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Analysis of the barrier properties of the initial lymphatics and the toxicity of lymph fluid during inflammation

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Publication Date
2007

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Analysis of the Barrier Properties of the Initial Lymphatics
and the Toxicity of Lymph Fluid During Inflammation

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Bioengineering

by

Patrick Michael Lynch

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2007
The dissertation of Patrick Michael Lynch is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2007
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ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Geert Schmid-Schönbein. His advice and guidance was invaluable throughout my time at UCSD, and his interest in the lymphatic system inspired me to pursue the field.

My deepest gratitude goes to Frank DeLano. Not only is he a world-class researcher, he is also a wonderful mentor and even better friend. His knowledge of experimental techniques is unmatched. Without his guidance in the lab, I would never have been able to perform the experiments that I describe here. Frank and I spoke daily about my experiments and results, and he was always full of good suggestions of how to produce stronger results and improve my experimental techniques. In addition, I would be remiss if I didn’t mention that he is also an outstanding chef.

I would also like to thank my good friend Nick Scheidler. We have been working towards the same goal together for over five years, and being able to both defend our dissertations within a month of each other is very rewarding. Congratulations.

Many others in my lab have provided me with technical expertise and overall guidance in my project. I would like to especially thank Dr. Lee Murfee, Dr. Hainsworth Shin, Mr. Jeff Rappleye, and Dr. Alex Penn for their help and advice.

My parents have supported me and my educational pursuits since I began elementary school. The importance that they placed on education drove me to always do my best in school, and is culminating in the completion of this degree. Without them, I am not sure I would have had the ambition or the confidence to pursue this goal.

Four years into my graduate studies, I met Ms. Valerie Lumpkin, the woman that
I will marry in just a few months. Her love, support, and compassion have carried me the last year and a half. I can not thank her enough for putting up with me on a daily basis, especially towards the end of my studies. Val, you make me feel like the luckiest guy in the world. I love you.

Chapter 2, in part, has been accepted for publication of the material as it appears in Lymphatic Research and Biology, Lynch, Patrick M.; DeLano, Frank A.; Schmid-Schönbein, Geert W., Mary Ann Liebert Inc, 2007. The dissertation author was the primary investigator and author of this paper.
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Lynch PM; DeLano FA; Schmid-Schönbein GW. “The primary valves in the initial lymphatics during inflammation.” Lymphatic Research and Biology, 2007. (in press)
Inflammation has a profound effect on the vascular circulation, but little is known about its effect on the lymphatics. In the first half of this dissertation, I examine the effect of inflammation directly on the barrier properties of the initial lymphatics in the rat spinotrapezius muscle. I hypothesize that fluid that travels from the interstitium into the initial lymphatics must travel through the primary valves, a unidirectional valve
system that allows fluid to flow into the lymphatics, but not back out. I determine that a particle with diameter 0.5 \( \mu \text{m} \) is able to pass through the primary valves, but particles with diameter 0.8 \( \mu \text{m} \) and larger are unable to. Acute inflammation does not change this property, although it does allow fluid to escape back into the interstitium at a rate greater than that in healthy animals. This suggests that during inflammation the initial lymphatics are compromised and the primary valves do not function as a unidirectional valve system.

In addition, I hypothesize that the lymphatic system is responsible for removing toxic peptides and lipids from the interstitium to limit their damage to the tissue. By doing this, the lymphatic endothelium, lymphocytes and dendritic cells collected by the lymphatics, and cells in the lymph node are being exposed to these toxic mediators. I provide evidence that lymph collected from the rat mesenteric lymph duct is toxic both under healthy conditions as well as under inflammatory conditions.
Chapter 1 Introduction

1.1 Lymphatic Anatomy

The lymphatic system has two major roles in the body. The lymphatics are an organ in the immune system whose function is to transport lymphocytes and dendritic cells from the tissue and present them to antigens in the lymph node. The second function is a route by which fluid can be transported from the interstitium to the blood. This transport is especially important for proteins, lipids, colloids, and cells, most of which are unable to be absorbed by the blood capillary. Most of the fluid that filters out of the capillaries is reabsorbed back into the blood stream. About one tenth of this fluid enters the lymphatics. This represents about 2 to 3 liters per day in the average human (Guyton, 2000).

Lymph is formed in the initial lymphatics, transported into the contractile lymphatics, and returned to the venous circulation via the thoracic duct. The initial lymphatics consist of a highly attenuated endothelial lining and allow fluid and small molecules to be transported from the interstitium into the lymphatic lumen. Initial lymphatics do not have their own smooth muscle media, and therefore rely on the expansion and compression of the surrounding tissue to provide pressure gradients for fluid transport into and along the length of the lymphatics (Aukland et al, 1993; Swartz et al, 1999). The initial lymphatics feed into the contractile lymphatics, which do have their own smooth muscle media. This smooth muscle functions both as a vascular smooth muscle and as a pump. The pumping mechanism is essential for the propulsion
of lymph against the pressure gradient that is present in the lymphatics. Lymph enters
the lymphatics at a pressure close to zero, and exits the lymphatics at the higher pressure
in the thoracic duct. The contraction velocity of the lymphatic smooth muscle is similar
to that of the cardiac muscle (Benoit et al., 1989). Interestingly, the lymphatic muscle
cells express contractile proteins that are a combination of cardiac, smooth, and skeletal
muscle protein isoforms (Muthuchamy et al., 2003). The pumping activity of the initial
lymphatics will be further described in Section 1.4.

1.2 Current Lymphatic Research

In the past, the lymphatic circulation received much less attention from
researchers than the blood circulation. One reason for this has been the lack of
molecular markers to help identify the lymphatics. In 1995, the fms-like tyrosine kinase
4 (FLT4), also known as the vascular endothelial growth factor receptor 3 (VEGFR3),
was discovered to be restricted to lymphatic endothelium and some high endothelial
venules in adult tissues. This receptor binds to the ligands VEGF-C and VEGF–D.
In developing tissues, however, FLT4 can be found on venous endothelia (Kaipainen
et al., 1995). In 1999, three additional molecular markers specific to the lymphatic
endothelium were discovered. The first is LYVE-1, which is a homologue of the CD44
glycoprotein. LYVE-1 is a hyaluronan receptor that is present on the lymphatic vessel
wall, but mostly absent in blood vessels (Banerji et al., 1999). The second is podoplanin,
a glomerular podocyte membrane protein (Breiteneder-Geleff et al., 1999). In the kidney,
podoplanin is involved in maintaining lamellar permeability and the shape of podocyte
foot processes in the kidneys (Al-Rawi et al, 2005). Its function in the lymphatics is still unclear. A third molecular marker is the prospero related homeobox gene-1 (Prox-1). Prox-1 activity is necessary for the differentiation of lymphatic endothelium from venous endothelium. This differentiation begins in the wall of the anterior cardinal vein. Prox-1 -/- knockout mice develop no lymphatic system and are embryonic lethal (Wigle et al, 1999). The origin of the lymphatic endothelium demonstrated by Prox-1 validates Sabin’s proposal of the venous origin of primary lymph sacs over 100 years ago (Sabin, 1902).

Most current research centers around lymphangiogenesis and the lymphatics’ involvement in cancer and metastasis. Since the discovery of the molecular markers described above, the number of publications in these fields has increased dramatically. Lymphangiogenesis research is centered around the embryonic development of lymph vessels (Karkkainen et al, 2004; Morisada et al, 2005) and how that relates to diseases of the lymphatic system such as Milroy disease, which is a result of a mutation in FLT4 (Karkkainen et al, 2000), and lymphedema-distichiasis syndrome, which is a mutation in the FOXC2 transcription factor (Dagenais et al, 2004). More recently, investigators have been studying the development of lymphatics in the adult animal. One interesting set of reports from Dr. Melody Swartz’s laboratory has reported on the role of interstitial fluid flow as a regulator of lymphangiogenesis (Boardman et al, 2003; Swartz et al, 1999). Additionally, Dr. Lee Murfee and Mr. Jeff Rappleye in our lab have recently investigated lymphangiogenesis and lymphatic sprouting in the rat mesentery. They observed what appear to be direct connections between blood vessels and lymphatics, implying that there may be similar mechanisms for lymphatic and blood vessel growth and patterning.
In addition, they noticed lymphatic vessels in areas devoid of blood vessels, indicating that lymphatic vessels may be able to develop without the presence of or need to communicate with blood vessels (Murfee et al, 2007a).

The lymphatic system is the primary route of metastasis for most cancers (Swartz et al, 2001). Recent evidence has shown that certain types of cancer can release VEGF-C, which then promotes the growth of lymphatics in and around the tumor (Skobe et al, 2001). While the functionality of these lymphatics is still unclear (Isaka et al, 2004; Padera et al, 2002), their presence appears to provide an easy route for the spread of tumor cells to the lymph nodes and other organs.

1.3 Initial Lymphatic Ultrastructure

The endothelium of the initial lymphatics is very thin. In the skeletal muscle, the thickness is on the order of about 0.2 \( \mu \)m. These cells are attached to the surrounding extracellular matrix via anchoring filaments, which consist mostly of fibrillin (Solito et al, 1997). The basement membrane is not always present, and when it is, it is very discontinuous along the length of the initial lymphatics (Leak et al, 1966). The endothelium around the vessel is continuous, though the cross-sectional area is often very non-circular (Figure 1.1).

The junctions between endothelial cells are much less uniform in the lymphatics than in the blood vasculature. Blood vessel endothelial cells are nearly always connected to each other with adherens and tight junctions. The lymphatic endothelial cells, on the other hand, do are not always connected with these junctions (Murfee et al, 2007b).
Figure 1.1  Micrographs of semi-thin sections of rat spinotrapezius muscle counter-stained with toluidine blue. Panel A is a low power image of a large venule (V) and arteriole (A) with three separate lymphatic vessels near them (L). The non-circular shapes of these vessels are typical for the initial lymphatics in the muscle. Panel B is a high power image of the lymphatic vessel on the left side of A. Note the very thin endothelial lining and the endothelial nuclei that are clearly visible (arrows).
Instead, two other morphologies between lymphatic endothelial cells are common. In some cases, the cells show an interdigitating pattern with each other. In other cases, one endothelial cell overlaps the other without any apparent junctional proteins (Casley-Smith, 1972; Skalak et al, 1984). It is at the location of these overlaps that the primary valves form (Figure 1.2).

SEM and immunohistochemical images show that lymphatic endothelial cells are often in the shape of an oak leaf (Castenholtz, 1984; Murfee et al, 2007b). This oak leaf pattern may facilitate the primary valve morphology described in the following section. The extensions of the leaf shape provide the location for the endothelial cells to overlap and for valves to form. Detailed examination of whole-mount immunohistochemical labeling of the lymphatics in the mesentery by Dr. Murfee in our laboratory indicate that the cell-cell junctions also show gaps in CD31 (Figure 1.3) and VE-Cadherin labeling. Most of these gaps in staining are less than 1 µm in length and occur at seemingly random locations along the junctions (Murfee et al, 2007b).

1.4 Primary and Secondary Valves

The formation of lymph and the propulsion of lymph through the lymphatics both occur in a unidirectional manner in healthy lymphatics. Once fluid is in the lymphatics, it must flow against a pressure gradient. Fluid enters the lymphatics at or near zero pressure, and exits the lymphatics at the thoracic duct at a pressure of approximately 5 mm Hg (Szabo et al, 1967). In addition to this pressure gradient, in humans, lymph from the lower body must travel upwards against the force of gravity. The lymphatics contain
Figure 1.2   Schematic of cell-cell junctions in vascular endothelium (A) and in lymphatic endothelium (B). The vascular endothelial cells are connected to each other with tight junctions, adherence junctions, and gap junctions (in red), which block fluid passage between endothelial cells. The lymphatic endothelial cells at the location of the primary valve overlap each other and contain no junctions between them. This allows fluid to flow from the interstitium to the vessel lumen, but not from the lumen to the interstitium.
Figure 1.3 Arteriole (A) and Lymphatic (L) in the rat mesentery, immunohistochemically labeled with CD31 (PECAM). CD 31 preferentially labels the cell borders. The endothelial cells in the lymphatic have an “oak leaf” pattern, consistent with other published reports. The extension of the leaf shape provides a location for the endothelial cell overlap necessary for the primary valves to form. The endothelial cells in the lymphatic are also larger in surface area than those in the arteriole (image courtesy of W.L. Murfee). Scale bar: 10 µm
valves in the lumen of the vessel in order to prevent fluid flow in the opposite direction. These valves are similar to those in the veins and are well described (Mazzoni et al, 1987). Briefly, these valves have a bileaflet structure. The valves are endothelial cells with no active smooth muscle. Therefore, due to the lymph flow having a small Reynolds number, pressure and viscous forces are responsible for the opening and closing of these valves (Mazzoni et al, 1987). For simplicity, we will call these valves the “secondary valves” as they are the second set of valves that lymph fluid encounters upon entering and passing through the lymphatics.

The issue that I am most interested in this dissertation is the unidirectional transport of fluid from the interstitium into the lymphatics. There have been multiple theories on lymph formation. One hypothesis proposed by Dr. Casley-Smith in the early 1970s was the osmotic aspiration theory (Casley-Smith, 1972). In brief, he proposed that fluid was drawn into the lymphatics osmotically because of a higher protein concentration in the initial lymphatics than in the surrounding tissue space. He suggested that this concentration gradient was produced when fluid exited the lymphatics during intermittent tissue compression. The endothelial cells during tissue compression were just far enough apart to allow fluid to pass through but excluded larger molecules. This left a higher concentration of proteins inside the lymphatic compared to the interstitium. However, no conclusive evidence can be found to support this theory. The gaps between endothelial cells proposed by Casley-Smith cannot be verified. In addition, further evidence regarding the protein concentrations in the initial and collecting lymphatics is much different than values proposed by the osmotic theory (Zawieja et al, 1987).
Another theory is what is referred to as the “retrograde pump,” proposed by Dr. Reddy (Reddy et al., 1975). This theory states that suction (i.e. a fluid pressure below that of the interstitial fluid) from the upstream contractile lymphatics draws fluid into the initial lymphatics. The contractile lymphatics, as previously stated, contain their own smooth muscle media for contraction of lymphangions and propulsion of lymph. As these lymphatics expand, Reddy contends that the pressure inside the contractile lymphatic becomes negative, effective sucking fluid from the interstitium through the initial lymphatics and into the contractile lymphatics. A mathematical model of this process was published in 1995 (Reddy et al., 1995). However, one striking result of this simulation was that in order for a lymphatic to be effective, its length can be no longer than six to eight times its diameter. In the rat spinotrapezius muscle, the diameter of the initial lymphatic vessel is typically no larger than 100 μm, but it is often two to four centimeters long for a length-to-diameter ratio of 200-400. While it is conceivable that this theory is plausible for initial lymphatics that are very close to contractile lymphatics, it is uncertain whether this method is effective for lymph formation in the initial lymphatics of most organs.

As an alternative to these theories, our laboratory has recently provided evidence for a mechanism that depends on transport by means of a two-valve system with “primary and secondary valves.” Our evidence suggests that the primary valve system allows flow into the lymphatics with no significant escape back into the interstitial space (Trzewik et al., 2001). This valve system is actually located at the location of the endothelial cells. In fact, we hypothesize that primary valves are created by neighboring endothelial cells overlapping one another. The locations of the anchoring filaments that connect the
endothelial cells to the extracellular matrix allow for one cell extension to deflect while
the other remains in place.

The opening and closing of these valves is determined by fluid pressure gradients
and viscous stresses during periodic expansion and compression of the initial lymphatics.
If, during expansion of an initial lymphatic channel, the pressure inside the lymphatic
lumen falls below the fluid pressure in the surrounding interstitium, the primary valves
open and allow fluid to enter. Conversely, if during compression, the pressure is larger in
the lymphatic lumen than in the interstitium, the valves close, preventing leakage of fluid
from the lymphatic lumen back into the interstitium (Figure 1.4).

According to this theory, the initial lymphatics require compression and expansion
cycles to promote lymph flow. By definition, the initial lymphatics do not contain their
own smooth muscle media; therefore, they must rely on another mechanism to provide
the force required change their lumen dimensions. The endothelium in the initial
lymphatics is firmly attached to its basement membrane that in turn is firmly attached to
interstitial fibers. Thus tension or compression generated by tissue fibers pull open and/
or compress the initial lymphatics. Physical activities by tissue surrounding the initial
lymphatics serve this function. Examples include skeletal muscle movement (Mazzoni
et al, 1990), arterial pulse and vasomotion (Skalak et al, 1984), intestinal peristalsis
(Simmonds, 1957), or skin massage (Brace et al, 1977; Ikomi et al, 1996). In many
organs there is a combination of such pump mechanisms depending on the tissue activity.
Lymph flow is frequency dependent: faster rates of expansion and contraction increase
the rate of lymph flow (Ikomi et al, 1996; Womack et al, 1988).
Figure 1.4  Diagram of a primary valve. The valve is generated by two endothelial cells overlapping each other. Because of the lack of cell-cell junctions and the gap in the anchoring filaments, the cell extension is free to deform into the lumen to allow fluid to flow from the interstitium towards the lymphatics. The opening and closing of the valves is determined by pressure gradients and viscous fluid stresses.
Figure 1.5  Schematic diagram of the primary and secondary valves. When the pressure in the interstitium is larger than in the initial lymphatics, the primary valves open and allow fluid to flow into the lymphatics (A). Conversely, when the pressure in the interstitium is less than in the initial lymphatics, the primary valves close and secondary valves open allowing fluid to travel along the length of the lymphatic (B). The opening and closing of both sets of valves is due to pressure and viscous fluid forces.
The primary valves and secondary valves must work together to ensure lymph formation and unidirectional lymph flow. The pump cycle consists of an expansion and a compression phase (Figure 1.5). During expansion by any of the mechanisms described above, the lumen of an initial lymphatic is pulled open. At this time, the primary valves are open and the secondary valves are closed since the pressure inside the lumen of the initial lymphatic falls below the pressure in the adjacent interstitial fluid. In contrast, during compression of the initial lymphatic lumen, the volume of the initial lymphatics decreases, its pressure rises above the fluid pressure in the adjacent tissue, and the primary valves close and prevent fluid from leaking back into the interstitium. Concurrently during compression, the secondary valves open and allow the lymph fluid to flow inside the lymphatics in a proximal direction towards the contractile lymphatics. Once in the contractile lymphatics, the unidirectional flow still depends on two subsequent valves working in concert in the longitudinal arrangement of lymphangions. In each lymphangion, flow is maintained in one direction by two secondary valves, one at its proximal and one at its distal end.

The two sets of valves must both work correctly in order to provide unidirectional flow into and along the lymphatics. One example of a possible defect in the lymphatic valves is in peritumor lymphatics. These lymphatics, when investigated using lymphangiography, show much different flow patterns than in healthy controls. The lymphatics near the tumor allow fluid, once in the lymphatic vessel, to flow in many different directions, while the lymphatics in the control tissue only allow flow in the caudal-medial to cranial-lateral direction (Isaka et al, 2004). This evidence may suggest
that the secondary valves in the tumor are defective and have lost the ability to properly close.

1.5 Spinotrapezius Muscle

All of the experiments carried out in Chapter 2 of this dissertation were performed in the rat spinotrapezius muscle. This tissue was chosen for several reasons. The first is that it is fairly easily accessible and is thin enough to be used for intravital microscopy. The muscle can be removed from the surrounding tissue, but still be attached to the proximal feeding arteriole and venule. This situation allows for blood and lymphatic flow through the muscle while the muscle lies over a viewing window for intravital observation.

The blood vasculature of the spinotrapezius muscle is well understood (Skalak et al, 1984; Schmid-Schönbein et al, 1987). The muscle is connected to the circulation via 4 to 6 arteriole-venule pairs that exit the muscle. The arterioles then branch into a meshwork of arcading vessels and are often paired with arcading venules. Due to this arcade, all but one of the feeding vessels can be ligated while still retaining perfusion to the entire muscle.

The lymphatic vasculature of the muscle is also fairly well described. Most of the initial lymphatics are located immediately adjacent to the arcading arterioles. Some venules that are not paired with arterioles also are paired with lymphatics. There are, however, some arteriole-venule pairs that are not associated with a lymphatic vessel at all (Skalak et al, 1984). The lymphatic vessels that run adjacent to these blood vessels can
also exhibit a network morphology, meaning that if two molecules of a fluorescent tracer enter the lymphatics and at the same point, they may travel through completely different lymphatics before exiting the muscle at the same location (Figure 1.6).

The lymphatics of the spinotrapezius muscle also contain secondary valves. These valves can be seen in histological cross-sections (Mazzoni et al, 1987). Their presence can also be inferred by the pattern of fluorescent albumin in the lymphatics (Figure 1.7). We are able to tell by the “blunt ended” structures in the fluorescent image that a valve is probably present. These valves cause lymph to flow either towards the proximal feeder or the distal feeder. Fluorescent albumin injected into the interstitial space in the center of the muscle travels through the lymphatics in both the proximal and distal directions. However, if the albumin is injected away from the center, once in the lymphatic, it only travels in the direction of the closest feeding blood vessels (my own observations).

The presence of the lymphatics at the edges of the muscle that is devoid of large arcading arterioles remains undetermined. Microinjection of fluorescent albumin in that part of the muscle has failed to show any lymphatic uptake. However, one would assume that in this region, which contains many capillaries and small arterioles and venules, lymphatics would be necessary for the reabsorption of fluid and proteins. A systematic histological or immunohistochemical analysis of this part of the muscle has not been performed.

The lymphatics in all skeletal muscles, including the spinotrapezius, are initial lymphatics, meaning they do not contain their own smooth muscle media (Skalak et al, 1984). Therefore, as described in Section 1.4, the lymphatics rely on extrinsic forces
Figure 1.6 Lymphatics in the spinotrapezius muscle filled with fluorescently labeled albumin showing a lymphatic network. The albumin was injected into the interstitium (arrow), then filled a lymphatic which diverged into two separate vessels. The vessels then converged forming a network-like structure, similar to that seen in the blood vasculature (Engelson et al., 1985). Scale bar: 500 μm
Figure 1.7  Micrograph of fluorescent albumin injected into the rat spinotrapezius muscle indicating the location of secondary valves. The albumin was injected at the left side of the image, entered the lymphatic vessel, then traveled through the lymphatic vessel from left to right. The albumin encountered a junction in the lymphatic vessel, but was only able to travel a short distance before it presumably hit a secondary valve and was not able to travel farther (arrow). Scale bar = 500 µm
to provide the expansion and compression needed for lymph formation and flow. The force that provides most of the flow is the deformation of the skeletal muscle. As the incompressible skeletal muscle fibers are stretched (muscle relaxation), the volume of the lymphatic vessel increases. The converse is true for muscle contraction. Increasing the rate of muscle contraction and relaxation increases the rate of lymph flow (Mazzoni et al., 1990; my own observations). Another mechanism of lymph formation in the skeletal muscle lymphatics is due to the association of the lymphatics with the arterioles. Since the lymphatics are often located adjacent to the arteriole, the diameter of the lymphatic is inversely proportional to the diameter of the arteriole. If the arterioles are pulsating rapidly, this causes enough of a volume oscillation in the lymphatic vessel to produce lymph fluid (Skalak et al., 1984).

1.6 Inflammation

Inflammation is typically associated with infections and the immune system. However, it is becoming more and more apparent that the inflammatory cascade is an important contributor to a range of diseases. Much is known about how inflammation affects the microcirculation. Blood vessels become more permeable, which causes tissue swelling. This increased permeability is due to the dissociation and rearrangement of endothelial cell-cell junctions (Schnittler, 1998). Leukocytes roll along, stick to, and eventually migrate through the vascular endothelium into the tissue (Carlos et al., 1994). In addition to the microcirculation, new information suggests that the contractile lymphatics respond to inflammatory stimuli (Amerini et al., 2004; Plaku et al., 2006; Wu
For example, in the contractile lymphatics of the mesentery, substance P causes a decrease in diameter, an increase in contraction frequency, and an increase in lymph pump flow. It is hypothesized that the increase in lymph flow serves to minimize the formation of edema during inflammation (Amerini et al., 2004).

Little is known about how inflammation affects the initial lymphatics. In this study, I examined the effects of acute inflammation on the barrier properties of the initial lymphatics and the properties of lymph fluid. N-formylmethionyl-leucyl-phenylalanine (fMLP) and platelet activating factor (PAF) are well known to induce inflammation. Both of these mediators “activate” a variety of cells, including platelets, macrophages, and vascular endothelial cells. They promote leukocyte extravasation and cause microvessels to become more permeable to fluid and proteins.

One method used to determine the inflammatory activity of a fluid is to expose it to naïve leukocytes. Leukocytes, along with other cell types, generate pseudopods when they are activated. Pseudopods are local cell projections caused by the reorganization of actin and other cytoskeletal proteins (Schmid-Schönbein, 2006). Pseudopods give leukocytes the ability to spread and migrate through the endothelium and through the interstitium (Zhelev et al., 1996). In addition to projecting pseudopods, neutrophils also may degranulate in response to an inflammatory stimulus. The granules in the neutrophil contain a large number of proteins important for their function as immune cells. These include bactericidal proteins, proteases, and defensins (Faurschou et al., 2003). The response of a neutrophil to an inflammatory stimulus depends on the specific molecule or molecules involved in the stimulus.
Another consequence of inflammation can be microvascular cell death. Endothelial cells in culture that are exposed to inflammatory mediators undergo cell death in a time- and concentration-dependent manner (Robaye et al., 1991). This cell death can be either apoptotic or necrotic, and can happen with or without the presence of inflammatory cells such as neutrophils (Schmid-Schönbein, 2006). Measurement of the amount of cell death may therefore be used as a tool for measuring the level of inflammatory activity in lymph or plasma.

1.7 Lymph fluid

In my second Specific Aim, I analyzed the toxicity of rat lymph during healthy and inflammatory conditions. The protein and lipid composition of lymph is different than that of plasma (Olszewski, 2003). Interestingly, the composition of lymph in the periphery can vary based on the amount and type of physical activity (Olszewski et al., 1977). The flow rate and composition of lymph in the mesentery can vary depending on the diet of the animal as well as the length of time lapsed since the animal last ate.

Dr. Lee Leak and his colleagues at the NIH have recently published a report on the proteomic analysis of lymph in sheep (Leak et al., 2004). He notes that the proteome of the lymph is derived from permeated plasma proteins, chylomicrons secreted into the lacteals, metabolic products of interstitial cells, and proteins secreted by the lymphatic endothelium. This study used SELDI-TOF-MS along with 2-D gel electrophoresis and MS to illustrate a method by which the proteomes of the lymph and plasma could be compared. Though only a small number of differentially expressed proteins were
identified, this represents a method that can be used not only to compare lymph with plasma, but also compare lymph samples from healthy and diseased specimens. In addition, Dr. Leak was able to identify low molecular weight peptides (<10 kD), which our laboratory believes to play an important role in inflammation (Kramp et al, 2003).

1.8 Specific Aims

My first Specific Aim is to examine the maximum sized particle that can passively flow through the lymphatic primary valves in the spinotrapezius muscle and to examine how acute inflammation affects this dimension and the permeability of the initial lymphatics. The mechanics by which fluid flows from the interstitium to the lymphatics is still not well understood. Evidence shows that a valve system at the cellular level allows fluid to enter the lymphatics and not escape, but little research has been performed to determine mechanical properties of these valves. In addition, it is unknown how inflammation affects the primary valves and the initial lymphatics.

Using intravital microscopy, I will use fluorescent particles injected into the intact rat spinotrapezius muscle to determine the “effective opening size” of the primary valves during normal and acute inflammatory conditions. This effective opening size is the maximum sized particle that can enter the lymphatics. I will also use fluorescent tracers to examine the permeability of the initial lymphatics under inflammatory conditions. Using frozen sections, I will analyze the location of the tracers compared to the location of the lymphatic vessels.
My second Specific Aim is to examine the inflammatory properties of lymphatic fluid. I hypothesize that one function of the lymphatics is to remove toxic substances from the tissue interstitium. However, the lymphatic endothelium is continuously exposed to this presumably toxic fluid as it travels to the lymph nodes. The cytotoxicity of this fluid has not been examined. I will compare the bioactivity of lymph in normal animals to those that have undergone acute inflammation.

I will collect lymph fluid from the mesenteric lymphatics of the rat. This vessel provides the largest volume of fluid within a reasonable time period. To examine its properties, it will be incubated with cultured lymphatic endothelial cells and with naïve human neutrophils. The endothelial cells will be examined for cell death, and the neutrophils examined for cell activation. I will also measure the concentrations of nonesterified fatty acids (NEFAs) in the mesenteric lymph. Current work being performed in our laboratory by Dr. Alex Penn suggests that NEFAs play an important role in cellular activation and cell death in one particular inflammatory state, physiological shock. Finally, I will examine the levels of activity of matrix metalloproteinases 2 and 9, which are important in the inflammatory cascade.
Chapter 2  Barrier Properties of the Initial Lymphatics

2.1 Objective

The objective of the first part of my thesis is to examine the maximum opening dimensions of the primary lymphatic valves by determining the entry of various sizes of microspheres that can passively flow through the lymphatic primary valves in the spinotrapezius muscle and to examine how acute inflammation affects the opening size and the barrier properties of the initial lymphatics. While many transmission electron microscopy (TEM) images of lymphatic valves have been published, they don’t provide an accurate picture of the actual “effective” opening size of the primary valves. There are three reasons why measurement of the opening size of valves on TEM images is ineffective. First, the processing required to produce these specimens does not retain completely accurate dimensions. Secondly, a single image of a fixed open valve does not necessarily show the valve in a fully open position. The valve may only be partially open. Finally, the valve opening size does not exactly correspond to the maximum sized particle that can fit through the valve. One must also consider properties of the surrounding tissue and the ability of a particle to be in the correct position to pass through the primary valve. Therefore, I will define the “effective opening dimension” of the primary valve as the diameter of the largest particle that can passively flow from the interstitium into the initial lymphatic.

Fluorescent tracers provide one of the best opportunities to visualize material entering the lymphatics. Biologically inert microspheres are selected because they have
few interactions with the lymphatic endothelium and surrounding extracellular matrix. If they enter the vessel, they will have to enter through a non-specific mechanism, namely via convective flow through the primary valves, driven by a pressure gradient. If they do not enter, they are too large to pass through the primary valve (Figure 2.1). Because of the small size of these valves, we are not able to view them directly with intravital microscopy. Therefore, this in vivo method, which looks at truly functioning lymphatics, is required to get an accurate measurement of the size of particle that can enter the initial lymphatics.

I am also interested in determining the effects of acute inflammation on the effective opening size of the primary valves and how inflammation affects the ability of the primary valves to prevent fluid flow from the lymphatic vessels back into the interstitium. In order to examine this, fluorescent tracers are allowed to enter the lymphatic vessels and, using a quantitative measurement technique, the amount of tracer that has leaked out of the lymphatic at a downstream location will be examined.

2.2 Materials and Methods

2.2.1 Animals

All in vivo experiments were approved by the University of California, San Diego Animal Subjects Committee. Male Wistar rats (300-400 g) were anesthetized by an intra-peritoneal injection of sodium pentobarbital (50 mg/kg). The left femoral vein was cannulated with PE-50 tubing for the administration of supplemental anesthesia (5 mg/kg) at intervals determined by a tail pinch response (Figure 2.2).
Figure 2.1   Diagram of microspheres entering the lymphatic through a primary valve. When the fluid pressure is larger in the interstitium than in the lumen of the lymphatic, the primary valve opens allowing fluid to pass into the lymphatics. If the microsphere is smaller than the opening (blue), it enters the lymphatic. If the microsphere is larger than the opening (red), it isn’t able to pass into the lymphatic.
Figure 2.2  Cannulation of the left femoral vein in the rat. With the rat lying on his back, the incision was made in the left leg (A). Polyethylene tubing was then inserted into the vein for the administration of sodium pentobarbital for anesthesia and euthanasia (B).
2.2.2 Spinotrapezius Muscle Preparation

The animal was placed on a water-heated (37°C) stage. The spinotrapezius muscle was exteriorized for intravital microscopy as described by Gray (1973), with modifications. With the rat lying on his stomach and his head to the surgeon’s right, an incision approximately 8 cm long was made in the skin along the middle of the spine. The skin to the rat’s right of this incision was separated from the connective tissue below it. A second perpendicular incision was made, originating in the middle of the first incision towards the right side of the rat approximately 3 cm long. Sutures were tied to the corners of the two flaps to pull the skin back and expose the tissue underneath the skin on the right side of the rat (Figure 2.3).

The fascial sheath on top of the spinotrapezius muscle was carefully cut away to expose the muscle. An incision was made through the left spinotrapezius and latissimus dorsi muscles as close to the spine as possible. This allows for preparation of the right spinotrapezius muscle with minimal damage to the medial side of the muscle. The rat is then placed on his left side, with his head now to the investigator’s left. Holding onto two sutures (size 6-0) tied to the strip muscle, the spinotrapezius is delicately separated from the underlying latissimus dorsi. The distal blood vessels were ligated and cut. Due to the complex network of blood vessels in this muscle (Engelson et al, 1985), the entire muscle remains perfused by the proximal arteriole and venule. The medial side of the muscle was cut away from the spine area, leaving the muscle only attached at the anterior edge. Sutures were placed around the edges of the muscle and used to hold it in place over the top of a 22-mm viewing window built into the stage (Figure 2.4). Using a dissection microscope, most of the connective tissue was removed from the
Figure 2.3 The exposed spinotrapezius muscle of the rat. An incision was made along the spine and second one was made perpendicular to the first. This skin was separated from the fascial sheath, which is then separated from the underlying spinotrapezius muscle. Sutures were used to hold the skin flaps open for easy access to the muscle. Smaller sutures were placed in the strip muscle parallel to the spinotrapezius to allow for easier separation of the spinotrapezius from the underlying latissimus dorsi muscle.
Figure 2.4  The spinotrapezius muscle separated from the latissimus dorsi muscle and laying over a viewing window. The distal arteriole/venule feeders were ligated and cut, allowing the muscle to be free enough from the rest of the animal to lie over the window (A). A small portion of connective tissue and the latissimus dorsi muscle (arrows) are still attached to the spinotrapezius (B). This was done to avoid any chance of accidentally cutting and damaging the edges of the spinotrapezius muscle.
spina trapezius in order to provide a clearer image through the intravital microscope.

During the procedure and all subsequent work, the muscle was suffused with Krebs-Henseleit bicarbonate-buffered solution. This solution consisted of NaCl (7.71 g/L), KCl (0.35 g/L), CaCl$_2$·2H$_2$O (0.29 g/L), MgSO$_4$·7H$_2$O (0.30 g/L), and NaHCO$_3$ (1.81 g/L) and is saturated with a 95% nitrogen – 5% carbon dioxide gas. Inflammation was induced by superfusion with a combination of fMLP and PAF in the buffer. As soon as the muscle was placed under the intravital microscope (time t=0), fMLP was added to the Krebs-Henseleit suffusate (10$^{-8}$ M final concentration). Thirty minutes later (t=30 min), PAF was added to the Krebs-Henseleit buffer (10$^{-8}$ M final concentration). Concentrations and the duration of suffusion were chosen on the basis of previous studies (Harris et al, 1998). Subsequent steps in the procedure begin an hour after the beginning of suffusion with fMLP (t=60 min).

2.2.3 Microinjection of fluorescent tracers

A glass micropipette (~10 µm tip diameter) was filled with fluorescently labeled albumin (Invitrogen, Carlsbad, CA), connected by plastic tubing to a glass Hamilton syringe with a threaded plunger, and placed on a micromanipulator (Figure 2.5). The albumin was then injected into the muscle within 100 µm of a large arteriole/venule pair. Once the albumin entered the lymphatic, fluorescent microspheres (Duke Scientific, Fremont, CA) were injected in a similar manner near the filled lymphatic. Each animal was randomly selected to be injected with microspheres with one of the following diameters: 0.2 µm, 0.5µm, 0.8µm, 1.0 µm, or 2.0 µm (n=3 each). The muscle was then stimulated via two electrodes for up to an hour to generate contraction and relaxation.
Figure 2.5  Microscope setup. The animal, still on the water-heated stage, was placed underneath the intravital microscope and the vasculature of the muscle was viewed through a water-immersion objective. The muscle remained suffused with Krebs-Henseleit buffer. The micropipette was held in place and maneuvered by a micromanipulator.
cycles. The muscle contractions serve to expand and compress the lymphatics (Mazzoni et al., 1990), thereby enhancing the fluid flow into and through these vessels. The different emission wavelengths of the fluorescent albumin and microspheres facilitated the detection of microspheres’ entry into the lymphatic. Bright field and fluorescent images were viewed through a 10X water immersion objective on a custom-made intravital microscope (Leica, Wetzlar, Germany), then recorded with a CCD camera (Optronics, Muskogee, OK) and stored on a videocassette recorder. To verify entry of the albumin into the lymphatics, the lymph node closest to the spinotrapezius muscle was harvested in a selected number of rats to confirm the presence of fluorescent albumin.

2.2.4 Image Analysis

Images were analyzed offline using digital analysis software (Image Pro). Fluorescent intensities of microspheres were measured as optical densities (0-255 gray levels) and expressed as \( I/I_s \), where \( I \) is the intensity of the microspheres inside the lymphatic vessel, and \( I_s \) the intensity of the microspheres in suspension. The exact location of the lymphatic vessel was identified by the location of the fluorescent albumin.

2.2.5 Quantum dot injection and tissue processing

I then examined the possibility that under inflammatory conditions, the initial lymphatic endothelium may permit reverse transport back into the interstitial space. In a separate group of animals, non-specific quantum dots (Invitrogen, Carlsbad, CA) with diameters of approximately 10 nm were microinjected adjacent to the muscle lymphatics similarly to the fluorescent albumin described above. We used quantum dots for this
purpose rather than albumin since their fluorescent intensity in the skeletal muscle tissue is easier to detect, especially on frozen tissue sections. Once the quantum dots entered the lymphatics, the vessel was occluded using an occlusion needle distal to the injection site near the location where the lymphatic channels exit the skeletal muscle. Five minutes later, the animal was euthanized. A portion of muscle that included the initial lymphatic in between the injection site and the occlusion site was harvested, rinsed in phosphate buffered saline (PBS), and snap frozen in liquid nitrogen. The tissue was then cut into 10 µm thick sections using a cryostat (Leica, Wetzlar, Germany).

2.2.6 Immunohistochemistry

To determine the proximity of the quantum dots to the lymphatics in the tissue, I attempted to label the frozen section with an antibody to a protein that is specific to the lymphatic endothelium. As discussed in Section 1.2, the most commonly used antibodies are for LYVE-1, VEGFR3, and podoplanin. I attempted to immunolabel each of these proteins in the frozen sections.

The antibodies for the LYVE-1 and podoplanin were raised in goat. Immunolabeling was carried out using the Vector ABC elite kit (Vector Laboratories, Burlingame, CA). After rinsing with PBS, frozen sections were treated with hydrogen peroxide (0.3%) for 5 minutes to reduce endogenous peroxidase activity. The tissue was then washed in PBS and incubated in normal blocking serum (provided in the kit) for 20 minutes, followed by incubation with either the LYVE-1 or podoplanin antibody (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for two hours at room temperature. Slides were rinsed in PBS then incubated with anti-goat biotinylated secondary antibody
(provided in kit) for 30 minutes. After another rinse in PBS, tissues were incubated with an avidin-biotin complex (provided in kit) for 30 minutes. Tissues were rinsed in PBS, incubated in Vector Nova Red substrate (Vector Laboratories) for 5 minutes, rinsed in tap water, dehydrated in ethanol, and allowed to air dry before being cover-slipped.

The antibody for the VEGFR3 (1:50 dilution Santa Cruz Biotechnology, Santa Cruz, CA) protein was raised in rabbit. Immunolabeling was performed using the Vector Inmpress kit (Vector Laboratories). The procedure is the same as with the ABC kit described above with the following difference. The steps involving the biotinylated secondary antibody and the avidin-biotin complex were eliminated. Instead, the slides were incubated in the ImmPRESS™ reagent for 30 minutes.

Some frozen sections were also immunolabeled with the endothelial receptor CD31 (PECAM). Though CD31 is present on both vascular and lymphatic endothelial cells (Ji, 2005), morphological examination of the frozen section serves to distinguish blood vessels from lymphatics. The procedure for the CD31 labeling was similar to the procedure using the ABC elite kit with a few exceptions. First, the CD31 antibody is already biotinylated (1:200 dilution, BD Pharmingen, San Jose, CA). Therefore, the steps involving the incubation in normal serum and in the biotinylated secondary antibody were eliminated. The incubation time for the Vector Nova Red substrate was increased from 5 to 20 minutes.

2.2.7 Analysis of frozen sections

Fluorescent and immunolabeled images of three non-sequential sections per animal were recorded. Because the immunolabeling for the lymphatic-specific proteins
LYVE-1, VEGFR3, and podoplanin did not work on the sections, the location of the lymphatic vessel was determined by CD31 staining. Using image-processing software (Image Pro), the number of pixels both inside and outside the lymphatic vessel with intensity (0-255 grey scale) greater than the background was counted. The background was defined as the intensity value that is larger than 98% of the pixels in the tissue at a location that does not have quantum dots. The number of pixels inside and outside the lymphatics was normalized to the cross-sectional area of the lymphatic and to the total number of pixels above the threshold. The normalization step was performed to account for the heterogeneity of the microinjection. Results are presented as a percentage of the control values.

2.2.8 Statistics

Values are shown as mean ± standard deviation. Comparisons were made using the Bonferroni t-test (for fluorescent microspheres) and student’s t-test (for frozen section analysis). Differences were considered statistically significant for p<0.05.

2.3 Results

2.3.1 Effective valve opening

The lymphatic vessels in the spinotrapezius muscle are consistently located near the large arteriole/venule pairs (Skalak et al, 1984). When fluorescent albumin was injected into the muscle near these vessel pairs, it immediately entered a lymphatic vessel. The injections of larger amounts of albumin or electrical stimulation of the
muscle caused the albumin to move rapidly along the lymphatic vessel, eventually leaving the muscle along one of the major lymphatic channels. The lymphatic vessels often wrap around the blood vessels, and there may be more than one lymphatic vessel running parallel to each of the arteriole/venule pairs.

Figure 2.6 shows the microvasculature of the spinotrapezius muscle (A,C) along with the microinjection of fluorescently labeled albumin (green) and fluorescent microspheres (red) (B,D). Both panels B and D show that albumin enters the lymphatics upon injection into the interstitium. Panel B shows that microspheres with diameter 0.2\(\mu\)m entered the lymphatics and traveled along the lymphatic in a similar pattern to the albumin. In contrast, panel D shows an injection of microspheres with diameter 0.8\(\mu\)m, which do not enter the lymphatics. These larger microspheres stay in a small pool at the injection site without any evidence for transport into the lymphatics. The albumin is present in the lymph node, confirming actual entry into the lymphatic vessel inside the muscle (Figure 2.7).

Microspheres with diameters of 0.2 \(\mu\)m and 0.5 \(\mu\)m entered the lymphatics immediately upon injection into the interstitium, while those with diameters of 0.8 \(\mu\)m, 1.0 \(\mu\)m, and 2.0 \(\mu\)m did not enter the lymphatics (Figure 2.8). Even after periods of up to an hour of muscle contraction by electrical stimulation, the 0.8 \(\mu\)m, 1.0 \(\mu\)m, and 2.0 \(\mu\)m microspheres did not enter the lymphatics. The contraction and relaxation of the muscle increased the flow rates of the albumin and the 0.2 \(\mu\)m and 0.5 \(\mu\)m microspheres, as demonstrated by the decrease in the fluorescent intensity at the injection site. However, this stimulation did not change the size of inert particles that was able to enter the initial lymphatics. Acute application of the inflammatory stimulators fMLP and PAF does not
Figure 2.6  Bright field micrographs of the rat spinotrapezius muscle microvasculature (A,C) and fluorescent microinjections (B,D). (A,B) Albumin (green) and microspheres (red) with diameter 0.2 µm were injected into the interstitium with a micropipette. Both readily entered the lymphatics and traveled along the same path towards the proximal end of the muscle. The arrow indicates yellow color where the two fluorescent signals overlap. (C,D) Albumin and microspheres with diameter 0.8 µm were injected into the muscle. While the albumin enters the lymphatics similarly to (B), the microspheres do not enter the lymphatic, even though the injection site is adjacent to the albumin-filled lymphatic. They did not enter the lymphatic even after 1 hour of electrical stimulation. Scale bar: 100µm.
Figure 2.7  Fluorescent micrograph of the lymph node. After the microinjection of fluorescently labeled albumin into the spinotrapezius muscle, the muscle was electrically stimulated for one hour to promote lymph formation. This lymph node is the first node that fluid exiting the muscle passes through. Most of the albumin still appears to be inside a distinct vessel within the node. Scale bar: 100 µm
Figure 2.8 Normalized fluorescent intensities of the microspheres inside the lymphatic vessels under control and inflammatory conditions. The fluorescent intensity of the microspheres inside the lymphatics is normalized by the intensity of the microspheres in suspension. Microspheres with diameters 0.2 µm and 0.5 µm readily entered the lymphatics, while those with diameters 0.8 µm, 1.0 µm, and 2.0 µm did not. * indicates that the values for the 0.2 µm and 0.5 µm microspheres are significantly different from the larger microspheres, but are not significantly different from each other. (p<0.05) There are no significant differences between control and inflammatory groups.
affect the maximum diameter value of the microspheres that can enter the lymphatics.

2.3.2 Permeability of the lymphatics during inflammation

Santa Cruz Biotechnology claims that its antibodies for LYVE-1, VEGFR3, and podoplanin work on rat tissue. However, I tried using all three and none of them worked effectively on frozen section of the rat (Figure 2.9). The LYVE-1 and podoplanin antibodies did not label the tissues any stronger than the background. The VEGFR3 antibody labeled both the lymphatics and blood vessels, despite extensive literature that claims this protein is not expressed on adult blood vessels (Kaipainen et al, 1995; Pepper et al, 2003). The ineffectiveness of these antibodies was independently verified by Dr. Lee Murfee, a post-doctoral researcher in our lab, on the rat mesentery.

Figure 2.10 shows frozen sections from muscles that have had quantum dots microinjected into the interstitium and transported into the lymphatics. Panel A is a micrograph of a control section immunolabeled with CD31. Darkly labeled structures are blood vessels, while the lighter stained vessel near the large blood vessels is a lymphatic vessel. Panel B shows the quantum dots in a serial section. In the control tissue, most of the quantum dots are inside or very close to the lymphatic vessel.

Panels C and D show micrographs of serial sections of a skeletal muscle that was exposed to acute inflammation. Panel C is labeled with CD31, and panel D shows the location of the quantum dots. The fluorescent tracer is much less localized near the lymphatic vessel when compared with panel B. The tissue section was taken sufficiently far from the site where the quantum dots were injected, suggesting that they have entered the lymphatic upstream of this section and leaked back through a permeable lymphatic
Figure 2.9  Frozen sections of rat muscle labeled with antibodies specific to LYVE-1 (A), Podoplanin (B), and VEGFR3 (C). Multiple investigators have shown that each of these proteins is specific to the lymphatics in adult tissues. However, in our hands, none of these antibodies worked as previously reported. The LYVE-1 and Podoplanin antibodies were not detectable above background levels. The VEGFR3 antibody appears to label the blood vessels in addition to possibly labeling the lymphatics. Arterioles and venules are labeled A and V, respectively. Scale bar = 50 µm.
Figure 2.10  Micrographs of frozen sections from control (A, B) and experimental (C, D) animals. The blood vessels and the lymphatic vessels are both positively stained with CD31 (A, C). The lymphatic vessels are distinguished from the blood vessels by their lighter immunolabeling and their non-circular shapes (in between the arrows). The fluorescent image from the control animal (B) shows that the quantum dots are mostly localized in the lymphatic. The tissue from the experimental animal (D) shows quantum dots spread much farther from the lymphatic vessel compared to the control (B). When the quantum dots leak from the lymphatics, they do not spread uniformly throughout the tissue. Instead, they follow paths of least resistance around the muscle fibers through the connective tissue. Scale bar: 100 µm.
Quantification of the fluorescent intensities inside and outside of the lymphatics shows that in the fMLP/PAF superfusion group, there are fewer quantum dots inside the lymphatic compared with the control group (Figure 2.11). Conversely, there are more quantum dots outside the lymphatics in this group, indicating more quantum dots have escaped from the lymphatic lumen back into the interstitium. The small number of quantum dots that leak from the lymphatics even in the control group is likely due to low levels of inflammation as a result of the surgical procedure.

2.4 Discussion

2.4.1 Effective valve opening

The maximum diameter of microspheres that can passively flow during active lymphatic pumping through the primary valves is between 0.5 µm and 0.8 µm in diameter in both control and in acute inflammatory conditions. This dimension is similar to the gap size of CD31 staining in the rat mesenteric lymphatics determined in our laboratory by Dr. Murfee (Murfee, 2007b). The possibility of fluid vesicular transport being responsible for the movement of the fluorescent microspheres across the lymphatic endothelium can be discounted by examination of a TEM image (Figure 2.12). First, the vesicles present in the endothelial cells are all approximately 0.1 µm in diameter, about five times smaller than the largest microsphere to enter the lymphatics. Second, if a vesicle with a diameter of 0.5 µm was to form, the vesicle would be twice the thickness of the cell, which would cause a pore to form in the endothelium. If pores such as this
Figure 2.11  Percentage of pixels brighter than the background inside (A) and outside (B) of the lymphatics compared with control. In the control group, there are more quantum dots inside the lymphatic, while there are fewer quantum dots outside the lymphatics when compared to the inflammatory group. (*p<0.05).
Figure 2.12  TEM image of two overlapping lymphatic endothelial cells. Vesicles are visible in the top endothelial cell (arrows). The size of these vesicles is much smaller than the microspheres that entered the initial lymphatic, confirming that vesicular transport cannot be the mechanism by which the microspheres traveled from the interstitium into the lymphatics. Also note that these cell extensions are very thin, both no more than 200 nm thick.
formed, the unidirectional transport into the initial lymphatics would be compromised.

The maximum size for the opening we found in the primary valves indicates that fluid and proteins can flow easily into the initial lymphatics. In contrast, immune cells, which are an order of magnitude larger than this effective opening, do not enter the lymphatics passively, even under inflammatory conditions. This suggests that there may be an unexplored active mechanism by which lymphocytes enter the lymphatics to be transported to lymph nodes. The mechanism that these cells use to exit the vascular circulation involves endothelial pore formation with actin depolymerization and lateral displacement of membrane adhesion proteins at cell junctions (Luscinskas et al, 2002; Schmid-Schönbein et al, 1995; Worthylake et al, 2001), but we have no indications of how similar or different this mechanism is to the migration of cells from the tissue to the lymphatics. One must keep in mind that leukocyte extravasation from the blood vessels occurs through an endothelial layer across which there is a constant pressure drop of approximately 20 mm Hg. Not only is the pressure drop across the lymphatic endothelium estimated to be about an order of magnitude less than this, the pressure drop oscillates between positive and negative values. Therefore, it is unlikely that lymphocytes are merely being “squeezed” through the primary valves due solely to a pressure gradient. Instead, we hypothesize that an “active” mechanism must exist to allow cells to enter the lymphatics.

In a similar manner, metastatic cancer cells may also use an active mechanism to enter the lymphatics (Pepper et al, 2003), and the current result may support this hypothesis. However, tumor-associated lymphatics often have very different morphologies than those in healthy animals (Isaka et al, 2004). In addition, it is still
unknown whether functional lymphatics are present inside the tumor or whether cancer cells that metastasize through the lymphatics must travel outside the tumor margin in order to spread to the lymph node (Padera et al., 2002). Further studies in tumor-associated lymphatics must be performed.

2.4.2 Permeability of the lymphatics during inflammation

The initial lymphatics rely on unidirectional transport from the interstitium into the lymphatic vessel in order to remove fluid and particulate material from the tissue. During periodic expansion and compression of initial lymphatic channels, fluid encounters open channels to travel from the interstitial space into the initial lymphatics. Fluid permeation outward is greatly reduced by the presence of a continuous, albeit highly attenuated, endothelium and closed inter-endothelial junctions. During inflammatory conditions, the endothelium in the initial lymphatics becomes permeable to particles the size of quantum dots, thus failing as a unidirectional barrier for particles the size of nanometers. This elevation of lymphatic endothelial permeability in an outward direction has two major effects. First, fluid is being cleared from the tissue less efficiently than if the lymphatics were not leaking. With the increased permeability of the blood vessels already causing more fluid in the tissue than normal, the decreased transport of the lymphatics causes this edema to increase even more. Second, leaking lymphatics allow inflammatory mediators to remain in the tissue longer than they would if the vessels didn’t leak. These mediators are typically small molecules that under normal conditions would be transported easily into the lymphatics and removed quickly from the tissue. When the lymphatics leak, these mediators remain in the tissue longer
and increase the inflammatory response.

The cause of the increased permeability during application of inflammatory mediators is still unresolved. One possibility is that the primary valves themselves aren’t functioning correctly. The primary valves are very short and thin cell extensions and could be susceptible to membrane stimulation. Also, the valves sometimes have minimal overlap, and if the volume of the lymphatic has increased due to the increased edema in the tissue, it is possible that the flap between neighboring cells may be stretched, opening a hole in the lymphatic (Figure 2.13). Another possibility is the formation of pores in the endothelial cells. The lymphatic endothelial lining is on the order of 0.2 µm (Figure 2.12), and the inflammation could cause pores to open up inside the endothelium (Schmid-Schönbein et al, 1995). Such pores would permit free fluid movement between the interstitium and the initial lymphatic channels, compromising the effectiveness of the primary valves.

Another possibility is that the mechanism by which the lymphatics leak could be similar to that of the vascular endothelium: a rearrangement of cadherins and tight junctions (Schnittler, 1998). Even though the primary valves have fewer molecular junctions between neighboring endothelial cells, there are locations along the length of the vessel that have endothelial cell-cell tight junctions (Casley-Smith, 1972; Collan et al, 1974; Skalak et al, 1984). They may be compromised by inflammatory mediators that have the ability to facilitate lateral motion of junctional proteins after actin depolymerization.

Several factors produce variability in the fluorescent intensity measurements within each group. One is the variation in the geometry of the lymphatic vessels. The
Figure 2.13 Two lymphatic endothelial cells showing very little overlap. This small overlap could easily be compromised by inflammation and render the primary valve ineffective, allowing fluid to move across the endothelial barrier in either direction.
cross section of the lymphatics in the direction parallel to the microscope objective will change the value of the fluorescent intensity that is measured. If the diameter in this direction is larger than average, there will be a larger volume of fluorescent tracer in the lymphatic, resulting in a brighter signal. Other factors that may increase the variability are the total volume of microspheres injected, the exact distance they are injected from the lymphatic vessel, and the direction of the injection in relation to the direction of flow in the lymphatic.

2.5 Acknowledgement

Chapter 2, in part, has been accepted for publication of the material as it appears in Lymphatic Research and Biology, Lynch, Patrick M.; DeLano, Frank A.; Schmid-Schönbein, Geert W., Mary Ann Liebert Inc, 2007. The dissertation author was the primary investigator and author of this paper.
Chapter 3 Toxicity of Lymph Fluid

3.1 Objective

The second aim is to examine the cytotoxicity of lymphatic fluid collected during control and inflammatory conditions. In the first aim, I showed that acute inflammation causes fluid to leak out of the initial lymphatics, therefore decreasing the efficiency by which they remove fluid from the tissue. In order to analyze the effect that this lymph has on the tissue, I want to examine its bioactivity and compare it with plasma from the same animal.

The fluid transported through the lymphatics is filled with proteins and lipids that are collected from the interstitium. This includes many waste products produced by cells. These waste products can be particularly harmful to the cells, and I hypothesize that one of the roles of the lymphatics is to transport them away as to reduce their damage. However, as the fluid is being transported through the lymphatics, lymphatic endothelial cells and the cells in the lymph node are being exposed to this fluid. Additionally, this fluid is eventually returned to the blood circulation at the thoracic duct. Any toxic factors that are in the lymph are then re-introduced to the tissue via the blood circulation.

In my analysis, I will measure the cytotoxicity of lymph by two methods. The first is to examine how lymph fluid affects naïve neutrophils. If there are inflammatory peptides or lipids in the lymph, the neutrophils may be activated. This activation may cause two changes in the neutrophils: pseudopod formation and degranulation. Both of these changes can be measured in large numbers of cells using a flow cytometer. The
flow cytometer passes cells via a laminar flow field through a laser beam. In addition to measuring fluorescence, the flow cytometer can detect how much light is scattered at low angles (forward scatter) and how much light is scattered at right angles (side scatter). The forward and side scatter of neutrophils has been previously shown to correlate to size and granularity, respectfully (Fernandez-Segura et al, 1995; Sklar et al, 1984).

The second aspect I will analyze is the effect of the lymph on cultured lymphatic endothelial cells. Lymph will be added to the culture medium, and cell death using the fluorescent marker propidium iodide (PI) will be assessed. This measurement gives an indication of the cytotoxicity of the lymph. The cultured cells that are being used are from the mesenteric lymphatics, and therefore compatible with the lymph collected from the mesenteric lymphatic duct. A similar study has been conducted in our lab examining the effect of lymph collected from animals in physiological shock on vascular endothelial cells (Miao et al, 2007).

Though there are many molecules in the lymph that may be toxic, I decided to determine levels of nonesterified fatty acids (NEFA), or free fatty acids, in the lymph and plasma samples. NEFAs are toxic to cells and their presence in the blood stream contributes to leukocyte activation and adhesion to the endothelium (Zhang et al, 2006). Most of the NEFAs in the blood are hydrophobically bound to proteins (mainly albumin) to decrease their toxicity (Leaf, 2001). Current work by Dr. Alex Penn in our laboratory suggests NEFAs may play a role as a toxic mediator in physiological shock (Penn, unpublished results).

Finally, in this aim I will examine the levels of Matrix Metalloproteinase 2 (MMP-2) and MMP-9 on the lymphatic endothelium in the mesentery. These proteases
are produced by endothelial cells. These two specific MMPs are considered to be
important in the inflammatory cascade (Okamoto et al, 2004), and I hypothesize that they
may be upregulated in response to an increase in cytotoxic mediators in the lymph fluid.

3.2 Materials and Methods

3.2.1 Animals

All in vivo experiments were approved by the University of California, San Diego
Animal Subjects Committee. Male Wistar rats (300-400 g) were anesthetized by an intra-
muscular injection of sodium pentobarbital (50 mg/kg IP). The left femoral vein was
cannulated with PE-50 tubing for the administration of supplemental anesthesia (5 mg/kg
IV) at intervals determined by a tail pinch response as previously described.

3.2.2 Collection of lymphatic fluid

The animal was placed on a water-heated (37°C) stage. Lymphatic fluid was
collected from the mesenteric lymphatic as previously described (Bollman et al, 1948;
Boyd et al, 2004) with modifications. With the rat lying on it’s back, a midline incision
approximately 4 cm long was made through the abdominal wall. Sutures were placed
through the skin and abdominal wall near the middle of the incision in order to retract
them out of the way as far as possible. Cotton swabs were used to displace the intestines
so the connection between the aorta and the superior mesenteric artery (SMA) is visible.
Care was taken to not damage the intestine during this process. Gauze soaked in warm
saline was used to displace the intestines and liver so the SMA remained visible.
The mesenteric lymphatic is immediately connected to the SMA. Typically, a large lymphatic is located adjacent to the artery in the cranial direction and a smaller lymphatic in the caudal direction. The lymphatic vessel in the cranial direction tends to produce more lymph than the smaller one. Using a stereomicroscope, the lymphatic and SMA were separated from the surrounding fat and connective tissue. They were not, however, separated from each other. A single 6-0 suture was looped underneath the artery/lymphatic pair and tied tight enough to collapse the lymphatic vessel, but not tight enough to occlude the artery.

The catheter used to collect lymph was size PE-10 and had been primed with heparinized saline for at least one hour before use and had one end beveled to a 45 degree angle. Before being inserted into the animal, the saline was blown out of the line as to not dilute the lymph. The catheter was passed through the right abdominal wall with the help of a 16-G needle. This was done to minimize the vertical distance that the lymph must travel while in the catheter. After tension is applied to the sutures around the lymphatic, the beveled end was inserted directly through the lymphatic endothelium about 5 mm into the vessel. Typically, a small amount of lymph began to flow into the catheter immediately. A small drop of superglue was applied to the lymphatic and catheter to hold them in place and stop any lymph that was leaking out of the lymphatic. The gauze was removed from the abdominal cavity and a piece of plastic wrap was placed on top of the incision to minimize fluid evaporation. Once the catheter was in place and collecting fluid, inflammation was induced by perfusion of fMLP through the catheter in the femoral vein (n=3). The dose of 0.3 mg/kg, determined from previous studies (Olsen et al, 1986), was given in a single bolus. Control animals without fMLP
received a similar dose of heparinized saline instead (n=3).

Lymph was collected in a 1.5 mL tube for 3 hours following the time in which the catheter is glued in place. After 3 hours, the collection was terminated. The rat was then heparinized (600 units per kg body weight) for the removal of blood. About 5 mL of blood was removed through the venous catheter. The blood and the lymph were centrifuged for 10 minutes at 800g in order to separate the cells from the fluid. The fluid was collected from the top of the centrifuge tube and frozen at -20°C for later use.

3.2.3 Human neutrophil purification

Neutrophils were isolated from 30 mL of human blood donated by a healthy volunteer (me). Blood was drawn into three 10 mL heparinized tubes at the UCSD Student Health Center. All the blood was pooled together in a 50 mL centrifuge tube to which 3 mL of 6% dextran was added (6 g of dextran 229 in 100 mL PBS). The tube was gently swirled and allowed to settle for approximately 40 minutes. The dextran cross-links with the erythrocytes and causes them to settle at the bottom of the tube. The plasma layer (which contains leukocytes and some erythrocytes) was removed and placed gently on top of 5 mL Histopaque – 1077 (Sigma-Aldrich, St. Louis, MO) in a 50 mL centrifuge tube. This solution was centrifuged at 600g for 20 minutes. After centrifugation, a small layer of red blood cells was at the bottom of the tube, followed by granulocytes, histopaque, lymphocytes and platelets, and finally plasma on top. The histopaque, lymphocytes and platelets, and plasma were all removed and discarded. The remaining pellet was resuspended in 5 mL of PBS.
Percoll was used to separate the remaining erythrocytes from the neutrophils. 3 mL of 55% percoll was put into a 15 mL centrifuge tube. The percoll was made isotonic by the addition of nine grams of sodium chloride to 100 mL of the stock solution of percoll, which was then diluted with isotonic saline. Using a syringe, 3 mL of 74% percoll was layered beneath the 55% percoll, being careful to maintain the interface between the two solutions. Finally, the resuspended pellet was added to the top of the 55% layer and the tube was centrifuged for 15 minutes at 600 g. The deceleration on the centrifuge must be slow in order to maintain distinct bands to form. The band of neutrophils formed around the 3 mL mark. This band was carefully removed and placed in a new 15 mL centrifuge tube that is then filled with PBS. The solution was gently mixed and centrifuged for 10 minutes at 100 g. The supernatant was discarded and the pellet resuspended in 5 mL of PBS. The yield from this procedure can be as high as 8*10^7 neutrophils for 30 mL of whole blood.

3.2.4 Analysis of the effect of lymph on isolated neutrophils

To determine the effect of lymph and plasma on the neutrophils, 100 µL of cells was combined with 100 µL of lymph, plasma, PBS (negative control) or fMLP (10^-6 positive control). After 15 minutes of incubation at room temperature, 100 µL of 3% glutaraldehyde (1% final concentration) was added to the cells. On the same day, the cells were analyzed by flow cytometry. The side scatter and forward scatter of 10,000 cells per sample were determined. Forward scatter is plotted as a function of side scatter. The results are gated to eliminate contamination from erythrocytes and eosinophils that were not completely removed during the neutrophil separation, as well as cells and
debris that may not have been separated by centrifugation following lymph and plasma collection. The median values of side scatter and forward scatter were used to compare samples.

3.2.5 Analysis of the effect of lymph on lymphatic endothelial cells

The cells used for this analysis were rat mesenteric lymphatic endothelial cells (RMLECs), which were a generous gift from Dr. David Zawieja at Texas A&M University (Hayes et al, 2003). They were isolated and grown from small mesenteric lymphatics that had been removed from the rat. I obtained the cells after they had already been passed. Cells were grown in gelatin (1%) coated flasks then transferred to gelatin coated 24-well plates for experiments. The media used was EGM-MV (Cambrex, East Rutherford, NJ). This is a basal media supplemented with bovine brain extract, heparin, hEGF, hydrocortisone, GA-1000 (Gentamicin, Amphotericin B), and fetal bovine serum. It has been developed specifically for microvascular endothelial cells.

Cells used for experiments were plated on gelatin-coated 24-well plates. They were allowed to grow three days to confluence. The cells were then rinsed with Dulbecco’s PBS and given fresh media. Ten percent of the media was replaced with either a lymph sample or a plasma sample that was collected from the rats. Controls included 10% of the media replaced with fMLP (final concentration 10^{-6} M), 10% replaced with dPBS, and 100% cell culture media. Each well also contained 8 µL of propidium iodide (PI, Sigma-Aldrich, St. Louis, MO) for a final concentration of 1 µM. PI is a fluorescent life/death indicator. The total volume in each well was 0.8 mL. Cells were
maintained in a humidified incubator at 37°C and 5% carbon dioxide except when images were being recorded.

Images of the cells were taken every hour for eight hours, then again 24 hours after the application of lymph and plasma samples. At each time point, one representative bright field image and four random fluorescent images were recorded. The cells were viewed through an inverted microscope (Leica, Wetzlar, Germany) with a 20X objective, then imaged with a CCD camera (Optronics, Muskogee, OK), and captured on a computer using image processing software (Image Pro).

The bright field images of the cells were used to qualitatively compare the cellular morphology of the experimental groups. The fluorescent images were used to determine endothelial cell death. The number of PI-positive cells was counted and averaged for each of the samples at each time point. PI-positive cells are easily distinguished in fluorescent images as bright spots in the nucleus of the cell.

3.2.6 Nonesterified fatty acid concentration in the lymph

The concentration of NEFAs in the lymph and plasma were measured using the NEFA-C kit from Wako Diagnostics (Richmond, VA). In a 96-well plate, 10 µL of sample were deposited in triplicate. Pilot studies indicated that both the lymph and plasma must be diluted in order for the concentration to be within the standard solutions. The lymph was diluted by 25 and the plasma by 5. A serial dilution of the standard solution (oleic acid) from 0 to 1 mM was made and pipetted into the plate in 10 µL aliquots in triplicate. 50 µL of reagent A was added to each well. 15 minutes later, 100 µL of reagent B was added. After 15 minutes more, the samples were read
colorimetrically at 560 nm. The samples, which were performed in triplicate, were averaged and compared to the standard curve to determine NEFA concentration.

3.2.7 Analysis of MMP-2 and MMP-9 activity on lymphatic endothelial cells

To measure the MMP-2 and MMP-9 activity in the mesenteric lymphatic endothelial cells, the rat was cannulated as described above. With the rat on its back, a midline incision approximately 4 cm long was made through the abdominal wall. The rat was then turned on its right side, causing the intestines to protrude through the opening in the abdomen. The cecum was located and pulled out of the body. The small intestine proximal to the cecum was slowly removed from the body and the mesentery was examined for lymphatic vessels as the intestine was removed (Figure 3.1). A mesenteric lymphatic vessel was deemed usable only when it was large and active enough to be visualized under light microscopy, but did not have too many adipose cells surrounding it as to obstruct the view of the endothelium. Not every rat contains a suitable lymphatic vessel in the mesentery.

Once an acceptable lymphatic had been found, the mesentery was stabilized using gauze. Care was taken to be sure that the intestine and mesentery outside the body are being continually suffused with Krebs-Henseleit bicarbonate-buffered solution as described in Chapter 2. To induce inflammation, fMLP (10^{-8} M final concentration) was added to the Krebs-Henseleit suffusate and applied to the mesentery for 30 minutes. Control tissues continued to be suffused with Krebs-Henseleit buffer. 0.5 mg of a fluorescent MMP substrate (EMD Biosciences, San Diego, CA) was dissolved in 30 mL of Krebs-Henseleit buffer. This substrate fluoresces in the presence of active
Figure 3.1 Exteriorization of the rat mesentery for intravital microscopy. An incision is made through the abdominal wall (A) and the intestine is gently pulled through the incision (B). Gauze is packed around the tissue to keep it from moving once it is under the microscope.
MMP-2 and MMP-9 proteins. The substrate is cleaved by these proteases causing
fluorescence under ultraviolet light excitation. The dissolved substrate was applied to
the mesentery topically for 15 minutes. During and after the application, fluorescent
images of the lymphatic vessel and surrounding blood vessels were viewed through a
10X water immersion objective on a custom-made intravital microscope (Leica, Wetzlar,
Germany), then recorded with a CCD camera (Optronics, Muskogee, OK) and stored on a
videocassette recorder.

3.2.8 Statistics

Values are shown as mean ± standard deviation. Comparisons were made using
the student’s t-test. Differences were considered significant for p<0.05.

3.3 Results

3.3.1 Collection of lymphatic fluid

The volume of lymph fluid collected from the six animals varied over the
three-hour collection time from 0.7 mL to 1.5 mL, with an average of 1.2 mL. Even
when normalized by the overall weight of the rat, these volumes showed no trend.
Additionally, the appearance of the lymph fluid was different between the rats. Most
fluid samples appeared very opaque and filled with fat to the unaided eye. At least one,
however, was only slightly cloudier than the plasma collected from that rat. None of the
lymph samples were noticeably more viscous than the plasma samples. The differences
in the lymph can most likely be attributed to how recently the rat has eaten, though
there is no obvious correlation between the opacity of the lymph and the time of day the collection was performed. During a pilot study, I mixed heavy cream in their water supply overnight. This caused the lymph to be much more white and opaque than my experimental rats, suggesting the dependence of diet on the consistency and constituents of mesenteric lymph fluid.

3.3.2 Effect of lymph on naïve human neutrophils

The flow cytometer has the ability to analyze thousands of cells in less than a minute. My results are based on 10,000 cells per data point. Figure 3.2 shows a forward scatter versus side scatter plot for control neutrophils (exposed to PBS, panel A) and cells exposed to fMLP (10⁻⁶ M, panel B). The dots representing neutrophils were gated (white box) and are the only data points analyzed. Points on the plot outside of this box are due to contamination of the sample, either from cells not being completely removed during neutrophil isolation or from debris present in the samples mixed with the neutrophils. The size and location of this box have been previously determined by Dr. Penn in our laboratory who has used this technique extensively (Penn et al, 2007). The median values for side scatter in these two plots are nearly identical (458 for control and 467 for fMLP). However, the median forward scatter for the control group is smaller than that of the fMLP group (279 compared to 392) (Figure 3.3).

Figure 3.4 shows a representative plot of forward scatter versus side scatter for neutrophils exposed to lymph (panel A) and to plasma (panel B) sample. The forward scatter values are similar between the two graphs, but the side scatter in the lymph is less than in the plasma sample (Figure 3.5). The average median value of side scatter for
Figure 3.2  Flow cytometer dot plots obtained from neutrophils exposed to PBS (A) and fMLP (B). The dots within the white boxes represent neutrophils. Each sample contains 10,000 data points within the white box. Forward scatter is on the abscissa and side scatter on the ordinate.
Figure 3.3 Side scatter (A,B) and forward scatter (C,D) histograms of neutrophils exposed to PBS (A,C) and exposed to fMLP (B,D). The median values of the side scatter are similar (458 and 467, respectively), but the median value of forward scatter of the fMLP cells is larger than for the control cells (392 compared to 279).
Figure 3.4  Dot plots for neutrophils exposed to lymph (A) and to plasma (B). There is a dramatic difference in the side scatter. Not one cell in the lymph sample has a side scatter value greater than 350, suggesting that all the cells have degranulated.
Figure 3.5  Side scatter (A,B) and forward scatter (C,D) histograms of neutrophils exposed to lymph (A,C) and exposed to plasma (B,D). The median value of the side scatter is significantly reduced in the cells exposed to lymph. The forward scatter histograms are similar.
Figure 3.6  Average median forward scatter and side scatter for neutrophils exposed to lymph and plasma. The average median side scatter for the cells exposed to lymph is significantly smaller than for the cells exposed to plasma (p<0.05).
the cells exposed to lymph is 217 while that for plasma is 440 (Figure 3.6). There is no
difference in the average of the median forward scatter values between the lymph and
plasma groups.

3.3.3 Effect of lymph on cultured lymphatic endothelial cells

The morphology of the endothelial cells exposed to plasma and lymph show no
noticeable differences during the first four hours of exposure to the sample (Figure 3.7). There are also very few dead cells (as indicated by propidium iodide fluorescence) at this
time point. After 4 more hours (t = 8 hours), some of the cells exposed to lymph begin to
round up and decrease in size (Figure 3.8). This change in morphology was subsequently
followed by an increase in PI labeling.

Cell cultures exposed to plasma had no more PI-positive cells than the controls
throughout the course of the experiment. However, the cells exposed to lymph began to
show more cell death near the six-hour time point (Figure 3.9). There is no data point at
24 hours for the cells exposed to lymph because there are actually no cells remaining. A
large amount of cellular debris was the only thing present in the wells that had contained
the lymph fluid. Cells in the three control groups (fMLP, PBS, and cell culture media)
were morphologically similar and there was no difference in the number of PI-positive
cells. Therefore, only data from the PBS group is shown.

At the time points used during this experiment, there is no statistical significance
between groups. When all the cell death values for lymph are compared with the values
for plasma at 8 hours, a paired student’s t-test returns a P-value of 0.061. At 24 hours,
there are no cells left in the lymph group to analyze, indicating 100% cell death. The
Figure 3.7  Lymphatic endothelial cells in culture after 4 hours of exposure to rat lymph (A,C) and plasma (B,D). The morphology of the cells is very similar at this time point. There are no dead cells as indicated by propidium iodide labeling (C,D). Scale bar: 100 µm.
Figure 3.8  Lymphatic endothelial cells in culture after 8 hours of exposure to rat lymph (A,C) and plasma (B,D). The cells exposed to lymph are smaller and have rounded up when compared with the same cells at 4 hours or when compared to the cells exposed to plasma, which look unchanged compared with 4 hours. There are over 50 PI-positive cells exposed to lymph (C) compared with just two exposed to plasma (D). Scale bar: 100 µm.
Figure 3.9  Time course of RMLEC death. The cells exposed to lymph start to show increased cell death at 6 hours. At 24 hours, there is no data point for these cells because all of the cells are dead and the only thing remaining is cellular debris.
Figure 3.10 Variability of cell death between individual animals following 8 hours of exposure to lymph and plasma. Four of the six animals showed increased cell death in the cells exposed to lymph compared to cells exposed to plasma.
high P-value at 8 hours is primarily due to very large standard deviations within the groups. One out of three animals in each group did not show increased endothelial cell death at this time point (Figure 3.10).

3.3.4 Nonesterified fatty acid concentration in the lymph

The concentration of NEFAs in the lymph is approximately six times the concentration in plasma (Figure 3.11). Similarly to the other assays performed, the standard deviation is again relatively large. However, the NEFA concentration in the lymph is consistently larger than in the plasma.

3.3.5 MMP-2 and MMP-9 activity on lymphatic endothelial cells

In all images recorded, both the vascular endothelium and the lymphatic endothelium have higher levels of MMP activity compared with the surrounding interstitial tissue (Figure 3.12). Visual inspection of multiple images reveals no difference in MMP activity between lymphatic vessels and blood vessels, as well as between control and inflammatory tissues. Due to the nature of the fluorophor and the limitations of our equipment, I was unable to quantify the fluorescent intensities as I had originally desired.

3.4 Discussion

3.4.1 Effect of lymph on naïve human neutrophils

There are many ways to evaluate biological activity of a neutrophil. These
Figure 3.11  Nonesterified fatty acid concentrations in rat lymph and plasma samples. The concentration of NEFAs in lymph is significantly higher than in plasma (p<0.05).
Figure 3.12  Fluorescent image of MMP-2 and MMP-9 activity in the rat mesentery. The lymphatic (L) and venule (V) both show MMP activity in the endothelial cells. The bright spot to the left of the lymphatic is a cluster of adipocytes that auto-fluoresce in the ultraviolet range. Scale bar: 100 µm
include pseudopod projection, degranulation, superoxide production, and CD11b expression, among others. I decided to use pseudopod projection and degranulation as they are measurements that have been used extensively in our laboratory. Neutrophils react differently to different inflammatory stimuli, making it important to use multiple tests for biological activity.

The amount of forward scatter produced by neutrophils exposed to lymph and plasma are nearly identical, with the value being approximately halfway between the value for control cells and for fMLP-stimulated cells. Dr. Penn has shown extensively that the forward scatter of neutrophils exposed to fMLP is larger than the forward scatter of control neutrophils (Penn et al, 2007). He has also shown through visualizing fixed cells under high power light microscopy that the increase in forward scatter corresponds to an increase in pseudopod formation, and is therefore an accurate measurement of biological activity.

The side scatter of the neutrophils exposed to lymph is significantly less than the side scatter of the cells exposed to plasma, suggesting that the neutrophils exposed to lymph have degranulated. This is an interesting observation when compared to the forward scatter results. There are multiple possible explanations for this. Because there is still an increase in forward scatter when compared with controls, there still may be an inflammatory mediator present in the lymph that causes pseudopod formation. Whether this is the same mediator that is present in the plasma is not certain. However, there is either a biological factor in the lymph that causes degranulation that is not present in the plasma, or this factor is also in the plasma but there is an additional protective molecule in the plasma that doesn’t allow the biological factor to degranulate the
neutrophils. More experiments must be performed to determine exactly what is causing the degranulation.

Under normal conditions, a neutrophil is present in the blood stream until it receives a signal to migrate into the interstitium. The cell uses pseudopods to migrate through the vascular endothelial wall and through the interstitium to the site of inflammation. This pseudopod formation begins in the blood stream, and therefore it is not surprising that an inflammatory stimulus from the plasma is able to cause the neutrophil to form pseudopods. Once the neutrophil reaches its target in the tissue, it degranulates and then undergoes apoptosis. Signals from the interstitium cause this degranulation. Because lymph fluid is very similar in composition to interstitial fluid, perhaps it should not be surprising that degranulation is caused by lymph and not by plasma.

A similar experiment was reported by Dr. Edwin Deitch and his colleagues. (2004). His group examined lymph from animals that had received a burn injury, different from my studies where they received an inflammatory mediator (fMLP). He also analyzed neutrophil activation differently than I, opting to measure the activation of CD11b. He saw an increase in CD11b activation in neutrophils that had been exposed to lymph from the burned animals, but did not see as much activation from control lymph. The control lymph did, however, activate neutrophils significantly more than cells exposed only to cell culture medium. It is difficult to compare our experiments due to a number of different conditions between them. One similarity, however, is that the neutrophils exposed to lymph are activated more than controls in both of our experiments. This indicates that there could be toxic mediators in the lymph even under
3.4.2 Effect of lymph on cultured lymphatic endothelial cells

Though no statistically significant differences were generated with the measurements I made, it is apparent that the lymph fluid causes more cell death in the lymphatic endothelial cells than plasma. As mentioned in the Results, after 24 hours of exposure, all that remained of the RMLECs was cellular debris. Three very interesting points present themselves as a result of my observations. First, the lymph fluid is toxic to the cells during both healthy and inflammatory conditions. Second, there is large variability between animals. Finally, the lymph is actually killing lymphatic endothelial cells, the same cells that are constantly exposed to lymph in the body.

The first point affirms my hypothesis that the lymphatics are important in the removal of toxic mediators from the tissue space. Even during healthy conditions, cells in the interstitium release waste products into the interstitium. These waste products, in addition to cellular debris from cells that have died, can be toxic to cells. The lymphatics appear to play a vital role in removing these from the tissue. The inflammation induced in my experiments does not appear to have any effect on this toxicity. One would presume that more toxic mediators would be produced during an inflammatory state, though this appeared to not be the case in my experiment. A more sensitive test or a more robust inflammatory model would be necessary to confirm this result.

The large variability among animals is not surprising. As I mentioned in Section 1.7, the composition of lymph fluid can vary due to a number of factors. In the mesenteric lymphatic duct, the variability is due in large part to diet. While all the rats
in my study ate the same food pellets, the amount they had eaten in the hours leading up to the lymph fluid collection varied. In order for the lymph composition to be more consistent between rats, I could have allowed the rats to fast for the night before the experiment. I didn’t do this for two reasons. First, the amount of lymph fluid collected would have been less. Second, the composition of the lymph is more physiologically “relevant” if the rat is allowed to be in its normal state and not required to fast. However, even though the rates at which the lymph caused the RMLECs to die were different, after 24 hours, all of the endothelial cells were dead.

Finally, one of the most interesting aspects of these results is that the lymph fluid actually kills lymphatic endothelial cells. I had originally postulated that the lymphatic endothelium may have mechanism that protects it from the toxins in the lymph fluid, but this could not be confirmed, at least in the case of RMLECs in culture. If a protective mechanism does exist, it must occur in vivo and not in the cultured RMLECs. One possible hypothesis could be that a glycocalyx is present in vivo that is not present in culture. The glycobiology of the lymphatic endothelial cell has yet to be explored. Another explanation for this phenomenon may be that the lymph fluid was altered as it was collected. The only processing done to the fluid after it was collected was centrifugation in order to remove any cells (presumably lymphocytes) that may be in the lymph. It is possible that these cells may have a protective effect that keeps the endothelium from being damaged.

3.4.4 Nonesterified fatty acid concentration in the lymph

The concentration of NEFAs in fasting plasma in humans averages around 0.3
mM (Jouven et al, 2001). The concentrations in the plasma that I collected from the rat are over three times this value (1 mM). This may be due, in part, to the fact that the rats were not fasting at the time of plasma collection. The average concentrations of NEFAs in the lymph were over six times as large as in the rat plasma (6.3 mM). While one may expect that there would be a large concentration of NEFAs in the mesenteric lymphatic due to the absorption of fat from the intestine, this is physiologically inaccurate. Dietary fats are emulsified into micelles in the duodenum and broken down into free fatty acids by pancreatic lipase. The fatty acids enter the intestinal epithelial cell where they are resynthesized into triglycerides and incorporated into chylomicrons. The chylomicrons then enter the intestinal lymphatics and are transported through the mesenteric lymphatics, the thoracic duct, and into the blood stream (Purves et al, 1998).

The NEFA-C kit measures the concentrations of free fatty acids only and does not measure the concentrations triglycerides and chylomicrons. One limitation to the kit, however, is that it cannot determine whether or not a NEFA is bound to albumin. A possible study to determine whether the NEFAs contribute to the cytotoxicity of lymph is to add an abundant amount of albumin to the lymph before incubation with neutrophils or lymphatic endothelial cells.

3.4.5 Analysis of MMP-2 and MMP-9 activity on lymphatic endothelial cells

MMP-2 and MMP-9 are inflammatory markers in the endothelial cell. A qualitative analysis in the rat mesentery indicates that the endothelial cells of both the blood vasculature and the lymphatics express more MMP activity than the surrounding interstitium. Another sign of inflammation, oxygen free radical production, has been
observed in the lymphatics in the mesentery as well (DeLano et al, 2005). An interesting complement to this observation would be to determine MMP activity in the collected lymph fluid. In addition, a general protease assay could determine if the lymph contains more active proteases than the blood. The presence of proteases in the lymph could have two implications. First, the proteases could be very damaging to the endothelial lining. Second, the proteases may actually have a protective effect by degrading toxic peptides that may be present in the lymph. Further studies would be needed in order to determine if, in fact, toxic peptides are present in the lymph fluid, and what contribution they have to the cytotoxicity that I have observed here.

3.5 Conclusions

The composition and cytotoxicity of lymph is a subject that has been investigated only to a limited degree. My results indicate that the lymph fluid has the potential to be toxic to various cell types even under control conditions. The exact components of the lymph that are toxic, as well as the mechanism by which they damage other cells, is a very interesting and currently unanswered question. This information will be very valuable in the lymphatic field in general.

First, understanding the biochemical components of lymph gives insight into the fundamental functions of the lymphatics. While much is known about the different roles played by the lymphatics, previously unexplored roles may exist as well. My hypothesis in this Chapter is that the lymphatics are important in removing toxic mediators from the interstitium in order to decrease potential damage to interstitial tissue cells. When
the lymphatics are not functioning properly, as in the case of lymphedema, this ability to remove toxins is compromised.

Secondly, the composition of lymph is similar to the composition of interstitial fluid. If the lymphatic transport is ineffective, such as in lymphedema, these proteins and lipids present in the lymph fluid would then be present in high concentrations in the tissue. For example, after breast cancer removal, patients can endure severe lymphedema in the arm (Stanton et al., 2003). If, in fact, the lymphatics serve to transport cytotoxic mediators out of the tissue, these patients may have elevated levels of these mediators in the arm that would presumably cause an increase in inflammatory activity. Understanding the components of lymph fluid as well as their effect on surrounding tissue could lead to a new level of understanding of lymphatic function.
Chapter 4 Conclusions

4.1 Overall conclusions

During the past 10 years, lymphatic research has made significant progress. Still, compared to blood vessels, the lymphatic vessels are not very well understood. One particular area that is relatively unexplored is the effect of inflammation on the lymphatics. In this dissertation, I have begun to examine how inflammation affects both the barrier properties of the initial lymphatics and the composition of lymph fluid.

Our lab has shown previous evidence that fluid enters the initial lymphatics through the primary valve system (Trzewik et al., 2001). In skeletal muscle lymphatics, these valves allow microspheres with diameters up to 0.5 µm to freely pass into the lymphatics, while microspheres with diameters of 0.8 µm and larger do not enter the lymphatics. Acute inflammation in the rat spinotrapezius muscle causes this valve system to fail. This may be due to the actual valves not functioning correctly, or may be due to defects in the thin endothelial lining. Either way, fluid is not removed from the tissue as efficiently as in healthy lymphatics, and inflammatory mediators are allowed to remain in the tissue for a longer period of time.

My analysis of lymph fluid in Chapter 3 indicates that the lymphatics do, indeed, carry toxic mediators from the tissue, even in healthy animals. This fluid causes neutrophils to degranulate and causes cell death in cultured lymphatic endothelial cells. The mechanism by which this occurs remains unclear. I measured nonesterified fatty acid concentration in the lymph and found it to be elevated compared to the plasma. NEFAs
have been shown to be toxic to a variety of cells. Whether or not they play a role in the cytotoxicity that I observe is to be determined. In Section 4.2.2, I describe future studies that could be carried out to further examine this mechanism.

This result indicates that a defect in the lymphatics, such as one described in Section 1.2, could cause an increase in the level of cytotoxic molecules in the tissue. For example, Dr. Rakesh Jain has shown that the tumor-associated lymphatics do not function properly (Isaka et al., 2004). If they are not able to remove fluid from near the tumor, toxic mediators that are released from the tumor are able to build up, further damaging the tissue. Understanding the nature of this fluid in a variety of conditions will be vital in the analysis of lymphatic function and disease.

4.2 Future Work

4.2.1 Analysis of primary valve structure

In collaboration with Dr. Chris Neal at the University of Bristol in the United Kingdom, we are working on a three-dimensional reconstruction of an open primary lymphatic valve in the spinotrapezius muscle using serial sectioning and transmission electron microscopy (TEM). I harvested and prepared muscle samples using the procedure described below.

The spinotrapezius muscle was exteriorized as described in Section 2.2.2. While the muscle was secured across the viewing window, it was electrically stimulated to promote lymph formation and movement of primary valves. One electrode was placed at the distal tip of the muscle, while the other was placed near the proximal end. The
muscle was stimulated with 8 volts at 4 Hz by an electric stimulator (Grass Technologies, West Warwick, RI). After 5 minutes of stimulation, a fixative was applied on top of the tissue while the electrical stimulation continued. After 5-10 minutes, the voltage pulse ceases to stimulate visible muscle contraction. At this point, the stimulator is turned off. Small specimens of muscle that include larger arterioles and venules are cut out and placed in fixative at 4°C. The fixative used is Cacodylate Karnovsky, which consists of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.3. Select specimens were also fixed in Cacodylate Karnovsky with 10 mg/mL tannic acid. All specimens were post-fixed in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer. Specimens were stored in 0.2 M sodium cacodylate buffer and sent to Dr. Neal. He embedded, cut, and imaged the tissues using standard electron microscopy procedures.

Initial images and a serial section reconstruction have recently been given to us (Figure 4.1). This reconstruction is not complete in that it does not consist of true serial sections, but it illustrates the approach. Once serial sections are complete, Dr. Neal will produce a three-dimensional reconstruction showing the morphology and dimensions of an open valve at the ultrastructural level.

Single TEM images are consistent with those previously published by our lab (Skalak et al, 1984). They show some endothelial-endothelial junctions with junctional molecules connecting them, while others have overlapping endothelial cells with no junctional molecules. The junctions I am most interested in showing are those which exhibit the open valve morphology that we hypothesize is the major route by which fluid and small particles enter the initial lymphatics.
Figure 4.1  Serial section reconstruction of an open primary lymphatic valve. Panel A shows a representation of 8 different TEM sections of an open lymphatic valve. This is an incomplete reconstruction, as the 8 sections are not consecutive, but instead are a sample of images from a total of 27 sections. Our collaborator, Dr. Chris Neal, is currently working on a complete reconstruction of an entire open primary valve. Panel B gives a sample TEM image with the color scheme used in panel A.
The ability to generate a full reconstruction of an open primary valve will be a
important advancement in the study of the morphology of primary valves. While the
experiments I have conducted with the microspheres give an indication of the minimum
sized particle that can enter the lymphatics, it doesn’t give a description of the three-
dimensional shape and size of the valves. Current TEM images and whole-mount
immunohistochemistry also do not adequately describe the morphology of an open valve.
Determination of the three-dimensional structure is needed to further analyze many
aspects of the primary valves. This analysis could provide valuable information about
the actual mechanics of the opening and closing of the valves. Proper functioning of the
primary valves is very important in various lymphatic diseases and in tumor-associated
lymphatic metastasis. This technique can be extended to tissues from a disease state,
and comparisons can be made with these and healthy primary valves. In addition, the
examination and potential mathematical modeling of the primary valves could help
predict possible defects in the ability of the valves to provide unidirectional transport.

4.2.2 Further analysis of the toxicity of lymph fluid

A number of experiments need to be performed to identify the effects of
inflammation on the cytotoxicity of lymph and to further analyze the mechanisms by
which lymph fluid damages cells. I will present a selected list.

1. Collect lymph for a shorter duration as a control. A three-hour collection was
used in order to accumulate sufficient volume of lymph in order to carry out multiple
assays. However, this long period of time in which the animal’s intestines have been
manipulated may cause spontaneous inflammation.
2. Examine lymph fluid in an animal model that has chronic inflammation, such as the Zucker rat. This will provide insight into the reaction of lymphatic endothelium under conditions that are analogous to chronic human diseases such as diabetes and hypertension.

3. Analyze the effects of lymph fluid on vascular endothelial cells to compare to lymphatic endothelial cells. This will help resolve whether the lymphatic endothelial cells have a mechanism that protects them from the toxicity of lymph fluid that vascular endothelial cells may not have.

4. Let the cells in the lymph fluid remain instead of removing them. This will help to determine whether the cells present in the lymph reduce its toxicity.

5. Collect fluid from different lymph vessels. While it is difficult technically to collect lymph from various lymphatics throughout the rat, it will be interesting to collect a small amount from pre-nodal lymphatics as well as at the thoracic duct just before the lymph returns to the blood stream to compare its cytotoxicity before and after passage through a lymph node.

6. Evaluate cell death of the lymphatic endothelium *in vivo*. This can be accomplished in the mesentery where lymphatics are easily visible using intravital microscopy. Inflammation can be induced and the cell death can be evaluated using PI in the living tissue. This will help in elucidating the differences between how cultured LECs and LECs *in vivo* are affected by lymph fluid.

7. Add albumin to lymph fluid before exposure to neutrophils and LECs. This will bind any NEFAs present in the fluid and will help determine if they are responsible for the cytotoxicity that I have demonstrated.
8. Run further assays to determine concentrations of other potentially toxic mediators such as proteases and lipases. Our laboratory has completed extensive work examining proteins such as these and we hypothesize that they are a major source of cell activation and cell death in inflammation.
Chapter 5 References


49. Penn AH, Hugli TE, Schmid-Schonbein GW: Pancreatic Enzymes Generate


