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Bearden, Melissa K., Lymphatic Pump Treatment Mobilizes Leukocytes from the Gastrointestinal Associated Lymphoid Tissue. Master of Science (Microbiology and Immunology), December, 2008, 39 pp., 4 tables, 5 illustrations, references, 40 titles.

Lymphatic flow and the migration of leukocytes are important to maintaining health. One treatment used to improve lymph flow is abdominal lymphatic pump treatment (LPT), which is thought to increase lymph return from the abdominal area. Previous research has shown the LPT increases lymph flow and leukocyte numbers in thoracic duct lymph, but further investigation into the specific cell types and their sources are needed. Through sampling the thoracic and intestinal lymph ducts, as well as the mesenteric lymph nodes (MLN), it was seen that there is an increase in cell output in the thoracic and intestinal ducts and the MLN during LPT as compared to baseline. This increase in cell output may help to augment the immune response during infection.

LYMPHATIC PUMP TREATMENT MOBILIZES LEUKOCYTES FROM THE GASTROINTESTIAL ASSOCIATED LYMPHIOD TISSUE

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Science

University of North Texas
Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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Fort Worth, Texas

December 2008

ACKNOWLEDGEMENTS

I would like to thank my mentor, Lisa Hodge, Ph.D. for her help in planning, reviewing and polishing this thesis. Her many hours are greatly appreciated. I would also like to thank my committee members, Jerry Simecka, Ph.D., H. Fred Downey, Ph.D., and Tina Machu, Ph.D. for their suggestions and corrections, which help to make this thesis a success.

I also wish to thank my fellow students in the lab, Monica Bellard and Jamie Huff, as well as research assistant Artur Schander, for their help with experiments and their support.

Finally, I want to thank my parents, Richard and Brenda Bearden, without whose time, patience, and encouragement none of this would have been possible.

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	Baseline	*LPT*	*Recovery*
Cell Concentrations (x 10^6 total leukocytes/ml)			
Neutrophils	0.24 ± 0.05	0.79 ± 0.23 *	0.43 ± 0.06
Monocytes	0.32 ± 0.07	0.92 ± 0.27 *	0.49 ± 0.10
Total Lymphocytes	9.34 ± 1.88	19.12 ± 4.24 **	13.4 ± 2.59
$CD4^+$ T cells	4.33 ± 0.46	10.8 ± 1.45 **	5.61 ± 1.26
$CD8^+$ T cells	1.96 ± 0.54	4.13 ± 1.11 **	2.23 ± 0.57
IgA^+	0.82 ± 0.17	2.22 ± 0.25 ††	0.79 ± 0.18
IgG^+	1.61 ± 0.44	3.26 ± 1.11 *	0.98 ± 0.17

Table 1. Lymphatic Pump Treatment Increases Leukocytes in Thoracic Duct Lymph.
Lymph was collected under 1) resting (Baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery).
Data are means ± SEM x 10^6 leukocytes/ml.
* ($P < 0.05$) denotes increase respective to baseline
** ($P < 0.05$) denotes increase respective to baseline and recovery
†† ($P < 0.001$) denotes increase respective to baseline and recovery
N = 6

	Baseline	*LPT*	*Recovery*
Cell Concentrations (x 10^6 total leukocytes/min)			
Neutrophils	0.27 ± 0.12	3.67 ± 0.96 **	0.75 ± 0.23
Monocytes	0.34 ± 0.14	4.24 ± 1.18 *	0.91 ± 0.25
Total Lymphocytes	10.32 ± 4.53	81.1 ± 22.2 **	18.3 ± 6.62
$CD4^+$ T cells	3.25 ± 0.62	43.7 ± 5.57 **	12.4 ± 4.74
$CD8^+$ T cells	1.24 ± 0.37	16.3 ± 4.12 *	5.31± 2.00
IgA^+	0.65 ± 0.18	9.02 ± 0.86 ††	1.48 ± 0.53
IgG^+	1.06 ± 0.21	13.4 ± 4.81 *	1.95 ± 0.45

Table 2. Lymphatic Pump Treatment Increases Leukocyte Flux in Thoracic Duct Lymph. Lymph was collected under 1) resting (Baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery).
Data are means ± SEM x 10^6 leukocytes/min.
* ($P < 0.05$) denotes increase respective to baseline and recovery
** ($P < 0.01$) denotes increase respective to baseline and recovery
†† ($P < 0.001$) denotes increase respective to baseline and recovery
N = 6

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	Baseline	*LPT*	*Recovery*
Cell Concentrations (x 10^6 total leukocytes/ml)			
Neutrophils	0.27 ± 0.06	1.87 ± 0.41 †	0.99 ± 0.24
Monocytes	0.25 ± 0.05	1.11 ± 0.25 *	0.56 ± 0.12
Total Lymphocytes	5.89 ± 0.66	16.1 ± 3.80 *	9.22 ± 1.75
$CD4^+$ T cells	2.76 ± 0.32	6.60 ± 1.80 **	3.69 ± 0.87
$CD8^+$ T cells	0.56 ± 0.07	1.42 ± 0.43 *	0.82 ± 0.19
IgA^+	0.37 ± 0.15	1.02 ± 0.56 *	0.62 ± 0.28
IgG^+	0.24 ± 0.07	0.72 ± 0.25 *	0.39 ± 0.22

Table 3. Lymphatic Pump Treatment Increases Leukocytes in Intestinal Duct Lymph. Lymph was collected under 1) resting (Baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery).
Data are means ± SEM x 10^6 leukocytes/ml.
* ($P < 0.05$) denotes increase respective to baseline
** ($P<0.05$) denotes increase respective to baseline and recovery
† ($P< 0.01$) denotes increase respective to baseline
N = 7

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	Baseline	*LPT*	*Recovery*
Cell Concentrations (x 10^6 total leukocytes/min)			
Neutrophils	0.09 ± 0.02	1.82 ± 0.58 †	0.50 ± 0.18
Monocytes	0.09 ± 0.02	1.11 ± 0.36 †	0.36 ± 0.14
Total Lymphocytes	1.90 ± 0.27	14.4 ± 5.19 *	6.68 ± 4.06
$CD4^+$ T cells	1.26 ± 0.32	8.40 ± 3.00 **	2.74 ± 1.03
$CD8^+$ T cells	0.25 ± 0.07	1.72 ± 0.48 **	0.64 ± 0.26
IgA^+	0.24 ± 0.12	3.54 ± 2.95 *	0.61 ± 0.42
IgG^+	0.12 ± 0.05	1.70 ± 1.12 *	0.45 ± 0.34

Table 4. Lymphatic Pump Treatment Increases Leukocyte Flux in Intestinal Duct Lymph
Lymph was collected under 1) resting (Baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery).
Data are means ± SEM x 10^6 leukocytes/ml.
* ($P < 0.05$) denotes increase respective to baseline
** ($P<0.05$) denotes increase respective to baseline and recovery
† ($P< 0.01$) denotes increase respective to baseline and recovery
N = 7

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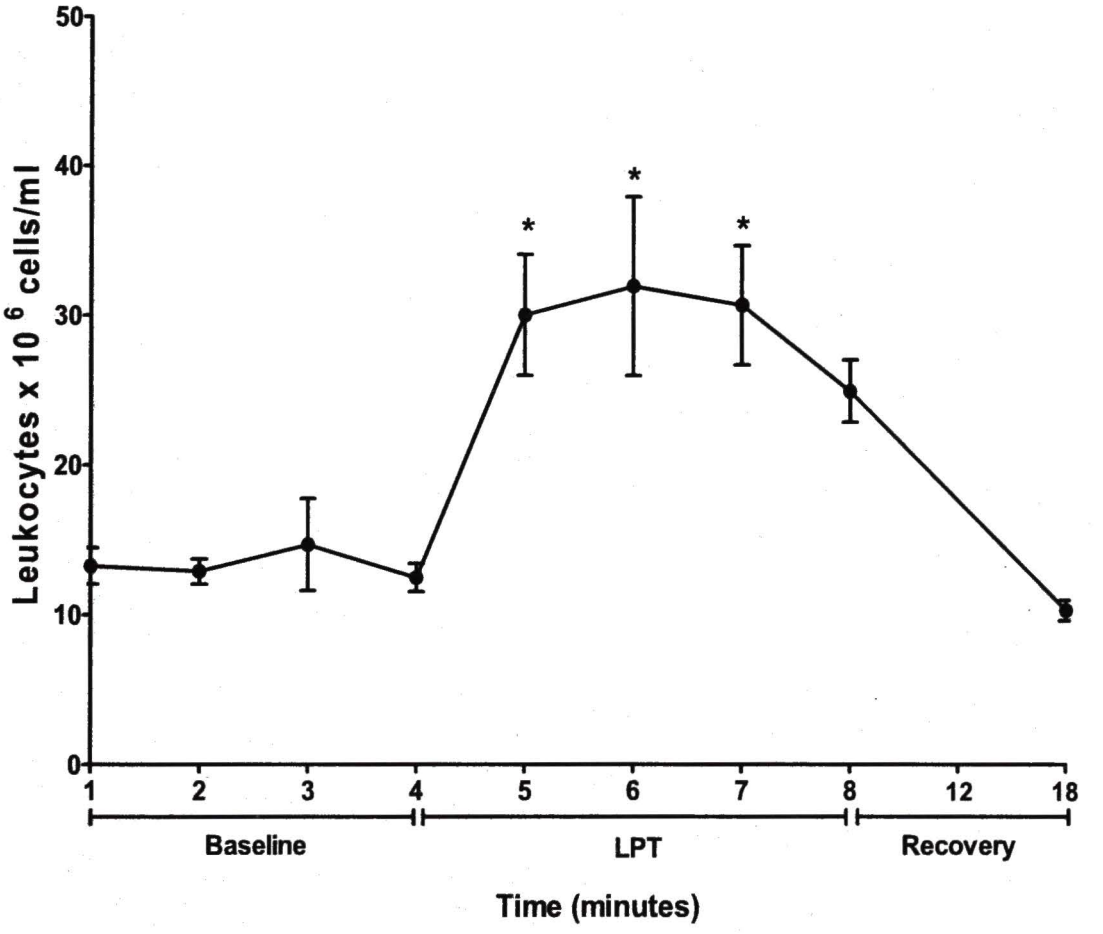

Figure 1. Abdominal LPT significantly increases total leukocyte count in thoracic duct lymph over baseline.
Data are means ± SEM x 10^6 leukocytes/ml.
* ($P < 0.05$)
N = 6

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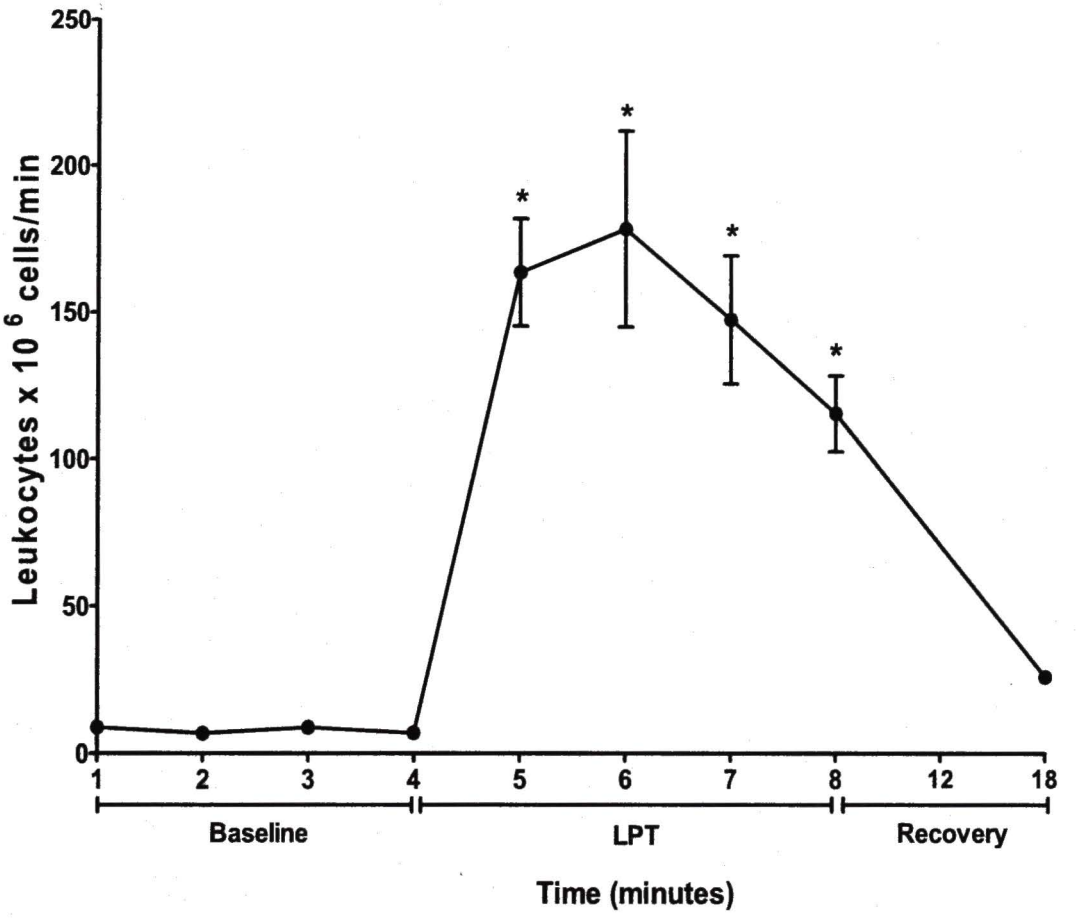

Figure 2. Abdominal LPT significantly increases total leukocyte flux in thoracic duct lymph over baseline.
Data are means ± SEM x 10^6 leukocytes/min.
* ($P < 0.01$)
N = 6

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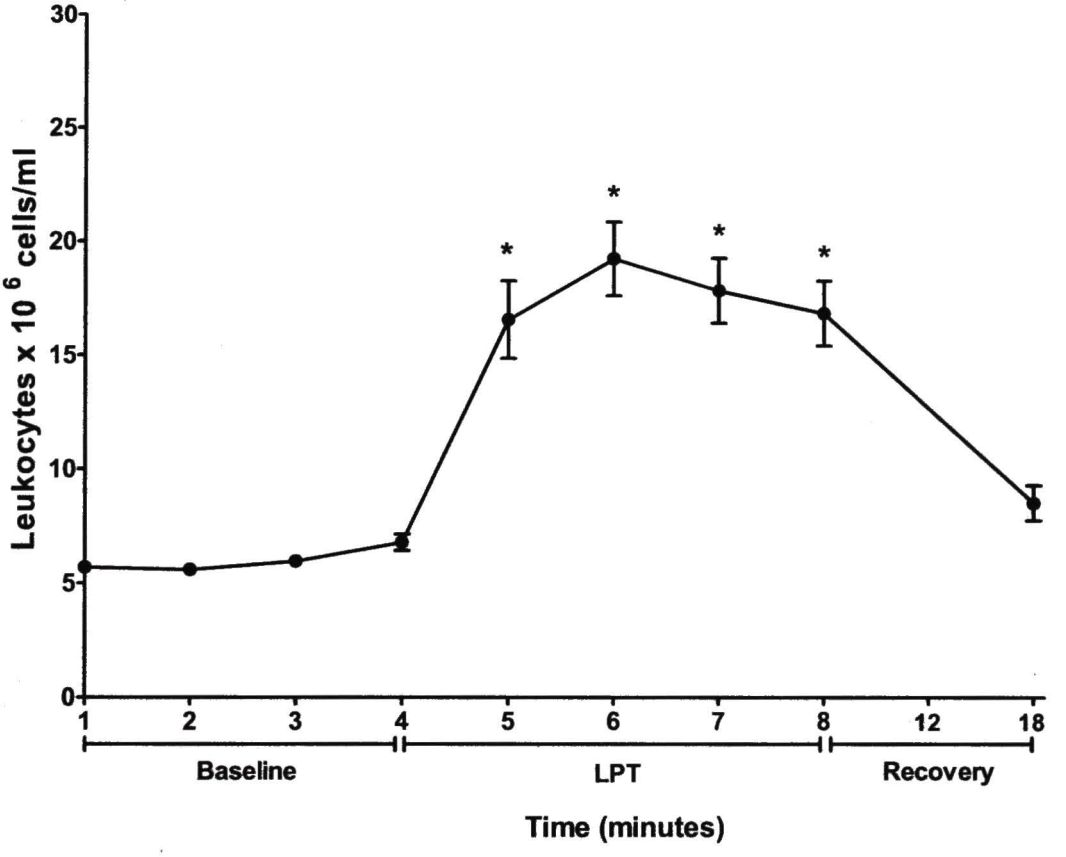

Figure 3. Abdominal LPT significantly increases total leukocyte count in the intestinal duct lymph over baseline.
Data are means ± SEM x 10^6 leukocytes/ml.
* ($P < 0.01$)
N = 7

x

x

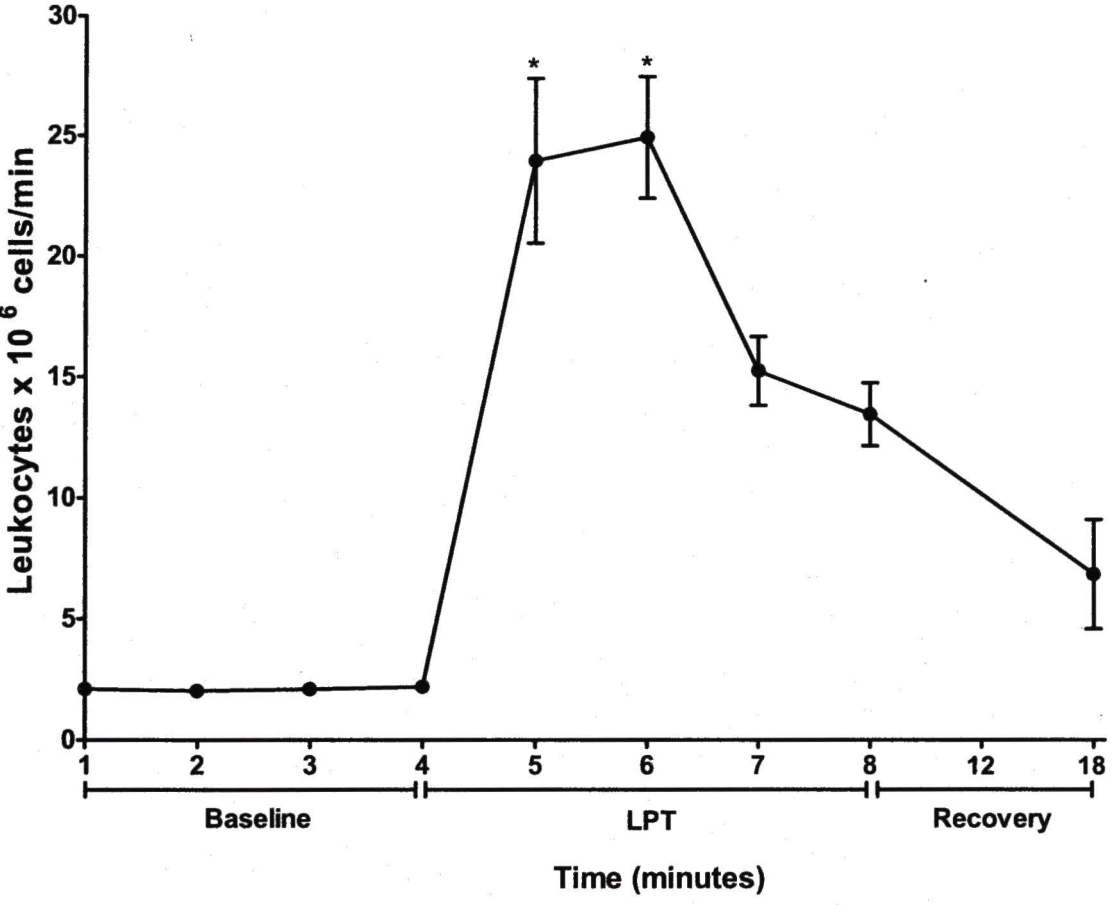

Figure 4. Abdominal LPT significantly increases total leukocyte flux in intestinal duct lymph over baseline.
Data are means ± SEM x 10^6 leukocytes/min.
* ($P < 0.01$)
N = 7

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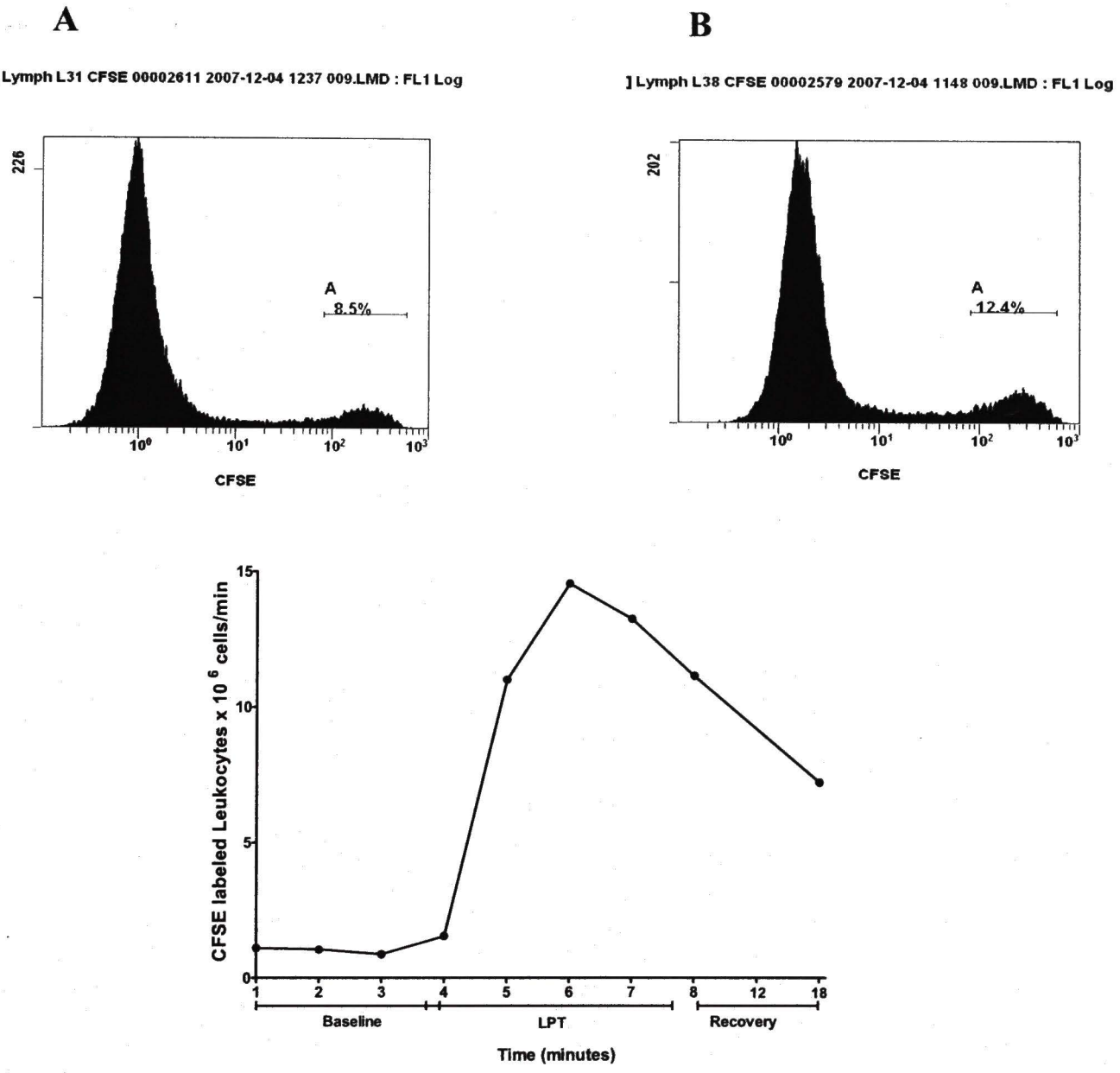

Figure 5. Abdominal LPT increases the percentage of CFSE-stained leukocytes from the MLN in the thoracic duct. (A) Four minutes baseline, (B) Four minutes LPT
Additionally, abdominal LPT increases CFSE-labeled leukocyte flux in the thoracic duct lymph. N = 1

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CHAPTER I

INTRODUCTION AND BACKGROUND

Lymphatics

As blood circulates, the plasma component seeps through the walls of capillaries and permeates the tissues. This interstitial fluid bathes tissue cells and contains metabolic wastes and toxins, as well as leukocytes that have entered the interstitial space. Much of this fluid returns to the blood through the venules, but the fluid that does not flows into lymphatic vessels (1). Lymphatic vessels lack tight junctions between endothelial cells. When skeletal muscle contracts, lymphatic vessels are distorted and spaces are opened between endothelial cells that permit protein, particles, and cells in the interstitial fluid to enter the lymphatics (2). The heart does not pump lymph through the lymphatic system. Instead, the low pressure flow of lymph, which is unidirectional, is caused by compression from the body's muscles, and by intrinsic lymphatic contractility under autonomic control (1, 3).

Afferent lymphatic vessels carry lymph from the tissues to highly organized lymphoid structures known as lymph nodes (1, 4). Efferent lymph flow carries lymph away from the lymph nodes and into the periphery (4). The thoracic duct is the largest lymphatic vessel in the

body. Most of the smaller lymph vessels from the body flow into the thoracic duct. Therefore, the fluid that is lost from the blood and travels through the lymphatic system then returns to the circulation (1).

Lymphedema is a disease that is caused by a failure in the transport of lymphatic fluid to the venous circulation, and leads to interstitial volume overload and lymph congestion. It most often occurs in the extremities (5, 6). Primary lymphedema occurs when there is an inherent defect in lymphatic vessels, while secondary lymphedema occurs because of acquired damage, such as a surgery, a tumor, or the removal of lymph nodes (6).

The population most at risk for secondary lymphedema is breast cancer patients, with an estimated 30% of patients developing some level of lymphedema of the arm (7, 5). The level of lymphedema relates to the type of surgery, radiation, and lymph node dissection or removal the patient had as treatment, but all levels lead to physical and emotional stress (8).

Symptoms of both primary and secondary lymphedema include swelling, heaviness, and weakness, as well as decreased flexibility and mobility of the affected limb. In addition to these symptoms, there is a risk of tissue damage and recurrent infections, such as cellulitis and lymphangitis, at the sites of edema due to skin breakdown (6). Therefore, therapies that improve lymphatic drainage are important to treat the symptoms of both primary and secondary lymphedema.

Therapies to improve lymphatic drainage

There are several treatments to improve lymph drainage and manage lymphedema. Elevation is probably the most common, and allows lymph drainage to flow unimpeded. Exercise enhances lymph flow, and is considered essential in the treatment of lymphedema. Compression bandaging and garments can help remove excess lymph that has accumulated, as

well as decrease interstitial fluid production to stop the progression of edema (5). In addition to bandaging and garments, pneumatic compression pumps can place intermittent outward force on areas of lymphedema, stimulating lymph flow and drainage. Pharmaceutical treatments of the past, such as diuretics, have not been shown to be helpful in managing lymphedema, but newer treatments with benzopryones, such as Coumarin, have been shown to be effective (6).

Manual therapies are also used to effectively treat lymphedema. Manual lymph drainage is a massage technique to siphon lymph fluid away from congested areas, and allow lymph to drain normally (6). Osteopathic physicians have developed a group of manipulations known as the lymphatic pump techniques that are designed to enhance lymph flow, which can also help treat lymphedema (9).

Treatments to increase lymph flow and lymphatic drainage can help to reduce the swelling and pain associated with lymphedema. When this congested fluid is dispersed, toxins and bacteria that accumulate are also dispersed, helping to decrease the possibility of secondary infection, which can make lymphedema worse (6). Importantly, relieving lymph edema can allow the redistribution of lymphocytes to areas where they are needed for immune responses.

Lymphocyte recirculation

Afferent lymphatic vessels carry antigen-bearing leukocytes from the tissues where antigen gains entry to the lymph nodes (1, 4). Lymph nodes are the site of B cell activation and proliferation in response to a specific antigen, and the site of T cell interaction with antigen presenting cells such as dendritic cells and macrophages, which leads to T cell activation. Efferent lymph flow carries activated lymphocytes away from the lymph nodes and into the periphery, where they can then perform their effector functions (4).

Afferent lymph carries only about 10% of the cells seen in the efferent lymph, and only about 5% of the cells seen in the efferent lymph are produced within the lymph node. The remaining 85% of efferent lymph cells come from the blood circulation into the lymph nodes via the high endothelial venules (HEV) (10). The extravasation of lymphocytes from the blood to the lymph nodes via the HEV is regulated by the expression of cell adhesion molecules on the surface of both the lymphocytes (integrins) and the HEV (ICAMs) (1). These molecules help determine the specificity of lymphocyte migration, and also help direct the tissues to which lymphocytes will traffic (10). The HEV are such a profound source of lymphocytes entering the lymph nodes, it has been estimated that 1.4×10^4 lymphocytes/second extravasate through the HEV into a single lymph node (1).

In the 1950's, pioneering studies of lymphocyte recirculation were performed by Gowans. In a rat model, when lymph was continuously drained from the thoracic duct, cell output remained constant for about the first 2 days, and then fell sharply on days 3 – 4. Output leveled off on day 5 to 20 – 30% of the initial value (11). Therefore, cell output could not remain constant when lymph was depleted from of lymphatic circulation. Gowans then designed a pump to reintroduce the drained lymph and lymphocytes into the femoral vein at the same flow rate as in the thoracic duct. When cell-free lymph was introduced, the output from the thoracic duct did not increase. However, when viable lymph and lymphocytes were continuously re-infused, the output from the thoracic duct remained almost constant for 4 days. This led to the conclusion that the continuous entry of viable lymphocytes from the blood may be necessary for constant cell output in the thoracic duct.

In a continuing study, Gowans drained thoracic duct lymphocytes of donor rats, radio-labeled them, and intravenously injected them into recipient rats (12). If the recipient's thoracic

duct had been drained prior to the transfer, transfusion restored the low levels of thoracic duct output in the recipient. This study showed that the infused labeled cells migrated to the thoracic duct at about 4 hours after transfusion, and demonstrated that lymphocytes can travel from the blood to the lymph. Importantly, this study led to the conclusion that the output of lymphocytes from the thoracic duct is maintained by the recirculation of lymphocytes from the blood.

In an additional study, Smith and Ford harvested thoracic duct lymphocytes from rats, radio-labeled them, and then passaged them through intermediate rats in order to establish an actual recirculating population of lymphocytes (13). The lymphocytes were then intravenously injected into recipient rats, and monitored for their appearance in lymphoid and non-lymphoid tissues. Results revealed that within 5 minutes of injection, 40% of labeled cells were in the blood, 40% were in the lung, and 10% of were in the spleen. Five – 30 minutes post injection the amount of labeled lymphocytes fell in the lungs and blood, but rose in the spleen, various lymph nodes, and Peyer's patches. During the 18 hours after injection, more labeled lymphocytes were found in the mesenteric lymph nodes than any other lymph nodes monitored, demonstrating that thoracic duct lymphocytes preferentially traffic to the mesenteric lymph nodes. Lymphocytes tend to preferentially recirculate back to their source tissue (10), so this indicates that the mesenteric lymph nodes may be a source of thoracic duct lymphocytes.

Furthermore, studies have shown there are two distinct pools of recirculating lymphocytes. For example, when cells were harvested from lymph draining the subcutaneous lymph nodes, radio-labeled, and intravenously reintroduced they were more readily found in the efferent lymph of the subcutaneous lymph nodes than the mesenteric lymph nodes. Likewise, when cells of the intestinal lymph duct were harvested, radio-labeled, and reintroduced i.v. they were found in higher concentrations in the lymph draining the mesenteric lymph nodes than the

lymph draining the subcutaneous lymph nodes (10). Again, this study suggests that lymphocytes traffic, or home, back to their original tissue source after they circulate.

In other early lymphatic studies, Mann and Higgins cannulated both the thoracic and intestinal lymphatic ducts in rats. They saw that the output of lymphocytes from the intestinal lymph duct was almost equal to the output of the thoracic lymph duct (14). Therefore, they deduced that thoracic duct lymphocytes are gathered from a "pool" drained by the intestinal duct, which drains the gastrointestinal mucosa, including the mesenteric lymph nodes and Peyer's patches.

In a study where thoracic duct lymphocytes were harvested, radio-labeled, and then injected intravenously, after 24 hours most of the cells were found in the intestine and in the mesenteric lymph nodes (15). Collectively, these studies suggest that thoracic duct lymphocytes are gathered from pools of lymphocytes in the gastrointestinal mucosa, and that these lymphocytes home back to their tissue source after being released into circulation.

There is further evidence that the mesenteric lymph nodes (MLN) are a source of lymphocytes that move through the lymphatics and then return to systemic circulation (16). When the MLN were fluorescently labeled *in vivo*, a maximum number of labeled cells were found in afferent lymph 48 hours after labeling. Afferent lymph is gathered from components seeping from blood circulation in the tissues (2). However, when the MLN were labeled and the intestinal lymph duct was catheterized, a maximum number of labeled cells were seen in the intestinal lymph after about 4 hours, but almost no labeled cells were found in the afferent lymph up to 72 hours after labeling. This suggests that cells from the MLN enter thoracic duct circulation via the intestinal lymphatics.

Collectively, these studies indicate that lymphocytes circulate between the lymph and the blood, and once lymphocytes are released into circulation, in the absence of inflammation, they tend to home back to their original tissue source. Additionally, thoracic duct lymphocytes are most likely gathered from the gastrointestinal mucosa, since they preferentially home to gastrointestinal tissues after recirculation.

Mucosal immunity and tissue specific homing

The mucous membranes, which line the respiratory, digestive, and urogenital tracts, are a major site of pathogen invasion (1). Mucosa-associated lymphoid tissue (MALT) is a loosely organized lymphoid tissue found at these sites. Specifically, the lymphoid tissue associated with the respiratory epithelium is known as the bronchus-associated lymphoid tissue (BALT), and that associated with the digestive tract epithelium is referred to as the gastrointestinal-associated lymphoid tissue (GALT). GALT includes the lamina propria, Peyer's patches, and the MLN (1, 2).

The lamina propria, which lies under the small intestine epithelia, contains macrophages, activated T helper cells, B cells, and plasma cells. Peyer's patches are lymphoid follicles within the submucosa where T cells and B cells can interact with pathogens. Peyer's patches can develop into secondary lymphoid follicles. M cells bring small samples of foreign antigen from the lumen of the gut into the GALT, where dendritic cells can then activate T cells and B cells (1).

Activated B cells within the GALT, and the collective MALT, produce IgA, a dimer immunoglobulin that is linked by the J chain. When IgA is secreted from plasma cells in the submucosa, the J chain binds to the poly-Ig receptor that is expressed on the basolateral surface of the mucosal epithelia. After binding, the receptor-IgA complex is transported across the

epithelium to the lumen via receptor-mediated endocytosis. The poly-Ig receptor is enzymatically cleaved and becomes the secretory component, which is bound to and released with the polymeric IgA. The secretory component helps mask sites on the IgA that are susceptible to proteolytic cleavage, protecting the IgA from degredation (1, 17).

After antigenic stimulation, effector and memory lymphocytes migrate back to the sites of antigen entry to mount immune responses, and this migration is determined by the cell surface expression of tissue-homing and chemokine receptors on lymphocytes and corresponding ligands in the postcapillary venules of specific tissues (18). Naïve lymphocyte migration into secondary lymphoid organs is through postcapillary HEV. The initial adhesion is brought about by the interaction of L-selectin, which is present on naïve lymphocytes, with ligands present on the HEV. The migration of lymphocytes into secondary lymphoid organs is a three step process, consisting of initial tethering or rolling, activation, and then finally firm adhesion (1, 18).

Dendritic cells and other microenvironment factors play a role in the homing of circulating lymphocytes to different secondary lymphoid organs and tissues. Naïve lymphocytes that encounter their specific antigen in the Peyer's patches or MLN upregulate expression of the integrin $\alpha 4\beta 7$ that interacts with the ICAM MadCAM-1, which is found in the mucosal tissue, and the chemokine receptor CCR9, which responds to CCL25, both of which direct recirculation of lymphocytes back into the GALT. Other factors such as retinoic acid and IL-4 have also been shown to cause the gut-homing of cells (18).

Within the lung, as within the GALT, there are adhesion molecules and chemokines that influence lymphocyte entry. The adhesion molecules LFA-1 and PSGL-1 and the chemokine-chemokine receptor combination CCL5-CCR5 have been shown to direct lymphocyte entry into

the lung (19). There have been several studies showing migration of lymphocytes between the GALT and the lung or BALT, as well as between the GALT and other mucosal sites.

To test the distribution of plasma cells after oral immunization, mice were orally immunized with ferritin. A 5:1 ratio of IgA versus IgG and IgM plasma cells were seen in the small intestine, mammary gland, salivary gland, and respiratory tract. In the spleen and peripheral lymph nodes, IgG and IgM plasma cells outnumbered IgA plasma cells (20). Therefore, this study demonstrates that IgA is the predominate antibody in mucosal tissues.

In another immunization model, both donor and recipient rats were immunized with intra-peritoneal (i.p.) injection of TNP-KLH with Freund's complete adjuvant, and then donors were intra-intestinally (i.i.) challenged and recipients were intra-tracheally (i.t.) challenged with TNP-KLH. Seven days later donors and recipients were challenged again in the same manner, and after three days thoracic duct lymphocytes (TDLs) were harvested from donors, CFSE-labeled, and transferred intravenously into recipients. Results showed an increased number of CFSE-labeled lymphocytes from i.i. challenged donors in the in the BALT of i.t. challenged recipients than in any controls (21). This suggests that TDLs from i.i immunized donors preferentially migrate into the BALT of i.t. challenged recipients.

An additional study immunized rats with cholera toxin by intra-colon, intra- duodenum, or intra-trachea injection, then challenged at the same and other mucosal sites. After five days, anti-toxin containing lymphocytes were found at both the challenge site and at distant mucosal sites as well. In another experiment, donor rats were primed by intra-colon injection, then donor TDLs were transferred to rats that had been challenged in the jejunum. Secondary anti-toxin responses were seen in the jejunum of the recipient rats (22), demonstrating that lymphocytes primed at a primary mucosal site can migrate to other mucosal sites upon challenge. This is a

property of the common mucosal immune system, which involves the trafficking of cells between different areas of the mucosal immune system (23).

In a separate study, donor rats were immunized by intra-Peyer's patch injection of non-typable *Haemophilus influenzae* (NTHI). TDLs were then harvested from the donors and given intravenously over four days to recipients that were sensitized intra-tracheally with NTHI. They found increased bacterial clearance in the TDLs recipients, as compared to recipients that received non-immune cells or rats that had no transfusion, and this was attributed to the homing of primed GALT lymphocytes, mainly T cells, from the donors to the respiratory tract of recipients (24). This study again shows that primed mucosal immune cells can migrate to other mucosal sites during a challenge.

A redistribution of primed effector or memory cells from a major source of lymphocytes like the GALT to the lung could be beneficial during a pulmonary infection such as pneumonia. If any cells from the GALT are antigen-specific for the invading pathogen, this could promote rapid interaction with the pathogen and a rapid immune response.

Osteopathic Manipulative Medicine

As the migration and circulation of lymphocytes are important to immune responses, and congestion of lymph flow can cause disease, Osteopathic physicians believe that removing obstructions to blood and lymph flow is one of the most effective ways to promote health (9). A collective group of manipulations known as the lymphatic pump techniques are designed to enhance lymph return from distal areas of the body. These techniques include the thoracic lymphatic pump, the liver, splenic, and pancreatic pumps, the pedal pump, and the abdominal lymphatic pump (9, 25). The increased lymph flow that occurs from these treatments is thought

to accelerate removal of cellular wastes, toxins, and bacteria from the interstitial fluid and also reduce edema (25).

Improving lymphatic flow to improve health is a technique that has been used by osteopaths since the founding of the profession. During the 1917-1918 influenza pandemic, the average mortality rate reported by allopathic physicians after influenza infection was 5-6%, and the mortality rate from influenza complicated by pneumonia was 25%. Osteopathic physicians during that time, treating patients with manual manipulation that included precursors to the lymphatic pump treatments, reported an influenza mortality rate of 0.25%, and a mortality rate of influenza complicated by pneumonia of 10% (26). This was the beginning of the anecdotal evidence that osteopathic manipulation, particularly those that stimulate lymphatic flow, can treat infection and disease to improve health.

In 1932, in one of the first studies to examine the effects of the lymphatic pump techniques on the immune response, Castillo and Ferris-Swift performed the splenic pump on 100 outwardly healthy individuals (27). Blood was taken from subjects as a baseline before treatment and at either 5 minutes and 30 minutes or 10 minutes and 60 minutes following treatment. They found that in 81% of subjects, the total leukocyte count was increased at some time point following treatment. When looking at differential counts, they found that neutrophils were increased in 76% of subjects after treatment. The opsonic index was also increased in 84% of subjects after treatment.

In 1934, the authors examined the effects of splenic pump on patients with acute infectious disease (28). Again, blood was taken as a baseline sample before treatment, and at two time points following treatment, varying from 5 minutes to 2 hours. It was seen that 73% of patients saw an increase in total leukocyte counts. In 84% of patients there was an increase in

mature neutrophils, as measured by a shift to the right of the Arneth index. There was also an increase in the opsonic index, agglutination, and bacteriolytic properties of the serum in patients following treatment.

Recently, further statistics were applied to the 1934 findings of Castillo and Ferris-Swift (29). Through this analysis it was seen that there was a statistical increase in total leukocyte count over baseline at 5 and 30 minutes post-treatment. A significant shift to the right of the Arneth index was also seen. They also confirmed enhanced agglutination, opsonic index, and bacteriolytic power following treatment.

During the 20th century, several studies were performed to examine the effect of osteopathic manipulation, including lymphatic pump, on assorted respiratory diseases. From 1961 to 1963, the effects of manipulation, antibiotics, or the two in combination were studied in children with a variety of respiratory illnesses (30). All children received supportive therapy, including fluids, pain medication, etc. Antibiotics were given at appropriate doses every 4-6 hours, and manipulative treatments were performed every 3-6 hours depending upon the patient's age. It was seen that while antibiotics alone promoted a quicker recovery, as measured by length of hospital stay, than manipulation alone, a combination of the two therapies resulted in a more rapid recovery than either treatment alone.

In the mid 1960's, the effects of thoracic pump on lower respiratory disease were examined (31). Patients were either part of a control group who received antibiotics, expectorants, sedatives, and fluids as needed, or an experimental group that received the same treatment, along with thoracic pump for 5 minutes, 3 times a day for 4-5 days. It was seen that thoracic pump-treated patients had a higher rate of sputum production and a shorter duration of

cough than control patients. Patients in the experimental group also had an increase in vital capacity at 5 days post-admission as compared to controls.

From 1996-1998, Noll et al examined the effects of manipulation, including thoracic lymphatic pump, on elderly patients hospitalized with acute pneumonia (32). Control patients received a standardized "light touch" sham protocol, while experimental patients received a standardized manipulation protocol. Each treatment was 10-15 minutes, twice a day, 7 days a week until an endpoint of hospital discharge, ventilator-dependent respiratory failure, or death was reached. The study showed that the experimentally treated group had a significantly shorter length of hospital stay, IV antibiotic use, and total antibiotic treatment as compared to the control group. It was also seen that on day 2 and 5 of the time course manipulation patients had an increase in temperature while control patients had a decrease. This could be indicative of an enhanced immune response.

A pilot study examined the effect of lymphatic pump treatment during hepatitis B vaccination (33). All subjects were given the hepatitis B vaccine at 0, 5, and 25 weeks. In addition, subjects in the experimental group were given patient-assisted and passive thoracic lymphatic pump as well as splenic pump on day 0, and on five days over the next two weeks. Results showed that the experimental group's average hepatitis B antibody titers were consistently higher from week 6 until the end of the study, and that they were significantly higher at week 25. At week 13, a statistically higher percentage of the experimental group achieved protective antibody titers as compared to the control group, and a consistently greater proportion of experimental subjects achieved protective antibody titers from weeks 5-18.

In a separate experiment, vaccination for pneumococcal pneumonia was followed by either no treatment or the thoracic lymphatic pump treatment for 5 minutes, twice a day for one

week (34). On day 14, it was seen that the treatment group had a significantly higher antibody response to three pneumococcal polysaccharides than the control group, indicative of a greater immune response.

While most studies of lymphatic pump treatment have been performed with humans, animal models have also been helpful in determining the treatment's effects. The first documented animal study of a pump technique was by Lane, who tested the effects of splenic pump manipulation on rabbits (35). Two rabbits were given three injections of sheep corpuscles over three days. After a week of rest, blood was taken for a baseline measurement, and then the animals were given varying lymphatic pump treatment regimens. It was seen that the treatment increased antibodies against the sheep corpuscles as compared to baseline.

To examine the effects of thoracic lymphatic pump treatment on lymph flow in anesthetized rats (36), a baseline blood sample was taken, and then a fluorescent probe was injected into the hind leg. Further blood samples were taken at 2, 4, 6, 9, 12, and 15 hours after the probe was injected. The control group received no treatment, while the experimental group was given 5 minutes of thoracic lymphatic pump treatment every hour until they awakened, typically at about 4 hours. In the first 9 hours, there was a significant increase in the amount of lymph flow, as measured by probe appearance in the blood, in the experimental group as opposed to the control group.

Recently, Knott et al measured the effect of abdominal LPT, thoracic LPT, and physical activity on thoracic duct lymph flow in a conscious canine model (37). After catheterization of the thoracic lymph duct, lymph flow was measured at baseline and during 30 seconds of either abdominal or thoracic LPT. In the physical activity experiments, lymph flow was measured during 90 seconds of 3 mph, 0% grade treadmill exercise.

The study found that abdominal LPT significantly increased thoracic duct lymph flow over baseline by approximately 4-5 ml/min. Thoracic LPT also caused a significant increase in thoracic duct lymph flow from baseline by about 2-3 ml/min. Physical activity caused the greatest statistical increase in thoracic duct lymph flow, by about 5-6 ml/minute over baseline. This was the first study to demonstrate that the lymphatic pump treatments enhance lymph flow in the thoracic duct.

As abdominal LPT had a greater effect on thoracic duct lymph flow than thoracic LPT, Hodge et al chose to use abdominal LPT in a study examining leukocyte count and flux in the thoracic duct lymph (38). Using an anesthetized canine model, lymph flow was measured and lymph samples were collected during baseline and 8 minutes of LPT.

Results showed that total leukocyte count significantly increased during application of LPT. Using differential staining and flow cytometry, it was seen that neutrophil, macrophage, T cell, and B cell counts also significantly increased during LPT. However, the percentage of the different cell types was not shifted, indicating that LPT does not preferentially stimulate the release of a particular cell type.

Consistent with Knott et al (37), lymph flow was significantly increased during application of LPT. When the increase in total leukocyte count was multiplied by the increase in lymph flow, the resulting flux of total leukocytes in the thoracic duct lymph significantly increased approximately 8-fold. There was also a significant increase in the flux of neutrophils, macrophages, T cells, and B cells.

While LPT has been used clinically for decades, and studies suggest it enhances the clearance of pulmonary infection, the exact mechanisms responsible for this protection have not been identified. The overall hypothesis of these studies is that by enhancing immune

surveillance LPT increases the number of leukocytes that can traffic to the lung during a pulmonary infection. The purpose of these studies was to determine if LPT induces any preferential shifts in lymphocyte subsets appearing in the thoracic duct, particularly IgA-bearing cells from mucosal tissues. Furthermore, we sought to determine if LPT mobilizes leukocytes from the gastrointestinal-associated lymphoid tissue (GALT). If a mucosal tissue, such as the GALT, is a source of the leukocytes mobilized during LPT, this may explain the clinical benefits seen in patients given LPT during pulmonary infection. Specifically, cells released from the GALT during LPT may migrate to other mucosal sites such as the lung during an infection and induce more protective immune responses at the site of infection.

CHAPTER II

LYMPHATIC PUMP TREATMENT MOBILIZES LEUKOCYTES FROM THE GASTROINTESTIAL ASSOCIATED LYMPHIOD TISSUE

Introduction

The release and circulation of leukocytes is important for the immune response against invading pathogens. In the lymphatic system, afferent lymph carries antigen-bearing leukocytes from tissues to the lymph nodes (1, 4), where lymphocytes are activated. Efferent lymph then carries activated lymphocytes into the periphery where they can perform their effector functions (4). The thoracic duct is the largest efferent lymph vessel in the body, with most of the smaller lymph vessels in the body flowing into it. The thoracic duct then empties into the left subclavian vein at the junction with the jugular vein (2). Therefore, activated lymphocytes enter blood circulation (1).

In early lymphatic studies, Mann and Higgins cannulated both the thoracic and intestinal lymphatic ducts in rats (14). They found that the lymphocyte output in the intestinal and thoracic ducts was almost equal. The authors therefore deduced that thoracic duct lymphocytes are gathered from a "pool" drained by the intestinal lymph duct. The intestinal duct drains the gastrointestinal mucosa, including the mesenteric lymph nodes (MLN) and Peyer's Patches.

Abdominal lymphatic pump treatment (LPT) is an Osteopathic manipulation designed to enhance lymph flow and promote health (9). The increased lymph flow from this treatment is

thought to accelerate the removal of toxins, wastes, and bacteria from the interstitial fluid as well as reduce edema (25). Previous work in our lab has shown that leukocyte count significantly increased during the application of LPT (38). Neutrophil, macrophage, T cell, and B cell counts were all significantly increased during LPT. However, the percentage of the different cell types was unaltered, indicating that LPT does not preferentially stimulate the release of a particular cell type. Lymph flow was significantly increased during the application of LPT as well. When the increase in total leukocyte count was multiplied by the increase in lymph flow, the resulting flux of total leukocytes in the thoracic duct lymph significantly increased approximately 8-fold. There was also a significant increase in the flux of neutrophils, macrophages, T cells, and B cells.

The mucosa-associated lymphoid tissue (MALT) is a loosely organized lymphoid tissue found at the mucous membranes, which are a major site of pathogen invasion (1). The lymphoid tissue associated with the digestive tract epithelium is known as the gastrointestinal-associated lymphoid tissue (GALT). GALT includes the lamina propria, Peyer's patches, and the MLN. There is also lymphoid tissue associated with the respiratory mucosal epithelium known as the bronchus-associated lymphoid tissue (BALT) (1, 2). Studies (20, 21, 22, 24) have shown that cells can migrate between these mucosal sites during an immune challenge. As Mann and Higgins deduced that the thoracic duct leukocytes are gathered from a "pool" drained via the intestinal lymph duct from the gastrointestinal mucosa, an increase in the release of thoracic duct lymphocytes during LPT may facilitate migration of these mucosal-derived cells to another mucosal site such as the respiratory tract and lungs. During an infection this influx of cells could be beneficial. This possible mechanism may explain the benefits seen in the clinical setting when patients with pulmonary infections were given LPT (30, 31, 32).

As the lab has previously shown an increase in the count and flux of T and B cells during LPT, we hypothesized that abdominal LPT will increase the count and flux of CD4+ and CD8+ T cells and IgG- and IgA-bearing cells in thoracic duct lymph. Additionally, if LPT mobilizes leukocytes from the GALT, we will seen an increase in the intestinal lymph duct leukocytes, including CD4+ and CD8+ T cells and IgG- and IgA-bearing cells. If a mucosal tissue is the source of these leukocyte increases, then an increase in the proportion of IgA-bearing cells may be seen. Since the mesenteric lymph nodes (MLN) comprise a part of the GALT, and leukocytes from the GALT are seen in the thoracic duct, we hypothesized that abdominal LPT will increase the number of leukocytes coming from the MLN into the thoracic duct.

Materials and Methods

Dogs. Adult mongrel dogs, which were free of clinically evident signs of disease, were used for this study. This study was approved by the Institutional Animal Care and Use Committee of UNTHSC, and was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication number 85-23, revised 1996).

Surgical Procedures. For catheterization of the thoracic lymph duct, six animals were anesthetized with sodium thiopental (5 mg/kg) and placed in a right, lateral, recumbent position. A left thoracotomy was made at the fifth intercostal space. The thoracic duct was dissected away from the aorta, isolated from connective tissue, and retracted with sutures. A small perpendicular incision was made in the thoracic duct and a PE50 or PE60 catheter was inserted into the duct and secured with a ligature. Pre-experiment lymph samples were collected to ensure that leukocyte counts were not elevated or reduced due to surgery, and that lymph flow was steady.

For catheterization of the intestinal lymph duct, 7 animals were anesthetized with sodium thiopental (5 mg/kg) and the abdominal cavity was opened. A small perpendicular incision was made in the large intestinal duct. A PE50 or PE60 catheter was inserted into the duct and secured with a ligature, and the abdomen was sutured closed. Pre-experiment lymph samples were collected to ensure that leukocyte counts were not elevated or reduced due to surgery, and that lymph flow was steady.

Lymphatic Pump Treatment. To perform LPT, the manipulator placed his/her hands below the costal margin on the dog's abdomen. The hands were moved at a frequency of 1-2 Hz towards the dog's back. The movement was done so that the recoil after a compression was immediately followed by another compression. This caused a pattern in which the recoil from one compression facilitated the next compression. Abdominal LPT was performed for 4 minutes.

Collecting Lymph Samples. Lymph samples were collected at 1 minute intervals into EDTA coated Vaccutainer tubes (Becton Dickinson). To calculate lymph flow, the pre-collection tube weight was subtracted from the post-collection tube weight. Samples were collected from the catheter during 4 minutes of baseline, 4 minutes of LPT, and 10 minutes of recovery.

Leukocyte Counts. The total leukocyte and differential leukocyte counts of each lymph sample were determined using either the Hemavet 950 blood analyzer (Drew Scientific) or a hemacytometer (Bright-Line) and microscope. To prepare lymph samples for enumeration by the hemacytometer, the lymph sample was vortexed and 1μL of lymph was added to 9μL of phosphate buffered saline (PBS) (Hyclone). 10μL of Trypan Blue (Sigma) was added to the lymph and PBS, and the solution was gently mixed. 10μL of the solution was placed on the hemacytometer slide, and the middle square was used to count the leukocytes.

Flow Cytometry. For flow cytometry staining of lymphocytes, the baseline samples were combined, as were the LPT and recovery samples. Separate 100μL aliquots of each of the samples (baseline, LPT, recovery) were washed two times with 2mL of staining buffer (PBS with 2% fetal bovine serum (FBS)) at 400g, 5 minutes, 4°C. After the final wash and removal of supernatant, the aliquots were stained with 10μL of either the anti-canine isotype control IgG2b, anti-canine CD3-FITC, anti-canine CD4-RPE, anti-canine CD8-AlexaFluor 647, anti-canine B cells-RPE, anti-canine IgA-FITC (1:20 dilution in staining buffer), or anti-canine IgG-FITC (1:20 dilution in staining buffer). Additionally, separate 100μL aliquots of each of the samples were stained with 10μL each of CD3 and CD4, CD3 and CD8, and CD4 and CD8. All flow cytometry antibodies were obtained from Serotec. One 100μL lymph aliquot was left unstained to serve as a negative.

The lymph aliquots were incubated in the dark at 4°C for 40 minutes, and were then washed once with 2mL of staining buffer at 400g, 5 minutes, 4°C. After the removal of supernatant, the lymph cells were fixed with 0.5% paraformaldehyde (Sigma) in PBS and stored at 4°C until flow cytometry analysis.

Flow cytometry was run on the Cytomics FC 500 (Beckman Coulter). Gates were established using the negative, unstained cells and cell populations were detected from peaks on a histogram or cell clusters on a dot plot. The population was shown as a percentage of cells in the sample. To determine the number of cells in a given lymphocyte population, the percentage was multiplied by the total number of leukocytes in the original lymph sample. Since the samples were combined, the mean leukocyte number was used. To determine the thoracic duct flux of a lymphocyte population, the cell population number was multiplied by the thoracic duct lymph flow.

CFSE-labeling of the Mesenteric Lymph Nodes. The thoracic duct of one animal was catheterized as described above, the abdominal cavity was opened, and ¼ to ½ of the mesenteric lymph nodes (MLN) were injected with 25 mg of lyophilized 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (BioChemika) dissolved in 5mL Dimethyl sulfoxide (DMSO) (Sigma). Each MLN was injected 4-5 times with about 100µl of the solution. The abdominal cavity was sutured closed and, after 2 hours, LPT was performed and lymph samples were taken during 4 minutes of baseline, 4 minutes of LPT, and 10 minutes of recovery as described previously. A pre-CFSE injection lymph sample was also collected to serve as a negative during flow cytometry. Leukocytes were enumerated using the hemacytometer as described previously.

To prepare for flow cytometry, 100µL aliquots were prepared from each lymph sample from each time point. The lymph aliquots were washed twice with 2mL of staining buffer (PBS with 2% FBS) at 400g, 5 minutes, 4°C. After the final wash and removal of supernatant, the lymph cells were fixed with 0.5% paraformaldehyde (Sigma) in PBS and stored at 4°C until flow cytometry analysis. Flow cytometry was performed as previously described. To determine the number of cells in a given population, the population percentage was multiplied by the total number of leukocytes in the original lymph sample. To determine the thoracic duct flux of a leukocyte population, the cell population number was multiplied by the thoracic duct lymph flow.

Statistical Analysis. For the thoracic duct experiments, Graphpad Prism was used to perform a repeated measures ANOVA with a Tukey post test to determine statistical differences between baseline, LPT, and recovery samples. For the intestinal duct experiments, Graphpad Prism was used to perform a Friedman test with a Dunn post test to determine statistical differences between baseline, LPT, and recovery samples. $P<0.05$ was considered statistically significant.

Results

Consistent with our previous study (38), total leukocytes significantly increased ($P< 0.05$) in the thoracic duct during LPT ($31.98 \pm 8.72 \times 10^6$ cells/ml) compared to baseline ($12.38 \pm 2.15 \times 10^6$ cells/ml) and recovery ($11.87 \pm 1.96 \times 10^6$ cells/ml) (Figure 1). In addition, total leukocyte flux significantly ($P< 0.01$) increased in the thoracic duct during LPT ($131.09 \pm 37.17 \times 10^6$ cells/min) compared to baseline ($12.38 \pm 2.15 \times 10^6$ cells/min) and recovery ($22.89 \pm 4.83 \times 10^6$ cells/min) (Figure 2).

Neutrophils, monocytes, lymphocytes, $CD4^+$ and $CD8^+$ T cells, and IgA- and IgG-bearing cells increased approximately 2-fold in the thoracic duct lymph during abdominal LPT (Table 1). The flux of neutrophils, monocytes, lymphocytes, $CD4^+$ and $CD8^+$ T cells, and IgA- and IgG-bearing cells in the thoracic duct lymph were also significantly increased approximately 12-fold during abdominal LPT (Table 2). However, there appears to be no preferential shift in any cell type released in the thoracic duct during LPT.

The number of total leukocytes in the intestinal duct lymph significantly increased ($P< 0.05$) during LPT ($16.87 \pm 3.32 \times 10^6$ cells/ml) compared to baseline ($6.15 \pm 0.55 \times 10^6$ cells/ml) and recovery ($9.26 \pm 1.42 \times 10^6$ cells/ml) (Figure 3) Additionally, intestinal lymph flux increased significantly ($P< 0.05$) during LPT ($27.97 \pm 12.75 \times 10^6$ cells/min) as compared to baseline ($2.75 \pm 0.66 \times 10^6$ cells/min) and recovery ($7.64 \pm 3.38 \times 10^6$ cells/min) (Figure 4). The number of neutrophils, monocytes, lymphocytes, $CD4^+$ and $CD8^+$ T cells, and IgA- and IgG-bearing cells increased approximately 4-fold in the intestinal duct lymph during abdominal LPT (Table 3). The flux of neutrophils, monocytes, lymphocytes, $CD4^+$ and $CD8^+$ T cells, and IgA- and IgG-

bearing cells in the intestinal duct lymph were also significantly increased approximately 10-fold during abdominal LPT (Table 4). As in the thoracic duct, there appears to be no preferential shift in cell type released in the intestinal duct during LPT.

To determine if LPT stimulates the release of leukocytes from the mesenteric lymph nodes (MLN), in one experiment the MLN were fluorescently labeled *in situ* with CFSE. LPT increased the percentage of total leukocytes coming from the labeled MLN from 8.5% to 12.4% as measured by CFSE-labeled cells. During the 4 minutes of LPT, flux of CFSE-labeled leukocytes from the MLN increased approximately 12-fold in the thoracic duct lymph (Figure 5).

Discussion

Consistent with our previous studies (38), we demonstrate that LPT significantly increases total leukocytes in the thoracic lymph duct approximately 2-3 fold over baseline values, and the flux of total leukocytes in the thoracic duct approximately 12-fold over baseline values. LPT also increases neutrophils, monocytes, lymphocytes, CD4+ and CD8+ T cells, IgA-bearing cells, and IgG-bearing cells. However, there is no preferential release of any lymphocyte subset into the thoracic duct during the application of LPT.

As is the thoracic duct, LPT also statistically increases total leukocytes in the intestinal lymph duct approximately 4-fold compared to baseline, and the flux of total leukocytes approximately 10-fold over baseline. Again, neutrophils, monocytes, lymphocytes, CD4+ and CD8+ T cells, IgA-bearing cells, and IgG-bearing cells are all increased during LPT, but there was no preferential increase in any of the lymphocyte subsets in the intestinal duct. Importantly, during the recovery phase, count and flux in both the thoracic and intestinal lymph ducts return to near baseline levels, indicating that the effects of LPT are transient. The clinical impact of this leukocyte release is still to be determined.

The intestinal lymph duct drains the gastrointestinal mucosa (14), so an increase in leukocytes in the intestinal duct most likely signifies that LPT is mobilizing leukocytes from the GALT into lymph circulation. In the one MLN experiment, we see that 4% of the cells released from the MLN during LPT enter the thoracic duct. This suggests that the GALT is a tissue source of the leukocytes released during LPT, but further experiments are needed to confirm this finding, especially since only about one-quarter to one-half of the MLN were CFSE-labeled.

When examining the data collectively, we see the mobilization of leukocytes in the thoracic duct increases from about 12 x 10^6 cells/min at baseline to about 170 x 10^6 cells/min at the height of LPT. In the intestinal duct, leukocyte mobilization increases from around 3 x 10^6 cells/min at baseline to a maximum of about 25 x 10^6 cells/min during LPT. While it is thought that most of the cells in the intestinal lymph duct travel to the thoracic lymph duct (14), during LPT the leukocytes in the intestinal duct are equal to only about 15% of the leukocytes seen in the thoracic duct. This indicates that that the intestinal lymph, and therefore the gastrointestinal mucosa that it drains, is not the only source of the increasing leukocytes seen during LPT. This is evidenced in the MLN data. The percentage of cells being released from the MLN increases during LPT, but these cells are still less than 15% of the total leukocytes released from the thoracic duct during LPT. Again however, not all of the MLN were labeled. Other possible tissue sources need to be examined for their leukocyte release during LPT. When discussing the intestinal lymph duct, it is also important to remember that there are multiple intestinal ducts, and only one duct is being examined in these experiments.

Previous studies (20, 21, 22, 24) have shown that primed mucosal lymphocytes can migrate to other mucosal areas during infection or inflammation. The leukocytes released during LPT could migrate to other mucosal tissues, increasing immune surveillance throughout the

MALT. In the event of a pulmonary infection, if any of the lymphocytes released during LPT are antigen-specific for the infecting pathogen, this could lead to a rapid protective immune response. Clinical studies have shown that LPT benefits patients with respiratory infections (30, 31, 32), and this could be one of the mechanisms of these positive outcomes.

CHAPTER 3

DISCUSSION

Conclusions

As in a previous report from our lab (38), these studies demonstrate that LPT significantly increases total leukocyte count and flux in the thoracic lymph duct. Neutrophils, monocytes, and lymphocytes, as well as $CD4^+$ and $CD8^+$ T cells and IgA- and IgG-bearing cells, are all significantly increased in the thoracic duct during the application of LPT. However, there is no preferential shift in the release of any cell type during LPT, suggesting that LPT equally releases leukocytes. We anticipated that there would be an increase in the proportion of IgA-bearing cells during LPT if a mucosal tissue, such as the GALT, was a source of the leukocytes released by LPT. This was not seen, but studies have shown that a proportion of the lymphocytes within the MALT and GALT are plasma cells that secrete the IgA which is found in mucosal secretions (1). Therefore, plasma cells do not have IgA on their surface and would not be identified as IgA-bearing cells during flow cytometry. So it is possible that the GALT is a source of the leukocytes that increase during LPT, even without an increase in the proportion of IgA-bearing cells. Studies to examine plasma cells that secrete IgA and cells that have intracellular IgA could determine if an increase in IgA occurs.

The significant increase in total leukocyte count and flux in the intestinal lymph duct during LPT confirms that the gastrointestinal mucosa, or GALT, is a source of the increased

leukocytes seen during LPT, since the intestinal lymph duct drains the gastrointestinal mucosa (14). As in the thoracic lymph duct, there is not a preferential increase in any cell type during LPT in the intestinal duct. Neutrophils, monocytes, and lymphocytes are significantly increased, as are the lymphocyte subsets of $CD4^+$ and $CD8^+$ T cells and IgA- and IgG-bearing cells, but in the same proportions.

The mesenteric lymph nodes (MLN), which are a component of the GALT, show increased leukocyte mobilization to the thoracic lymph duct during LPT as compared to baseline. This preliminary experiment suggests that the GALT is a source of the leukocytes mobilized into thoracic duct lymph during LPT. However, the MLN are not the only source of the increasing leukocytes seen during LPT, because they make up less than 15% of the total number of leukocytes detected in the thoracic duct lymph during LPT. It is important to remember however that not all of the MLN were labeled for this experiment, and that the MLN have venous drainage, so not all of the leukocytes from the MLN will move directly into lymph circulation. This experiment needs to be repeated to verify that this particular tissue is a source of the increased leukocytes.

Studies have shown that the output of lymphocytes from the thoracic and intestinal ducts are almost equal, which has led to the belief that most thoracic duct lymphocytes come from the intestinal duct (14). However, the data show differences between the outputs of leukocytes in the two ducts, especially at the LPT time points. At the maximum point of intestinal leukocyte flux, the output is only about 15% of the maximum thoracic leukocyte flux. It is possible that some leukocytes leave the intestinal lymph duct and go directly into blood circulation. It is also important to remember that there are multiple intestinal lymph ducts, and that while the large duct was catheterized for these experiments, the smaller ducts do also flow into the thoracic

lymph duct. Of course, there are almost certainly other tissue sources that contribute to the increasing leukocytes observed during LPT, and these sources need to be studied. Also, while the numbers of leukocytes in the thoracic and intestinal lymph ducts should be very similar, differences could be attributed to the fact that the thoracic lymph duct drains most of the lower extremities and the abdomen, not only the intestinal lymph (1).

A limitation to these studies is that while leukocytes do increase in the intestinal and thoracic duct lymph, this increase cannot be readily detected in the blood, even though the thoracic duct empties directly into venous blood circulation. This is due, most likely, to the vast amount of immune cells already circulating within the blood. This makes systemic leukocyte increases due to acute application of LPT hard to measure. We do estimate, however, that the release of the approximately 600,000,000 cells during LPT will increase the blood leukocyte concentration by approximately 4%.

Future directions

As was stated before, there are most likely other sources for the increased leukocytes seen with LPT than the intestinal lymph duct and the MLN that were labeled. Firstly, the MLN experiment needs to be repeated, to confirm the results that were seen. Also, more MLN need to be labeled in the future to determine if labeling all, or most, of the MLN increases the proportion of labeled MLN leukocytes appearing in the thoracic duct during LPT.

In future experiments, other lymphoid tissues will be examined during LPT to see if they increase leukocyte output during LPT. Current experiments in the lab have catheterized the splenic vein to monitor the output of leukocytes from the spleen during baseline, LPT, and recovery. Preliminary data has shown that, as in the thoracic and intestinal lymph ducts, LPT increases the output of leukocytes in the splenic vein over baseline and recovery values.

Interestingly, when examining neutrophils, monocytes, and lymphocytes, it is seen that lymphocytes are most increased in cell type in the spleen during LPT (unpublished data).

While leukocyte increases in the splenic vein enter into venous blood circulation, it is unlikely that these cells are the same ones that are seen in the thoracic or intestinal ducts during LPT. Leukocytes remain in the blood for approximately 30 minutes before they migrate to the lymph nodes or other tissues (1). As the increases in the thoracic and intestinal ducts are seen within 1-2 minutes of beginning LPT, the leukocytes from the spleen should not have enough time to enter venous circulation, extravasate into lymph nodes, and then enter the efferent lymph which makes up the intestinal and thoracic lymph. We anticipate that at 4-18 hours the leukocytes from the spleen would appear in the lymph, and that LPT may facilitate their appearance.

Currently in the lab, there have been labeling experiments to determine the destination of labeled thoracic duct leukocytes. When cells were labeled *in vivo* and injected in the jugular vein, it was seen that one of the first tissues that these cells migrated to was the lungs. They could also still be found in the blood soon after injection. This is consistent with previous studies of the migration of thoracic duct lymphocytes (13). Future experiments include labeling of tissue sources, whether these are the GALT, spleen, or other sources, that increase leukocyte output to the intestinal and thoracic ducts during LPT, to determine the distribution of these released cells. These labeled leukocytes can be monitored for not only when they enter lymph, but also when they enter systemic circulation, and biopsies can then be taken to determine what tissues these cells enter and when. To determine if the leukocytes that are released during LPT have different migration patterns than those released during baseline, cells can be collected during baseline and during LPT. These cells can be labeled *in vitro* with two different

fluorescent dyes, and then intravenously injected. Blood samples can be taken over a set period of time and tissue biopsies can be taken at the end of the experiment to locate the destination of these labeled leukocytes.

Leukocytes released during LPT may be more activated than the leukocytes released during baseline. Currently in the lab, cells collected at baseline and during LPT are being stimulated with LPS to see if there is a difference in activation between the two. The cells are also being labeled *in vitro* with CFSE to see if leukocytes that are released during LPT are more proliferative then the leukocytes released at baseline.

Clinically, LPT is used during times of infection and illness, so it is important to study the effects of LPT during an immune challenge. It will be seen if LPT treated animals have a decrease in disease severity and an enhancement of the immune response as compared to animals that are challenged and do not receive LPT.

The possible effects of LPT

Many studies have shown that mucosal-derived leukocytes can migrate from their source tissue to other mucosal areas in the event of an immune challenge (20, 21, 22, 24). Since LPT mobilizes more leukocytes from the GALT into the thoracic and intestinal lymph ducts than are present at baseline, it is possible that these increased cells can migrate to other mucosal tissues, especially if inflammation is present.

Studies in humans have shown that the thoracic duct has a large population of memory T cells that home to the gastrointestinal mucosa (39). In the event of a pulmonary infection, it is possible that these leukocytes released during LPT could migrate to the lung as part of immune surveillance. If any of the lymphocytes are antigen-specific memory cells for the invading pathogen, this could lead to an enhanced immune response.

The clinical benefits of LPT in patients with respiratory infections have been noted for decades. In revealing that LPT mobilizes leukocytes from the mucosal GALT, which can possibly enhance immune surveillance, these studies give credence to the use of LPT by Osteopathic physicians

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then empties into the left subclavian vein at the junction with the jugular vein (2).