mediated inhibition of rat mesenteric collecting lymphatic vessel pumping

by

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ABSTRACT

Lymphatic contractility is modulated by transmural pressure and shear stress exerted by the lymph on the lymphatic vessel wall. It has previously been shown that in rat mesenteric collecting lymphatic vessels, lymph flow inhibits lymphatic contractility in an endothelial nitric oxide synthase (eNOS) dependent manner. However, the molecular elements responsible for the activation of this signaling pathway are not known. TRPV4 is a non-selective cation permeable channel ubiquitously expressed throughout the body. It promotes calcium entry and has been shown to contribute to blood flow dependent vasodilation but its role in lymphatics under conditions of flow has yet to be determined. It is expressed in lymphatic vessels and promotes the entry of Ca^{2+} upon stimulation with classical NO-dependent pathway agonists, resulting in endothelial cell depolarization. This is imperative since lymph flow and transport is required to maintain tissue fluid homeostasis within the body. In our study, we postulated that TRPV4 contributes to flow-induced collecting lymphatic relaxation in a NO-dependent, endothelium-dependent manner. In the current study qPCR was used to determine TRPV4 gene expression along with the expression levels of other mechanosensitive TRP channels. Immunofluorescence was used to determine TRPV4 protein expression within whole lymphatic vessels and in rat lymphatic endothelial cells. Pressure myography allowed for the determination of TRPV4 function under conditions of flow within lymphatic vessels and the implications of TRPV4 agonism under no flow conditions at a set transmural pressure. The results obtained implicate the expression of TRPV4 on both the smooth muscle and endothelial layers with agonism triggering a biphasic response. They also indicate a role for TRPV4, nitric oxide, and dilatory prostaglandins in rat mesenteric lymphatic vessels in modulating flow-induced decreases in contractile activity.
ACKNOWLEDGEMENTS

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and better critical thinker. He always took the time to listen, and he was really approachable with any problems and even mistakes that were made in the lab. I am thankful that I had the privilege to work in this lab environment with all of these wonderful people.

DEDICATION

I would like to dedicate this thesis to my Lord and Savior Jesus Christ. I have been blessed with an incredible life and capabilities. I would also like to dedicate this to my family, who are God’s gift to me and shown me how to do everything excellently.

To my wife Lize-Mari, thank you for loving me as I am, making my life easy and supporting me in everything I do.

To my mother and father, and my mother-in-law and father-in-law, thank you for your unconditional love for me and support through everything.

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It is not your business to succeed, but to do right; when you have done so, the rest lies with God.

*C. S. Lewis, Yours Jack*
1. CHAPTER 1: BACKGROUND

1.1. History of the Lymphatic System

Ancient Greek scholars, including Hippocrates and Aristotle, were the first to describe the lymphatic system, and further contributions were made by anatomists from the Alexandrian school in the subsequent years (Loukas et al., 2011). Hippocrates created the term chylos (chyle) and described the axillary lymph nodes (Natale et al., 2017). Galen of Pergamum made many contributions to anatomy, with one of them being the description of the lacteal vessels and mesenteric lymph nodes within animals (Loukas et al., 2011). But after these early observations, there were no significant discoveries made about the lymphatic system until the 16th and 17th centuries. Gaspare Aselli made the most well-known discovery in 1622 during the dissection of a live dog’s abdomen. He had intended to study the diaphragm but noticed a network of white-colored vessels throughout the abdomen (Figure 1). He called these vessels “lacteal vessels” due to the white color of the solution which they were carrying. He also noted that these vessels had valves, making them similar to veins and postulated that they prevented backflow (Loukas et al., 2011). After this, the thoracic duct was discovered by Bartolomeo Eustachius and the cisterna chyli by Jean Pecquet, thereby expanding the known network of vessels. Thomas Bartholin, in 1653, observed that distinct lymphatic vessels came from the gut and the liver and that both of these vessels drained independently into the thoracic duct. The vessels from the intestine contained a milk-like fluid, while those from the liver contained clear liquid resulting in Bartholin calling them “lymphatics,” hence giving the lymphatic system its name (Natale et al., 2017). Around this time, the Peyer’s patches were discovered in the small intestine by Johann Conrad
Peyer, and the function of the lymphatic valves was described using wax and suet injections by Jan Swammerdam (Loukas et al., 2011, Natale et al., 2017). In 1745 Johann Nathanael Lieberkühn discovered that lacteal vessels originated from intestinal villi through the injection of corrosive agents into the gut (Natale et al., 2017).

During the mid-1700s, the Hunter brothers contributed new knowledge to the function of the lymphatic system. William Hunter determined that the arteries were continuous with veins but that the lymphatic system was not. Through the injection of dye into arteries, he observed that it was only present in the veins when arteries were not damaged, but if the arteries were injured, the dye showed up in the lymphatics. From this, he concluded that lymph was derived from the extracellular, interstitial fluid, and that the movement of lymph was not dependent on the heart (Natale et al., 2017). In 1787, Paolo Mascagni published his work on the lymphatic system, demonstrating its presence throughout the body and within inner organs. Mascagni further elucidated the existence of superficial and deep lymphatics, describing their topographical location. He also proposed that lymphatics originate in the interstitium and consist of blind vessels with microscopic pores. Similarly, Mascagni made observations of deep and superficial lymph nodes and observed that all lymphatic vessels entered a lymph node at some point. Based on this observation, he distinguished between the afferent and efferent lymphatic vessels. His last discovery in the lymph nodes was that the composition of the lymph changed after passing through a lymph node. He thus concluded that lymph nodes slowed the flow of lymphatic fluid to induce composition changes (Natale et al., 2017).

In the 19th and 20th centuries, many contributions were made through research on lymphatics. During this time, Ernest Henry Starling discovered that lymph came from
the fluid that had passed through the capillary walls due to differences in pressure, and this work was expanding upon by Cecil K. Drinker and Joseph M. Yoffey. They showed that differences in protein composition impacted the formation of lymph (Loukas et al., 2011; Natale et al., 2017). More recently, during the 1950s, John Bernard Kinmonth introduced lymphangiography allowing for the visualization of the lymphatic system using x-rays (Natale et al., 2017). Since then, there have been numerous improvements in the display of lymphatic vessels with an explosion of information on the molecular characteristics of lymphatic vessels and lymphangiogenesis occurring from the 1990s on (Natale et al., 2017). The study of the lymphatic system has a long history, but there remains much to be elucidated.
Figure 1: A photograph of the intestinal lymphatic vessels filled with white lymph due to high-fat content in a human patient. Image to represent what Gaspare Aselli might have seen during his live dissection of a dog’s abdomen. Figure adapted from Colorado State University Hypertexts for Biomedical science. LN indicates the lymph node, and the arrows are pointing to the mesenteric collecting lymphatics.
1.2. Lymphatic System Development

The lymphatic system exhibits a substantial amount of plasticity and heterogeneity within the body, reflecting a tissue-specific specialization (Ulvmar & Mäkinen, 2016). All lymphatics within the body express Prox1 (Prospero homeobox 1), Podoplanin, VEGFR3 (vascular endothelial growth factor receptor 3), and Nrp2 (neuropilin 2). They also express the endothelial-specific junction proteins PECAM-1, Claudin 5, and VE-Cadherin, though the organization pattern in initial and collecting lymphatic vessels are distinct (Ulvmar & Mäkinen, 2016).

Florence Sabin did the first study on the origin of lymphatic vessels in 1902 on embryonic pigs. She found that the two primitive jugular lymph sacs originated from endothelial cells sprouting off large veins, followed by lymphatic vessel sprouting from the lymph sacs (Sabin, 1902). Prox1 expression was first observed around day E9.5 in mice within a subpopulation of blood endothelial cells (ECs) in the anterior cardinal vein (Wigle & Oliver, 1999). These Prox1 positive ECs budded off the anterior cardinal vein and formed the embryonic lymph sacs. The lymph sacs gave rise to the lymphatic vasculature in nearby tissues through sprouting, proliferation, and migration. The jugular lymph sac gave rise to the lung lymphatics and posterior lymph sacs to the mesenteric and posterior organ lymphatics (Figure 2) (Srinivasan et al., 2007). Prox1 deletion resulted in the lack of an embryonic lymphatic system, indicating it has a master transcriptional role in lymphatic development (Srinivasan et al., 2007). Recently two non-venous origins for lymphatic vessels have been discovered within mouse embryos. The two subsets are postulated to be hemogenic endothelial cells within the cardiac
lymphatics, showing an arterial source while the other was of non-endothelial origin contributing to the formation of dermal lymphatics (Klotz et al., 2015; Martinez-Corral et al., 2015). These findings have shed light on the possible source of heterogeneity in the lymphatic system between organs, opening the door to further exploration of this area.

VEGF-C is integral to the development of the lymphatic system. The migration and survival of the Prox1 expressing endothelial cells and the formation of the lymph sacs are both dependent on VEGF-C signaling. The deletion of VEGF-C resulted in a lack of lymphatic vessels leading to embryonic lethality (Karkkainen et al., 2004). Once the lymphatic system has been established, Foxc2 (Forkhead Box C2), calcineurin, and NFATC-1 (nuclear factor of activated T cells) are vital in the maturation process. Foxc2 expression is also required for the formation and maintenance of lymphatic valves and vessel integrity during postnatal life in collecting lymphatics (Aspelund et al., 2016). In more recent work, there have also been numerous other molecules identified to be involved in the lymphatic maturation process.
Figure 2: Illustration of the development of the lymphatic system from veins within the mouse embryo. The embryonic veins give rise to endothelial cells expressing Prox1 which will lead to the formation of the lymph sacs which give rise to the lymphatic plexus. The lymphatic plexus provides the foundation from which to build the lymphatic network. This process depends on a variety of transcription factors as seen above. CV denotes cardinal vein, ISV = intersomitic veins. Figure adapted from Yang & Oliver, 2014.
1.3. Components of the Lymphatic System

The lymphatic system is mainly composed of four different components: initial lymphatic vessels, otherwise known as lymphatic capillaries, pre-collecting lymphatic vessels, collecting lymphatic vessels and lymph nodes (Aspelund et al., 2016). Collecting lymphatic vessels can be split into two different types, afferent lymphatics that carry the lymph towards a lymph node and the efferent lymphatics that transport the lymph away from the lymph node back into the central circulation. When classifying vessels as afferent or efferent, it is crucial to note which lymph node is the reference point due to the presence of multiple lymph nodes between the periphery and where the lymphatic system joins central circulation. The lymphatic system is present throughout the body with all tissues containing lymphatics except for bone marrow and neural tissue (Aspelund et al., 2016). Recently there has been the discovery of the lymphatic system within the central nervous system, revealing that it is more widespread than previously thought (Louveau et al., 2015). Absorption of lymph from the interstitial space occurs via initial lymphatic vessels and then transported through pre-collecting and afferent collecting lymphatic vessels to the lymph node. At the lymph node, lymph flow slows, allowing for antigen sampling and the induction of an immune response. The lymph then exits the lymph node via efferent collecting lymphatics. After passing through a few other lymph nodes and converging with other efferent lymphatic vessels, it drains into the right duct or the larger thoracic duct. Lymph enters venous circulation at the junction of the internal jugular and subclavian veins through four lymphovenous valves via a combination of extrinsic forces, intrinsic pumping and pressure fluctuations in the venous system (Aspelund et al., 2016).
1.3.1. Initial Lymphatics

Initial lymphatics are blind-ended and consist of a single layer of endothelial cells lacking smooth muscle cells, pericytes, and a basement membrane layer. The expression of LYVE-1 (Lymphatic vessel endothelium hyaluronan receptor 1) and CCL 21 (Chemokine C-C motif ligand 21) are indicative of the initial lymphatic vessel (ILV) phenotype differentiating them from collecting lymphatic vessels. The endothelial cells are shaped like oak-leaves and do not form a tight, impermeable cell layer but rather are overlapping and joined together by button-like junctions. These button-like junctions are where adjacent endothelial cells join together and are rich in vascular endothelial cadherin (VE-cadherin) and tight junction-associated proteins (Aspelund et al., 2016). The outside endothelial cells attach to the extracellular matrix with anchoring filaments, which are essential in lymphatic drainage (Aspelund et al., 2016). The filaments prevent the collapse of the outer endothelial cells as interstitial pressure is increased (Figure 3). This structure allows the endothelial cells to act as a primary valve that regulates the entry of interstitial fluid and immune cells into the initial lymphatics and prevents backflow out. Under conditions where interstitial fluid pressure is higher than that of the lymph inside, these flaps’ part, creating gaps between endothelial cells 2-3 µm in size (Liao & von der Weid, 2015). These flaps remain open, allowing interstitial fluid entry until the pressure of the fluid inside the ILV is high enough to push the flaps closed again. The number of open flaps in the initial lymphatics depends on location in the body, with areas exposed to more mechanical forces or pressure variations containing more open flaps. Both injury and the formation of edema in the tissue do not impact the
number of valves present in the ILVs (Zawieja et al., 2008). Lymph movement through the initial lymphatic network strictly relies on extrinsic forces such as the pressure gradient. Lacteals are a unique type of ILV that is within the small intestinal villi through which dietary lipids and lipid-soluble substances are absorbed (Karkkainen et al., 2004).

1.3.2. Pre-collecting Lymphatics

Pre-collecting lymphatic vessels are the transition point from initial to collecting lymphatics. They express a hybrid phenotype of initial and collecting lymphatics, sharing the presence of button-like junctions with initials, making them permeable (Figure 3) (Schulte-Merker et al., 2011). They share the presence of intraluminal one-way valves and basement membrane coverage with collecting lymphatic vessels but are smaller in size (Pan et al., 2010; Schulte-Merker et al., 2011). The valves are in closer proximity and expressed at higher levels, especially close to branch points in the pre-collectors (Zawieja et al., 2018). Pre-collectors can also connect independent initial lymphatic networks in the head and neck region of humans (Pan et al., 2010).

1.3.3. Collecting Lymphatics

Collecting lymphatic vessels (CLVs) are the functional pumps within the lymphatic system, driving the movement of lymph towards the draining lymph node and back into central circulation (Gashev, 2008). Collecting lymphatic vessels consist of two cell layers, a tube of endothelial cells that is surrounded by a layer of lymphatic smooth muscle cells. The endothelial cells make contact with one another via zipper-like junctions creating a tightly sealed impermeable layer, unlike the initial lymphatics, which
containing button-like junctions that act as a primary valve within the system, making initial lymphatics permeable. The CLV endothelial cell layer also contains a continuous basement membrane providing structural support to these vessels (Aspelund et al., 2016). Collecting lymphatic vessels consist of units called lymphangions that are comprised of two intraluminal, one-way valves on either end of a chamber that is actively contracting in the middle (Figure 3). These valves, coupled with the contractile nature of collecting lymphatics, ensure that lymph movement occurs unidirectionally towards the lymph node (Zawieja et al., 2008). These valves do not seem to seal perfectly instantaneously but rather allow for a small amount of backflow that aids in the ultimate closing of the valves (Munn, 2015). CLVs converge in a tree-like network in which two lymphatic vessels merge upstream to become a single, more prominent collector downstream. Due to this structure, the coordination of contractions between lymphangions is essential to ensuring efficient transport instead of having the system “fight itself” (Munn, 2015). Intrinsic pumping is not the sole manner of lymph transport as external forces, such as skeletal muscle contractions and respiratory movements, also play a role in the lymph movement. As previously stated at the start of this section, collecting lymphatic vessels can be categorized into afferent and efferent lymphatics based on their location to a specified lymph node.

1.3.4. Lymph Node

A collagen-rich capsule with an underlying layer of lymphatic endothelial cells (LECs) is what comprises the two outermost layers of a lymph node. These LECs form both the subcapsular sinus (SCS) and medullary sinus (MS) that interact with all the
incoming lymph. Both the SCS and MS contain macrophages that incorporate in between LECs and sample all incoming lymph for antigens and pathogens (Liao & von der Weid, 2015). The LECs in the lymph node can present self-antigens and are involved in the process of peripheral tolerance. The lymph node contains a system of collagen conduits that provides the scaffolding for lymph node structure and allows for the entry of fluid and small antigens within the lymph node (Liao & von der Weid, 2015). It also has distinct B-cell follicles in the cortex, that are separated by interfollicular zones. The paracortex contains both the T cell zone and high endothelial venules (Ulvmar & Mäkinen, 2016). High endothelial venules provide a site for the entry of lymphocytes from blood circulation before they migrate to the B and T cell zones. The lymph node then drains into efferent lymphatic vessels that transport immune cells and lymph to central circulation (Figure 4) (Liao & von der Weid, 2015).
Figure 3: The lymphatic system is composed of three types of lymphatic vessels: initial (capillaries), pre-collecting and collecting lymphatic vessels. Each vessel type has unique characteristics and a vital role in the transport of lymph to the lymph node. Adapted from Hu et al., 2019.
Figure 4: a cross-section shows the lymph node structure through the lymph node. The lymph node consists of distinct cell populations with different roles in the initiation of an immune response. It slows lymph flow and samples antigens present to induce protection from the pathogen. Figure adapted from Schudel et al., 2019.
1.4. Roles of the Lymphatic System

The lymphatic system has four significant roles within the body: 1) maintenance of fluid balance, 2) the absorption of dietary fats 3) the facilitation of the host’s immune response through the immune cells and antigen trafficking and 4) the maintenance of peripheral tolerance (Aspelund et al., 2016; Liao & von der Weid, 2015).

1.4.1. Maintenance of Fluid Balance

Under physiological conditions, fluid exchange occurs in capillary beds throughout the body. In this process, fluid leaves the capillaries through the semipermeable endothelial cells and fills the extracellular space where it becomes interstitial fluid (Munn, 2015). Interstitial fluid usually contains water, proteins, and antigens along with other solutes. Absorption of the interstitial fluid can occur through the venules and continue in the systemic circulation, or through the initial lymphatics in the tissue beds. The lymphatic system maintains fluid balance through the absorption of the interstitial fluid, and it is a vital function for sustaining life. Previous work by Levick & Michel showed that the entire plasma volume of 3L extravasates from circulation every 9 hours (Levick & Michel, 2010). Daily 4L of lymph is returned to central circulation via the right, and thoracic ducts and another 4L returns at the lymph nodes (Renkin, 1986; Adair & Guyton 1983). Ablation of lymphatic vessel function in mice usually causes embryonic edema, which is fatal (Karkkainen et al., 2004). These results prove the necessity of the lymphatic system to sustain life, and disruption during adulthood usually results in the formation of lymphedema (Aspelund et al., 2016).
1.4.2. Absorption of Dietary Fats

The small intestine is vital to the absorption of nutrients and dietary fats. Absorption occurs via the villi found throughout the length of the small intestine (Bernier-Latmani et al., 2017). These villi contain blood vessels, nerves, and lymphatic vessels known as lacteals. Where all other initial lymphatics consist of solely button-like junctions, lacteals have a combination of both button-like and zipper-like junctions (Figure 5) (Karkkainen et al., 2004; Bernier-Latmani et al., 2017; Bernier-Latmani et al., 2015). Lacteals absorb chylomicrons, formed in the enterocytes, that consist of long-chain fatty acids. They also absorb cholesterol and fat-soluble vitamins such as vitamins A, D, E, and K (Bernier-Latmani et al., 2017). Transportation of cholesterol can also occur as HDLs (high-density lipoproteins), which are also mainly taken up by the lacteal (Bernier-Latmani et al., 2017). Both molecular complexes enter the lymphatics via the intracellular flap valves that are created by the discontinuous, button-like junctions between lymphatic endothelial cells (Tso & Balint, 1986; Baluk et al., 2007). Lymph movement in lacteals transpires by the contractions of the surrounding villus smooth muscle. These contractions occur in a vertical motion through the shortening of the villi resulting in lymph propulsion (Choe et al., 2015). This group also observed smooth muscle perpendicular to the villi smooth muscle that surrounding the lacteals and postulated that they may allow for lacteal contractions independently of the villus contraction.

Lacteals drain into the submucosal lymphatic network in the gut wall consisting of solely initial lymphatic vessels. The submucosal plexus drains into mesenteric lymphatic vessels that transport the lymph back to the central circulation, circumventing
the liver, and first-pass metabolism (Unthank & Bohlen, 1988; Bernier-Latmani et al., 2017). Bypassing the first-pass metabolism makes the creation of lipophilic drugs that specifically target the lymphatics highly appealing since avoidance of the first-pass metabolism would result in higher systemic concentrations (Trevaskis et al., 2015).

1.4.3. Facilitation of the Host’s Immune Response

The lymphatic system is required to generate an immune response. Initial lymphatics, found throughout the body, allow antigen and antigen-presenting cell (APC) entry and transport to the lymph node (Liao & von der Weid, 2015). T cells are the most common cell in healthy afferent lymph with dendritic cells (DCs) and B cells accounting for most of the remaining cells. During inflammation, the numbers of T cells and DCs increase dramatically, and the upregulation and transport of other cell types such as neutrophils and type I and II macrophages also occur (Jackson, 2019; Bellingan et al., 1996; Gorlino et al., 2014). Most studies on immune cell entry focus on DCs in the dermis and epidermis of mice. The DCs showed a random migration pattern until they were within 90 µm of an initial lymphatic vessel. At that point, they start migrating in a unidirectional manner to the initial lymphatic vessels (Weber et al., 2013). DCs migrate from the surrounding tissue to lymphatic vessels via a CCL21 (Chemokine C-C motif ligand 21) gradient, entering lymphatic vessels via the flap-like valves. DC entry can occur in an integrin-independent, proteolysis-independent process, and in an integrin-dependent manner involving ICAM-1 and VCAM-1 upregulation (Lämmermann et al., 2008; Johnson et al., 2006). LYVE-1, along with the Mannose receptor, ALCAM, L1CAM, and PECAM-1 are also required for DC transmigration. LYVE-1 also plays a
vital role in the formation of the ICAM-1 and VCAM-1 containing cups that facilitate DC entry (Johnson et al., 2017; Jackson, 2019). CCL21 is not the only molecule involved in the chemotactic guidance of immune cells. Both sphingosine-1-phosphate (S1P) and lymphotoxins are known to facilitate immune cell migration at initial lymphatics (Pham et al., 2010; Brinkman et al., 2016). Lymphatic endothelial cells (LECs) express CCL21 in a gradient manner where initial lymphatics express higher amounts than collecting lymphatics (Liao & von der Weid, 2015). Due to the high expression of CCL21 in the initial lymphatics, DCs attach to the initial lymphatic wall and crawl in both the same and opposite directions of flow (Tal et al., 2011). As the initials transition to collecting lymphatics, there is a decrease in permeability and an increase in flow rate. At this point, DCs detach from the vessel wall and flow to the lymph node (Liao & von der Weid, 2015; Jackson, 2019). T cells enter the initial lymphatics via the use of numerous receptors and adhesion molecules. These molecules include the mannose receptor coupled with CD44, CLEVER-1, CCR7 (Salmi et al., 2013; Karikoski et al., 2009; Debes et al., 2005). CCR7 is the receptor that binds to CCL21 indicating DCs and T cell migration occur similarly. Once within the initial lymphatic vessels (ILVs), these T cells can interact with the DCs present in the ILVs. These interactions happened during both contact hypersensitivity and delayed-type hypersensitivity, with the latter resulting in longer interaction times (Hunter et al., 2019). This group also observed T cell egress from the ILVs into the surrounding tissue, while DCs did not egress at any point (Hunter et al., 2019). These results indicate a role for the ILVs in the stimulation of an adaptive immune response and immune modulation. Once at the lymph node, the DCs first need to migrate across the subcapsular sinus. Upon crossing the sinus, gradients of chemokines
CCL21 and CCL19 regulate the DC migration guiding them to the T-cell zone where they survey naïve T cells (Cyster, 2005; Liao & von der Weid, 2015). T cells do not enter through the SCS, but are rather passively transported to the MS. At the MS T cells cross into the lymph node parenchyma in a CCR7-independent manner but migrate towards the paracortical T cell zones in a CCR7-dependent way (Braun et al., 2011). Free antigens act differently depending on their size with those smaller than 70kDa flowing along the conduits into the lymph node parenchyma where DCs capture them or flowing straight to the B cell zones. The lymph node sinus contains macrophages that restrict and sample bulkier antigens upon lymph node entry. When activated, these macrophages can release IL-18 and activate NK cells and γδ T cells to express IFNγ preventing the systemic spread of pathogens (Kastenmüller et al., 2012). They are also able to transport the antigens to B cells resulting in the activation of an adaptive immune response (Liao & von der Weid, 2015). Once activated, immune cells egress via an S1P gradient into the efferent lymphatic vessels that transport them back into the central circulation. In the efferent lymph T cells make up the majority of transported immune cells with 90% entering the lymph node through the high endothelial venules (Smith et al., 1970).

1.4.4. Maintenance of Peripheral Tolerance

The final important role that the lymphatic system plays within the body is the maintenance of peripheral tolerance. In the gut, it is essential for the tolerance of harmless commensal bacteria and food antigens while maintaining peripheral tolerance to self-antigens (Pabst & Mowat, 2012; Aspelund et al., 2016). Peripheral tolerance depends on semi-activated DCs that carry self-antigen to the lymph node. These DCs interact with
self-reactive T cells that have escaped central immune tolerance and cause anergy or clonal depletion (Steinman et al., 2003). In the lymph node, LECs and fibroblastic reticular cells can also cross-present self-antigens to self-reactive CD8\(^+\) T cells resulting in clonal depletion (Cohen et al., 2010; Tewalt et al., 2012). Thus, the lymphatic system is not only crucial in the initiation of immune responses but also the maintenance of peripheral tolerance.
Figure 5: Lipid absorption occurs within the small intestine through the lacteals within the villi located on the luminal side of the intestinal wall. Lacteals have a unique combination of button-like and zipper-like junctions between endothelial cells allowing for the entry of long-chain fatty acids in the form of chylomicrons (CMs). Contractions by the surrounding smooth muscle and the villus itself drive lymph movement. The enteric neurons modulate contractions, and a dense network of blood vessels surrounds the lacteal. Lymph drains into the submucosal plexus, which transports lymph to the mesenteric lymphatic vessels. Image adapted from Xia et al., 2019.
1.5. Lymphatic Dysfunction and Disease

The lymphatic system plays a significant role in the progression of a large variety of diseases. Dysfunction of lymphatic vessels is associated with an exacerbation of atherosclerosis, a slower recovery post-myocardial infarction, inflammatory bowel disease (IBD), multiple sclerosis (MS) and Alzheimer’s (Bernier-Latmani et al., 2017; Louveau et al., 2015). The disease that it is most well-known for causing is lymphedema. Lymphedema is a consequence of inadequate clearance and transport of protein-rich interstitial fluid resulting in edema, inflammation, fibrosis, distension, and fatty tissue proliferation (International Society of Lymphology, 2013; Schulte-Merker et al., 2011). It is generally thought to be incurable and usually results in the swelling of limbs but can also take place in the head, neck, and breast (Tiwari et al., 2013). There are two types: primary (hereditary) or secondary lymphedema. Primary lymphedema occurs due to genetic mutations, while secondary lymphedema occurs due to factors such as damage from surgery or UV radiation (Sukumaran et al., 2013).

There are many genetic mutations leading to primary lymphedema with VEGFR3 being the first gene identified and accounting for about 50% of lymphedema cases (Aspelund et al., 2016). Other genes from the VEGFC/VEGFR3 signaling axis, such as CCBE1, PTPN14 (tyrosine phosphatase), and FOXC2 result in varying types of lymphedemas (Aspelund et al., 2016). The most common forms of primary lymphedema are Milroy and lymphedema-distichiasis-syndrome with Milroy being present at birth or occurring shortly after, due to a VEGFR3 missense mutation in the tyrosine kinase domain. The onset of lymphedema-distichiasis occurs during late childhood and is due to loss of function mutations in the
FOXC2 gene. Individuals with lymphedema-distichiasis have ordinary vessel density but exhibit lymph reflux due to incompetent valves (Schulte-Merker et al., 2011).

Secondary lymphedema happens in response to surgery, radiation, trauma, or infection (Schulte-Merker et al., 2011). It is a common occurrence after the surgical removal of cancer and irradiation of the surrounding tissue in first world countries (Tiwari et al., 2013). In tropical areas such as Africa, South America, and Southeast Asia, lymphatic filariasis is the cause for 40 million cases of secondary lymphedema. Filariasis occurs by the transmission of parasitic nematodes that specifically dwell in lymphatic vessels from mosquitoes (Aspelund et al., 2016). Once in the lymphatic vessels, these nematodes use the lymphatics to travel throughout the body, often severely damaging vessels. The holes that they create in lymphatic vessels lead to inadequate drainage of the lymph resulting in lymphedema. Currently, the only treatment available to those who suffer from lymphedema is Decongestive Lymphatic Therapy (DLT). DLT consists of manual lymphatic drainage by trained therapists, careful skincare, and constant wearing of compression garments (Moore & Bertram, 2018).

The lymphatic system is also involved in other vascular anomalies such as Gorham disease and Kaposi sarcoma. Gorham’s disease is rare and characterized by local vascular proliferation alongside bone resorption. (Schulte-Merker et al., 2011). These individuals also have systemic dysfunction of lymphatic vessels (Radhakrishnan & Rockson, 2008). Kaposi sarcoma (KS) is caused by infection with human herpesvirus 8 (HHV8) or KS-associated herpesvirus. It exhibits leaky and highly proliferative vessels that express a combination of markers for blood endothelial cell (BEC) and lymphatic endothelial cell (LEC) lineage (Mesri et al., 2010). Interestingly BECs show a transcription shift to a LEC phenotype, and
LECs show a shift to more of a BEC phenotype (Schulte-Merker et al., 2011). These are just two examples of numerous vascular anomalies in which there is lymphatic involvement.
1.6. Lymphatic Vessel Modulation of Pumping

1.6.1. Extrinsic Forces

Extrinsic force impacts the pressure and flow patterns in the lymphatic vessels throughout the body and may result in dramatic differences in lymphatic vessel structure within the same portion of the body (Gashev, 2008). These forces are passive due to their autonomy from active contractions of muscle cells in the vessel wall. However, they contribute to the lymph movement by inducing lymphatic contractility and providing pressure gradients leading to flow. Extrinsic driving forces include lymph formation and central venous pressure fluctuations, which impact lymph flow throughout the body. Other extrinsic forces, such as cardiac and arterial pulses, skeletal muscle contractions, respiration, and gastrointestinal peristalsis, also impact lymph movement (Gashev, 2008). These forces contribute to the pressure gradients observed throughout the body and play an essential role in local modulation of lymph flow.

1.6.2. Intrinsic Forces

Active contractions of lymphangions are the source of intrinsic forces experienced by lymphatic vessels. It is vital for lymph flow in most mammals, including humans (Gashev, 2008). Due to the absence of a centralized pump as in the circulatory system, each lymphangion is responsible for the short-distance movement of lymph into the adjacent lymphangions (Gashev, 2008). This coordination of contractions is crucial to lymph movement over long distances in the body, and these contractions can occur in a peristaltic wave-like manner along the vessels (McHale & Roddie, 1976). Each
contraction results in a positive pressure gradient at the downstream front of the contraction, facilitating lymph flow. At the same time, there is a negative pressure gradient at the upstream edge causing transient backflow, closing the upstream valves (Gashev, 2008). Two notable factors modulate the intrinsic contractile activity of lymphatic vessels: transmural pressure and shear stress due to lymph flow. It is difficult to uncouple the impact of these two factors due to the phasic nature of lymphatic contractions. These two factors are most likely involved in an intricate interplay to control lymph flow via the contraction-relaxation dynamics provided by collecting lymphatic vessels (Kunert et al., 2015).

1.6.3. Pacemaking and the Initiation of Lymphatic Contractions

The contractile activity of lymphatic vessels is known to be initiated by spontaneous transient depolarizations (STDs) (van Helden, 1993). This phenomenon was first described within veins and resulted in the initiation of an action potential (van Helden, 1991). STDs were determined to occur independently of nerve and endothelium function, in a myogenic manner, and suggested to be the lymphatic pacemaker (van Helden, 1993). Low chloride (Cl\(^{-}\)) concentrations and cytosolic Ca\(^{2+}\) chelated with BAPTA AM inhibited the formation of STDs, signifying an indispensable role for the intracellular release of Ca\(^{2+}\) from the SR (Van Helden, 1993). The inhibition of STDs with 2-aminoethoxydiphenyl borate (2-APB) and xestospongin C indicated intracellular Ca\(^{2+}\) release occurred in an IP\(_3\)R-dependent manner (von der Weid et al., 2008). Voltage-dependent calcium channels (VDCCs) are known to be critical in modulating pacemaker activity in lymphatic vessels with T-type Ca\(^{2+}\) being responsible for the regulation of
contraction frequency in Sprague-Dawley rat mesenteric lymphatic vessels (Lee et al., 2014). L-type channels were responsible for the strength of contractions observed with low concentrations of inhibitors diltiazem and nifedipine, exclusively impacting the amplitude of contractions and high concentrations inhibiting contractions completely. T-type Ca\textsuperscript{2+} channels, when inhibited with low concentrations of Ni\textsuperscript{2+} and mibebradil, resulted in a decreased change in contraction frequency as transmural pressure increased (Lee et al., 2014). These results indicated that L-type channels played a similar role in the excitation-contraction coupling as in other tissues and that the T-type channels regulated contraction frequency.

In mice, the differential expression of L-type channels occurs throughout the body, and this impacts the pumping behavior of the lymphatic vessel (Zawieja et al., 2018). They found L-type channel expression throughout the lymphatic system. However, the action potentials of visceral lymphatics were more “hill-shaped,” and they postulated that it was due to differences in L-type Ca\textsuperscript{2+} expression (Zawieja et al., 2018). The electrical coupling of the smooth muscle layer allows for the quick propagation of electrical changes (Hald et al., 2018). Local cell events trigger action potentials in the smooth muscle cells inducing the propagation to the adjacent cells, thus requiring less energy. A heterogeneity of the smooth muscle layer allows for easier propagation of the action potential in the smooth muscle layer while preventing oscillations in membrane potential without the triggering of an action potential. Though no specific pacemaking cells have been defined, they may be present in lymphangions, with the location at either end of the lymphangion allowing for the most efficient propagation of action potentials (Hald et al., 2018). STDs regulating pacemaking activity are known to involve Ca\textsuperscript{2+}-
dependent Cl⁻ channels but the identity of the channel was not known (von der Weid et al., 2008). Recently anoctamin 1 (ANO1), a Ca²⁺-dependent chloride channel, has been implicated as crucial in mediating lymphatic contractility. ANO1 deletion resulted in the ablation of the pressure-dependent increase in contractile activity (Zawieja et al., 2019). It is activated by increases in intracellular Ca²⁺ due to Ca²⁺ entry through L-type Ca²⁺ channels and Ca²⁺ release from the SR through IP₃Rs (Zawieja et al., 2019). Within the past year, new evidence implicating L-type Ca²⁺ channels as the essential pacemakers has emerged. To and colleagues exhibited that T-type Ca²⁺ channel knockout mice showed no significant changes in frequency, amplitude, or fractional pump flow of collecting lymphatics compared to wild-type (WT) mice. Inhibition of the L-type Ca²⁺ channels resulted in the complete abolition of spontaneous lymphatic contractions. They lastly showed mibefradil, and Ni²⁺-dependent inhibition occurred due to off-target effects on L-type Ca²⁺ channels (To et al., 2020). These results indicate the dependence on multiple ion channels to initiate the contractile response in lymphatic vessels.

The endothelial and smooth muscle cell layers are electrically distinct from one another in lymphatic vessels. The absence of IKCa and SKCa channels in endothelial cells results in a lack of endothelium hyperpolarization in response to ACh stimulus (Behringer et al., 2017; von der Weid & Van Helden, 1997). The presence of high myoendothelial gap junction resistance within collecting lymphatic vessels make it highly unlikely that these two cell layers are electrically coupled (Hald et al., 2018). Since there is a lack of electrical coupling and a lack of endothelium hyperpolarization, secreted factors from the endothelium such as nitric oxide (NO) and prostaglandins PGI₂ and PGE₂ play a crucial role in inhibiting the contractile activity of lymphatic smooth muscle
cells (Gashev et al., 2002; Rehal et al., 2009). However, other prostanoids, such as thromboxane A\textsubscript{2} increase lymphatic contractility (Johnston et al., 1983; Mizuno et al., 1998). NO is synthesized in the endothelium by eNOS, while PGI\textsubscript{2}, PGE\textsubscript{2} and TxA\textsubscript{2} are synthesized in the endothelium by COX-1 (Gashev et al., 2002; Rehal et al., 2009). NO diffuses to the smooth muscle where it binds soluble guanylate cyclase (sGC) trigger its pathway resulting in PKG-mediated relaxation. The dilatory prostanoids bind their respective G-protein coupled receptors (GPCRs) on the cell surface and trigger PKA-dependent relaxations within smooth muscle cells. Both increases in transmural pressure and lymph flow induce the release of these factors resulting in the modulation of collecting lymphatic vessel contractility.

1.6.4. Transmural Pressure

Transmural pressure impacts lymphatic contractility by modulating both inotropic (strength of contraction) and chronotropic (frequency of contraction) effects (Gashev, 2008). Both in vivo and in vitro studies, exhibited that increasing transmural pressure induced positive inotropic and chronotropic effects (McHale & Roddie, 1976; Gashev et al., 2007; Lee et al., 2014). In bovine mesenteric lymphatics, lymphatic vessels increased stroke volume and contraction frequency up to a pressure of 4-5 cmH\textsubscript{2}O, reaching the maximum for lymph movement. Further increases in transmural pressure increased frequency, but the decrease in stroke volume generated a decrease in the total lymph movement (McHale & Roddie, 1976). Typically changes in transmural pressure create a logarithm-shaped curve for the pressure to pumping relationship that is species and region-specific (Figure 6) (Gashev, 2008; Gashev et al., 2004). Smaller lymphatic vessels
located more peripherally exhibit maximums in lymphatic contractility at higher transmural pressures than more centrally located vessels (Gashev et al., 2007). The increased activity at higher pressures may be due to the higher outflow resistance that needs to be overcome by these vessels. The result would be shifting the optimal pumping pressure range to more elevated values of pressure. Though distension of the lymphatic wall by changes in transmural pressure is fundamental in modulating the contractile activity of lymphatics, it is not mandatory for pacemaking (Gashev, 2008).

1.6.5. Shear Stress and Lymph Flow

Lymph flow is a significant factor that impacts the functioning of lymphatic vessels through the induction of shear stress. Differences in pressure between the point of lymph entry and downstream lymphangions may result in a pressure gradient that produces lymph movement or flow. Chambers adjacent to one another modulate the contractions occurring to ensure they function in a coordinated manner. This coordination takes place in an endothelium-dependent manner, and shear-stress inhibits the intrinsic pacemaker activity of the lymphatic system (figure 7A, B) (Kornuta et al., 2015). In male Sprague-Dawley rats, findings show that an increase in flow results in a decrease in contraction frequency and ejection fraction meaning less lymph is actively being moved (Gashev et al., 2002). The decrease in contraction frequency transpired immediately upon induction of flow, while the reduction in amplitude of contractions occurred minutes after the change. The passive flow of lymph may be increased under these conditions through lymphatic valves being open and the lymphatic vessel acting similarly to a pipe. The decrease in contraction amplitude may result in the conservation of energy by vessels
when passive lymph movement is high enough to maintain physiological conditions while also decreasing the outflow resistance (Gashev, 2008).

The same group also found that after an initial period of no contractions, contraction frequency increased until reaching equilibrium after 3 minutes at the specified amount of flow (Gashev et al., 2002). This desensitization also happens in the thoracic ducts, cervical and femoral lymphatic vessels isolated from male Sprague-Dawley rats, where under conditions of constant transmural pressure, an increase in shear-stress took place (Gashev et al., 2007; Gashev et al., 2004). Results show that the flow created by contractions in the thoracic duct played a vital role in the regulation of the contractile cycle (Gasheva et al., 2006). Contractile and non-contractile portions of the thoracic duct were isolated and compared. The contractile portions possessed significantly less tone, and the addition of an endothelial nitric oxide synthase (eNOS) inhibitor L-NAME resulted in the restoration of tone. They also found the reduction in tone improved diastolic filling, increased strength of contractions, increased ejection fraction while decreasing the contraction frequency (Gasheva et al., 2006). These findings suggest that lymph flow is a highly dynamic variable playing a fundamental role in lymphatic vessel function.
Figure 6: Frequency to transmural pressure response curve of collecting mesenteric lymphatic vessels within male Sprague-Dawley rats. Lymphatic contraction frequency increased until about 8cmH₂O pressure before plateauing exhibiting the typical flattening of the curve in response to increases in pressure. Figure adapted from Lee et al., 2014.
Figure 7: Flow within lymphatic vessels results in a decrease in contraction frequency and amplitude. Collecting mesenteric lymphatic vessels were isolated, cannulated, and pressurized from male Sprague-Dawley rats for these results. Under baseline conditions of constant transmural pressure (A), the contraction amplitude and frequency are high. The induction of flow (B) results in a decrease in the contraction amplitude and contraction frequency. Figure adapted from Gashev et al., 2002.
1.7. Endothelium-Dependent Relaxation Pathways

Flow within lymphatic vessels plays a fundamental role in the modulation of contraction frequency and induces a decrease in the contraction frequency of lymphatic vessels. In blood vessels flow causes a relaxation of the vessel resulting in dilation, and there are three major endothelial-dependent relaxation pathways, while in lymphatics there are only two major pathways. The first pathway is dependent on nitric oxide (NO) production via endothelial nitric oxide synthase (eNOS). The second requires prostaglandins produced via the cyclooxygenase 1 (COX-1) enzyme. The third, and final, pathway in blood vessels occurs through hyperpolarization caused by the activation of small and intermediate Ca^{2+}-regulated K^+ channels (SK and IK channels) (Edwards et al., 2010). While this occurs in blood vessels, it does not occur in lymphatic vessels due to a lack of electrical coupling via gap junctions between endothelial and smooth muscle cells and a lack of SK and IK channel expression in endothelial cells (von der Weid & Van Helden 1997; Behringer et al., 2017). Due to a lack of support for this pathway in lymphatic vessels it will not be addressed in the scope of the current project. The lack of coupling enables the membrane potentials in the endothelium and the muscle to remain distinct from one another. In lymphatics, NO plays an indispensable role in the modulation of lymphatic contractions and tone under conditions of flow (Gashev et al., 2002). Though the focus of this project is lymphatic vessels, discussion of the endothelium-dependent relaxation pathways will focus on how they function in blood vessels due to the depth of understanding in this field. Where appropriate the pathways will be compared and contrasted to what occur within lymphatic vessels resulting in a complete understanding of how these pathways function.
1.7.1. eNOS-dependent Nitric Oxide (NO) Production

The discovery of this pathway occurred in 1980 when Furchgott and Zawadzki found that the arterial endothelium produced a factor that mediated a relaxation response upon acetylcholine (ACh) treatment. Removal of the endothelium resulted in the ablation of the relaxation observed. However, when present, it resulted in a vasodilatory response leading to the proposal of an endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980). This EDRF was then defined as nitric oxide in 1987 by Ignarro through determining that EDRF and NO had the same properties resulting in the relaxation of arteries and veins (Ignarro et al., 1987). NO-induced relaxations led to the paradigm that the endothelium was a significant source of mediators for both relaxation and constriction.

Nitric oxide can be produced by three different nitric oxide synthases (NOS) with endothelial NOS playing a crucial role in vascular function, neuronal NOS in neuronal cells, and inducible NOS expression occurring in a wide variety of cells (Tejero et al., 2019). All three of these NOS are dependent on L-arginine and molecular oxygen (O₂), as indicated by the cells inability to produce NO under anoxic or hypoxic conditions (Tejero et al., 2019). L-arginine concentrations are heavily dependent on dietary intake, with biosynthesis being inadequate to compensate for insufficient in-take (Hecker et al., 1990). All three forms of NOS are dimeric, multidomain enzymes that require multiple co-factors to support the synthesis of NO (Daff, 2010). The dimeric nature is due to the transfer of electrons occurring between the reductase domain and oxygenase domains of the two different monomers, thus preventing the formation of NO in monomeric forms of the NOS (Tejero et al., 2019).
eNOS is the constitutively active form of NOS within endothelial cells and is the primary source of NO production within the vascular system (Tejero et al., 2019). The NO produced modulates not only the blood flow in the vascular system but also decreases the expression of inflammatory adhesion molecules and platelet activation (Tejero et al., 2019). Erythrocytes (red blood cells) can absorb the NO produced acting as a sink for excessive NO while also releasing NO at distant sites, where required (Klinger & Kadowitz, 2017). eNOS phosphorylation is imperative to its activity within the body with phosphorylations at Ser 1177 and Tyr 81 enhancing NO production and phosphorylations at Thr 495 and Tyr 657 inhibiting NO production (Tejero et al., 2019). Caveolin can also modulate eNOS activity by sequestering eNOS until sufficient concentrations of Ca\(^{2+}\) are present in the cell. Improper modulation by caveolin results in too high concentrations of eNOS, resulting in the uncoupling of eNOS and the production of superoxide instead of nitric oxide (Kone et al., 2003; Klinger & Kadowitz, 2017). The activation of eNOS is calcium (Ca\(^{2+}\)) and calmodulin (CaM)-dependent. Increases in intracellular Ca\(^{2+}\) usually result in the dephosphorylation of Thr 495, promoting the activity of eNOS. Cytosolic Ca\(^{2+}\) binds CaM, and the activated CaM binds to eNOS, enabling dimerization. Dimerization aligns the oxygenase and reductase domains in enzymes, prompting the dissociation from caveolin (Kone et al., 2003). The phosphorylation of Ser 1177 can occur in response to agonist addition (bradykinin), shear stress, or activation with VEGF, with all of these pathways increasing eNOS activity (Bauer et al., 2003; Dimmeler et al., 1999; Fulton et al., 1999). Modulation of eNOS activity can transpire by glutathionylation, s-nitrosation, acetylation, and glycosylation. eNOS localization depends in part on both myristoylation and palmitoylation (Tejero et al., 2019).
The NO relaxation pathway, as described in blood vessels, starts with an influx of Ca\textsuperscript{2+} into the endothelial cell when a shear stress sensor is triggered at the surface by luminal fluid movement. The Ca\textsuperscript{2+} that enters the cell and the Ca\textsuperscript{2+} released from the SR binds to CaM, and the activated CaM allows for the dimerization and activation of eNOS. Activated eNOS then uses O\textsubscript{2} and L-arginine as substrates to synthesize NO and L-citrulline (Klinger & Kadowitz, 2017). The co-factors nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and heme are required to carry electrons during the oxidation of L-arginine to L-citrulline and NO (Klinger & Kadowitz, 2017). eNOS activity can be inhibited by the blocker N\textsubscript{w}-nitro-L-arginine (L-NNA) in a non-enzyme specific manner (Tejero et al., 2019). It mimics L-arginine, competing with it for the binding site in eNOS and upon binding inhibits the production of NO.

In blood vasculature, the NO produced in the endothelial cell diffuses to the adjacent vascular smooth muscle cells where it acts on soluble guanylate cyclase (sGC). sGC is the canonical receptor of NO and located within the cytosol as a heterodimer (Tejero et al., 2019). There are two possible \(\alpha\) and \(\beta\) subunits, and they possess a different distribution throughout the body (Budworth et al., 1999). The \(\beta\) subunit contains the heme domain essential to NO binding, resulting in a change in conformation. The change in conformation activates the C-terminal catalytic domain facilitating the conversion of GTP into cGMP (Winger & Marletta, 2005). cGMP can target phosphodiesterases, cGMP-dependent kinases, and cGMP-gated ion channels downstream (Münzel et al., 2017; Münzel et al., 2003; Warner et al., 1994). cGMP acts on PKG, which has a myriad of downstream targets that modulate the Ca\textsuperscript{2+} concentration.
in smooth muscle cells, facilitate K\(^+\) efflux, and modulate the activity of myosin light chain phosphatase. PKG inhibits L-type Ca\(^{2+}\) channels on the plasma membrane and IP\(_3\)R channels on the sarcoplasmic reticulum (SR) via IRAG upregulation. G-protein coupled Ca\(^{2+}\) activation is inhibited through the upregulation of RGS-2 (regulator of G-coupled signaling 2), further depleting intracellular Ca\(^{2+}\). PKG lastly promotes Ca\(^{2+}\) removal from the cytoplasm through upregulation of the SERCA Ca\(^{2+}\) pump on the SR (Kass et al., 2007; Klinger & Kadowitz, 2017; Munn, 2015). PKG activation also promotes K\(^+\) efflux through the activation of BK\(_{Ca}\) channels leading to hyperpolarization of smooth muscle cells and relaxation. PKG activity also impacts myosin light chain (MLC\(_{20}\)) phosphorylation. Myosin light chain phosphatase (MLCP) activity is increased through the inhibition of RhoA (Ras homolog member A) and the downregulation MYPT (myosin phosphatase targeting subunit) through phosphorylation at Ser 695 (Klinger & Kadowitz, 2017). Enhancing MLCP activity results in the relaxation of the vascular smooth muscle and an inhibition of the contraction response (Figure 8A).

The NO-dependent pathway can also be stimulated with the classical agonists ACh or bradykinin. ACh acts in a G-protein coupled receptor-dependent manner (GPCR) through the M3 muscarinic receptor and G\(_q\) or G\(_{13}\) coupling with phospholipase-C\(\beta\) (PLC\(\beta\)). PLC\(\beta\) activity cleaves phosphatidylinositol 4’,5’ bisphosphate (PIP\(_2\)) into di-acyl glycerol (DAG), and inositol 1’, 4’, 5’ tris-phosphate (IP\(_3\)). IP\(_3\) triggers Ca\(^{2+}\) release from the SR through the activation of the IP\(_3\) Receptors (IP\(_3\)Rs), and the subsequent increase in intracellular Ca\(^{2+}\) enables the formation of the Ca\(^{2+}\)-CaM complex that activates eNOS activity in endothelial cells (Tejero et al., 2019). Due to this, ACh can be used to determine if the endothelium is indeed functional before exposing vessels to flow.
(Sukumaran et al., 2013). The eNOS-dependent production of NO triggers a complex pathway that plays a vital role in mediating the activity of both arteries and lymphatic vessels (Sukumaran et al., 2013; Behringer et al., 2017). eNOS-dependent production of NO in lymphatic vessels is generally similar to blood vessels, though there is one notable difference. While BK$_{Ca}$ channels are integral in regulating resting membrane potential in the blood vasculature, they do not play a leading role in lymphatic vessels (von der Weid, 1998; von der Weid & Van Helden, 1996). Instead, K$_{ATP}$ channel inhibition with 10 µM Glibenclamide resulted in the abrogation of ACh-induced, NO-dependent hyperpolarization in the lymphatic smooth muscle cells (von der Weid, 1998). These results indicate that the K$_{ATP}$ channels may be fulfilling the role of the BK$_{Ca}$ channel in lymphatic vessels. A more likely mechanism involves the action of both of these channels in regulating the membrane potential of the smooth muscle layer within collecting lymphatic vessels.

1.7.2. Prostaglandin Synthesis

The prostaglandin synthesis pathway has previously shown to play an integral role in mediating relaxation within both arteries and lymphatic vessels (Moncada et al., 1976; Hanley et al., 1989; Rehal et al., 2009). Shear stress alters the plasma membrane structure stimulating the release of prostaglandins in an endothelium-dependent manner. It is a well-understood pathway and will be described in blood vessels. Changing the plasma membrane conformation prompts the cleavage of phospholipids to form DAG and IP$_3$. Phospholipase-A$_2$ cleaves DAG after it is formed from plasma membrane to form arachidonate. Arachidonate has many possible
downstream products depending on the cleavage that occurs. If cleaved by 12-lipoxygenase, it can form 12-HETE, and 15-lipoxygenase activity results in lipoxins. The activity of 5-lipoxygenase produces 5-HPETE, and cleavage by Cyp450 causes EET (epoxyeicosatrienoic acids) formation, though it can also form other vasoactive mediators such as leukotrienes. The EETs can activate smooth muscle BKCa channels in an endothelium hyperpolarizing factor (EDHF)-dependent manner contributing to the relaxation response in arteries. Cyclo-oxygenase (COX) is the first enzyme involved in prostaglandin synthesis, and its inhibition prevents prostaglandin synthesis (Randhawa & Jaggi, 2015; Rang et al., 2007). There are two COX enzymes, COX-1 and COX-2. COX-1 is constitutively active and present in most body tissues, while inflammation or injury stimulates COX-2 expression and activity (Lucas, 2016). The COX enzymes convert arachidonic acid (AA) into prostaglandin G2 (PGG2) and subsequently prostaglandin H2 (PGH2). PGH2 peroxidation can result in the production of thromboxanes (Tx’s) or one of the D, E, I, or F prostaglandins (Hata & Breyer 2004). Treatment with the competitive inhibitor indomethacin represses COX enzyme activity preventing prostaglandin synthesis. Indomethacin competes with AA for active site binding, and when bound, prevents access to the active site, blocking AA cleavage. Indomethacin has a higher potency than other NSAIDs such as naproxen and ibuprofen, inhibiting COX activity to a greater extent (Lucas, 2016).

Prostaglandin I2 (PGI2) is the primary prostaglandin involved in the relaxation response, acting on the IP-receptors located mainly on the surface of smooth muscle cells (Blindt et al., 2002). The Gs protein facilitates IP-receptor and adenylate cyclase coupling, permitting the conversion of adenosine triphosphate (ATP) into cyclic
adenosine monophosphate (cAMP) (Pluchart et al., 2017). IP-receptors can also activate PLC through coupling with the G_q protein. cAMP targets protein kinase A (PKA), and PKA activation targets a myriad of downstream effectors. PKA activity promotes K^+ efflux through BK_{Ca} channels and the inhibition of Ca^{2+} release from the SR through IRAG (IP_3R-associated cGMP kinase substrate) repressing IP_3R (inositol 1', 4', 5' trisphosphate receptor) activity (Lyle et al., 2017). Within lymphatic vessels PKA promotes K^+ efflux through K_{ATP} channels, though recent evidence indicates that there are also contributions from the BK_{Ca} channels in CLVs (To et al., 2020). PKA activity also inhibits MLCK activity preventing the subsequent phosphorylation of MLC (Figure 8B) (Miller, 1983). All of these downstream targets stimulate the vasorelaxant response.

The IP receptor can also associate with the TP receptor and form a heterodimer (Woodward et al., 2011). In this case, when PGI_2 activates IP, it can result in vasoconstriction. Overall, the synthesis of prostaglandins and specifically PGI_2 within the endothelium play an essential role in mediating an endothelium-dependent relaxation response within both arteries and lymphatic vessels.
Figure 8: The two main flow mediated endothelium-dependent relaxation pathways that occur in blood vessels and contribute to lymphatic vessel pumping inhibition. A) The NO-dependent pathway that is dependent on eNOS activity in the endothelium producing NO that diffuses to the smooth muscle layer and causes relaxation. B) The prostaglandin synthesis pathway in which PGI$_2$ synthesized in the endothelium diffuses and acts on the IP receptor on the smooth muscle resulting in downstream signaling and relaxation.
1.8. Shear Stress and Mechanosensitive Ion Channels in the Vasculature

1.8.1. Shear Stress Sensors

Blood flow within the cardiovascular system plays an imperative role in the modulation of endothelial function. There are two main types of flow, laminar and disturbed flow, with laminar flow resulting in endothelial cell alignment with the direction of flow and the upregulation of anti-inflammatory genes (as reviewed in Givens & Tzima, 2016; Chatzizisis et al., 2007). Flow within the vasculature results in shear stress, which is sensed by a myriad of mechanosensors located on the luminal, junctional, or basal sides of the endothelial cells. The apical side of the cells expresses shear stress sensors such as primary cilia, the glycocalyx, GPCRs, caveolae, and mechanosensitive ion channels (as reviewed in Givens & Tzima, 2016). At the junctional points between cells PECAM-1, VE-Cadherin and VEGF receptors play a role. At the same time, on the basal portion of the membrane, integrins are crucial in mediating shear stress-dependent responses (as reviewed in Givens & Tzima, 2016). Within the cardiovascular system, TRPV4 and Piezo1, two mechanosensitive ion channels, play an essential role in sensing shear stress. In the following sections, the functions of TRPV4 will be discussed due to the large amount of literature supporting its role in vasodilation.

1.8.2. TRP Channel Family

The initial discovery of Transient Receptor Potential (TRP) channels took place in Drosophila melanogaster (fruit fly) that carried mutations in the Trp gene (reviewed in Yue et al., 2015). These mutations resulted in light-sensing mutants that showed a transient voltage response resulting in an impaired visual adaption. This development led
to the discovery of other TRP channel families in different animals such as zebrafish (*Danio rerio*), worms, and mammals (Yue et al., 2015; Tang et al., 2018). Currently, the TRP channel superfamily consists of more than 30 cation channel members, 28 in mammals, classified into seven different subfamilies. These subfamilies include the TRPC (canonical) family, TRPM (melastatin) family, TRPV (vanilloid) family, TRPP (polycystin) family, TRPML (mucolipin) family, TRPA (ankyrin) family and the TRPN or NOMPC family (Figure 9) (reviewed in Yue et al., 2015; Hantute-Ghesquier et al., 2018). The structure encoded by these genes are similar to the voltage-gated ion channel superfamily, with a major difference being the lack of a voltage sensor domain on trans-membrane segment 4, and they all contain at least six transmembrane domains (Christensen & Corey, 2007; Yue et al., 2015). The pore-forming domain is in between transmembrane segments 5 and 6, with both the C and N terminus ends located in the cytoplasm (Yue et al., 2015).

All members of the TRP channel family are permeable to Ca$^{2+}$ except for TRPM4 and TRPM5, which are monovalent, cation-selective channels. Only TRPV5 and TRPV6 are highly selective for Ca$^{2+}$ cations with a $P_{Ca}/P_{Na}$ value of greater than 100 (reviewed in Nilius et al., 2007). TRP channels are involved in the transduction of a wide range of stimuli, including nociception, taste perception, olfaction, osmolarity sensing, thermosensation, and chemosensation (Kaneko & Szallasi, 2014; Yue et al., 2015). The transient receptor potential vanilloid (TRPV) family encompasses six mammalian members: TRPV1-6. TRPV1-4 all function as heat-activated channels that are modestly permeable to Ca$^{2+}$, while being non-selective cation channels. TRPV5 and 6 are both highly Ca$^{2+}$ selective channels that are activated by low intracellular Ca$^{2+}$ concentrations
(Nilius et al., 2007). It was named the vanilloid family due to the identification of TRPV1 being sensitive to capsaicin (a vanilloid) (Kaneko & Szallasi, 2014). The general structure of TRPV channels includes 3 to 5 ankyrin repeats in the N-terminal, cytosolic side of the channel alongside the previously mentioned six transmembrane domains, and conducting pore (Hantute-Ghesquier et al., 2018).

There are a few significant flow sensitive and mechanosensitive TRP channel family members. The most well documented is TRPV4, which can sense shear stress by itself, in conjunction with TRPC1, or through heterotetramer formation with both TRPC1 and TRPP2 (Mendoza et al., 2010; Ma et al., 2010; Du et al., 2014). TRPV1 displays a very similar structure to TRPV4 and can facilitate Ca\(^{2+}\) entry in response to shear stress as well. TRPC6 is a mechanosensitive TRP channel that responds to increases in pressure, contributing to myogenic tone (Nilius et al., 2007). It can interact in series with TRPM4 to mediate myogenic constriction in response to pressure (Gonzales et al., 2014).

1.8.3. TRPV4

The TRPV channel that is of particular interest to us is the TRPV4 channel. In vitro TRPV4 is known to be activated in the range of 24-34 °C, but it can also be constitutively active at 37 °C (Güler et al., 2002; Kauer et al., 2009; White et al., 2016). It is a polymodally activated channel that responds to osmolarity, mechanical stimuli, and arachidonic acid products (Darby et al., 2016). Activation of TRPV4 in both the preoptic area and the skin results in hyperthermia, while inhibition resulted in hypothermia, justifying its role in thermoregulation (Yadav et al., 2017; Vizin et al., 2015). There are five known splice variants of TRPV4, with only two of them being full-length variants.
expressed on the cell surface. The remaining three are incompletely formed and restricted to the endoplasmic reticulum (ER) (Arniges et al., 2006). TRPV4 expression occurs in a variety of tissues, including the heart, endothelium, vascular smooth muscle, brain, kidneys, salivary glands, lungs, liver, spleen, and within DRG neurons (Randhawa & Jaggi, 2015; Balemans et al., 2019). TRPV4−/− mice are viable and fertile, exhibiting only minor phenotypes that include altered osmosensation, compromised vascular endothelial function, and defective bladder wall stretch sensation (Cortright & Szallasi, 2009; Sonkusare et al., 2012; Everaerts et al., 2010). Improper functioning of TRPV4 has been shown to result in pulmonary hypertension and impaired control of blood pressure in the vasculature (Yang et al., 2012; Nishijima et al., 2014). It also plays a role in visceral hypersensitivity during IBD and respiratory diseases such as acute lung injury and hydrostatic pulmonary edema (Balemans et al., 2019; Goldenberg et al., 2015).

TRPV4 is activated endogenously by epoxyeicosatrienoic acids (EETs) in an anandamide and an arachidonic acid-dependent manner (White et al., 2016). EETs bind a specific site on TRPV4 resulting in the activation of the channel allowing for calcium entry and the triggering of relaxation pathways (Vriens et al., 2005; Berna-Erro et al., 2017). It can pharmacologically be activated by the phorbol ester 4α-phorbol 12,13 didecanoate (4α-PDD) and by the selective agonist GSK1016790A. Inhibitors previously thought to be selective for TRPV4, gadolinium, and ruthenium red, have multiple off-target effects with newer inhibitors proving to be more selective (Grace et al., 2017). HC067047 potently, selectively and reversibly inhibits human, rat, and mouse TRPV4 function within transfected HEK293 cells with IC50 values of 48 ± 6 nM, 133 ± 25 nM and 17 ± 3 nM, respectively (Everaerts et al., 2010). RN-1734 with an IC50 of 5.9
µM, and the orally bioavailable GSK2193874 are other commonly used TRPV4 inhibitors (Vincent et al., 2009; Grace et al., 2017). Recently the pharmacokinetics and safety of a new TRPV4 inhibitor, GSK2798745, were determined in healthy controls and patients with stable heart failure. In healthy subjects, there were no clinically significant safety concerns for doses up to 12.5mg one-time doses and 5mg/day doses for 14 days with these doses being sufficient to provide near-complete inhibition of TRPV4 function (Goyal et al., 2019).

In previous research conducted in rat pulmonary arteries, TRPV4 played a principal role in mediating endothelium-dependent vasodilation. Two significant findings were that TRPV4 was responsible for “calcium sparklets” in the blood vessel endothelium within mice and that it was involved in artery dilation at low pressures within rats (Bagher et al., 2012; Sonkusare et al., 2012). These “sparklets” were transient increases in Ca^{2+} flux through TRPV4 channels that acted cooperatively. The authors also found that as few as 3-8 TRPV4 channels were sufficient to induce maximal arterial relaxation (Bagher et al., 2012). The second study used rat cremaster arterioles and found that TRPV4 mediated the increase in intracellular calcium in endothelial cells during conditions of low pressure leading to a dilation. Inhibition of TRPV4 in these situations resulted in an increase in myogenic tone instead of the relaxation previously observed (Sonkusare et al., 2012). Work by Mendoza et al. showed TRPV4 function was vital in flow-dependent relaxation via the NO-dependent and EDHF-dependent pathways in small mesenteric arteries (Mendoza et al., 2010). In another study, rat pulmonary arteries were precontracted with phenylephrine and subsequently treated with the TRPV4 agonist, GSK1016790A, resulting in a relaxation of the artery (Sukumaran et al., 2013). Similar
results occurred in a study utilizing rat cremaster arterioles. They found that TRPV4 was involved in endothelium-dependent vasodilation in response to shear-stress. This group also found that TRPV4 interacted with acetylcholine receptors (AChR) after periods of shear stress (Darby et al., 2018).

Cell surface expression of TRPV4 depends on protein synthesis and correct folding in the ER, glycosylation, recycling, and degradation. Through all of this, the molecular mechanism of TRPV4 mechanosensation is not clearly understood and requires elucidation. Recently TRPV4 modulation under conditions of flow and stimulation with the selective agonist GSK1016790A was determined. Using Human Umbilical Vein Endothelial Cells (HUVECs), Baratchi and colleagues determined that shear stress sensitized TRPV4 to its selective agonist GSK1016790A (GSK) (Baratchi et al., 2015). They also showed that TRPV4 surface-level expression increased after shear stress stimulation in an intracellular Ca\(^{2+}\) dependent manner as inhibition of TRPV4 function with HC067047 and GSK2198374 did not impact trafficking to the cell surface. They lastly determine that TRPV4 trafficking to the cell surface occurred in an actin cytoskeleton dependent manner (Baratchi et al., 2015). In more recent work, they showed that TRPV4 resided in clusters under conditions lacking shear stress, but upon shear stress induction, the clusters dissociated into individual channels. Cell surface expression of TRPV4 was determined to occur in an integrin \(\alpha 4\beta 1\) and \(\alpha 5\beta 1\)-dependent manner (Baratchi et al., 2017). Upon GSK stimulation of TRPV4 channels in HUVECs, there was a transient increase in surface expression that coincided with a transient increase in Ca\(^{2+}\) entry. TRPV4 upregulation at the surface occurred via complete or partial vesicle fusion, and GSK stimulation significantly increased the partial vesicle fusion. Surface
expression of TRPV4 was downregulated within 20 minutes of stimulation and took place in a Ca$^{2+}$-dependent manner. The decrease in surface expression of TRPV4 occurred due to the translocation to recycling endosomes (Baratchi et al., 2019).

In collecting lymphatic vessels, TRPV4 plays an important role in ACh-induced relaxation of lymphatic vessels (Behringer et al., 2017). ACh is a neurotransmitter that acts on the vasculature in a biphasic manner. It has receptors that are present on both the endothelial cells and on smooth muscle cells with endothelial stimulation resulting in a relaxation response and muscle stimulation in constriction or contraction (Tousoulis et al., 2014). ACh-induced relaxations occur in response to calcium entry into the endothelial cell, initiating eNOS activity. eNOS activation, as previously mentioned, results in the production of NO, leading to the relaxation of the vessel. The ACh response observed in popliteal lymphatic endothelial cell tubes (LECTs) isolated from TRPV4$^{-/-}$ mice differed significantly from WT mice. TRPV4 ablation resulted in a significant decrease in the Ca$^{2+}$ entry into the LECTs, and depolarization was attenuated by 70% in the TRPV4$^{-/-}$ mice (Behringer et al., 2017). The decreased Ca$^{2+}$ entry indicates that TRPV4 may be playing a role in an eNOS-dependent manner in the endothelium-dependent relaxation pathway in lymphatic vessels.

TRPV4 can also act in a COX-dependent manner, regulating vascular contractions through thromboxane receptor activation. In mouse aortas, TRPV4 was capable of regulating Ca$^{2+}$ entry in response to GPCR activation that stimulates cytosolic phospholipase A$_2$ (cPLA$_2$) (Saifeddine et al., 2015). They also found local activation of GPCRs such as PAR1, PAR2 or AT$_1$ receptors resulted in the augmentation of the TRPV4-dependent constriction response. In other work, TRPV4 activation coupled with
eNOS inhibition resulted in a vasoconstriction response in mouse mesenteric arteries (Mendoza et al., 2010). In this study, they did not try and elucidate the pathway through which it was occurring but noted that it occurred in an endothelium-dependent manner. Supporting these results, TRPV4 activation within endothelium-denuded mesenteric arteries resulted in a vasoconstriction response (Sukumaran et al., 2013). TRPV4 can also be activated by 5-HT (serotonin), resulting in a contraction in pulmonary arteries (Xia et al., 2013). These responses contribute complexity to the regulation of vascular tone via TRPV4-dependent mechanisms.
Figure 9: Single subunit structure of the six different transient receptor potential (TRP) channel families expressed in mammals. All TRP channels require tetramer formation for the channel to be functional. TRP channels can form both homotetramers and heterotetramers. These heterotetramers can be formed by channels from the same or different families. All Families have the pore formation occurring between transmembrane segments 5 and 6 and have the N and C terminal segments within the cytoplasm of the cell. Figure adapted from Yue et al., 2015.
2. CHAPTER TWO: HYPOTHESIS AND RATIONALE

2.1. Rationale

The lymphatic system plays a vital role in the maintenance of fluid balance, the absorption of dietary fats, and the facilitation of an immune response while being essential to sustain life (Aspelund et al., 2016). Fluid transport is mainly dependent on the active contractile activity of the collecting lymphatic vessels, though extrinsic forces do contribute to lymph movement (Gashev, 2008). Modulation of the collector lymphatic vessel’s ability to contract occurs by both transmural pressure and intraluminal fluid flow, which produces shear stress. Increases in transmural pressure increase the contractility of lymphatic vessels, while increases in fluid flow result in a decrease in contractility (McHale & Roddie, 1976; Lee et al., 2014; Gashev et al., 2002, Gashev et al., 2004). Flow-induced decreases in lymphatic contractility are known to be partially dependent on nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS). The elevation of eNOS activity is dependent on an increase in cytosolic calcium (Ca\textsuperscript{2+}) concentrations, with both the entry of external Ca\textsuperscript{2+} and the release of Ca\textsuperscript{2+} from internal stores contributing to the cytosolic concentration.

TRPV4 is a polymodally activated cation selective, Ca\textsuperscript{2+} permeable channel found in both endothelial and smooth muscle cells (Darby et al., 2016; Mendoza et al., 2010). Within the vascular system, it contributes to flow-induced, endothelium-dependent relaxation (Sonkusare et al., 2012; Mendoza et al., 2010). Flow-induced relaxations occur through contributions by both the endothelium-derived hyperpolarizing factor (EDHF) and the eNOS-dependent NO production pathways (Sonkusare et al., 2012; Mendoza et al., 2010). Within the lymphatic system, it mediates the endothelial cell depolarization due to the
endothelium-dependent relaxation response upon acetylcholine (ACh) treatment, which is a classical agonist of the NO-dependent relaxation pathway (Behringer et al., 2017). Due to a lack of electrical coupling between endothelial and smooth muscle cells in lymphatics, and a lack of IKCa and SKCa expression, the electrical coupling portion of the EDHF relaxation pathway does not contribute to smooth muscle relaxation in collecting lymphatic vessels (von der Weid & Van Helden 1996; Behringer et al., 2017). TRPV4 may thus be contributing to the NO-dependent relaxation that occurs within lymphatic vessels in response to flow.

The characteristics of TRPV4 and its known function within the vascular system make it a prime candidate that contributes to flow-induced vessel relaxation and decreases in contractile activity. The increase in Ca^{2+} through this channel and its known function upstream of eNOS make it a logical choice to pursue within the scope of this project. In this project, we investigated the role that TRPV4 plays in the flow-induced decrease in contractile activity within collecting lymphatic vessels of the rat mesentery.
2.2. Hypothesis

TRPV4 plays a key role in mediating the flow-induced inhibition of lymphatic pumping via eNOS-dependent NO production. Inhibition of TRPV4 activity will result in a significant restoration of the decrease in contractile activity observed in response to intraluminal flow within rat mesenteric lymphatic vessels.

Figure 10: Schematic of the project hypothesis.
2.3. Aims

2.3.1. **Aim 1:** Determine TRPV4 gene and protein expression, and protein localization within rat mesenteric collecting lymphatic vessels.

2.3.2. **Aim 2:** Investigate the role of TRPV4 in flow-induced inhibition of lymphatic contractility
3. CHAPTER THREE: METHODS

3.1. Animal care

Adult male Sprague-Dawley rats aged 8 to 12 weeks (150-300g, Charles River Labs, Senneville, QC) (n = 40) were used for all experiments. Rats were housed in an enriched environment containing a shelter and bedding at 20 °C with light: dark cycles of 12:12 and access to water and standard rat chow *ad libitum*. The University of Calgary Animal Care and Ethics Committee reviewed and approved of all the protocols used. This rat model was used because the mesenteric lymphatics display a robust phasic contractile activity, similar to humans, that can be studied via pressure myography.

3.2. Solutions

A 0.5% MOPS-APSS (albumin containing physiological saline solution) was used to store the gut removed from Sprague-Dawley rats and for all subsequent dissection and vessel isolation procedures performed at room temperature. The solution had a pH 7.40 ± 0.01 at room temperature (21°C), and contained (in mM): 145 NaCl, 4.7 KCl, 2 CaCl₂, 5 dextrose, 1.17 MgSO₄, 3 MOPS (3-(N-morpholino)-propane sulfonic acid), 1.2 NaH₂PO₄, 0.02 EDTA and 2 sodium pyruvate with 0.5% w/v BSA (bovine serum albumin) added to the solution meaning (5g of BSA per 1L of MOPS-PSS). The same solution was made, with the pH of 7.40±0.01 determined at 37 °C to be used for pressure myography *ex vivo* pumping experiments. This solution was used to fill the pipettes on which lymphatic vessels were mounted. It was also superfused into the vessel-containing chamber at a physiologically relevant temperature of 36-37 °C. Calcium free MOPS-PSS was made containing (in mM): 145 NaCl, 4.7 KCl, 5 dextrose, 1.17 MgSO₄, 3 MOPS, 1.2 NaH₂PO₄, 3
EDTA and 2 sodium pyruvate and had a pH of 7.40 ± 0.01 at 37 °C and allowed for the determination of the maximal dilation of the lymphatic vessels (Davis et al., 2009). The only difference between this solution and other PSS solutions used were the replacement of CaCl\textsubscript{2} with 3mM of EDTA and the lack of BSA.

3.3. Lymphatic Vessel Isolation

Rats were euthanized by an anesthetic overdose with isoflurane and sacrificed using decapitation. The stomach was then cut open, and the small intestine was exposed and moistened with room temperature (RT) 0.5 % MOPS-APSS. Sutures were tied at the terminal ileal and proximal duodenal ends, just before the cecum and after the stomach, respectively. The small intestine was then removed from the rat and placed in RT 0.5 % MOPS-APSS. The small intestine was transferred to a dissecting dish containing fresh RT 0.5 % MOPS-APSS. 3-5 mesenteric arcades, containing an artery, vein, lymphatic vessels, and some fat, were isolated from the ileal portion of the intestine (Figure 11). The first arcade isolated was immediately pinned to the bottom of a Sylgard-coated dish filled with RT 0.5% MOPS-APSS. The remaining arcades were pinned in dishes containing DMEM: F12 (1:1) media supplemented with antibiotics and stored in a 37 °C incubator with 5 % CO\textsubscript{2} until use (ThermoFisher Scientific, Waltham, MA). After pinning the first arcade, lymphatic vessel isolation occurred by gentle removal of the surrounding fat cells and connective tissue with forceps exposing an 8 – 10 mm long portion of the vessel. This vessel would then be excised and flushed for either real-time quantitative polymerase chain reaction (RT-qPCR) or pressure myography. Vessels were isolated in the same manner from the stored arcades were more contracted (less inflated) than those from the first arcade.
Figure 11: Rat mesenteric arcade isolated from the ileal portion of the small intestine pinned in a Sylgard-coated dish. Arcade from a male Sprague-Dawley rat showing the artery and vein in the middle (white arrows) with the clear collecting lymphatic vessels within the fat on either side (red arrows). It is oriented with the portion adjacent to the small intestine at the bottom of the picture and the lymph node adjacent portion at the top.
3.4. Endpoint RT-qPCR

Due to the low cell numbers and RNA yield from individual mesenteric lymphatic vessels, 4-6 vessels of about 4-6 mm in length were isolated and pooled from the ileal portion of the intestine of a total of 7 animals. Vessels were mounted on a small glass pipette, with a diameter of about 70 µm, and then gravity flushed with a pressure of about 10 cmH₂O at the inflow pipette with Dulbecco’s Phosphate Buffered Saline (DPBS). The outflow pipette was not pressurized but was used to mount the downstream portion of the vessel and facilitate the flow of fluid through the vessel. Vessels were stored in 350 µL buffer RLT containing 1 % β-Mercaptoethanol at -20 °C until mRNA extraction. The vessels were homogenized, and RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA), as stated in the manufacturer's protocol. The purity and yield of RNA were determined using a Nanodrop 2000c Spectrophotometer. cDNA synthesis occurred using 5X All-In-One RT Master Mix (Applied Biological Materials Inc., Canada) as recommended by the supplier. The sample was diluted to a concentration of 1 ng/µL and stored at –20 °C until qPCR was performed.

RT-qPCR was performed on the samples for TRPV4 (transient receptor potential vanilloid 4), TRPV1 (transient receptor potential vanilloid 1), TRPC1 (transient receptor potential canonical 1), TRPC6 (transient receptor potential canonical 6), TRPM4 (transient receptor potential melastatin 4), TRPM8 (transient receptor potential melastatin 8), ANO1 (Anoctamin 1), BEST1 (Bestrophin 1) and on the housekeeping genes GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and HPRT1 (hypoxanthine phosphoribosyltransferase 1). All results for channels other than TRPV4 will be presented in the appendices. qPCR was done using 10 µL total volume, as stated in the manufacturer's protocol (ThermoFisher Scientific). The first step was 10 minutes at 95 °C to denature the
DNA before 40 cycles of 15 seconds at 95 °C for denaturation, 30 seconds at 60 °C for annealing and 30 seconds at 72 °C for elongation and the final cycle being 5 min at 72 °C. Using the 72 °C step instead of just 60 °C for a full minute resulted in better replication since this is the temperature best suited for replication. The resulting products were run on a 2% agarose gel, stained with SYBR-Safe (ThermoFisher Scientific), for 60 minutes to allow for adequate resolution. The bands were viewed with UV transillumination using a gel imaging system (Chemidoc XRS, Bio-Rad). The TaqMan probes used are listed below (Table 1) and purchased from ThermoFisher Scientific.

Table 1: Gene transcripts and corresponding qPCR TaqMan primer sets used to determine gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>TaqMan Probe</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV4</td>
<td>Rn00576745-m1</td>
<td>59</td>
</tr>
<tr>
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<td>Rn00585625-m1</td>
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<td>TRPM4</td>
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<tr>
<td>BEST1</td>
<td>Rn01451107-m1</td>
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<td>Rn01775763-g1</td>
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<tr>
<td>HPRT1</td>
<td>Rn01527840-m1</td>
<td>64</td>
</tr>
</tbody>
</table>
3.5. Pressure Myography

Vessels were excised and mounted on glass pipettes that were size matched within 4 μm outer diameters of one another, ranging from 83-102 μm and the corresponding inner diameters being about 20 μm smaller in both pipettes due to the thickness of the glass capillaries. The pipette with the smaller diameter of the two was placed at the upstream portion, the end that will be exposed to the highest pressure once flow is applied. This was done to avoid an accumulation of the fluid within the vessel since if the rate of fluid entry was higher than exit then fluid building pressure may affect the contractility. Vessels were mounted on the upstream side and tied with a suture before being gravity flushed with about 10 cmH₂O pressure to remove immune cells and lymph within the lymphatic vessel. After flushing, the vessel was mounted on the downstream pipette and secured in place with a second suture. The vessels mounted had two valves between the pipettes, equivalent to one full lymphangion in the middle, and all parameters were tracked on this middle lymphangion. The pipettes were connected to independent reservoirs containing 37 °C 0.5% MOPS-APSS allowing for independent manipulation of the pressure experienced at the inflow and outflow pipettes and thus the ability to induce a pressure gradient. The chamber in which the vessels were mounted contained the same solution (37°C 0.5 % MOPS-APSS pH 7.40 ± 0.01). The solution was constantly superfused in at a rate of 3 mL/min after being heated to a physiological temperature of 36-37 °C. A peristaltic pump determined the rate of superfusion, and the solution was heated using a water bath and jacket that heated the solution until it entered into the chamber (Figure 12).

Once mounted and sutured on either end, vessels were set to their approximate in situ length by increasing the transmural pressure up to 10 cmH₂O transiently. The pressure
was subsequently decreased to 3 cmH\textsubscript{2}O by lowering both the inflow and outflow reservoirs and allowed to equilibrate for 20-25 minutes, similarly to what was done in the lab previously (Lee et al., 2014). After equilibration, the pressure was increased to 5 cmH\textsubscript{2}O for 5 minutes before the addition of 10 \textmu M acetylcholine (ACh) for 3 minutes to determine if the endothelium is present and functional. When the endothelium was functional the response to this treatment would be a decrease in the contraction frequency of the lymphatic vessel and possibly an extended period of time with no contractions. The vessel was then allowed to equilibrate for 10-15 minutes until a consistent contraction frequency observed before being subjected to a flow ramp. The vessel needs to be contracting faster than the contraction frequency at 3 cmH\textsubscript{2}O, a minimum of 5 contractions, and a maximum of 25 contractions/min. The flow ramp consisted of 3 differences in pressure, 1, 5, and 9 cmH\textsubscript{2}O, with each exposure being 5 minutes long, resulting in a total time of 15 minutes for the ramp. The pressure difference was created by lowering the reservoir at the outflow pipette and raising the reservoir at the inflow pipette the equivalent amount (Figure 13). The vessel was then allowed to equilibrate at 5 cmH\textsubscript{2}O transmural pressure and 0 cmH\textsubscript{2}O pressure difference for 10 minutes before the addition of pharmacological agents. There was variability in the contraction frequency of the lymphatic vessels post flow ramp with some showing a small decrease and others a large decrease in contraction frequency at constant pressure compared to the frequency before the flow ramp. Due to the lower contraction frequency for the second ramp, parameters such as the frequency and the fractional pump flow (FPF) were calculated as percentages of the baseline frequency. The compounds were eNOS inhibitor L-NNA (100 \textmu M), TRPV4 inhibitor HC067047 (HC) (1 \textmu M or 3 \textmu M), COX-1/2 inhibitor Indomethacin (10 \textmu M) or combinations of these treatments for 20 minutes (Table 1). After the 20-minute
administration at constant transmural pressure, the vessel was subjected to a second flow ramp of 1, 5, and 9 cmH₂O pressure difference, as stated previously. Upon ramp completion, vessels were allowed to equilibrate at 5 cmH₂O pressure for 10 minutes before a 3-minute exposure to 10 µM ACh. The vessel was allowed to recover for 7 minutes before the 10-minute addition of calcium-free MOPS-PSS to determine the maximal diameter of the lymphatic vessel. The data obtained for the ACh response was collected every 2 minutes from the last 2 minutes before exposure until 7 minutes after the washout started. Flow ramp data came from the final 3 minutes of the exposure to the particular pressure difference since the vessel has stabilized at this point, this length of time is slightly longer in duration than other groups have previously done (Gashev et al., 2002).

The first vessel was isolated and mounted within an hour of rat euthanization, and the subsequent vessels were left in their arcades and stored in a 37 ºC incubator, as stated in Lymphatic Vessel Isolation. Despite these measures, inconsistent responses to flow ramps occurred for the first and second vessels, though a flow response was never observed in the third vessel of the day. Due to this, the second and third vessels were used to determine the response to TRPV4 agonism. In these instances, lymphatic vessels were exposed to increasing concentrations of TRPV4 agonist GSK 1016790A (GSK101) starting at 0.1 nM up to 1 µM (0.1, 0.3, 1, 3, 10, 30, 100, 300 nM and 1 µM) to construct concentration-response curves. The vessels were mounted as before and allowed to equilibrate at 3 cmH₂O pressure for 20-25 minutes. Vessels were subjected to 10 µM ACh for 3 minutes before the washout of ACh and allowed to recover for 10-15 minutes. Then naïve vessels with a functional endothelium were exposed to GSK101 concentrations as previously stated for 5 minutes at each point in a cumulative concentration-response protocol meaning the resulting ramp took
45 minutes. The duration of GSK101 applications was chosen to account for the time taken for GSK101 to exhibit its effects, often being 3 minutes. After the concentration-response curve, the GSK was washed out, and the vessel was allowed to recover for 10 minutes before the addition of 10 μM ACh for 3 minutes. After ACh washout, the vessel recovered for 8 minutes before the addition of calcium-free MOPS-PSS for 10 minutes. Second and third vessels that did show a response upon the first stimulation with ACh were allowed to recover for 10-15 minutes before the addition of L-NNA, Indomethacin, HC, or a combination of treatments as listed (Table 2) for 20 minutes. The GSK concentration-response curve and subsequent treatments were done as in the naïve vessels that solely received GSK101 treatment. Data for the concentration-response curves came from the final 2 minutes at each concentration since it takes a couple of minutes for the agonist to act at each concentration.

3.6. Lymphatic Vessel Parameters

Tracking of lymphatic vessel contractions with LabVIEW software resulted in the output of 4 basic parameters: frequency, amplitude, systolic and diastolic diameters. All lymphatic vessels were tracked in one location, approximately in the middle of the intact lymphangion (bordered by valves) set between the two pipettes. All parameters were measured using this single point and due to this may not be as accurate as using multiple measurements along the lymphangion. Two additional parameters were calculated to determine more active measures of lymph movement, ejection fraction (EF) and fractional pump flow (FPF). EF was done in the manner described by Benoit and colleagues, using the diameters during both diastole and systole and the FPF was determined by multiplying the EF by the contraction frequency (Benoit et al., 1989, Scallan et al., 2016). The EF was
calculated by tracking a single point of the lymphangion for the duration of the experiment. The full lymphangion was then treated as a pipe and the amount of lymph expelled with each contraction was determined and represented as the ejection fraction (EF). The EF was calculated with the following formula.

\[
EF = \frac{(\text{diastolic diameter})^2 - (\text{systolic diameter})^2}{(\text{diastolic diameter})^2}
\]

The formula is based on the premise of calculating the area of a circle using the formula \(\pi \times r^2\). Since \(\pi\) is a constant in both portions of the formula it is removed and the areas for diastolic (relaxed) and systolic (contracted) diameters are calculated using only the diameter squared. The value for the systolic diameter squared is subtracted from the value for the diastolic diameter squared and the corresponding value is divided by the diastolic diameter squared to produce a normalized difference. The normalized difference is then used as the parameter to display the ejection fraction (EF). This simple formula has consistently been used to account for EF in a manner similar to stroke volume for cardiac function. Due to the simplified nature of this formula, it may not account for difference in contraction properties in different portions of the lymphangion, such as the middle compared to the two edges next to valves.

The amount of active lymph movement was presented as fractional pump flow (FPF), similar to cardiac output. FPF was determined by multiplying the contraction frequency by the EF and as stated is indicative of the active lymph movement due to contractions.

\[
FPF = \text{Frequency} \times EF
\]
FPF was normalized to a baseline value at the start of each of the ramps due to the decrease in contraction frequency after exposure to a flow ramp. Doing this allowed us to compare the impact that a specific drug treatment had on the FPF vs the control ramp.
Figure 12: Schematic representing the setup used for all ex vivo pressure myography experiments. Cannulation of the lymphatic vessel on two small glass pipettes, connected to two independently adjustable reservoirs, one to the inflow pipette and one to the outflow pipette. Vessel visualization took place using a microscope, and the contractile activity was tracked on the computer using a specialized LabVIEW program (National Instruments). To maintain the temperature in the recording chamber at a physiologically relevant temperature of 37°C the new solution is warmed up using a water bath. The superfusion of the fresh solution into the bath occurred at a constant rate of 3 mL/min using a peristaltic pump. To maintain temperature and a constant level of the solution in the bath, excess solution is removed and disposed of in a waste container. Adapted from Lawton et al., 2019.
Figure 13: The transmural pressure is maintained throughout the flow ramp by creating a pressure difference between the inflow and outflow pipettes. The transmural pressure is maintained by raising the inflow pipette a set amount and lowering the outflow pipette by the equivalent amount. At all points, the mean transmural pressure is 5 cmH₂O, and the pressure gradient induced to create flow can be 0, 1, 5, or 9 cmH₂O.
Table 2: Flow response ramps in the presence of either treatment for the control or under conditions of TRPV4, eNOS, and COX inhibition. Inhibition of TRPV4 with concentrations of 1 µM or 3 µM HC067047, eNOS with 100 µM L-NNA and COX-1 with 10 µM indomethacin. An X denotes treatment in the corresponding box, with the corresponding color of the graph in the results described on the left.

<table>
<thead>
<tr>
<th>Color Bars</th>
<th>Control</th>
<th>1µM HC067047</th>
<th>3µM HC067047</th>
<th>100µM L-NNA</th>
<th>10µM Indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grey</td>
<td>X</td>
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<td></td>
<td></td>
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<td>Purple</td>
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</table>

Table 3: GSK ramps were conducted in the absence or presence of HC067047, L-NNA, or Indomethacin. The following combinations were used to determine the differential impact that these antagonisms have on lymphatic contractility. All ramps consist of cumulative additions of 0.1 – 1000nM GSK doses in 0.5 log scale doses.

<table>
<thead>
<tr>
<th>Color Bars</th>
<th>No Treatment</th>
<th>3µM HC067047</th>
<th>100µM L-NNA</th>
<th>10µM Indomethacin</th>
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<tr>
<td>Red</td>
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<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Dark Blue</td>
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<td>X</td>
<td>X</td>
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<td>X</td>
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<td>X</td>
<td></td>
</tr>
<tr>
<td>Light Blue</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grey</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>
3.7. Western Blots

Western blots were carried using a two-step process for antibody binding. Lymphatic vessels were isolated and lysed in extraction buffer and sonicated 3 times for 5 seconds before being loaded on 10% polyacrylamide gels, containing both a stacking and resolving portion, and run at 120 V for 100 minutes via gel electrophoresis. Proteins in the resolving gel were transferred to 0.2 µm nitrocellulose membranes using a 90-minute transfer at 120 V in transfer buffer containing 20% methanol (v/v) using a Bio-Rad PowerPac Basic (041BR65330). The blots were then removed from the transfer sandwich and cut to the appropriate sizes before being subjected to a Ponceau red stain for 10 minutes to determine the presence of protein on the blot. The Ponceau red was washed off before an hour exposure to blocking solution consisting of 5 % (w/v) skim milk in 0.1 % TBS-T was used to minimize non-specific binding of the antibodies. TBS-T is tris-buffered saline with the corresponding percentage of Tween 20 added. The membranes were incubated with anti-TRPV4 antibody (ab39260; Abcam, Cambridge, United Kingdom) at a 1:1000 dilution in the blocking solution overnight of the 1mg/mL stock. Three 10-minute washes in 0.1 % TBST were conducted and the appropriate secondary for TRPV4 (HRP conjugated donkey anti-rabbit) was added and incubated in the blocking solution for an hour. The membranes were thoroughly washed three times for 10 minutes in 0.1 % TBST before incubating the blot in ECL media or femto ECL media for 30 seconds to a minute and the detection of the chemiluminescence signal.
3.8. Immunohistochemistry

For the whole-mount preparations, an arcade was isolated, and the connective tissue and fat were gently removed from around one lymphatic vessel using fine tipped forceps. The whole-mount arcades were fixed with 4% PFA for 1 hour and they were subsequently washed three times in DPBS for 10 minutes each. Arcades were incubated with a blocking solution which consists of PBS (phosphate-buffered saline) supplemented with 0.3% Triton X-100 and 2% BSA for 1 hour at room temperature (21 °C). Primary antibodies for TRPV4 and VEGFR3 at concentrations of 1 mg/mL and 0.2 mg/mL respectively were added at a dilution of 1 in 200 µl and left overnight at 4°C. The primary unconjugated antibodies used were rabbit anti-TRPV4 antibody (ab39260; Abcam, Cambridge, United Kingdom) and goat anti-VEGFR3 antibody (AF743; R&D Systems, Minneapolis, MN). These were washed off with three 10-minute PBST (phosphate-buffered saline with 0.3% Triton X-100) before the addition of primary conjugated α-SMA (C6198; Sigma-Aldrich, St. Louis, MO), TRPV4 secondary antibody Alexa Rb647 (A21443; Life Sciences, Eugene, OR) and VEGFR3 secondary antibody Alexa Gt488 (A11055; Life Sciences) at a dilution of 1 in 400 µl for 1 hour. Upon completion, three 10-minute washes in PBST were done before incubating with DAPI for 5 minutes in PBST at a dilution of 1 in 500µl. Images were obtained using an inverted confocal fluorescence microscope (Leica TCS SP8).

For rat lymphatic endothelial cells, a similar protocol was used to characterize the cells. These cells are primary cultured cells that were cultured from duodenal and ileal lymphatic vessels in these rats. Cells were obtained by digesting a single duodenal or ileal lymphatic vessel and allowed to grow to confluence in a 35 mm dish before transferring to a T75. Vessels were dissected in dissection buffer containing (in mM): 137 NaCl, 5 KCl, 1
MgCl₂, 10 HEPES and 10 glucose along with 0.1 % BSA. Vessels were transferred to the
dissociation buffer before being transferred to dissociation containing 6.2 mg/mL Papain, 15
mg/mL Collagenase H and 20 mg/mL Dithioerythritol (DTE) and being placed in in a 37 °C
incubator for 25 minutes. Cold dissociation buffer was added to dilute the enzymes and cool
the plate before being transferred to the gelatin coated 35mm dish and stored at 37 °C in the
incubator before topping up with media 4 hours later. Cells from Passage 3-6 were
characterized using an eight-well chamber slide to determine the changes occurring in the
cell culture over time. Cells were fixed in 4% PFA for 20 minutes before being washed three
times for 10 minutes with PBS. Blocking solution consisting of PBS supplemented with
0.1% Triton X-100 and 2% BSA was added to the cells for 1-2 hours to prevent non-specific
binding of antibodies. Primary antibodies for TRPV4, VEGFR3, CCL21, Prox1, Foxc2, and
LYVE-1 were added at a dilution of 1 in 200 µl in blocking solution overnight at 4 °C (Table
3). After overnight incubation, three 10-minute washes were done, and the appropriate
secondary antibodies were added to each well (Table 3). Three 15-minute washes were done
with PBST before counterstaining with Phalloidin Alexa 488 in PBS for 15 minutes. One 15-
minute wash in PBS was done before mounting and imaging using the confocal fluorescence
microscope, as stated previously.
Table 4: Antibodies used for lymphatic endothelial cell characterization and the function of each antibody.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV4 (ab39260) Abcam (Cambridge, United Kingdom)</td>
<td>It binds to the C-terminal portion of TRPV4, and expression occurs throughout the cell. It is raised against recombinant mouse TRPV4 (Rabbit anti-mouse, validated for rat and horse)</td>
</tr>
<tr>
<td>VEGFR3 (AF743) R &amp; D Systems (Minneapolis, MN)</td>
<td>Mark the C-terminus end of the channel and localizes to the surface or cytoplasm of the cell (Goat anti-mouse)</td>
</tr>
<tr>
<td>LYVE-1 (11-034) Angio Bio Co (Del Mar, CA)</td>
<td>Binds hyaluronan on the surface in initial lymphatic endothelial cells \textit{in vivo} (Rabbit anti-mouse)</td>
</tr>
<tr>
<td>Prox1 (PA585552) ThermoFisher Scientific (Waltham, MA)</td>
<td>Pan lymphatic endothelial cells specific marker for a transcription factor localized to the nucleus</td>
</tr>
<tr>
<td>Foxc2 (AF689) R &amp; D Systems (Minneapolis, MN)</td>
<td>Transcription factor specific to lymphatic endothelial cells localized to the nucleus (sheep anti-mouse)</td>
</tr>
<tr>
<td>CCL21 (AF457) R &amp; D Systems (Minneapolis, MN)</td>
<td>Binds to the cytokine CCL21 with staining throughout the cell (Goat anti-mouse with high cross-reactivity with rat)</td>
</tr>
<tr>
<td>(\alpha)-SMA (C6198) Sigma-Aldrich (St. Louis, MO)</td>
<td>1º conjugated marker of smooth muscle actin and used as a negative control to ensure there are no myocytes in culture with the endothelial cells (anti-mouse)</td>
</tr>
<tr>
<td>Alexa 647 anti-rabbit 2º Ab (A21443) Life Sciences (Eugene, OR)</td>
<td>2º antibody used to label TRPV4, Prox1, and LYVE-1 1º antibodies</td>
</tr>
<tr>
<td>Alexa 647 anti-goat 2º Ab (A21469) Life Sciences (Eugene, OR)</td>
<td>2º antibody used to label VEGFR3 and CCL21 1º antibodies in cell culture</td>
</tr>
<tr>
<td>Alexa 647 anti-sheep 2º Ab (A21448) Life Sciences (Eugene, OR)</td>
<td>2º antibody used to label Foxc2 1º antibody</td>
</tr>
<tr>
<td>Alexa 488 anti Goat 2º Ab (A11055) Life Sciences (Eugene, OR)</td>
<td>2º antibody used to label VEGFR3 1º antibody in the whole-mount preparations</td>
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</tbody>
</table>
3.9. Pharmacological Treatments

The following reagents were used in both the pressure myography experiments. GSK1016790A (17289), HC067047 (20927) and Acetylcholine-HCl (23829), were purchased from Cayman Chemical (Ann Arbor, MI). NG-nitro-L-arginine (L-NNA) (N6601-6G), Indomethacin (I7378-5G), Bradykinin (B3259-1MG) were all purchased from Sigma-Aldrich (St. Louis, MO).

3.10. Statistical Tests

For the flow graphs shown as two sets of bars at four different pressure differences, statistical significance was assessed using a repeated measures two-way analysis of variance (ANOVA) with Dunnett’s post hoc to determine if flow had a significant impact on the parameter compared to the baseline value established at 5 cmH₂O pressure and with Sidak’s post hoc to determine if there were differences between treatments at the same amount of flow. For the line graphs showing TRPV4 agonism in a concentration-response curve, a two-way ANOVA with Sidak’s post hoc test was used to determine statistically significant differences between treatment groups and the EC₅₀ was determined using non-linear regression graphing the log (agonist) against the response (frequency) with a variable slope. Significant differences in EC₅₀ values between groups were determined using a one-way ANOVA with a Tukey’s post hoc test. For the impact of a flow ramp and the addition of inhibitors on the contractile frequency and contraction of amplitude, two-tailed student’s paired T-tests were performed to determine statistical significance. A p < 0.05 was used to determine significant differences for all tests. All average data are presented ± standard error of the mean (SEM). All statistical analyses were calculated with PRISM 9.1.0.
4. CHAPTER FOUR: RESULTS

4.1. TRPV4 Expression

4.1.1. *TRPV4 gene and protein expression occur within Sprague-Dawley rat mesenteric collecting lymphatic vessels.*

Gene expression of TRPV4 and other mechanosensitive TRP channels, TRPV1, TRPC1, TRPC6, TRPM4, and TRPM8, and Ca$^{2+}$-dependent Cl$^{-}$ channels Anoctamin 1, and Bestrophin 1 were determined using qPCR (Appendix A). Bands were difficult to observe after one round of PCR, thus requiring a second round of amplification (Figure 14B). The second round of amplification allowed for the determination of the respective product band sizes (Figure 14C). TRPV4 protein expression transpired throughout the collecting lymphatic vessels, occurring in the endothelium and smooth muscle in comparable amounts. Both immune cells and the adventitia around the lymphatic vessels also exhibited TRPV4 expression (Figure 15A, Appendix B). Notably, substantial expression of TRPV4 was observed in regions subjected to high levels of shear stress, such as the valves (Figure 15B).
Figure 14: TRPV4 is expressed at low levels basally in whole lymphatic vessels, and there may be splice variants present. (A) TRPV4 gene expression occurs at low levels in whole lymphatic vessels isolated from the ileal portion of male Sprague-Dawley rats. Relative expression was determined compared to housekeeping genes HPRT1 (hypoxanthine phosphoribosyltransferase) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). RU = relative units and is the amount of expression in comparison to the average expression of the two housekeeping genes with an n = 5, (B) Representative figure in which TRPV4 products obtained after one round of qPCR, due to low concentrations or amounts of the product visualization was challenging for an n = 4. C) Representative figure of TRPV4 products after reamplification of the original qPCR products with the same primers with an n = 4. Each experiment utilized material from a single animal.
Figure 15: TRPV4 protein expression takes place throughout the rat, collecting mesenteric lymphatic vessels. (A) TRPV4 protein expression occurs in rat mesenteric lymphatic vessels, each sample consists of four to ten vessels pooled from the ileal portion of a single rat (n = 6). (B) A lymphangion of a rat mesenteric lymphatic vessel with TRPV4 being expressed on the endothelium (a), on the smooth muscle layer (b) and in immune cells around the vessels (c). (C) TRPV4 protein expression at a valve site, the white lines are showing the valve’s structure, and (d) is showing TRPV4 expression on the valve. DAPI was used to mark cell nuclei in grey,
TRPV4 in green, VEGFR3 marked the endothelium in blue, and α-SMA marked the smooth muscle in red. These are representative figures for an n=5.
4.2. Pressure Myography Flow Responses

4.2.1. Conditions of flow under a constant mean transmural pressure resulted in a significant decrease in contractile activity.

Lymphatic vessels experienced a flow ramp that consisted of 5-minute exposures to 1 cmH₂O, 5 cmH₂O, and 9 cmH₂O pressure gradients totaling 15 minutes. The baseline was first established at 5 cmH₂O transmural pressure and 0 cmH₂O pressure gradient (flow), and upon completion of the ramp, baseline conditions were re-established. The contraction frequency was significantly decreased by flow in a magnitude-dependent manner with a significant decrease observed by 1 cmH₂O of flow (Figure 16A). Baseline conditions resulted in a frequency of 14.36 ± 0.92 contractions per minute (con/min), and this decreased to 12.30 ± 0.90, 9.97 ± 0.81, 8.09 ±0.75 and 10.47 ± 0.64 con/min for 1, 5, 9 and 0 cmH₂O flow. The amplitude of contractions during baseline conditions of 0 cmH₂O flow and 5 cmH₂O pressure was 82.02 ± 4.24 µm and it increased significantly to 86.06 ± 4.06 µm at 1 cmH₂O flow. Values at 5, 9 and 0 cmH₂O flow were 85.86 ± 4.01, 76.93 ± 4.95 and 88.88 ± 3.44 µm respectively, though none of these differed significantly compared to baseline (Figure 16B). The diastolic diameter at baseline was 150.30 ± 5.20 µm and it increased significantly at 1 cmH₂O flow to 152.2 ± 4.97 µm. The subsequent values at 5, 9 and 0 cmH₂O flow were 147.20 ± 4.98, 132.60 ± 6.29 and 154.40 ± 5.14 µm with the decrease at high flow and the increase at the new baseline being significant (Figure 16C). The systolic diameter under baseline conditions was 66.85 ± 3.28 µm and this decreased to 66.69 ± 3.224, 62.09 ± 3.08, 57.76 ± 2.76 and 65.50 ± 3.52 µm for 1, 5, 9 and 0 cmH₂O flow with both 5 and 9 cmH₂O flow being significantly lower than baseline values (Figure 16D). The ejection fraction was 0.76 ±
0.02 at baseline conditions and this changed to $0.78 \pm 0.02$, $0.79 \pm 0.02$, $0.75 \pm 0.04$ and $0.81 \pm 0.01$ at 1, 5, 9 and 0 cmH$_2$O flow, respectively. Ejection fractions at both 1 and 0 cmH$_2$O flow were significantly higher than at baseline conditions (Figure 16E). The fractional pump flow (FPF) decreased significantly throughout the flow ramp in a magnitude-dependent manner similar to the changes in the frequency. The initial FPF was $10.59 \pm 0.62$ min$^{-1}$ and this decreased to $9.30 \pm 0.60$, $7.79 \pm 0.63$, $6.37 \pm 0.67$ and $8.45 \pm 0.52$ min$^{-1}$ for 1, 5, 9 and 0 cmH$_2$O flow with all values being significantly lower than baseline conditions (Figure 16F). The contraction frequency never fully recovered to pre-ramp conditions upon re-establishing the baseline conditions of 5 cmH$_2$O transmural pressure and 0 cmH$_2$O pressure difference, resulting in a significantly decreased contraction rate (Figure 16A). Due to this decrease in contraction frequency all experiments consisting of two successive flow ramps have both the contraction frequency and the FPF expressed as a percentage of the baseline values. Baseline values for frequency and FPF were obtained through averaging the frequency and the FPF at the 0 cmH$_2$O flow and 5 cmH$_2$O transmural pressure point before the first flow ramp. All subsequent points were determined using the average and the result were shown as a percentage of baseline.
**Figure 16: Flow induced through a pressure gradient decreases the contraction frequency and active lymph movement of rat mesenteric lymphatics.** All graphs were represented as raw data with each n number representing one vessel per rat. Changes in the (A) contraction frequency, (B) amplitude of contractions, (C) diastolic diameter, (D) systolic diameter, (E) ejection fraction, and (F) fractional pump flow under conditions of flow. Statistical difference determined using a 1-way repeated measures ANOVA with Dunnett’s post hoc test to determine significant changes compared to the control value. Treatment groups were compared to the control group of 0 cmH₂O pressure gradient with significant differences signified as p < 0.05 *, p < 0.01 **, p < 0.001 *** and p < 0.0001 **** for an n = 33. All error bars are ± SEM.
4.2.2. *The flow-induced decrease in contractile activity is reproducible over two flow ramps.*

Lymphatic vessels were subjected to two 15-minutes ramps with pressure gradients (flow) of 1 cmH\(_2\)O, 5 cmH\(_2\)O, and 9 cmH\(_2\)O for 5 minutes each. The baseline was established at 5 cmH\(_2\)O pressure and 0 cmH\(_2\)O pressure gradient before each ramp. Vessels showed a decrease in the contraction frequency and in the amplitude of contractions as flow increased for both the first and second control ramps (Figures 17A & B). There were significant decreases in the contraction frequency in the first ramp at 5 and 9 cmH\(_2\)O with the frequency decreasing from 100 ± 23.44 % to 87.83 ± 17.30 %, 63.01 ± 21.50 %, and 47.09 ± 24.67 % for 1, 5 and 9 cmH\(_2\)O flow, respectively. In the second ramp the frequency changed from 100 ± 9.1 % to 83.6 ± 13.2 %, 70.2 ± 18.7 %, and 56.7 ± 30.1 % for 1, 5 and 9 cmH\(_2\)O flow, respectively. Though there was a trend towards a decrease in the second ramp it did not reach significance due to a bit more variability in the flow response (Figure 18A). The amplitude of contractions did not show significant differences for either ramp throughout flow or between the ramps. For the first ramp at 0, 1, 5 and 9 cmH\(_2\)O flow were 99.4 ± 13.6, 103.3 ± 13.7, 107.7 ± 14.9, and 74.8 ±13.1 µm, respectively. The second ramp showed a similar response with amplitudes of 102.3 ± 6.3, 105.8 ± 7.0, 107.5 ± 16.2 and 70.4 ± 11.9 µm being observed for 0, 1, 5 and 9 cmH\(_2\)O flow, respectively (Figure 18B). The diastolic diameter showed no significant changes in either ramp, though there was a decrease in diameter at high flow. The diastolic diameters were 177.2 ± 14.6, 174.7 ± 14.6, 169.6 ± 14.0 and 136.5 ± 16.4 µm and 177.9 ± 14.0, 178.1 ± 13.7, 168.3 ± 16.0, and 135.8 ± 15.8 µm for the first and second ramps respectively (Figure 18C). The systolic diameter decreased significantly at during the first flow ramp at 9 cmH\(_2\)O flow but showed no significant changes during the
second flow ramp. The diameter decreased from 77.8 ± 4.7 to 71.4 ± 5.2, 62.0 ± 4.8 and 61.7 ± 3.6 during the first ramp and from 75.7 ± 8.2 to 72.2 ± 7.6, 60.8 ± 3.2 and 65.0 ± 4.4 in the second ramp (Figure 18D). The ejection fraction (EF) showed no significant changes for either ramp with the first ramp’s EFs being 0.80 ± 0.03, 0.82 ± 0.03, 0.86 ± 0.03, and 0.77 ± 0.04, and the second ramp being 0.82 ± 0.01, 0.84 ± 0.01, 0.86 ± 0.02, and 0.75 ± 0.03 (Figure 18E). The FPF showed a significant decrease in the first ramp at 5 cmH₂O flow, with it decreasing from 100 ± 20.4 % to 91.0 ± 13.2, 67.8 ± 21.5, and 51.1 ± 27.1 %. The second ramp showed no significant changes for the duration of flow with the FPF decreasing from 100 ± 8.2 % to 84.8 ± 12.6, 72.9 ± 18.7 and 56.4 ± 29.8 % (Figure 18F). The contraction frequency at 0 cmH₂O flow upon completion of the first ramp showed no difference with the frequency changing from 12.8 ± 2.9 con/min to 12.8 ± 1.2 con/min (figure 19A). The amplitude of contractions increased slightly from 100.2 ± 13.7 to 103.3 ± 10.8 µm, though this was not significant (Figures 19B). During the 30-minute equilibration period between ramps, no significant changes occurred in either the contraction frequency or the amplitude of contractions with the frequency changing from 12.8 ± 1.2 con/min to 11.4 ± 1.0 con/min and the amplitude changed from 103.3 ± 10.8 µm to 103.4 ± 6.8 µm (Figures 19C and D).
Figure 17: Representative traces showing the final three-minute portion at each pressure difference experienced by vessels for both flow ramps. (A) The trace for the first flow ramp conducted on lymphatic vessels. These results were reproducible in the second ramp (B), with the contraction properties of both ramps being very similar.
Figure 18: Flow induction via a pressure gradient caused significant decrease in contraction frequency, and active lymph movement in the first ramp that was reproducible in a second ramp. Changes in the (A) contraction frequency (B) amplitude of contractions, (C)
diastolic diameter, (D) systolic diameter, (E) ejection fraction, and (F) fractional pump flow in response to two sequential flow ramps. Graphs for frequency and FPF are corrected to a baseline value due to variations in the baseline frequency of lymphatic vessels. Statistical difference determined using a 2-way repeated measures ANOVA with Dunnett’s post hoc test to determine differences within a ramp and Sidak’s post hoc test to determine differences between ramps. Significance compared to 0 cmH₂O within a ramp shown as P < 0.05 *, p < 0.01 **. All error bars are ± SEM for an n = 5 experiments.

Figure 19: Exposing lymphatic vessels to a flow ramp had no impact on the contraction frequency or amplitude. (A) Contraction frequency and (B) contraction amplitude before and 10 minutes after the vessels were subjected to a 15-minute flow ramp. (C) Contraction frequency and (D) contraction amplitude in response to 30-minute equilibration period. Statistical difference determined using a paired two-tailed Student’s t-test for an n = 5 with error bars being ± SEM.
4.2.3. **Lymphatic vessel treatment with a low concentration of HC067047 did not significantly restore contractile activity under conditions of flow.**

Lymphatic vessels experienced a flow ramp and, upon completion, were given 10 minutes to stabilize before the addition of 1 µM HC067047 (HC) for 20 minutes. After 20 minutes of treatment, the vessels underwent subsequent exposure to a second flow ramp. Representative traces of lymphatic vessel contractile activity for the control ramp showed a significant decrease in frequency and a decrease in vessel diameter (Figure 20A). Treatment with 1 µM HC partially restored contraction frequency without restoring vessel diameter (Figure 20B). There was a slight, but insignificant, prevention of the decrease in contraction frequency under flow conditions upon 1 µM HC addition. In the control ramp the frequency decreased significantly from 100 ± 15.2 % to 81.7 ± 13.2 %, 64.2 ± 12.3 % and 48.1 ± 12.0 %. Treatment with 1 µM HC ablated these significant decreases with the frequency being 100 ± 15.3 %, 87.1 ± 17.4 %, 74.9 ± 8.6 % and 63.1 ± 7.8 % (Figure 21A). The contraction amplitude showed no significant changes in the control ramp, while in the 1 µM HC treated ramp there was significant increase in amplitude at 1 cmH₂O flow and a significant decrease at 9 cmH₂O flow. In the control ramp the amplitude changed from 74.8 ± 8.6 µm at 0 cmH₂O flow to 78.9 ± 7.4 µm, 72.6 ± 5.6 µm and 59.5 ± 8.9 µm at 1, 5 and 9 cmH₂O flow, respectively. The amplitude of the 1 µM HC treated ramp changed from 88.8 ± 4.4 µm at 0 cmH₂O to 92.1 ± 4.4 µm, 83.3 ± 7.5 µm and 59.3 ± 8.9 µm at 1, 5 and 9 cmH₂O flow, respectively (Figure 21B). The diastolic diameter did not change significantly in either ramp or did not differ significantly between ramps. The diastolic diameters for the duration of the two ramps were 142.2 ± 7.1 µm, 143.2 ± 6.8 µm, 132.5 ± 8.0 µm and 109.6 ± 10.2 µm, and 150.0 ± 6.1 µm, 149.7 ± 6.2 µm, 139.8 ± 8.5 µm and 112.4 ± 13.3 µm (Figure 21C). At the same time, the systolic diameter decreased significantly in the 1 µM HC
treated ramp at 1 cmH₂O. The systolic diameters for the control and 1 µM HC treated ramp were 67.4 ± 9.7 µm, 64.3 ± 8.7 µm, 59.9 ± 9.0 µm and 50.1 ± 5.3 µm, and 61.3 ± 8.3 µm, 57.6 ± 8.6 µm, 56.4 ± 7.3 µm and 53.1 ± 8.5 µm, respectively (Figure 21D). The ejection fraction significantly increased at 1 cmH₂O flow in the 1 µM HC treated ramp, while showing no significant changes in the control ramp. The ejection fractions for the control and 1 µM HC treated ramps were 0.76 ± 0.06, 0.78 ± 0.05, 0.79 ± 0.04 and 0.77 ± 0.05, and 0.83 ± 0.03, 0.85 ± 0.03, 0.82 ± 0.04 and 0.75 ± 0.06 at 0, 1, 5 and 9 cmH₂O flow, respectively (Figure 21E).

Fractional pump flow (FPF) showed significant decreases at 1 and 9 cmH₂O in the control ramp and no significant changes in the 1 µM HC treated ramp. The FPF for the control and 1 µM HC treated ramps were 100 ± 11.1 %, 85.3 ± 10.6 %, 68.2 ± 12.2 % and 51.7 ± 14.7 %, and 100 ± 14.3 %, 87.8 ± 15.9 %, 76.3 ± 10.7 % and 57.7 ± 8.8 % at 0, 1, 5 and 9 cmH₂O flow, respectively (Figure 21F). Exposure to flow resulted in a decrease in contraction frequency from 16.7 ± 2.5 con/min to 11.5 ± 2.0 con/min and an increase in the amplitude of contractions from 74.9 ± 8.6 µm to 88.8 ± 6.1 µm, though these changes were not significant (Figures 22A and B). The addition of 1 µM HC067047 (HC) did not significantly the contraction frequency or the contraction amplitude. The contraction frequency decreased from 11.5 ± 2.0 con/min to 10.4 ±1.6 con/min and the amplitude remained unchanged (88.8 ± 6.1 µm and 88.8 ± 4.4 µm) (Figures 22C and D).
Figure 20: Representative traces showing the final three-minute portion at each pressure difference experienced by vessels for the control and 1 µM HC067047 treated flow ramps. (A) The trace for the control flow ramp conducted on 1 µM HC067047 treated lymphatic vessels showed both a decrease in frequency and lymphatic vessel diameter. Administration of 1 µM HC067047 during the second flow ramp (B) resulted in a partial restoration of contraction frequency but not diameter during flow regardless of the magnitude of flow.
Figure 21: Treatment with 1µM HC067047 did not result in significant restoration of contractile activity under conditions of flow. (A) Contraction frequency, (B) amplitude of contractions, (C) diastolic diameter, (D) systolic diameter, (E) ejection fraction, and (F)
fractional pump flow under conditions of flow and in response to treatment with 1 \( \mu \text{M} \) HC067047 under conditions of flow. Graphs for frequency and FPF are corrected to a baseline value due to variations in the baseline frequency. Statistical difference determined using a 2-way repeated measures ANOVA with a Dunnett’s post hoc test to determine significant differences within a ramp compared to no flow conditions and Sidak’s post hoc test to determine significant differences between treatments. Significance shown as \( p < 0.05 \) * \( p < 0.01 \) ** \( p < 0.001 \) *** compared to the baseline of 0 cmH\(_2\)O flow for the respective ramp with an \( n = 7 \) and the error bars being \( \pm \) SEM.

Figure 22: Both exposures to flow and 1 \( \mu \text{M} \) HC067047 did not significantly impact the contractile activity or the amplitude of contractions. Comparison of contraction frequency (A) and contraction amplitude (B) before, and ten minutes after being subjected to a 15-minute flow ramp. (C) Contraction frequency and (D) contraction amplitude before and after 1 \( \mu \text{M} \) HC067047 treatment. Statistical difference determined using a paired two-tailed Student’s T-test for an \( n = 7 \) with error bars being \( \pm \) SEM.
4.2.4. Treatment with 3 µM HC067047 resulted in the ablation of the flow-dependent decrease in contractile activity.

Lymphatic vessels were subjected to a flow ramp, and upon completion, 3 µM HC067047 addition occurred as with 1 µM HC. Representative traces of lymphatic vessel contractile activity for the control ramp showed a significant decrease in frequency in response to flow (Figure 23A). 3 µM HC treatment ablated the decrease in frequency (Figure 23B). There was a significant decrease in contraction frequency in the control ramp at 5 cmH₂O, while the 3 µM HC treated ramp showed no significant changes. The frequency for the control ramp and 3 µM treated ramps were 100 ± 11.7%, 91 ± 14.2%, 75.2 ± 11.3% and 63.0 ± 7.2%, and 100 ± 13.2%, 102.7 ± 20.5%, 120.6 ± 19.7% and 127.1 ± 19.6% at 0, 1, 5 and 9 cmH₂O flow, respectively. Though there was a large difference in the response between ramps at 9 cmH₂O flow, it did not reach significance, and this may be due to the low n number used since the p values was 0.1 (Figure 24A). There were no significant changes in the amplitude of contractions, or significant differences between ramps. Control and 3 µM HC treated ramp amplitudes were 87.7 ± 3.7 µm, 91.4 ± 3.6 µm, 92.1 ± 1.5 µm, and 96.4 ± 4.4 µm, and 91.7 ± 3.7 µm, 99.6 ± 4.4 µm, 102.7 ± 7.7 µm and 100.9 ± 7.7 µm (Figure 24B). The diastolic diameter increased significantly in the 3 µM HC treated ramp at 1 cmH₂O but not the control ramp. The diastolic diameters in the control and 3 µM HC treated ramps were 151.7 ± 11.4 µm, 154.7 ± 12.0 µm, 154.8 ± 12.7 µm and 153.5 ± 13.7 µm and 156.0 ± 13.6 µm, 158.6 ± 13.5 µm, 158.1 ± 13.6 µm and 157.0 ± 13.5 µm at 0, 1, 5 and 9 cmH₂O in the respective ramps (Figure 24C). The systolic diameter showed no significant changes compare to no flow conditions in either ramp or between ramps. The systolic diameters in the control
and 3 µM HC treated ramps were 64.0 ± 13.1 µm, 63.4 ± 13.8 µm, 62.7 ± 12.6 µm and 57.0 ± 10.2 µm and 64.4 ± 12.4 µm, 59.0 ± 10.2 µm, 55.4 ± 9.7 µm and 56.2 ± 8.5 µm at 0, 1, 5 and 9 cmH2O in the respective ramps (Figure 24D). The ejection fractions showed no significant differences for either ramp and for the control and 3 µM HC treated ramps were 0.68 ± 0.08, 0.69 ± 0.09, 0.70 ± 0.08 and 0.72 ± 0.07, and 0.68 ± 0.08, 0.72 ± 0.08, 0.74 ± 0.08 and 0.73 ± 0.08 for 0, 1, 5 and 9 cmH2O flow, respectively (Figures 24E). There was a significant decrease in the fractional pump flow for the control ramp at 9 cmH2O flow and a significant increase when treated with 3 µM HC at 5 and 9 cmH2O flow, though there were no significant differences between treatments. The FPF decreased in the control ramp from 100 ± 10.5 % to 90.2 ± 11.6 %, 75.6 ± 6.0 % and 69.8 ± 11.1 % at 1, 5 and 9 cmH2O, respectively. The FPF increased in the 3 µM HC treated ramp from 100 ± 16.8 % to 106.5 ± 21.6 %, 127.5 ± 22.7 % and 133.5 ± 21.5 % at 1, 5 and 9 cmH2O, respectively (Figures 24F). Conditions of flow resulted in a decrease in contraction frequency from 13.1 ± 1.5 con/min to 8.4 ± 0.8 con/min, though not significant, while not impacting the amplitude of contractions (87.6 ± 3.7 µm to 89.3 ± 5.8 µm) (Figure 25A and B). The decrease in contractile activity after exposure to flow prevented restoration to the initial baseline contraction frequency, even though frequency increased during exposure to the second ramp. The addition of 3 µM HC067047 resulted in a significant decrease in contraction frequency from 8.4 ± 0.8 con/min to 7.2 ± 0.9 con/min while not impacting the amplitude of contractions (89.3 ± 5.8 µm to 91.7 ± 3.7 µm) (Figure 25C and D). Interestingly this response solely occurred after conditions of flow as the addition of 3 µM HC to naïve vessels had no significant impact on the frequency (Appendix C).
Figure 23: Treatment with 3 µM HC prevented the flow-induced inhibition of lymphatic pumping. (A) The trace for lymphatic vessel activity of the control flow ramp conducted before the addition of 3 µM HC067047 and subsequent ramp showed a magnitude-dependent decrease in contraction frequency. Administration 3 µM HC067047 during the second flow ramp (B) resulted in the ablation of the flow-induced decrease in contraction frequency. It is important to note that the contraction frequency before the second ramp is less than the frequency before first ramp and this was observed in all vessels.
Figure 24: Treatment with 3 µM HC067047 prevented the flow-induced decrease in contraction frequency. Graphs are corrected to a baseline value for frequency and FPF due to variations in the baseline frequency of lymphatic vessels. (A) Contraction frequency, (B)
amplitude of contractions, (C) diastolic diameter, (D) systolic diameter, (E) ejection fraction, and (F) fractional pump flow under conditions of flow and in response to treatment with 3 µM HC067047 under conditions of flow. Statistical difference determined using a 2-way repeated measures ANOVA with Dunnett’s post hoc test when compared to no flow and a Sidak’s post hoc test to determine significant differences between treatment groups at the same flow. p < 0.05 *, p < 0.01 ** compared to the baseline of 0 cmH₂O flow for the respective ramp for an n = 5 with the error bars being ± SEM.

Figure 25: Exposure to flow did not significantly impact contraction frequency, and the amplitude of contractions though 3 µM HC067047 addition significantly decreased contraction frequency. Comparison of contraction frequency (A) and contraction amplitude (B) before, and ten minutes after being subjected to a 15-minute flow ramp. (C) Contraction frequency and (D) contraction amplitude before and after 3 µM HC067047 treatment. Statistical difference determined using a paired two-tailed Student’s t-test for an n = 5. p < 0.01 ** for a significant decrease due to 3 µM HC067047 addition and error bars are ± SEM.
4.2.5. Treatment of lymphatic vessels with NOS inhibitor L-NNA prevents the flow-dependent decrease in contractile activity.

Lymphatic vessels were subjected to a flow ramp and, upon completion, were given 10 minutes to stabilize before the addition of 100 µM L-NNA for 20 minutes, followed by exposure to a second flow ramp. Representative traces of lymphatic vessel contractile activity for the control ramp showed a significant decrease in frequency in response to flow (Figure 26A), which was ablated by the addition of 100 µM L-NNA, resulting in an increase in frequency in response to flow (Figure 26B). In the control ramp there was a significant decrease in contraction frequency by 5 cmH$_2$O flow and in the L-NNA treated ramp there was a significant increase in frequency at 9 cmH$_2$O flow. Though differences between ramps are obvious this did not reach statistical significance at 9 cmH$_2$O with a p value of 0.06. Conducting a power test revealed that a sample size of $n = 8$ would be required to determine significant differences between groups due to variability in the response to 100 µM L-NNA. The frequency for the control ramp and L-NNA treated ramps were 100 ± 14.0 %, 85.9 ± 14.1 %, 73.8 ± 10.9 % and 67.5 ± 8.7 %, and 100 ± 11.8 %, 96.0 ± 13.7 %, 102.7 ± 14.6 % and 131.8 ± 18.4 % at 0, 1, 5 and 9 cmH$_2$O flow, respectively (Figure 27A). There were no significant changes in the amplitude of contractions, diastolic diameter, systolic diameter or ejection fraction in response to flow or between control and L-NNA treated ramps (Figures 27B-E). Control and L-NNA treated ramp amplitudes were 82.4 ± 10.2 µm, 87.9 ± 7.9 µm, 79.3 ± 10.8 µm and 80.6 ± 10.3 µm, and 87.9 ± 8.5 µm, 91.3 ± 8.6 µm, 84.0 ± 10.1 µm and 77.9 ± 12.1 µm (Figure 27B). The diastolic diameters in the control and L-NNA treated ramps were 143.8 ± 10.5 µm, 148.2 ± 9.5 µm, 140.2 ± 10.2 µm and 137.8 ± 10.2 µm and 150.5
±10.6 µm, 152.3 ± 9.9 µm, 144.9 ± 8.8 µm and 140.2 ± 10.8 µm at 0, 1, 5 and 9 cmH2O in the respective ramps (Figure 27C). The systolic diameters in the control and L-NNA treated ramps were 61.4 ± 3.9 µm, 60.3 ± 3.3 µm, 60.9 ± 4.8 µm and 57.1 ± 4.0 µm, and 62.6 ± 4.8 µm, 61.0 ± 4.0 µm, 60.8 ± 5.6 µm and 62.3 ± 4.9 µm at 0, 1, 5 and 9 cmH2O in the respective ramps (Figure 27D). The ejection fractions for the control and L-NNA treated ramps were 0.80 ± 0.03, 0.83 ± 0.02, 0.79 ± 0.05 and 0.81 ± 0.05, and 0.82 ± 0.02, 0.83 ± 0.02, 0.81 ± 0.05 and 0.77 ± 0.06 for 0, 1, 5 and 9 cmH2O flow, respectively (Figures 27E). There was a significant decrease in the fractional pump flow for the control ramp at 9 cmH2O flow and no changes in the L-NNA treated ramp, with no significant differences between ramps. The FPF decreased in the control ramp from 100 ± 13.6 % to 88.7 ± 14.1 %, 75.7 ± 12.7 % and 69.8 ± 10.5 % at 1, 5 and 9 cmH2O, respectively. The FPF changed in the L-NNA treated ramp from 100 ± 12.3 % to 96.8 ± 13.6 %, 102.6 ± 15.9 % and 125.6 ± 20.4 % at 1, 5 and 9 cmH2O, respectively (Figures 27F). Conducting a power test on this data determined that an experimental group size of 13 would be required to observed statistically significant differences. Exposure to flow significantly decreased the contraction frequency from 14.2 ± 2.0 con/min to 9.3 ± 0.6 con/min 10 minutes after completion of the ramp, while there was no significant change in the amplitude of contractions (82.4 ± 10.2 µm to 90.6 ± 6.5 µm) (Figures 28A and B). The addition of 100 µM L-NNA resulted in a slight decrease in contraction frequency from 9.3 ± 0.6 con/min to 8.2 ± 0.9 con/min and no change in the amplitude of contractions (90.6 ± 6.5 µm to 87.8 ± 8.3 µm) (Figures 28C and D).
Treatment with 100 µM L-NNA ablated the flow-induced decrease in contraction frequency resulting in an increase in frequency as flow increased. (A) The trace for lymphatic vessel activity of the control flow ramp conducted before the addition of 100 µM L-NNA and subsequent ramp showed a decrease in contraction frequency. Treatment with 100 µM L-NNA during the second flow ramp (B) resulted in the ablation of the flow-induced decrease in contraction frequency. L-NNA treatment increased frequency to levels higher than the baseline.
Figure 27: L-NNA treatment results in the ablation and reversal of the flow-induced decrease in contractile activity. (A) Contraction frequency, (B) amplitude of contractions, (C) diastolic diameter, (D) systolic diameter, (E) ejection fraction, and (F) fractional pump flow.
under conditions of flow and in response to treatment with 100 µM L-NNA under conditions of flow. Graphs are corrected to a baseline value for frequency and FPF due to variations in the baseline frequency of lymphatic vessels. Statistical difference determined using a 2-way repeated measures ANOVA with a Dunnett’s post hoc test for differences compared to the no flow conditions and a Sidak’s post hoc test to determine differences between treatments. p < 0.05 * compared to the baseline of 0 cmH₂O flow in the respective ramps for an n = 6 with error bars shown as ± SEM.

Figure 28: Exposure to flow significantly decreases contraction frequency, while L-NNA treatment does not significantly impact contraction frequency or amplitude of contractions. Comparison of contraction frequency (A) and contraction amplitude (B) before, and ten minutes after being subjected to a 15-minute flow ramp. (C) Contraction frequency and (D) contraction amplitude before and after 100 µM L-NNA treatment. Statistical difference determined using a paired two-tailed Student’s t-test for an n = 6. With p < 0.05 * for a significant decrease compared to the pre-ramp baseline due to flow ramp exposure and error bars shown ± SEM.
4.2.6. **Lymphatic vessel treatment with Indomethacin results in the partial restoration of contractile activity at high flow conditions.**

Lymphatic vessels were subjected to a flow ramp, and upon completion, 10 µM Indomethacin addition occurred as with previous inhibitors. Representative traces of lymphatic vessel contractile activity for the control ramp showed a decrease in frequency in response to flow (Figure 29A), which was ablated by the addition of 10 µM Indomethacin, resulting in the restoration of contraction frequency to baseline levels (Figure 29B). Contraction frequency significantly decreased at 9 cmH₂O for the control ramp, and 10 µM Indomethacin treatment ablated the significant decrease but showed no significant differences compared to no flow conditions. The frequencies for the control and Indomethacin ramps were 100 ± 18.2 %, 79.0 ± 16.2 %, 71.9 ± 19.2 % and 65.0 ± 13.5 %, and 100 ± 14.1 %, 87.9 ± 16.9 %, 80.5 ± 18.1 % and 100.6 ± 12.6 %, respectively (Figure 30A). The amplitude of contractions increased significantly in the control ramp at 1 cmH₂O flow and showed no significant differences in the Indomethacin treated ramp (Figure 30B). Control and Indomethacin treated ramp amplitudes were µm, 82.0 ± 10.6 µm, 88.6 ± 11.2 µm, 88.4 ± 6.0 µm and 72.0 ± 18.6 µm, and 81.5 ± 4.3 µm 84.9 ± 3.2 µm, 73.9 ± 13.6 µm and 66.0 ± 15.2 µm, respectively (Figure 30B). The diastolic diameter, systolic diameter, ejection fraction and fractional pump flow showed no significant differences in either treatment (Figures 30C-F). The diastolic diameters in the control and Indomethacin treated ramps were 151.5 ± 12.0 µm, 153.9 ± 10.7 µm, 149.4 ± 6.2 µm and 140.2 ± 12.5 µm, and 155.7 ± 8.8 µm, 155.8 ± 7.4 µm, 145.0 ± 12.6 µm and 143.6 ± 14.6 µm at 0, 1, 5 and 9 cmH₂O in the respective ramps (Figure 30C). The systolic diameters in the control and Indomethacin treated ramps were 67.0 ± 4.0
µm, 66.8 ± 4.6 µm, 62.1 ± 4.5 µm and 66.0 ± 5.4 µm, and 73.1 ± 6.4 µm, 70.8 ± 5.3 µm, 68.7 ± 5.9 µm and 73.2 ± 4.2 µm at 0, 1, 5 and 9 cmH₂O in the respective ramps (Figure 30D). The ejection fractions for the control and Indomethacin treated ramps were 0.80 ± 0.02, 0.81 ± 0.02, 0.83 ± 0.02 and 0.70 ± 0.12, and 0.78 ± 0.02, 0.80 ± 0.01, 0.74 ± 0.07 and 0.68 ± 0.09 for 0, 1, 5 and 9 cmH₂O flow, respectively (Figures 30E). There were no significant changes in the FPF in either ramp or between ramps and the FPF decreased in the control ramp from 100 ± 17.5 % to 82.5 ± 17.0 %, 76.8 ± 20.8 % and 61.2 ± 18.8% at 1, 5 and 9 cmH₂O, respectively. The FPF changed in the Indomethacin treated ramp from 100 ± 14.4 % to 89.6 ± 16.7 %, 77.0 ± 19.4 % and 84.8 ± 18.3 % at 1, 5 and 9 cmH₂O, respectively (Figures 30F). The fractional pump flow showed a similar trend to the frequency with Indomethacin treatment resulting in a partial restoration to baseline values (Figure 30F). Exposure to flow resulted in a significant decrease in contraction frequency from 14.3 ± 2.6 con/min to 11.1 ± 1.3 con/min. However, it did not impact the amplitude of contractions (87.5 ± 9.3 µm to 88.9 ± 8.6 µm) (Figures 30A and B). The addition of 10 µM Indomethacin did not significantly impact the contraction frequency (11.1 ± 1.3 con/min to 11.0 ± 1.6 con/min) or the amplitude of contraction (88.9 ± 8.6 µm to 83.6 ± 5.0) (Figures 30C and D).
Figure 29: Treatment with 10 µM Indomethacin resulted in the partial restoration of contraction frequency in response to flow. (A) The trace for lymphatic vessel activity of the control flow ramp conducted before the addition of 10 µM Indomethacin showed a small decrease in contraction frequency. Treatment with 10 µM Indomethacin during the second flow ramp (B) resulted in the partial restoration of the flow-induced decrease in contraction frequency. It restored the contraction frequency to baseline levels across all magnitudes of flow.
Figure 30: Indomethacin prevented the significant decrease in contractile activity under conditions of flow. (A) Contraction frequency, (B) amplitude of contractions, (C) diastolic diameter, (D) systolic diameter, (E) ejection fraction, and (F) fractional pump flow under

Control  n = 5  10µM Indomethacin
conditions of flow and in response to treatment with 10 μM Indomethacin under conditions of flow. Graphs are corrected to a baseline value due to notable variations in the baseline frequency and diameter of lymphatic vessels. Statistical difference determined using a 2-way repeated measures ANOVA with a Dunnett’s post hoc test for differences compared to the no flow conditions and a Sidak’s post hoc test to determine differences between treatments. p < 0.05 * compared to the baseline of 0 cmH₂O flow in the respective ramps for an n = 5 with error bars shown as ± SEM.

Figure 31: Neither flow nor the addition of 10 μM Indomethacin resulted in significant changes to contraction frequency or amplitude of contractions. Comparison of contraction frequency (A) and contraction amplitude (B) before, and ten minutes after being subjected to a 15-minute flow ramp. The addition of 10 μM Indomethacin did not significantly impact the (C) contraction frequency or (D) contraction amplitude. Statistical difference determined using a paired two-tailed Student’s t-test for an n = 6 with error bars showing ± SEM.
4.2.7. Combined treatment with L-NNA and Indomethacin did not impact the flow-induced decrease in contraction frequency.

Lymphatic vessels experienced a flow ramp, and upon completion, 10 µM Indomethacin and 100 µM L-NNA were added together in the manner previous inhibitors were. The lymphatic vessel response to increases in flow can be observed in the representative traces for the control and dual treated ramps (Figures 32A and B). Representative traces of lymphatic vessel contractile activity for the control ramp showed a decrease in frequency in response to flow (Figure 32A), which not impacted by the addition of 10 µM Indomethacin and 100 µM L-NNA (Figure 32B). Contraction frequency significantly decreased by 5 cmH₂O flow in the control ramp and this was not observed in the 10 µM Indomethacin and 100 µM L-NNA treated group. The frequencies for the control and dual treated ramps were 100 ± 16.4 %, 97.8 ± 24.8 %, 71.8 ± 14.5 % and 50.3 ± 10.5 %, and 100 ± 35.7 %, 90.3 ± 22.7 %, 66.3 ± 3.0 % and 54.0 ± 10.7 %, at 0, 1, 5 and 9 cmH₂O respectively (Figure 33A). The amplitude of contractions showed a significant increase in the control ramp that was ablated with Indomethacin and L-NNA treatment. Control and dual treated ramp amplitudes were µm, 61.4 ± 15.0 µm, 66.2 ± 15.8 µm, 81.8 ± 15.2 µm and 84.3 ± 18.5 µm, and 65.6 ± 16.9 µm 64.7 ± 20.5 µm, 77.0 ± 20.9 µm and 71.9 ± 21.5 µm, respectively (Figure 33B). Diastolic diameter showed a significant increase at 1 cmH₂O flow in the control ramp, while there was a significant decrease in the systolic diameter at 9 cmH₂O flow in the control ramp but not the dual treated ramp (Figures 32C and D). The diastolic diameters in the control and dual treated ramps were 141.8 ± 24.4 µm, 144.7 ± 24.5 µm, 148.8 ± 24.7 µm and 140.0 ± 32.0 µm and 137.9 ± 30.0 µm, 133.2 ± 30.4 µm, 131.5 ± 33.8 µm and 143.6 ± 14.6 µm at 0, 1, 5
and 9 cmH$_2$O in the respective ramps (Figure 33C). The systolic diameters in the control and dual treated ramps were 80.4 ± 9.7 µm, 78.6 ± 11.3 µm, 67.0 ± 11.4 µm and 55.7 ± 13.5 µm, and 72.3 ± 14.1 µm, 68.5 ± 13.6 µm, 60.9 ± 10.2 µm and 59.7 ± 13.0 µm at 0, 1, 5 and 9 cmH$_2$O in the respective ramps (Figure 33D). Ejection fraction showed a similar trend to the increase in the amplitude of contractions though it never reached significance in either ramp. In contrast, the fractional pump flow showed a similar trend to the contraction frequency, reaching a significant decrease in the control ramp at 9 cmH$_2$O (Figures 33E and F). The ejection fractions for the control and dual treated ramps were 0.65 ± 0.05, 0.69 ± 0.05, 0.80 ± 0.03 and 0.85 ± 0.01, and 0.71 ± 0.04, 0.71 ± 0.04, 0.78 ± 0.04 and 0.77 ± 0.05 for 0, 1, 5 and 9 cmH$_2$O flow, respectively (Figures 30E). There were no significant changes in the FPF in either ramp or between ramps and the FPF decreased in the control ramp from 100 ± 18.7 % to 100.5 ± 24.2 %, 85.1 ± 15.5 % and 64.0 ± 13.0 % at 1, 5 and 9 cmH$_2$O, respectively. The FPF changed in the dual treated ramp from 100 ± 33.0 % to 89.8 ± 19.5 %, 74.7 ± 4.7 % and 61.4 ± 14.1 % at 1, 5 and 9 cmH$_2$O, respectively (Figures 30F). Exposure to flow resulted in a significant decrease in contraction frequency from 15.1 ± 2.5 con/min to 10.8 ± 2.4 con/min, while not impacting the amplitude of contractions (61.4 ± 15.0 µm to 67.4 ± 13.8 µm) (Figure 34A and B). The addition of 10 µM Indomethacin and 100 µM L-NNA resulted in a slight decrease in contraction frequency from 10.8 ± 2.4 con/min to 9.5 ± 3.4 con/min, while not impacting the amplitude of contractions (67.4 ± 13.8 µm to 65.6 ± 16.9 µm) (Figure 34C and D).
Figure 32: Treatment with both L-NNA and Indomethacin did not impact the flow-induced decrease in contraction frequency. (A) The trace for lymphatic vessel activity of the control flow ramp that was conducted before the addition of 100 μM L-NNA and 10 μM Indomethacin showed a decrease in contraction frequency as flow increased. Administration of 100 μM L-NNA and 10 μM Indomethacin during the second flow ramp (B) did not impact the contraction frequency under conditions of flow.
Figure 33: Treatment with both L-NNA and Indomethacin did not significantly restore contractile activity in response to flow. (A) Contraction frequency in (B) amplitude of contractions, (C) diastolic diameter, (D) systolic diameter, (E) ejection fraction, and (F)
fractional pump flow under conditions of flow and in response to treatment with both 10 µM Indomethacin and 100 µM L-NNA under conditions of flow. Graphs are corrected to a baseline value due to notable variations in the baseline frequency and diameter of lymphatic vessels. Statistical difference determined using a 2-way repeated measures ANOVA with a Dunnett’s post hoc test for differences compared to the no flow conditions and a Sidak’s post hoc test to determine differences between treatments. p < 0.05 *, p < 0.01 ** compared to the baseline of 0 cmH₂O flow in the respective ramps for an n = 5 with error bars shown as ± SEM.

Figure 34: Flow results in a significant decrease in contraction frequency. The addition of 10 µM Indomethacin and 100 µM L-NNA did not result in significant changes to contraction frequency or amplitude of contractions. Comparison of contraction frequency (A) and contraction amplitude (B) before, and ten minutes after being subjected to a 15-minute flow ramp. (C) Contraction frequency or (D) contraction amplitude before and after 10 µM Indomethacin and 100 µM L-NNA treatment. Statistical difference determined using a paired two-tailed Student’s t-test for an n = 4. p < 0.01 ** for a significant decrease due to flow ramp exposure with error bars shown as ± SEM.
4.3. TRPV4 Agonism Concentration-Response Curves

4.3.1. Assessment of endothelium-dependent responses

Lymphatic vessels were isolated from the rat mesentery and mounted on glass pipettes and maintained at 3 cmH₂O pressure for 22-25 minutes before being stimulated with 10 µM acetylcholine (ACh) to determine the functional activity of the endothelium. After ACh treatment vessels were allowed to equilibrate before the subsequent addition of the antagonists and TRPV4 agonist GSK1016790A (GSK101). Vessels containing a functional endothelium showed a transient cessation of contractions in response to 10 µM ACh addition (Figure 35). Washout of ACh resulted in a return to pre-treatment frequency.
Figure 35: Lymphatic Vessel trace showing a decrease in contraction frequency in response to treatment with 10 µM acetylcholine (ACh) that were used for subsequent GSK101 control ramps. Vessels were subjected to a three minute exposure to acetylcholine which resulted in a transient decrease in contraction frequency before being restored to baseline contraction frequency. Changes in contraction characteristics were not significant after the washout of ACh.
4.3.2. *TRPV4 agonism in lymphatic vessels results in a biphasic contractile response.*

After establishing the functionality of the endothelium, Lymphatic vessels underwent a cumulative concentration-response curve of TRPV4 agonist GSK101 consisting of concentrations ranging from 0.1 nM to 1 µM. Eight experimental groups were established, and each one will be presented in the following sections. Administration of GSK101 to untreated, control vessels led to a biphasic contractile response. A decrease in contraction frequency occurred at low concentrations of GSK101 (0.1 – 3 nM), with the peak relaxation taking place at 1 nM GSK101. At concentrations 30 nM and higher, an increase in contraction frequency took place with the peak contraction frequency observed at 300nM GSK101 as shown in Figure 36.
Figure 36: Representative trace of lymphatic vessel contractility when subjected to increased GSK101 concentrations. GSK101 was added sequentially from 0.1 nM to 1 μM, each concentration lasting 5 minutes. The final two minutes of each exposure shown on the trace above. Representative for an n = 5.
4.3.3. Contribution of endothelial TRPV4 to the GSK101 response

To determine the contributions of the two major endothelium-dependent relaxation pathways, NO from eNOS and dilatory prostaglandins from COX, the inhibitors L-NNA and Indomethacin were used. Each pathway was inhibited individually and together to determine if they functioned in an additive manner. In vessels with a functional endothelium, treatment with L-NNA (100 µM, orange line) did not ablate the decrease in contraction frequency at low GSK101 concentrations but did shift the EC\textsubscript{50} slightly to the left, while also increasing the maximal contraction frequency (Figure 37A). Treatment with Indomethacin (10 µM, purple line) prevented the decrease in contraction frequency at low GSK101 concentrations. It did result in a small rightward shift in the increase in contraction frequency while only increasing the maximal contraction frequency a small amount at 100 and 300 nM (Figure 37B). Dual treatment prevented the decrease in contraction frequency at low GSK101 concentrations and resulted in the highest peak contraction frequency with the peak being reached at 300 nM. Dual treated vessels showed no shift in the EC\textsubscript{50} value for the increase in contraction frequency (Figures 37C & D). The EC\textsubscript{50} values for the control, 100 µM L-NNA treated, 10 µM Indomethacin treated and dual treated groups were 2.1 x 10\textsuperscript{-8}, 1.4 x 10\textsuperscript{-8}, 2.3 x 10\textsuperscript{-8} and 2.1 x 10\textsuperscript{-8}, respectively. The concentration-response curve non-linear regressions for the EC\textsubscript{50}’s can be found in Appendix D.
Figure 37: Treatment with L-NNA, Indomethacin or a combination of both treatments alter the contractile properties of lymphatic vessels. (A) Treatment with 100 µM L-NNA (orange line), (B) treatment with 10 µM Indomethacin (purple line), (C) Dual treatment with and 100 µM L-NNA and 10 µM Indomethacin (blue line), and (D) all four treatment groups. All changes were baseline corrected to the values obtained in the final two minutes in the respective inhibitors before the addition of GSK101. The baseline values were then denoted as 100% (dotted line). Statistical difference determined using a 2-way ANOVA followed by a Sidak’s post hoc test for (A – C) and Tukey’s post hoc test for (D). p < 0.05 for significant differences.
between control and 100 µM L-NNA and 10 µM Indomethacin treated groups. p < 0.05 @ for significant differences between 100 µM L-NNA and 10 µM Indomethacin, and 10 µM Indomethacin treated groups. Results for an n = 5 for GSK treatment alone, an n = 5 for 100 µM L-NNA, an n = 6 for 10 µM Indomethacin and an n = 8 for 100 µM L-NNA and 10 µM Indomethacin.
4.3.4. **TRPV4 agonism in the presence of the antagonist HC067047**

In order to inhibit TRPV4 function, we used the selective inhibitor HC067047 (HC). Combinations of treatments of HC with the eNOS inhibitor L-NNA and the COX inhibitor Indomethacin were used to determine if TRPV4 agonism was acting through either of these pathways. Treatment with 3 µM HC (black line) resulted in a rightward shift in both portions of the biphasic response to GSK101 treatment. The decrease in frequency occurred at 10 and 30 nM before an increase in frequency took place at 100 nM with the peak being reached at 300 nM and the 1 µM GSK101 being significantly higher than control vessels (Figure 38A). Dual treatment with 3 µM HC and 100 µM L-NNA (light blue line) resulted in a further rightward shift of the concentration-response curve. There was a decrease in frequency from 10 to 30 nM GSK101 and the peak was reached at 1 µM GSK101, though there were no significant differences compared to the control ramp (Figure 38B). Treatment with 3 µM HC and 10 µM Indomethacin (grey line) cause a rightward shift in the concentration-response curve while reaching peak contraction frequency at 300 nM GSK101. These vessels showed a depressed contraction frequency for the duration of the GSK101 concentration response curve, though no points were significantly different from the control vessel (Figure 38C). Treatment with all three inhibitors (green line) resulted in a rightward shift of similar size to the HC and L-NNA co-treated group. It exhibited a similar curve to the treatment with HC and Indomethacin exhibiting a small decrease in contraction frequency at concentrations of 0.3 to 100 nM GSK101, though this was not significant (Figures 38D & E). The EC\textsubscript{50} values for the 3 µM HC treated, 3 µM HC and 100 µM L-NNA treated, 3 µM HC and 10 µM
Indomethacin treated and triple treated groups were $1.0 \times 10^{-7}$, $2.3 \times 10^{-7}$, $1.1 \times 10^{-7}$ and $3.4 \times 10^{-7}$, respectively. (Appendix D).
Figure 38: Inhibition of TRPV4 agonism with 3 µM of the selective antagonist HC067047 (HC) results in a rightward shift of both the decrease and increase in contraction frequency. All changes were baseline corrected to the values obtained in the final two minutes in
the respective inhibitors before the addition of GSK101. The baseline values were then denoted as 100% and the corresponding line across the graph indicates the baseline values before treatment. In (A), inhibition with 3 µM HC (black line) compared to control, (B) treatment with both 3 µM HC and 100 µM L-NNA (light blue line), (C) treatment with both 10 µM Indomethacin and 3 µM HC (grey line), (D) treatment with all three inhibitors (HC, L-NNA, Indomethacin) (green line). (E) All 3 µM HC treated ramps compared to the control ramp (red line) Statistical difference determined using a 2-way ANOVA with Sidak’s post hoc test for (A – D) and with Tukey’s post hoc test for (E). p < 0.05 *, p < 0.01 **, p < 0.001 *** for significant differences between 3 µM HC treated and control-treated vessels. p < 0.01 >>, for significant differences between control and triple treated vessels. p < 0.05 %, p < 0.01 %%, p < 0.0001 %%%% for significant differences between 3 µM HC treated, and triple treated vessels. p < 0.01 ++ for significant differences between 3 µM HC treated, and 100 µM L-NNA and 3 µM HC treated vessels. p < 0.01 &&, p < 0.001 &&& for significant differences between 3 µM HC treated, and 10 µM Indomethacin and 3 µM HC treated vessels. Results for an n = 5 for GSK Treatment alone, an n = 3 for 3 µM HC treated vessels, an n = 4 for 100 µM L-NNA, 10 µM Indomethacin, and 3 µM HC treated vessels, an n = 3 for the 100 µM L-NNA and 3 µM HC treated vessels, and an n = 3 for 10 µM Indomethacin and 3 µM HC treated vessels.
4.3.5. Changes in amplitude, diastolic diameter, systolic diameter, ejection fraction and fractional pump flow

Vessels with a functional endothelium (red line) exhibited the smallest decrease in contraction amplitude, diastolic diameter, and ejection fraction at high concentrations of GSK101 (Figures 39A, B and D). These vessels exhibited a significantly smaller decrease in contraction amplitude and ejection fraction than the 3 µM HC treated vessels at 300 nM and 1 µM, while also showing a significantly smaller decrease in diastolic diameter at 300 nM. It showed a more substantial increase in the systolic diameter than all three the other groups, 100 µM L-NNA and 10 µM treated (blue line), 3 µM HC, 100 µM L-NNA and 10 µM Indomethacin (triple) treated (green line) and 3 µM HC treated vessels (black line) (Figure 39C). Lastly it showed a similar trend to the frequency for the fractional pump flow (FPF) with a significantly lower FPF than the 100 µM L-NNA and 10 µM Indomethacin treated group at 30 and 100 nM GSK101 and a significantly higher FPF than the 3 µM HC treated group at 300 nM GSK101 (Figure 39E). Both the 100 µM L-NNA and 10 µM Indomethacin treated and triple treated vessels showed similar decreases in contraction amplitude, diastolic diameter and ejection fraction (Figures 39A, B and D). The 100 µM L-NNA and 10 µM Indomethacin treated vessels showed a larger increase in the systolic diameter at high GSK concentrations though this was not significant (Figure 39C). The 100 µM L-NNA and 10 µM Indomethacin treated group had a significantly higher FPF compared to the triple treated vessels from 10 – 300nM (Figure 39E). 3 µM HC treated vessels (black line) showed the largest decreases in contraction amplitude, ejection fraction and diastolic diameter (Figures 39A, B and D). This group had a significantly higher contraction amplitude and ejection fraction than the
100 µM L-NNA and 10 µM Indomethacin treated vessels at 100 nM. They also had a significantly lower contraction amplitude and ejection fraction at 300 nM than 100 µM L-NNA and 10 µM Indomethacin and triple treated vessels (Figures 39A & D). These vessels exhibited a significantly higher FPF than the triple treated vessels at 100 nM while being significantly less than the dual treated vessels at 10, 30 nM and 300 nM GSK101. The other four treatment groups showed responses similar to the Indomethacin and L-NNA co-treated ramp (blue line) and the triple treated ramp (green line) (data not shown).
Figure 39: Inhibition of TRPV4 or endothelial function significantly impacted the contraction properties of rat mesenteric lymphatic vessels. All changes were baseline corrected to the values obtained in the final two minutes in the respective inhibitors before the
addition of GSK101. The baseline values were then denoted as 100% and the corresponding line across the graph indicates the baseline values before treatment. Changes in the (A) amplitude (B) diastolic diameter, (C) systolic diameter, (D) ejection fraction and (E) fractional pump flow, are also shown for the four treatment groups. Statistical difference determined using a 2-way ANOVA with Tukey’s post hoc test. p < 0.01 **, p < 0.001 ***, p < 0.0001 **** for significant differences between 3 µM HC treated and control-treated vessels. p < 0.05 #, p < 0.001 ###, p < 0.001 ### for significant differences between 100 µM L-NNA and 10 µM Indomethacin treated and triple treated vessels. p < 0.05 ^, p < 0.01 ^^ for significant differences between control and 100 µM L-NNA and 10 µM Indomethacin treated vessels. p < 0.05 $, p < 0.01 $$, p < 0.001 $$$, p < 0.0001 $$$$$ for significant differences between 3 µM HC and 100 µM L-NNA and 10 µM Indomethacin treated vessels. p < 0.05 %, p < 0.01 %%, p < 0.001 %%%, p < 0.0001 %%%%% for significant differences between 3 µM HC and triple treated vessels. Results for an n = 5 for GSK Treatment alone, an n = 3 for 3 µM HC treated vessels, an n = 8 for the 100 µM L-NNA and 10 µM Indomethacin pre-treated vessels and an n = 4 for 100 µM L-NNA, 10 µM Indomethacin, and 3 µM HC treated vessels.
4.4. Endothelial Cell Characterization

4.4.1. Primary cultured rat mesenteric lymphatic endothelial cells express classical endothelial markers and TRPV4 during passages three through six.

Lymphatic endothelial cells were isolated from the duodenal portion of the intestine and cultured. The expression of common lymphatic markers was determined through passages 3-6. The negative control showed no staining, while Prox1 showed both nuclear and peri-nuclear staining within confluent sheets of endothelial cells (Figures 40A & B). LYVE-1 expression was lacking and is indicative of initial lymphatic vessels, while Foxc2 expression occurred within the nuclei (Figures 40 C & D). Lastly, VEGFR3 expression occurred on the cell surface, while CCL-21, which is highly expressed within initial lymphatic vessels, expression was not observed (Figure 40 E & data not shown). These cells also did not express α-SMA allowing for confidence that there is no contamination with smooth muscle cells (data not shown). TRPV4 expression occurred throughout the cells in copious amounts, showing expression within the cytoplasm and at the cell surface (Figure 40F). These results indicate that the cells are collecting vessel lymphatic endothelial cells and remain phenotypically the same over the first six passages. These cells also stably express TRPV4 throughout passages justifying their use in future studies to determine TRPV4 function within LECs.
Figure 40: Lymphatic endothelial cells isolated from the duodenum’s collecting lymphatic vessels show consistent expression of lymphatic specific markers. (A) Control image showing secondary antibody control staining. (B) Prox1 staining within the nucleus and in the perinuclear region. (C) LYVE-1 expression observed at low levels in cultured cells. (D) CCL-21 was not
observed within the cultured lymphatic endothelial cells. (E) Foxc2 showed nuclear staining and is a marker specific to collecting lymphatic endothelial cells. (F) VEGFR3 expression occurred on the cell surface and is observed throughout the lymphatic system. (G) TRPV4 expression observed throughout endothelial cells. (H) α-SMA is a smooth muscle marker, and its expression was not observed at any point. An n = 1 for four passages with representative images from passage 6. All images were obtained using a confocal microscope with the same settings for laser power and length of time for all stains.
Both transmural pressure and luminal flow modulate lymphatic vessel activity (McHale & Roddie, 1976; Lee et al., 2015; Gashev et al., 2002). How these parameters modulate, lymphatic contractility is dependent on the species and the location of the lymphatic vessel within the body (Zawieja et al., 2018; Gashev et al., 2004). Increasing pressure increases the contraction frequency until reaching a plateau at about 10 cmH₂O transmural pressure. In comparison, the amplitude of contractions increased until 4 cmH₂O transmural pressure before decreasing in a magnitude dependent manner within rat mesenteric collecting lymphatic vessels (Lee et al., 2014). The frequency modulation occurs through T-type Ca²⁺ channels and the amplitude or strength of contractions by L-type Ca²⁺ channels (Lee et al., 2014). However, a recent study found L-type Ca²⁺ channels to be indispensable in modulating contraction frequency (To et al., 2020). The calcium-dependent anion channel ANO1 also is vital in the pressure-dependent increase in contractile activity (Zawieja et al., 2019).

Flow decreases both the contractile frequency and the amplitude of contractions of rat mesenteric lymphatic vessels. This decrease occurred in an eNOS-dependent manner since treatment with 100 µM L-NMMA, an NOS inhibitor, resulted in a partial restoration of the frequency and amplitude of contractions (Gashev et al., 2002). The flow-dependent decrease in contractility occurs in the thoracic duct, cervical, and popliteal lymphatic vessels (Gashev et al., 2002; Gashev et al., 2004). Of these four types, mesenteric and popliteal lymphatics were less sensitive to flow, not showing a significant decrease in contraction frequency until the flow gradient reached 5 and 4 cmH₂O, respectively (Gashev et al., 2004). Conditions of
flow within afferent iliac lymphatic vessels can result in the synthesis of contractile prostaglandins (Koller et al., 1999). Indomethacin inhibited the production of contractile prostaglandins resulting in a decrease in the contraction frequency. Though a flow-dependent decrease occurs in a NO-dependent manner in collecting lymphatics, the exact molecular mechanisms or sensors are unknown. Based on work done in blood vessels, TRPV4 has emerged as an important mediator or contributor to the endothelium-dependent relaxation response. Though this is the case in arteries and blood vessels the role it plays in collecting lymphatic vessels during flow is unexplored.

The present study focused on the role of TRPV4 in mediating the flow-dependent decrease in contractile activity of rat mesenteric collecting lymphatic vessels. TRPV4’s role in endothelium-dependent relaxation within the vascular system has been substantially explored, but its role in lymphatic vessels remains undetermined. Calcium entry through TRPV4 is known to contribute to both the NO-dependent and EDHF-dependent relaxation pathways within arteries (Mendoza et al., 2010; Sukumaran et al., 2013; Bagher et al., 2012; Sonkusare et al., 2012). Due to the lack of electrical coupling between the lymphatic endothelial and smooth muscle layers and lack of IKCa and SKCa expression, the classical EDHF-dependent relaxation pathway does not contribute to the relaxation responses observed in lymphatic vessels (von der Weid & Van Helden 1997; Behringer et al., 2017).

To interrogate the potential role of TRPV4 in lymphatic vessel response to flow we first assess its expression in the rat mesenteric lymphatic vessels. Both western blot and immunofluorescent staining, indicate that TRPV4 channels expression occurs in both the endothelial and smooth muscle layers of lymphatic vessels.
TRPV4 agonism of whole vessels resulted in a biphasic contractile response with a decrease in contraction frequency taking place at low concentrations and an increase at high concentrations. Treatment with both L-NNA and COX inhibited the decrease in contraction frequency implicating the contributions of both these pathways to decrease contraction frequency in response to low concentrations of GSK101. TRPV4 may be involved mediating the flow-dependent decrease in mesenteric lymphatic vessels. To determine if TRPV4 inhibition resulted in a significantly different response a few more experiments would be conducted since this would increase the power of the statistical test. Based on the power calculation two more experiments would be required to determine if TRPV4 does play a significant role in the flow-induced reduction in contraction frequency. Treatment with both L-NNA and Indomethacin resulted in the prevention of the flow-dependent decrease in contractile activity though neither treatment was significantly different from the control ramp. TRPV4 agonism with GSK1016790A resulted in a biphasic response with the relaxation at low concentrations with NO, dilatory prostaglandins, and another pathway seeming to contribute to this response. This study has shown that TRPV4 is expressed in lymphatic vessels and contributes to the flow-dependent decrease in contractile activity similarly as in arteries. The molecular mechanisms responsible for the decrease in contraction frequency in response to flow remain to be elucidated within both endothelial and smooth muscle cell types.
5.1. Gene Expression of TRPV4 and other Mechanosensitive TRP Channels

Gene expression determination occurred through quantitative real-time PCR (RT-qPCR) utilizing TaqMan probes for the transient receptor potential channels TRPV4, TRPV1, TRPC1, TRPC6, TRPM4, and TRPM8. Gene expression of the Ca\(^{2+}\)-sensitive Cl\(^{-}\) channels anoctamin 1 (ANO1) and bestrophin 1 (BEST1) were also determined. Rat mesenteric lymphatic vessels are small in size, and the RNA yield from a single vessel is quite low. Thus, 4 – 6 vessels were pooled from the ileal portion of every rat, with the vessels being taken from the five arcades upstream of the terminal ileal arcade. Vessels were flushed to remove the immune cell containing lymph, ensuring the observed gene expression came from the lymphatic vessel smooth muscle and endothelial layers. Immune cells can be embedded into the lymphatic wall either on the endothelial or smooth muscle layer and contribute to gene expression profiles. Both macrophages and neutrophils located in the lung express TRPV4, and activation of these inflammatory cells result in enhanced production of reactive oxygen species and migration. (Hamanaka et al., 2010; Damann et al., 2009; Goldenberg et al., 2017). In rat mesenteric lymphatics, TRPV4 gene expression was quite low, and only 5 out of 7 rats showed TRPV4 gene expression (Figure 14A). There are low levels of TRPV4 gene expression in collecting lymphatic vessels, while there was considerably higher expression within the kidney. The kidney was used instead of the lung as a positive control due to expression TRPV4 at higher levels on the mRNA level as a whole tissue (Figure 14A, Appendix A) (Kunert-Keil et al., 2006).

TRPC1 was the most consistently expressed ion channel with expression observed in 6 of 7 rats while TRPV1, TRPM4, ANO1, TRPC6, and BEST1 expression occurred in 3, 5, 5, 1, and 1 rat respectively. The fact that gene expression was not consistently observed was
most likely due to sample transfer rather than true gene expression, especially since ANO1 is crucial to lymphatic vessel pumping/pacemaking. Low and inconsistent gene expression of these channels may be due to the local conditions’ differences within the gut of each animal. Some of these channels may perform a redundant function, making them dispensable in lymphatic vessels, or may be expressed within immune cell types that are variably present or embedded in certain vessels.

TRPC1 can form flow-sensitive complexes with TRPV4 both in vitro and in vivo (Ma et al., 2010; Greenberg et al., 2017). HEK293 cells transfected with TRPV4 showed a transient increase in intracellular Ca²⁺ in response to flow, and when co-transfected with TRPC1, this response was prolonged, and the same occurred in HUVECs (Ma et al., 2010). In the vasculature system, TRPC1 and TRPV4 can form heterotetramers. The inhibition of either channel resulted in a marked decrease in the flow-dependent increase in NO production (Greenberg et al., 2017). Interestingly, in the latter study, TRPV4 stimulation with GSK1016790A was partially inhibited with the TRPC1 blocking antibody T1E3 indicating the interaction of these channels is necessary for modulating flow sensitivity in arteries (Greenberg et al., 2017). The expression of TRPC1 in collecting lymphatic vessels supports the idea that it may act in the same manner within lymphatic vessels to modulate the response to flow. To test the function of TRPC1 the externally binding antibody T1E3 would have to be used in conjunction with proximity ligation assays to determine the physiological impact and the proximity to TRPV4 channels within the vessels. The other TRP channels contribute to the flow-dependent and pressure-dependent responses but will not be discussed further.
The variability in the mRNA-based detection analysis for all tested TRP channels may be due to the local microenvironment’s differences. It may also be that these channels are long-lived, meaning once produced, the protein can persist for long periods without the need for upregulation in gene expression (Baratchi et al., 2019). Previous work by Baratchi and colleagues showed that TRPV4 stimulation with its selective agonist GSK101 resulted in an increase in TRPV4 trafficking to the cell surface (Baratchi et al., 2019). Half an hour after stimulation there was a decrease in the cell expressing TRPV4, while there was an increase in the endosomal TRPV4, lending support to the idea that these channels are long-lived and may be recycled. Different regions of the gut may also exhibit different expression levels. The duodenal portion is where most of the lipid absorption occurs while in the colon, a more substantial amount of fluid absorption occurs. Due to the increased volume and increased osmolarity or viscosity of the lymph in these areas they may be impacting TRPV4’s response. TRPV4 has been shown to play an important role in sensing the osmolarity and thus differences in the make-up of the lymph may impact the observed response. The use of a nested PCR in which there is a second set of primers in between the first set of primers may allow for increased yield in gene expression allowing for the determination of the presence or absence of these channels with greater confidence. In all cases, TRPV4 expression occurred in meager amounts compared to the housekeeping genes of HPRT1 (hypoxanthine phosphoribosyltransferase) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). When qPCR products were run on a 2% agarose gel, bands were tough to visualize with faint or no bands visible after the initial qPCR. The amplification of the products a second time using the same primers, resulted in multiple observed bands for TRPV4. There are five known splice variants of TRPV4 within humans, with only two of
these forms being functional and expressed at the plasma membrane as a mature channel (Arniges et al., 2006). The three chief bands were isolated for sequencing, and only the strongest band was sequenced due to the low yield obtained from each band, and it was, in fact, TRPV4. The TaqMan probe for TRPV4 spans the 5th and 6th exons, only binding properly spliced TRPV4 and being released when DNA is replicated. Due to location of the probe, all fluorescence observed is indicative of TRPV4 specifically.

5.2. TRPV4 Protein Expression

TRPV4 protein expression occurred at low levels within rat mesenteric lymphatic vessels and vessels were pooled from the ileal section of each rat. In our western blots there was variability between samples, and this may be due to variability in TRPV4 expression within lymphatic vessels or the presence of imbedded TRPV4 expressing immune cells. TRPV4 protein expression occurred in both the endothelial and smooth muscle layers of lymphatic vessels. Our study supported the expression of TRPV4 in both the smooth muscle and endothelial layers of pulmonary and cerebral arteries (Sukumaran et al., 2013; Xia et al., 2013; Marrelli et al., 2007). TRPV4 was also expressed on immune cells and in cells located in the adventitial layer. Immune cell expression is supported by the fact that both neutrophils and macrophages express TRPV4 in the lung (Hamanaka et al., 2010; Damann et al., 2009). We observed robust TRPV4 expression at endothelial valves. These valves are where high amounts of shear stress are occurring supporting the idea that TRPV4 contributes to shear stress or flow-dependent responses (Moore & Bertram, 2018). NO production also takes place in higher amounts at the valve sites, contributing to vessel
relaxation (Bohlen et al., 2011). Thus, TRPV4 may be playing an essential role in the production of NO and the regulation of endothelium-dependent relaxation within lymphatic vessels.

It is important to note that the TRPV4 antibody was not tested on the lymphatic smooth muscle cell culture or cells that do not express TRPV4 as a negative control. It would be important to use TRPV4 knockout animal tissue samples, or TRPV4 deficient cell types as a negative control for Western Blots. TRPV4 expression in HEK cells or endothelial cells from TRPV4-deficient mice would also be a good negative control for the validation of immunofluorescent staining of TRPV4 in lymphatic vessels and lymphatic endothelial cells.

5.3. Pressure Myography and Flow Ramps

The use of pressure myography allowed for the determination of the impact of flow on rat mesenteric collecting lymphatic vessels. Pressure myography allows the manipulation of the transmural pressure and the magnitude of induced flow on lymphatic vessels. Parameters such as the contraction frequency, amplitude of contractions, systolic and diastolic diameters can be tracked and determined throughout the experiment. The ejection fraction (EF) and fractional pump flow (FPF) can be determined using these parameters (as shown in Methods Section 3.7). The EF indicates the strength of contractions, the percentage of lymph in a lymphangion ejected with every contraction and is expressed as a normalized value. The FPF is a measure of the active movement of lymph, the amount moved by the intrinsic contractile activity of the lymphangion and may not be an accurate
representation of the total lymph moved due to the contributions of external forces to lymph movement.

Pressure myography allows us to study the response of isolated lymphatic vessels in response to transmural pressure, fluid movement (flow), and agonists and antagonists. It cannot fully recapitulate the physiological conditions that lymphatic vessels experience in vivo, including contributions from surrounding immune cells and other cell types. It does not account for the external pressure the lymphatic vessels experience, the mediators present in the lymph and the interstitium, or the dynamic changes in pressure and flow that the lymphatic vessels encounter. Thus, ex vivo pumping experiments will never provide us with a complete understanding of lymphatic contractility and its dynamic modulation within the body. However, it does allow us to visualize the vessels and monitor changes that occur in response to isolated stimuli. It is challenging to visualize lymphatics, especially in the abdominal cavity in vivo. Due to this in vivo imaging and ex vivo vessel contractility experiments should be used in a combinatorial manner to provide an accurate picture of what is taking place under physiological conditions.

5.3.1. Control Flow Response

In our study, flow caused a magnitude-dependent decrease in contraction frequency that was significant by 1 cmH₂O flow. Vessels that did show a flow response responded in a magnitude-dependent manner, with greater amounts of flow resulting in a greater decrease in contraction frequency. There were significant increases in contraction amplitude
at low (1 cmH₂O) flow, while the ejection fraction showed increases at 1 and 0 cmH₂O flow. Associated with these changes, the systolic diameter decreased significantly by moderate amounts of flow and the diastolic diameter increased significantly at low flow and the new baseline conditions, while decreasing significantly at high levels of flow, contrary to what happens in blood vessels at high levels of flow (Bubloz et al., 2012). The increase in myogenic tone may allow the lymphatic vessel to act more like a pipe without jeopardizing the vessel’s integrity when experiencing high levels of force. By increasing the tone under conditions of flow, it may act as blood vessels do when exposed to transmural pressure to conserve vessel function (Gonzalez et al., 2014). The fractional pump flow (FPF) showed a similar response to the frequency with the significant decrease occurring by 1 cmH₂O of flow. Interestingly, neither the frequency nor the FPF of lymphatic vessels increased to the pre-ramp baseline levels after experiencing a flow ramp. Though the mesenteric collecting lymphatic vessels did show a flow response, it occurred inconsistently. About a third of the vessels exposed to flow responded with a decrease in contractile activity. Since these lymphatic vessels responded inconsistently only those that showed a decrease in contraction frequency in response to flow were used. We used solely these vessels due to our interest in how TRPV4 contributes to the flow-dependent decrease in contraction frequency. This inconsistency may be due to large fluctuations in pressure and flow these vessels were consistently exposed to in the gut. These fluctuations depend on many factors with food ingestion being one. Substantial changes in the fluid flow or pressure within the gut may impact the vessels’ sensitivity or predispose them to respond in a particular manner. Increases in pressure may result in an increase in contractile activity of these vessels, while also contributing to an increased external pressure. The complex
interplay of these factors may cause vessels to be more sensitive to flow, resulting in a decrease in contraction frequency, or increasing contraction frequency in response to flow. TRPV4 expression and localization within endothelial cells have been shown to be dependent on flow leading to different results between vessels (Baratchi et al., 2017). There may also be variability in the endothelium functionality, though endothelial function was tested by exposing vessels to a 3-minute treatment with 10 μM ACh. Vessels used for these experiments had to exhibit a decrease in contraction frequency in response to ACh treatment to be utilized for a flow ramp. Unfortunately decreases in frequency in response to ACh treatment did not correlate with flow responses resulting in a decrease in contraction frequency indicating complexity in the regulation of the flow-dependent decrease in contraction frequency.

To ensure the results observed were reproducible, we exposed the vessels to a second flow ramp half an hour after the first one. Our results indicated that response to the second flow ramp was very similar to the first, though it showed a bit more variability than the first ramp. Due to this variability, the frequency under flow conditions was not significantly different from the baseline, at a constant transmural pressure within the second ramp. There were no significant changes in the amplitude of contractions, diastolic diameter, systolic diameter or the ejection fraction in the vessels that experienced two flow ramps. The FPF significantly decreased in response to flow in the first ramp but not the second, exhibiting a similar response to that observed for contraction frequency. Having established that the flow response was reproducible, the impact of pharmacological treatments on flow could be determined. A key finding from flow ramp exposure was that the contraction frequency never returned to the same levels as before the first flow ramp.
Our lymphatic vessels showed variability in their ability to re-establish a similar contraction frequency to the one before the flow ramp was conducted. The decreased contractile frequency after the flow ramp may be due to acute changes in the lymphatic vessel as a response to shear stress or due to more permanent changes. This may impact the response to the treatments used in this study, but further elucidation would be required. It would be important to look at actin fibre remodeling and cell structure in response to flow via a pressure gradient within these lymphatic vessels to determine which proposed mechanism was contributing more significantly. Even though the subsequent baseline was significantly less than the initial baseline in most cases, the overall trend was similar between the two ramps. The decreased contractile activity for the second baseline compared to the first contributed to normalizing the baseline parameters at 5 cmH$_2$O transmural pressure and 0 cmH$_2$O pressure difference (flow) before each ramp.

In previous work, rat mesenteric vessels decreased contractile activity in response to flow (Gashev et al., 2002). The decrease was partially mediated by nitric oxide (NO) derived from endothelial nitric oxide synthase (eNOS), with 100 µM L-NMMA treatment, resulting in a 20-30% restoration of contraction frequency and 35-40% restoration of contraction strength. This partial restoration suggested that the NO-dependent relaxation pathway was not the only pathway playing a role in the endothelium (Gashev et al., 2002). In their experiments, they only conducted a single ramp for each lymphatic vessel (experiment type). Thus, they did not test the reproducibility of the flow-dependent decrease in contractile activity. Prostaglandin synthesis can also contribute to the relaxation responses in lymphatic vessels (Hanley et al., 1989; Rehal et al., 2009). Both prostaglandin E$_2$ (PGE$_2$) and prostaglandin I$_2$ (prostacyclin) contribute to endothelium-dependent
relaxation. They acted through the EP4 and IP receptors located on the smooth muscle, respectively, with PGE2 contributing to more significant amounts of relaxation (Rehal et al., 2009).

Induction of lymph flow on lymphatic vessels results in changes in contractile activity. Exposure of rat mesenteric lymphatic vessels to flow induced by 1, 3, 5, and 7 cmH2O pressure gradients resulted in a magnitude-dependent decrease in contraction frequency (Gashev et al., 2002). The decrease in contractile activity was significant by 1 cmH2O of flow, while the amplitude of contractions decreased significantly by 3 cmH2O. These changes translated into significant decreases in the fractional pump flow (FPF) and ejection fractions (EF) at the 3 cmH2O flow (Gashev et al., 2002). In another study, Gashev and colleagues compared lymphatic vessels from four locations within rats in response to increases in transmural pressure and flow (Gashev et al., 2004). The second study showed that flow caused a decrease in contractile activity in all four vessel types: mesenteric, hindlimb (popliteal), cervical, and thoracic ducts. The most significant decreases occurred in the cervical lymphatics and the thoracic duct with almost complete ablation of contractions. At the same time, the other two tissues showed decreases, but to a lesser extent. In all four vessel types, there were significant decreases in the contraction frequency by 5 cmH2O. The systolic diameter increased significantly by 3 cmH2O flow, while the ejection fraction and FPF decreased significantly by 3 and 4 cmH2O, respectively (Gashev et al., 2004). In our study the frequency and FPF decreased in a manner similar to both Gashev studies, but we did not observe any decrease in the ejection fraction. Our study showed a magnitude-dependent decrease in contraction frequency that was significant by 1 cmH2O of flow. The decrease in contraction frequency produced similar changes in the FPF
with significance observed by 1 cmH$_2$O, and both these results were similar to those observed in mesenteric lymphatics (Gashev et al., 2002; Gashev et al., 2004). We did not observe an increase in the systolic diameter but rather a decrease at both 5 and 9 cmH$_2$O flow indicating larger contractions at high amounts of flow. The lymphatic vessel may be compensating for the decrease in frequency, trying to work as efficiently as possible to conserve energy. The diastolic diameter increased at 1 cmH$_2$O and the new baseline indicated a decrease in tone, leading to an increase in cross-sectional area possibly leading to the more efficient flow of the lymph through the vessel. The diastolic diameter decreased at 9 cmH$_2$O, which was unexpected. The vessel may have been increasing its tonic contraction to maintain the structure, acting more like a pipe. In previous work by Wang and colleagues on rat mesenteric lymphatics, lymphatic tone decreased as transmural pressure increased from 1 to 5 cmH$_2$O. This decrease was dependent on the action of MLC$_{20}$, with the ratio of phosphorylated to unphosphorylated MLC$_{20}$ decreasing with the decrease in tone (Wang et al., 2009). In our system the role of MLC$_{20}$ phosphorylation remains unelucidated and flow may be regulating the phosphorylation in a different manner. Koller and colleagues observed that flow increased contraction frequency and decreased contraction amplitude of rat iliac lymphatic vessels (Koller et al., 1999). In their work, they found that contractile prostaglandins increased the contraction frequency in response to flow. Based on both sets of findings, the flow response seen in our rat mesenteric collecting lymphatic vessels are in line with what would be expected though it may not be the best vessel type to induce a reduction in contraction frequency.
5.3.2. TRPV4’s Role in Flow

Having established a flow-dependent decrease in contraction frequency, we wanted to determine the role of TRPV4. In our results, treatment with 1 µM of the selective TRPV4 inhibitor, HC067047 (HC), did not significantly alter lymphatic vessel contractility. It did eliminate the significant decrease in contractility observed throughout the control ramp leading to no significant differences in frequency within the ramp, though there were no significant differences compared to the control ramp. These results indicate that 1 µM HC did not have a significant impact on the flow-dependent decrease in frequency. These results also indicate that TRPV4 may not be playing an important role in the lymphatic vessel flow response. In a study by Baratchi and colleagues, 1 µM HC treatment did not completely inhibit TRPV4 activation within HUVECs with high concentrations of GSK101 (300nM) (Baratchi et al., 2019). Though flow is most likely triggering a different signaling pathway 1 µM HC may not be enough to completely inhibit TRPV4 action. Complete vasodilation within third order mesenteric arteries from mice have been shown to be dependent on as few as three functional TRPV4 tetramers (Sonkusare et al., 2012). This supports that notion that the concentration of HC may be insufficient to completely inhibit TRPV4 action and a higher concentration may be required. The increase in amplitude and ejection fraction within the 1 µM HC ramp were unexpected. The increase was due to a significant decrease in the systolic diameter, indicating an increase in the contraction size/strength. Vessels may be using the pressure gradient to increase passive flow, while optimizing the lower energy required to move the lymph. Though 1 µM HC treatment ablated the significance of the flow response, none of the changes were significant, indicating that TRPV4 does not play an integral role in the flow-dependent decrease in
contraction frequency. The use of 1 μM HC ensured that minimal off-target effects occurred. HC has an IC$_{50}$ of 133 ± 33 nM for rat TRPV4 in transfected HEK293 cells. It shows high selectivity for TRPV4 with TRPM8 and the hERG channels being the only other channels showing IC$_{50}$’s below the micromolar range (Everaerts et al., 2010). In their experiments, 1 μM HC resulted in the complete inhibition of mouse TRPV4 activity (Everaerts et al., 2010). HC concentrations used to inhibit TRPV4 activity have varied from 300 nM at the low end to 10 μM at the high end (Darby et al., 2018; Bagher et al., 2012). Due to these notable variations in HC concentration used and the lack of TRPM8 expression within our mesenteric rat lymphatics, a slightly higher concentration of 3 μM was subsequently used further to elucidate the function of TRPV4 in lymphatic vessels.

TRPV4 inhibition with 3 μM HC resulted in the restoration of contraction frequency during flow. There was a significant decrease in the contraction frequency within the control ramp, and 3 μM HC treatment completely ablated the decrease in frequency. Though the contraction frequency in the presence of 3 μM HC looks different from the control ramp, this was not significant. This is most likely due to the low n number of experiments and variability in the degree of inhibition between lymphatic vessels. Conducting a power test on the results indicate that two more experiments would be required to observe significant differences between the ramps. Compared to the baseline at 0 cmH$_2$O flow, there was no significant difference at any point on the 3 μM HC treated ramp, and this was most likely due to an insufficient number of replicates. Treatment with 3 μM HC significantly increased the diastolic diameter at low flow conditions compare to no flow conditions within the same ramp. This was unexpected, though TRPV4 has been shown to be involved in pulmonary artery constrictions with TRPV4 inhibition ablating this
response (Xia et al., 2013). Treatment with 3 µM HC did not significantly impact the amplitude of contractions, the ejection fraction, or the systolic diameter of lymphatic vessels. The FPF was not significantly different from control ramp though it showed a very similar response and trend to the frequency. Treatment with 3 µM HC ablated the significant decrease in FPF observed in the control treated ramp and rather resulted in a significant increase in FPF as flow was increased. This increase in frequency and FPF were due to a large decrease in contraction frequency upon the completion of the first flow ramp. Vessels usually showed a 40% reduction in frequency upon completion of the first flow ramp and this may have created the conditions that allow the lymphatic vessels to increase their frequency in response to flow when treated with 3 µM HC. These results suggest that TRPV4 may contribute to the flow-dependent decrease in contraction frequency, though two more experiments would need to be conducted to determine the veracity of this statement.

Within lymphatic vessels, TRPV4 mediates the Ca\(^{2+}\) entry in response to ACh stimulation leading to the depolarization of endothelial cells. TRPV4 null mice showed a significantly decreased endothelial cell depolarization and Ca\(^{2+}\) entry in response to ACh treatment (Behringer et al., 2017). These results indicate TRPV4 contributes to facilitating Ca\(^{2+}\) entry in response to classical eNOS pathway agonists. In arteries, TRPV4 is heavily implicated in regulating tone and vasodilation in response to flow. In mice, TRPV4 activation occurs at low pressures and upon the induction of fluid flow (Bagher et al., 2012; Mendoza et al., 2010). When expressed in a heterotetramer with TRPC1, there is an increased sensitivity to flow in second-order mesenteric arteries of rabbits (Greenberg et al., 2017). Aged rats show decreased TRPV4 expression compared to young rats coupled with a
decreased dilation in response to flow. Increasing TRPV4 expression in mesenteric arteries of aged rats resulted in the significant restoration of flow-induced vasodilation. It also resulted in increased Ca²⁺ entry and NO production in mesenteric arterial endothelial cells (MAECs) (Du et al., 2016). These results, coupled with ours, support the idea that TRPV4 is essential in mediating flow responses within both blood and lymphatic vessels.

5.3.3. NO Contributions to Flow-induced Changes

The flow-dependent decrease in contractile activity is dependent on eNOS-induced production of NO to some extent. eNOS inhibitor L-NMMA (100 µM) resulted in a 20-30% restoration of contraction frequency and 35-40% restoration of contraction strength under conditions of flow (Gashev et al., 2002). In the case of an increase in contraction frequency upon flow exposure, 100 µM L-NAME did not impact the contractile activity (Koller et al., 1999). Both these results support the notion that eNOS contributes to the flow-induced relaxation response in lymphatic vessels. In our experiments, the control ramp resulted in a significant decrease in contractile activity by 5 cmH₂O flow and a significant decrease in FPF at 9 cmH₂O, though none of the other parameters changed significantly. Treatment with 100 µM L-NNA ablated the decrease in contractile activity, significantly increasing the frequency by 9 cmH₂O flow. It also prevented the significant decrease in FPF at 9 cmH₂O, though there were no significant differences between treatments. It is important to note that the two treatment groups did show results that looked different even
though they were not significantly different. The p value for the frequency at 9 cm H₂O was 0.06 and using a power test it was determined two more experiments would be required to determine significant differences.

Furthermore, 100 µM L-NNA treatment resulted in a significant increase in contraction frequency at 9 cm H₂O flow compared to the second ramp’s baseline. This increase indicated the importance of NO in negatively regulating chronotropic changes. The increase in frequency compared to the baseline was unexpected, and no previous reports implicate these results. These results may be due to the regional variations in lymphatic contractility based on the surrounding environment. It may also be due to how lymphatic vessels respond to flow. As shown, flow ramp treatment results in the frequency never returning to the frequency observed pre-ramp. This decrease in frequency may lead to vessels being more likely to increase their frequency, since it may be closer to the original baseline frequency. Using only L-NNA without looking at the impact on molecular mechanisms prevents us from fully asserting that all the observed changes are due to eNOS function. If the treatment did function as specified, the observed results could be concluded to be due to eNOS inhibition.

The similarity of response to 100 µM L-NNA treatment and 3 µM HC treatment may be due to TRPV4 function in the eNOS-dependent production of NO, since this has been shown in lymphatics and blood vasculature (Behringer et al., 2017; Mendoza et al., 2010; Bagher et al., 2012; Sonkusare et al., 2012). As previously stated, TRPV4 contributes to both the EDHF and NO-dependent relaxation pathways in arteries (Mendoza et al., 2010; Bagher et al., 2012; Sonkusare et al., 2012). The lack of electrical coupling and IKCa and
SK_{Ca} channel expression in lymphatic endothelial cells eliminate the classical EDHF pathway as a pathway that significantly contributes to vasodilation in lymphatic vessels (von der Weid et al., 1998; Behringer et al., 2017). Our results support the notion that the function of TRPV4 may contribute to NO-dependent relaxation pathway within lymphatic vessels.

5.3.4. Prostaglandin Synthesis in Flow

Treatment with 10 µM Indomethacin resulted in the partial restoration of contractile activity at low flow and complete restoration at high flow. The control ramp showed a significant decrease in contractile activity at 9 cmH₂O flow, and this was ablated with 10 µM Indomethacin treatment. The FPF showed a similar trend to the contraction frequency though it did not reach significance. The two groups do show responses that do look different, but the lack of a significant decrease can be attributed to the size of the error bars for 10 µM Indomethacin treated ramp due to the variability in responses between vessels. There were no significant changes in the amplitude of contractions or the diastolic and systolic diameters when comparing the control and 10 µM Indomethacin treated ramps. These results indicated that COX-derived dilatory products may contribute to chronotropic changes though not the inotropic changes due lymph flow. Prostaglandin synthesis within lymphatic vessels can contribute both pro-contractile and pro-relaxation molecules (Johnston et al., 1983; Rehal et al., 2009). PGE₂ and PGI₂ both contribute to relaxation responses occurring in lymphatic vessels (Rehal et al., 2009). Under conditions of flow, both thromboxane A₂ (TxA₂) and prostaglandin H₂ (PGH₂) contribute to the observed
increase in contractile frequency and contractile tone within iliac lymphatic vessels (Koller et al., 1998; Mizuno et al., 1998). Inhibition of TxA$_2$ production resulted in a reversal of AA-induced constrictions to dilations indicating that dilatory prostaglandin production occurs in lower levels in these vessels. PGE$_2$ treatment also resulted in a dose-dependent dilation of the rat iliac lymphatic vessels used (Mizuno et al., 1998). These results support our observations that dilatory prostaglandins are produced by the lymphatic endothelium and contribute to flow-induced decreases in contractile activity. Further confidence in these results would be obtained by determining the individual effects of PGE$_2$ and PGI$_2$ to the observed decrease in contractile frequency. To do this, vessels could be treated with specific inhibitors for PGE$_2$ and PGI$_2$ during flow conditions or the impact of PGI$_2$ and PGE$_2$ concentration-response curves could be determined.

5.3.5. eNOS and COX-1 Contributions to Flow

The use of both 100 µM L-NNA, and 10 µM Indomethacin, resulted in less of an impact than either treatment alone. These results were surprising since both the eNOS-dependent and COX-dependent pathways contribute to contraction frequency decreases (Gashev et al., 2002, Gashev et al., 2004, Rehal et al., 2009). They also exhibit crosstalk resulting in an augmentation of the other pathway (Salvemini et al., 1993, Salvemini et al., 2013, Pérez-Cremades et al., 2017). There was a significant decrease in contraction frequency by 5 cmH$_2$O flow in the control ramp that was prevented in the dual treated ramp. There was a slight restoration in the flow-dependent decrease in contraction frequency for all three flow points with three vessels showing no impact and one vessel
showing a strong inhibition of the response. Interestingly there was a significant increase in the contraction amplitude at 5 cmH$_2$O and 9 cmH$_2$O during the control ramp and this was ablated in the treated ramp. These results indicate that both NO and Indomethacin may contribute to differences in contraction strength. This increase in amplitude was due to a decrease in systolic diameter that reached significance at 9 cmH$_2$O in the control ramp while the diastolic diameter significantly increased at 1 cmH$_2$O flow in the control ramp. Control ramps tended to show some variability in the strength of the flow-mediated decrease in contraction frequency. The differences between vessels make it imperative to have a large enough sample size to account for this. The significant decrease in systolic diameter at 9 cmH$_2$O in the control ramp resulted in the corresponding increase in ejection fraction at 9 cmH$_2$O, though this was not significant. Due to the complex nature and crosstalk between the NO-dependent and prostaglandin-dependent pathways inhibition of both pathways may be triggering compensatory mechanism. The FPF showed a similar response or trend to the change in frequency in response to flow. The reasons for this occurring are unclear, though there may be differences in the number of constrictor and relaxation prostanoids released during flow. Arachidonic acid metabolism inhibition can result in the decreased production of TxA$_2$, PGH$_2$, PGI$_2$, and PGE$_2$ (Mizuno et al., 1998). Previous results from the iliac lymphatic vessels of rats indicate that COX-1 inhibition with Indomethacin resulted in a decrease in contractile frequency during flow (Koller et al., 1999). There may also be another molecule released from the lymphatic endothelium contributing to the relaxation response. This molecule could be 5, 6-EET since it a known endothelium-derived hyperpolarizing factor in the cardiovascular system. It is primarily metabolized by COX-1 to its vasoactive from and soluble epoxide hydrolases contribute to
the degradation of 5, 6-EETs to its less vasoactive form (Carroll et al., 1992; Vriens et al., 2005; Behm et al., 2009). There are three other EETs, 8, 9-EET, 11, 12-EET, and 14, 15-EET, and all three also contribute to the relaxation of arteries (Behm et al., 2009; Earley et al., 2005). These are most likely not contributing as much as 5, 6 EET since soluble epoxide hydrolases has a far higher affinity for these substrates, meaning they are converted to their less vasoactive forms quicker. Even though they may contribute less, there is a known EET binding site on TRPV4 and all the EETs contribute to relaxation response in arteries (Behm et al., 2009).

Indomethacin treatment and TxA\textsubscript{2} and PGH\textsubscript{2} receptor antagonism abolished the flow-induced increase in contractile activity (Koller et al., 1999). These two results indicate that constrictor prostanoids may contribute to the contractile responses observed in lymphatic vessels. In our case, lymphatic vessels may release more contractile prostaglandins at baseline conditions, and Indomethacin treatment may be more important to the production of contractile prostanoids than relaxation ones. Though the NO and prostaglandin-dependent pathways are the main focus of this study, histamine also contributes to the relaxation response in mesenteric lymphatics (Nizamutdinova et al., 2014). Nizamutdinova and colleagues found that inhibition of eNOS or histamine production alone did not completely inhibit the flow-induced relaxation of lymphatic vessels. It was only with dual inhibition that complete ablation of the flow-dependent relaxation response occurred. They also found that histidine decarboxylase was present in endothelial cells and that it resulted in the production of histamine that could act as an endothelium-derived relaxation factor (EDRF) (Nizamutdinova et al., 2014). These results
indicate that the response we observed may be due to compensatory mechanisms, and the role of histamine within our system remains to be determined.

5.4. Pressure Myography and TRPV4 Agonism

The selective agonist of TRPV4, GSK1016790A (GSK101), is commonly used to activate TRPV4. GSK101 treatment results in an increase in Ca\(^{2+}\) entry and TRPV4 trafficking to the cell surface (Baratchi et al., 2019). Stimulations also lead to increases in NO production within freshly isolated rabbit mesenteric arterial endothelial cells (Greenberg et al., 2017). Within mouse, rat and rabbit arteries, TRPV4 stimulation with GSK101 resulted in a vasodilation response (Mendoza et al., 2010; Bagher et al., 2012; Sonkusare et al., 2012, Greenberg et al., 2017; Darby et al., 2018). When arteries were pre-treated with the selective antagonist HC067047, a significant attenuation of the relaxation responses occurred (Bagher et al., 2012; Sonkusare et al., 2012; Greenberg et al., 2017; Darby et al., 2018). TRPV4 stimulation in lymphatic vessels increases cytosolic Ca\(^{2+}\) concentrations causing membrane depolarization (Behringer et al., 2017). The lack of electrical coupling between the endothelium and smooth muscle layers allows for different resting potentials and responses to occur in either cell layer (von der Weid & Van Helden 1996; Behringer et al., 2017). Thus, even though a depolarization occurred in the endothelium, it is not transmitted to the smooth muscle via an electrical connection, preventing a contraction from occurring (Behringer et al., 2017). Endothelium derived relaxing factors such as NO, EETs and prostaglandins and contractile factors (thromboxanes) can act on the smooth muscle layer to regulate the membrane potential.
5.4.1. TRPV4 Agonism of Whole Lymphatic Vessels

Our results indicate that TRPV4 expression occurs on both the endothelium and the smooth muscle layers of lymphatic vessels. High levels of TRPV4 expression occur within the endothelial cells of the vascular system and the smooth muscle of mesenteric, pulmonary and cerebral arteries within mice, rats and rabbits (Mendoza et al., 2010; Bagher et al., 2012; Greenberg et al., 2017; Sukumaran et al., 2013; Xia et al., 2013; Marrelli et al., 2007). These cells may exhibit different responses upon TRPV4 stimulation. As previously stated, TRPV4 stimulation on the endothelium results in a concentration-dependent relaxation within pulmonary arteries (Sukumaran et al., 2013). Endothelium removal resulted in the ablation of this response in precontracted arteries. In isolated rat pulmonary arteries, TRPV4 stimulation of whole vessels resulted in a decrease in tone, while stimulation of endothelium-denuded vessels resulted in a significant increase in tone. The increases in tone were significantly more in the endothelium-denuded vessels by 30 nM of GSK101 (Sukumaran et al., 2013). These results indicate that TRPV4 can mediate Ca\(^{2+}\) entry on smooth muscle cells, increasing tone.

In our study stimulation of TRPV4 in intact vessels resulted in a biphasic response. There was a decrease in the contraction frequency at low concentrations of GSK101 (0.1 - 3 nM), while at concentrations of 30 nM and higher, an increase in contraction frequency occurred. The maximal frequency observed in GSK101 (control) treated vessels occurred at 300 nM with the frequency not exceeding double the baseline contraction frequency. Interestingly these vessels showed the smallest decrease in contraction amplitude and
ejection fraction. This may be due to the stimulation of TRPV4 on the endothelium. Stimulation of endothelial TRPV4 can result in a relaxation response within arteries, decreasing the tone (Sukumaran et al., 2013). The decrease in tone may then contribute to the prevention of the decrease in contraction amplitude and ejection fraction. This may be due to the relaxation response facilitated by the endothelium preventing over activation of the smooth muscle layer. This biphasic response may depend on the differential expression and sensitivity of TRPV4 on the smooth muscle and endothelium. If TRPV4 expression occurs at higher levels in the endothelium, activation may occur at lower concentrations than smooth muscle. Similarly, greater trafficking of TRPV4 channels within endothelial cells may also result in the increased sensitivity in endothelial cells compared to smooth muscle cells. Activating the endothelium significantly more could result in the triggering of downstream relaxation through eNOS or COX dependent mechanisms. The downstream factors could diffuse to the smooth muscle and result in a relaxation leading to a decrease in contractile activity. As the concentration increased, TRPV4 on the smooth muscle was triggered, resulting in smooth muscle depolarization and contraction. High concentrations of GSK101 eliciting the smooth muscle’s depolarization would overpower any relaxation response due to the endothelial TRPV4. The increased stimulation of the smooth muscle contractile response could also account for the decrease in contraction amplitude observed at high concentrations of GSK101. As the GSK101 concentration increased, the amplitude of contractions decreased. This decrease was due to an increase in the systolic diameter with small decreases in the diastolic diameter for the duration of the experiment except in the 3 µM HC treated group, which showed a large decrease in the diastolic diameter. These results indicate a constant stimulation of the lymphatic smooth muscle cells resulting in a
fibrillation-like contraction. The contractions decrease in amplitude and this is due to the inability to fully contract and expel the lymph present in the vessel. All of this resulted in less efficient active movement of the lymph due to the overactivation of the smooth muscle layer. These results indicate that TRPV4 function contributes to lymphatic vessel contraction.

Many studies on TRPV4 activation within endothelial cells, arteries and arterioles have taken place. Concentrations of 100 nM GSK101 and higher resulted in endothelium damage of both cremaster arterioles and mesenteric arteries. The damage resulted in the loss of local control of Ca$^{2+}$ influxes, and an increase in global Ca$^{2+}$ currents (Bagher et al., 2012). The loss of local Ca$^{2+}$ dynamics control may cause the consistent decrease in contraction frequency observed at a concentration of 1 µM of GSK101 in our experiments. The damage caused by this concentration could result in irreparable cell damage and possibly death accounting for observation of lower contraction frequencies than at 100 or 300 nM GSK101 (Figures 37A-D and Figure 38D). Upon washout of GSK101 the vessels showed varying responses with contraction frequency decreasing in part but never re-establishing the frequency before the ramp. GSK101 has an EC$_{50}$ of 2.1 nM for humans and 18 nM for mice, indicating that nanomolar concentrations are enough to observe a response (Thorneloe et al., 2008). In cell cultures, the use of 10 nM GSK101 to stimulate TRPV4 is standard, while in arteries, concentrations range from 0.1 - 100 nM (Jin et al., 2011; Bagher et al., 2012; Darby et al., 2018; Sukumaran et al., 2013). In pulmonary arteries and cremaster arterioles, the maximal relaxation response to GSK101 occurred by a concentration of 30 nM with a plateau in relaxation response observed at higher concentrations (Sukumaran et al., 2013; Darby et al., 2018). The GSK101-induced
relaxation response within the blood vasculature occurred in an endothelium-dependent manner with NO production and EDHF being the major pathways within blood vessels (Sukumaran et al., 2013; Mendoza et al., 2010). To determine how TRPV4 acts on the endothelium, the role of the NO-dependent and prostaglandin-dependent relaxation pathways were investigated using the selective inhibitors L-NNA and Indomethacin.

5.4.2. Endothelium-dependent TRPV4 Agonism in Lymphatic Vessels

Our study used three different inhibitory treatments to demonstrate the action of crucial endothelium-dependent relaxation pathways. Treatment with 100 µM L-NNA targeted eNOS activity, 10 µM Indomethacin inhibited COX, and a combination of both treatments acted on both pathways. L-NNA treatment did not ablate the decrease in contraction frequency at low GSK101 concentrations ranging from 0.1 - 3 nM but did decrease the maximal relaxation observed at 1 nM GSK101, though this was not significant. These vessels exhibited a higher peak contraction frequency at concentrations of 100 and 300 nM GSK101, though this was not significant. Based on these results the NO-dependent relaxation pathway does not seem to be significantly contributing to lymphatic vessel modulation via TRPV4 stimulation with GSK101. Indomethacin treatment prevented the decrease in contraction frequency at low GSK101 concentrations ranging from 0.1 to 1 nM, though none of the changes were significant. These vessels showed a peak contraction frequency similar to control vessels. These results indicate that TRPV4 is likely not modulating lymphatic vessel contractility via the prostaglandin-dependent pathway. Dual treatment had an additive effect with the ablation of the decrease in
contraction frequency at low concentrations and an increase in frequency similar to L-NNA treated vessels. Interestingly by inhibiting both pathways all relaxation responses were inhibited making proposing the possibility that there are no other relaxation pathways involved, though this needs to be further elucidated.

In mouse pulmonary arteries, 100 nM of GSK101 resulted in a significant increase in endothelial cell cytosolic Ca\(^{2+}\) and vasodilation response. The addition of L-NAME to inhibit eNOS activity resulted in a significant decrease in vasodilation in response to 100 nM GSK101 (Mendoza et al., 2010). In GSK101 concentration-response curves done on rat pulmonary arteries, a concentration-dependent relaxation occurred. Inhibition of eNOS with 100 µM L-NAME or soluble guanylate cyclase (sGC) with 10 µM ODQ prevented the relaxation. In contrast COX-1 inhibition with Indomethacin did not inhibit the relaxation response (Sukumaran et al., 2013). Lymphatic vessels seem to be behaving differently from arteries within our study. Nitric oxide production does not seem to contribute to the observed relaxation in response to TRPV4 stimulation. Neither do dilatory prostaglandins such as PGE\(_2\) and PGI\(_2\). Both of these prostaglandins are known to contribute to a decrease in contraction frequency under baseline conditions at a constant transmural pressure (Rehal et al., 2009). Contractile prostaglandins have also been shown to contribute to contraction frequency resulting in an increase in response to flow (Mizuno et al., 1999, Koller et al., 1998). Based on these results along with ours it is reasonable to expect that there is a complex interplay between both the NO-dependent and prostaglandin-dependent relaxation pathways. These pathways do not act solely distinct from one another but rather have the ability to interact and modulate one another leading to compensatory actions by each pathway (Pérez-Cremades et al., 2017). Though both these pathways seem to contribute to
the relaxation response observed they may not be the only players in the complex process of regulating lymphatic vessel contractility.

Arachidonic acid (AA) is synthesized from the cleavage of membrane phospholipids by PLA₂ within cerebral arteries and numerous other cell types (Marrelli et al., 2007, as reviewed in Leslie, 2016). Prostaglandins and thromboxane are not the only AA metabolites found in the body. AA can also be cleaved with lipoxygenases and by the cytochrome P450 (Cyp450) enzymes to form vasoactive compounds. There may be contributions by epoxycosatrienoic acids (EETs) that are produced by Cyp450 from arachidonic acid, making this pathway COX-independent (Romashko et al., 2018). Though it is COX-independent, 5, 6-EETs are metabolized by COX-1 to produce another vasoactive EET derivative (Carroll et al., 1992). Interestingly, EETs can act on TRPV4, allowing for calcium entry. It would be important in the future to determine the impact of EETs in TRPV4-dependent regulation of lymphatic vessel contractility. There may be some overlap between the COX-dependent and Cyp450-dependent pathways and targeting one pathway may result in a compensatory mechanism being triggered in the other. Since both pathways are dependent on phospholipid cleavage and the formation of AA, inhibition of one pathway may result in an increase in AA availability triggering the other pathway to a greater extent.

In lymphatics the classical EDHF response is not present as it is in arteries (von der Weid & Van Helden, 1997). There can still be contributions from the non-classical EDHF response through the release of hyperpolarizing factors, such as those that act on the BKCa channels within arteries (Edwards et al., 1998; Edwards et al., 2010). EETs can diffuse to
the smooth muscle as a hyperpolarizing factor resulting in the activation of the BK$_{Ca}$ channel on smooth muscle cells (Darby et al., 2016; Earley et al., 2005). In guinea pig lymphatic vessels, the BK$_{Ca}$ channels do not act as they do in arteries, rather it seems that K$_{ATP}$ channels fulfill the role of membrane hyperpolarization (von der Weid, 1998). The exact role of BK$_{Ca}$ channels within rat lymphatics remain undefined and may possibly be contributing to the non-classical EDHF response. Thus, in our system it would be important to determine if EETs are acting directly on K$_{ATP}$ or on BK$_{Ca}$ channels to induce smooth muscle hyperpolarization. The results obtained in our study indicate that the NO-dependent relaxation pathway and the prostaglandin-dependent pathway do not significantly contribute to the relaxation of lymphatic vessels in response to TRPV4 stimulation. There also seems to be a contribution by any other pathways involved though this remains to be elucidated.

5.4.3. TRPV4 Agonism When Inhibited with HC067047

Having established the role of TRPV4 in endothelium-dependent relaxation and the contributions of both eNOS and COX-1, we wanted to selectively inhibit TRPV4 function and determine its impact on lymphatic contractility. The inhibition of TRPV4 occurred through the use of the selective inhibitor HC067047 (HC). Vessels were treated with HC alone, HC and L-NNA, HC and Indomethacin, and HC, L-NNA, and Indomethacin. Administration of 3 µM HC resulted in a rightward shift of the GSK101 concentration-response curve, suggesting inhibition of TRPV4 activity. As stated in the introduction HC is a relatively selective TRPV4 inhibitor with sub micromolar IC$_{50}$’s on only the TRPM8 and
hERG channels and no know targets downstream of TRPV4 (Everaerts et al., 2014). It prevented the decrease in contraction frequency until 10 nM GSK101 and the increase in frequency until a concentration of 100 nM of GSK101 compared to an increase at 30 nM when only the endothelium function was inhibited with L-NNA and Indomethacin. Interestingly, there was also an increase in the maximal contraction frequency reached by the lymphatic vessels. This may be due to the triggering contractile prostaglandins or an inability for the smooth muscle to be modulated by endothelial TRPV4. GSK101 does impact the trafficking of TRPV4 to the surface within endothelial cells, but the impact on TRPV4 trafficking within smooth muscle cells is unknown (Baratchi et al., 2019). The fact that endothelial function is impaired at concentrations of GSK101 100 nM or higher may also contribute to the inability to control or inhibit the contraction frequency. Lastly, there may be a toxic effect of GSK101 at concentrations above 100 nM. This may lead to the loss of Ca\(^{2+}\) regulation and possibly death of endothelial cells and possibly smooth muscle cells and this may result in a lack of control of contractile activity, resulting in a high contraction frequency observed. The results obtained seems to indicate that there might be a differential sensitivity to TRPV4 agonism in endothelial and smooth muscle cells with endothelial cells being more sensitive to TRPV4 stimulation though the mechanisms remain to be further elucidated.

There was a further rightward shift in vessels treated with a combination of 3 µM HC and 100 µM L-NNA, indicating an additive effect of NO synthesis inhibition. The dual inhibition prevented the decrease in frequency until 10 nM with the maximal decrease at 30 nM being less than half the maximal decrease observed in control vessels. These results were unexpected and may indicate the triggering of other off-target effects or that the
concentration of HC used was insufficient to inhibit TRPV4 activity completely when treated with GSK101. The latter situation seems unlikely since, in previous reports, concentrations of 1 and 3 µM HC were found to mostly inhibit TRPV4 activity (Everaerts et al., 2010; Baratchi et al., 2019). It is possible that by inhibiting TRPV4 function with HC off-target or non-specific effects of GSK101 have been unmasked, though there is no mention of this in previously published literature. It would be important to also test another TRPV4 inhibitor, such as RN1734, since this would allow us to determine if these effects were HC specific or not. These vessels attained a maximal contraction frequency similar to the control treated vessels and about half of the 3 µM HC treated vessels at 1 µM GSK101. Based on these results TRPV4 seems to not only be acting in a NO-dependent manner but is rather contributing to multiple pathways. In vessels treated with HC and Indomethacin the contraction frequency was depressed below baseline values until 300 nM GSK101. This is strongly influenced by a vessel that showed no response to GSK101 treatment, though all three vessels were prone to decreases in frequency. This, once again, may be due to non-specific actions of GSK101 that are being unmasked, though this would have to be tested. The increase in contraction frequency occurred at 300 nM indicating a rightward shift that was similar in magnitude to the HC and L-NNA treated vessels. Interestingly the maximal frequency never reached control vessel frequency, indicating that TRPV4 agonism may be acting in a contractile prostaglandin dependent manner. Based on the decrease in frequency and lower peak observed it seems that both contractile and dilatory prostaglandins may be acting in response to TRPV4 stimulation, but this would need further elucidation. In the vessels treated with all three inhibitors, HC, L-NNA, and Indomethacin, there was a steady decrease in contraction frequency until 100 nM GSK101. This was unexpected and may be
due to the triggering of more EET synthesis resulting in relaxation (Vriens et al., 2005). It could also possibly be due to the role that TRPV4 plays on the smooth muscle contraction since it does allow for the entry of Ca\(^{2+}\), that is required for contractions. At concentrations of 300 nM and 1 µM GSK101, there were increases in contraction frequency, though they did not significantly exceed the control vessels' frequency and showed a similar profile to the HC and L-NNA treated vessels.

In previous work, 1 µM HC inhibited Ca\(^{2+}\) entry into endothelial cells by 93% when treated with 10 nM GSK101 (Sonkusare et al., 2013). In a concentration-response curve done on cremaster arterioles, 300 nM of HC resulted in the inhibition of relaxation in response to GSK101 until a concentration of 300 nM GSK101 (Darby et al., 2018), while in HUVECs, both 300 nM and 1 µM HC inhibited calcium entry in response to GSK101 until a concentration of 300 nM (Baratchi et al., 2019). Based on these observations, the rightward shift in our concentration-response curve fits with previously established data. In conclusion, our results indicate that TRPV4 stimulation results in the activation of the COX-dependent and eNOS-dependent pathways. There also seems to be another pathway that contributes to TRPV4 activation. This pathway may consist of the Cyp450 enzyme-dependent synthesis of EETs. TRPV4 has a known EET binding domain, and 5, 6-EET binds directly to TRPV4 (Vriens et al., 2005; Berna-Erro et al., 2017). Inhibiting Cyp4502C9 specifically and EET synthesis with sulfaphenazole and 14, 15-EEZE, respectively, would be important in determining EETs contribution to GSK101 induced activation of TRPV4. EET actions within blood vessels have been elucidated, but they remain unknown within the lymphatic system. Our experiments also allowed us to determine that 3 µM HC was a well-suited concentration to use for the flow ramp experiments. Based on these results, TRPV4
activation by GSK101 occurs on both the smooth muscle and the endothelium, with HC selectively inhibiting this response. Both COX-1 and eNOS activity seems to contribute along with another pathway, possibly EETs.

5.5. Rat Collecting Lymphatic Endothelial Cells (LEC) Gene Expression

Rat duodenal collector lymphatic endothelial cells (LECs) showed constant expression of classical endothelial cell markers during the passages three through six. We observed substantial Prox1, VEGFR3, and Foxc2 expression with low expression of LYVE-1 and no expression of CCL21. TRPV4 expression occurred consistently throughout passages of the endothelial cells. Prox1 expression, commonly used to differentiate between blood and lymphatic endothelial cells, was consistently observed with nuclear and perinuclear localization. Prox1 also plays a master transcriptional role in lymphatic development with its presence, indicating that the cells are indeed lymphatic endothelial cells (LECs) (Srinivasan et al., 2007). VEGFR3 is the receptor for VEGF-C and is integral to the development of the lymphatic system (Karkkainen et al., 2004). The expression within our LECs occurs on the cell surface and is consistent with the expected profile (Karkkainen et al., 2004). Foxc2 is a transcription factor expressed by lymphatic endothelial cells and essential in forming functional valves (Petrova et al., 2004). Mutations result in lymphedema, and loss of function results in a lack of lymphatic and venous valves (Sabine & Petrova, 2014; Petrova et al., 2004). LYVE-1 expression at low levels is consistent with the fact that it is primarily an initial lymphatic marker (Aspelund et al., 2016). The presence of LYVE-1 staining may also be due to the “unmasking” of the receptor since hyaluronic
acid usually binds it. The lack of CCL21 expression in these LECs indicates that they are most likely collecting lymphatic endothelial cells since CCL21 is expressed at high levels in initial lymphatic endothelial cells to aid in dendritic cell entry (Aspelund et al., 2016; Johnson et al., 2017). TRPV4 expression throughout all passages indicates that these cells are consistent with previous studies on TRPV4 in lymphatics (Behringer et al., 2017). The use of low passage numbers and the consistent expression of TRPV4 allow for confidence that these cells can be used to elucidate further TRPV4's function on a molecular level within lymphatic endothelial cells. Another important experiment would be the characterization of the lymphatic smooth muscle cells (LSMCs) and determining TRPV4 expression within these cells at the protein level.

5.6. Limitations

This section of the manuscript will address the limitations of this study and the experiments required to address these issues. I will be addressing issues regarding the use of TRPV4 antibodies and pressure myography.

5.6.1. TRPV4 Antibody

TRPV4 protein expression was determined using a rabbit polyclonal antibody raised against a mouse-derived peptide of TRPV4 that was also reactive with rat and horse TRPV4. This antibody's polyclonal nature increases the chance that it will be successful for western
blot and immunofluorescent analysis. The TRPV4 channel structure is different in these two scenarios, and the difference in 3D structure may impact TRPV4 binding between assays. The disadvantage of using antibodies like this is the possibility of non-specific binding. Non-specific binding of antibodies makes the presence of the proper negative controls integral to the interpretation of results. The lack of negative control, such as tissue from a TRPV4 deficient mouse, makes it difficult to determine if TRPV4 binding is specific. When using mouse tissue, the same tissue should also be used from a wild-type mouse to ensure that the mice and rats' results are the same. Even with positive and negative controls, an IgG control would account for non-specific staining or bands on the blot.

The lack of certainty that the antibody is specific to TRPV4 raises questions about the immunofluorescent images' significance. Non-specific binding of secondary antibodies could be excluded. Still, the non-specific binding of primary antibodies was not confirmed and is dependent on the results obtained in the western blot and using tissue samples that are TRPV4 deficient. Accounting for these limitations restricts the assertion that TRPV4 protein expression occurs in both cell layers. Incorporating the previously mentioned controls will go a long way in confirming the presence of TRPV4 at the protein level within these vessels.

5.6.2. Pressure Myography

Using isolated lymphatic vessels has its benefits, as previously mentioned. Pressure myography allows us to determine the impact of different drug treatments, transmural
pressures, and flow on lymphatic vessel contractions. With that said, there are limitations to this method.

The limitations include observing a phenotype in response to treatment without understanding the underlying mechanism, the lack of cells surrounding the vessel, and the lack of dynamic, physiological conditions. Molecular methods such as calcium imaging and nitric oxide imaging need to be used on both endothelial cells and isolated lymphatic vessels to determine action mechanisms. Without these experiments, it is impossible to claim that the inhibitors used are functioning as advertised and that the results are dependent on these molecular pathways. These are included in the future directions portion of the document and are required to complete the study. The second and third issues stem from the rat lymphatic vessel's isolation from the animal and the surrounding tissue. Supplementing these experiments with intravital imaging would be indispensable to determining what impact TRPV4 agonism or antagonism has on lymphatic function in dynamic, physiological conditions. The results obtained from intravital imaging would allow us to construct a more precise role for TRPV4 in lymphatic vessels. Addressing these limitations entirely or in part would be very important to strengthening this study, leading to conclusive results.

5.7. Future Directions

This investigation’s results indicate that TRPV4 contributes an essential role in the flow-induced decrease in contractile activity. TRPV4 protein expression occurs in both the
endothelium and the smooth muscle, but the molecular manner in which it functions requires further inquiry.

Our study established that TRPV4 contributes to the endothelium-dependent relaxation within mesenteric lymphatic vessels. To further uncover the specific manner in which TRPV4 functions in the endothelium, changes in the cytosolic Ca\(^{2+}\) and NO concentrations need to be determined. The characterized rat duodenal and ileal lymphatic endothelial cells (LECs) would be used during early passages, while lymphatic specific markers and TRPV4 expression remain constant. First, the impact of TRPV4 activation with GSK on changes in cytosolic Ca\(^{2+}\) concentrations would be determined using a fluorescent dye such as Fluo-4. We would expect that TRPV4 agonism would increase cytosolic Ca\(^{2+}\) in these cells, as shown in HUVECs and transfected HEK293 cells (Baratchi et al., 2019). Establishing that TRPV4 activation does result in increases in cytosolic Ca\(^{2+}\), the role of TRPV4 during conditions of flow would be determined. The use of Ibidi flow chambers allows for specific manipulation of the amount of flow LECs experience. In previous experiments using human dermal lymphatic endothelial cells (HDLECs), shear stress in the range of 0.1 – 10 dynes/cm\(^2\) was used, and we would use similar values. Determination of the changes in cytosolic Ca\(^{2+}\) in response to flow would occur, and the impact of TRPV4 inhibition on this response would be determined. Cytosolic Ca\(^{2+}\) activates eNOS activity within endothelial cells producing NO. Due to this, NO production in response to TRPV4 activation and flow would be determined. By using DAF-FM imaging, the determination of changes in intracellular NO concentration in response to TRPV4 activation can occur. Determining the role TRPV4 plays in NO production will allow for a more accurate picture of the position of TRPV4 on the pathway. The difficulty with using DAF-FM dye is that it
requires oxidizing conditions to work. In the absence of oxygen, there is no observed increase in fluorescence in response to NO production. Other dyes such as DAN-1-EE and DAR-1 or 2 can be used instead, but as a general rule, DAF-FM is used to determine the changes in NO concentrations within cells (Cortese-Krott et al., 2012). If live imaging seems unfeasible with DAF-FM, cell treatments with TRPV4 agonist and other drugs could be conducted, and the total NO produced measured. Another possible method would consist of the use of a NO-sensing probe. The only draw-back of this method is that there is an initial decrease in NO production detected under conditions of flow due to the removal of NO from the local environment (Andrews et al., 2010).

Using Fura-red and DAF-FM concurrently would allow us to visualize the dynamic changes in intracellular Ca\(^{2+}\) and NO production at the same time. Using this the temporal changes in both concentrations could be determined. Both primary cells and cultured cells can be used for this method with primary cells having the advantage of being phenotypically similar to \textit{in vivo} and the cultured cells forming a confluent monolayer. Endothelial cells become very flat in culture and are not the same as under physiological conditions (Baratchi et al., 2017). An interesting thing to do in this case would be to isolate endothelial tubes from the lymphatic vessels and then image the changes in Ca\(^{2+}\) or NO when these tubes are exposed to flow. Another possible variation of these experiments consists of using mice popliteal vessels instead of rat mesenteric vessels. The reason being the availability of transgenic mouse models that allow for the incorporation of Ca\(^{2+}\) sensitive dyes within the cells or the deletion of TRPV4 activity. Using these methods, the dynamic changes in Ca\(^{2+}\) and NO occurring in a TRPV4-dependent manner in response to flow could be determined.
TRPV4 can interact with TRPC1 in the blood vasculature and form a flow-sensitive ion channel in the endothelial layer (Ma et al., 2010; Greenberg et al., 2017). It can also form a complex with both TRPC1 and the BK$_{Ca}$ channel (K$_{Ca}$.1.1) on the smooth muscle layer to form a channel that aids in hyperpolarization (Ma et al., 2014). However, the role of TRPC1 and TRPV4 heterotetramer formation within lymphatics remains undetermined. We have shown that TRPC1 gene expression can be found in whole mesenteric lymphatic vessels, but do not know if TRPC1 protein expression colocalizes with TRPV4.

Determination of TRPC1 protein expression and location within lymphatic vessels would happen through immunofluorescence. The resolution of TRPV4 and TRPC1 co-expression would occur through proximity ligation assays and immunohistochemistry. Immunofluorescence would allow for an easy determination of co-expression, while the proximity ligation assay would support the findings. In previous work showing co-expression of TRPV4 and TRPC1 proximity ligation assays only worked when the two channels were within 50 nanometers of one another, providing strong support for co-expression of these channels (Greenberg et al., 2017). Proximity ligation assays start out similarly to immunofluorescence with the binding of primary antibodies. These primary antibodies are labeled with secondary antibodies labelled with oligonucleotides that are joined to one another with connector oligonucleotides to form a complete circle with ligase. The oligonucleotide act as a primer for the polymerase added leading to rolling circle amplification and the formation of a concatemer. Lastly fluorescently labelled oligonucleotides bind to the DNA sequence allowing for the visualization of these complexes using fluorescent microscopy (Greenberg et al., 2017). If the channels did form heterotetramers, the use of pressure myography experiments would determine the functional
relevance. The application of the TRPC1-specific antibody during flow would allow for the determination of its role in the lymphatic endothelium. A concentration-response curve conducted with the TRPV4 agonist GSK in the presence of TRPC1 antagonism would further support heterotetramer formation. Both changes in cytosolic Ca\textsuperscript{2+} and cytosolic NO production experiments, conducted as stated previously, would allow for the determination of the molecular pathway in which the heterotetramers act.

In HUVECs, TRPV4 trafficking occurs in an α5β1 integrin-dependent manner (Baratchi et al., 2017). HUVEC treatment with GSK results in an increase in TRPV4 recruitment to the cell surface, and flow acts in the same manner (Baratchi et al., 2019; Baratchi et al., 2017). The problem with the flow experiments on HUVECs within the culture is that the cells form a very flat surface and no longer have lateral portions. The cell layer is thinner than in vivo, and the cells only contain basal, apical and basolateral portions due to their flat morphology (Baratchi et al., 2017). The difference in cell shape and structure from physiological conditions make it difficult to determine if the same would occur within blood or lymphatic vessels. Determining what controls TRPV4 trafficking within a more physiological environment such as arteries or lymphatic vessels has not been done. Targeting the α5β1 integrin function within lymphatic vessels will allow us to determine if TRPV4 is acting as a shear stress sensor itself or acting downstream of a shear stress sensor. Inhibiting the α5β1 integrin function before treating a lymphatic vessel with a flow ramp would clarify how TRPV4 activation occurs within lymphatic vessels. To understand the molecular mechanism, LECs could be used to determine the impact of α5β1 integrin function on the production of cytosolic Ca\textsuperscript{2+} and NO.
The role of epoxyeicosatrienoic acids (EETs) in endothelium-dependent relaxation under conditions of flow would be determined. There are four EET regioisomers, 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET. All four are vasoactive compounds, contributing to relaxation responses within the vasculature (Behm et al., 2009). These compounds are metabolized to their less vasoactive compounds primarily by soluble epoxide hydrolases (sEH), though 5,6-EET can be metabolized by COX-1 to its more vasoactive form (Vriens et al., 2005; Carroll et al., 1992). 5,6-EET has a known binding site on TRPV4 that contributes to channel opening and stabilization during pore opening (Berna-Erro et al., 2017). Thus, it would be imperative to determine the role of EETs in response to the flow-induced decrease in contraction frequency and TRPV4 stimulation. EET function may explain the decreased efficacy in inhibiting flow-dependent decreases in contraction when both 100 µM L-NNA and 10 µM Indomethacin are used. For these experiments, selective EET antagonists such as 14,15-EE-(Z)-E would be used to prevent EET production in response to shear stress. These experiments would be conducted as previously done using pressure myography. While focusing on the EET function, the gene and protein expression levels of specific CYP450 enzymes would be targeted. CYP2C8 and CYP2J2 enzymes are essential in the synthesis of the EETs within the vascular system (Lee et al., 2010). Determining the contributions of these enzymes to EET production within lymphatic vessels will lead to a better understanding of the similarities and differences between lymphatic vessels and arteries.

Lastly, understanding the differences between lymphatic beds is essential. In mice, visceral and peripheral lymphatics have different contractile properties dependent on L-type channel function to some extent (Zawieja et al., 2018). Due to the variable expression in L-type channels and the impact on the cytosolic Ca²⁺ concentrations, the hypothesis that
TRPV4 expression differs between lymphatic beds is reasonable. Understanding the differential expression and function of TRPV4 in different beds would allow for a more accurate understanding of the role of TRPV4 and the ramifications of improper function. To conduct this study, the use of mice would be beneficial due to the existence of a TRPV4\(^{-/-}\) mouse line. The TRPV4\(^{-/-}\) mouse line would allow us to determine if there were any compensatory mechanisms for the flow response in lymphatics and what they might be. In rats, different lymphatic vessel beds show differing responses to flow (Gashev et al., 2004). TRPV4 expression may be contributing to this and elucidating its function in each bed would be valuable.

5.8. Conclusions

The current study focused on the role of TRPV4 in flow-induced changes in contraction frequency of rat mesenteric collecting lymphatic vessels. Our results indicate that TRPV4 gene expression occurs within whole lymphatic vessels, and protein expression occurs in both the endothelial and smooth muscle cells. Both eNOS and COX-1 activity contribute to the endothelium-dependent relaxation that occurs in response to flow. TRPV4 inhibition results in the ablation of the flow-dependent decrease in contractile activity in a similar manner to eNOS inhibition. 1 \(\mu\)M HC067047 does not entirely inhibit TRPV4 activity, while 3 \(\mu\)M does significantly inhibit TRPV4 within lymphatic vessels. Lastly, the stable expression of TRPV4 occurs in primary cell cultures from duodenal lymphatic endothelial cells. These cells show a constant expression of common lymphatic specific markers indicating that they will be valuable tools in the study of TRPV4 function within the
lymphatic endothelium. There also seems to be a third major relaxation pathway that is involved in the flow-dependent decrease in contraction frequency. The pathway may involve EETs since they bind TRPV4 directly and are involved in the EDHF-dependent relaxation response in arteries. This study has furthered our knowledge and understanding of the role TRPV4 plays under conditions of flow. Understanding its role will allow for better-targeted therapies to alleviate the symptoms of those who live with various lymphatic dysfunctions.
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7. Appendices

Appendix A: RT-qPCR analysis of TRP channel and Calcium-activated chloride channel expression within rat mesenteric lymphatics, and TRPV4 expression in varying tissues. (A) Gene expression of flow-sensitive TRP channels TRPV4, TRPC1, and TRPV1, mechanosensitive TRP channels TRPM4 and TRPC6, and Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels ANO1 and BEST1. Relative expression determined in comparison to the housekeeping genes HPRT1 and GAPDH for an n = 7. (B) TRPV4 is expressed in mesenteric lymphatic vessels, the kidney, and LECs, but not in LSMCs grown in culture, possibly due to the formation of a synthetic phenotype and the loss of the contractile phenotype.
Appendix B: TRPV4 staining negative controls. The primary antibodies result in minimal off target staining and show relatively high specificity for TRPV4. All images are at 63X magnification and 1.3x zoom. (A) whole mount image of rat mesenteric lymphatic vessel showing minimal non-specific staining, using only secondary antibodies for TRPV4 and VEGFR3. The (B) fully stained collecting lymphatic vessel shows robust VEGFR3 expression on the endothelium in blue and TRPV4 expression throughout the vessel in green. (C) Zoomed in section of the secondary antibody control group showing no non-specific binding. (D) Zoomed in section of the fully stained collecting lymphatic vessel showing expression of both VEGFR3 and TRPV4. For an n = 3.
Appendix C: The addition of 3 µM HC had no significant impact on any of the contraction parameters of rat mesenteric lymphatic vessels. The (A) contraction frequency showed a small, insignificant decrease, while the (B) amplitude of contractions showed an insignificant increase. The (C) diastolic diameter was unaffected, (D) systolic diameter decreased slightly, and
the (E) ejection fraction increased similarly to the (B) amplitude of contractions. The FPF showed no changes whatsoever. Significant differences were determined using a one way ANOVA with Tukey’s post hoc tests for an $n = 4$. 
Appendix D: Concentration-response curves constructed to determine the EC50 values for each treatment. Inhibition of eNOS, COX enzymes or TRPV4 function resulted in rightward shifts in the EC50 values compared to the control vessels that only received GSK101 stimulation. The EC50 values for the control treated, 100 µM L-NNA treated, 10 µM Indomethacin treated and 100 µM L-NNA and 10 µM Indomethacin treated groups were $2.1 \times 10^{-8}$, $1.4 \times 10^{-8}$, $2.3 \times 10^{-8}$ and $2.1 \times 10^{-8}$, respectively. The EC50 values for the 3 µM HC treated, 3 µM HC and 100 µM L-NNA treated, 3 µM HC and 10 µM Indomethacin treated and triple treated groups were $1.0 \times 10^{-7}$, $2.3 \times 10^{-7}$, $1.1 \times 10^{-7}$ and $3.4 \times 10^{-7}$, respectively.