Lymphatic Pump Treatment Enhances the Lymphatic and Immune System and Ameliorates Disease Severity in a Rat Model of Respiratory Infection

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Schander, Artur, Lymphatic Pump Treatment Enhances the Lymphatic and Immune System and Ameliorates Disease Severity in a Rat Model of Respiratory Infection. Doctor of Philosophy (Cell Biology and Immunology), May 2014, 197 pp., 18 tables, 30 illustrations, bibliography, 279 titles.

The purpose of these studies was to explore the benefits, effects, and mechanisms of LPT in both a healthy and a diseased animal model, and hence provide scientific rationale for the clinical application of LPT.

Novel findings in this dissertation demonstrate that in anesthetized canines: 1) LPT mobilizes leukocytes from the GALT into lymphatic circulation; 2) LPT mobilizes inflammatory mediators into lymphatic circulation; and 3) repeated application of LPT increases lymph flow, concentration of leukocytes, and flux of inflammatory mediators into lymphatic circulation. In addition, this dissertation for the first time demonstrates: 1) the development of a novel lymph enhancing rodent model, in which LPT increases leukocyte flux in the cisterna chilii, predominantly from the GALT; and 2) that LPT facilitates the clearance of pneumococcal respiratory infection and suggests a mechanism by which LPT might facilitate the clearance of pneumococcal pneumonia. Our studies demonstrated that LPT transiently mobilized leukocytes from the mesenteric lymph nodes. We found a significant increase in the concentrations of MCP-1 and flux of IL-6 flux in TDL and MDL in anesthetized dogs. Interestingly, both IL-6 and MCP-1 were present in BALF of rats infected with pneumococcus, and LPT significantly increased IL-6 and moderately increased MCP-1 concentrations compared to Sham and Control
animals, which supports our notion that LPT may increase cytokine/chemokine redistribution from the mesentery to the lung.

We demonstrated that LPT enhanced the clearance of *S. pneumoniae* after 3 consecutive daily treatments and found that LPT increased the concentrations of SP-D, IL-6, IL-12p70, and IL-17 in BALF and enhanced the release of NO$_2^-$ and IL-6 by AM 4 days post-infection. Collectively these studies suggest, that LPT re-distributes inflammatory mediators to the lung, enhances the recruitment of macrophages and neutrophils to the lung and skews alveolar macrophages towards a M1 phenotype, all of which may be responsible for and promote the clearance of *S. pneumoniae*. 
LYMPHATIC PUMP TREATMENT ENHANCES THE LYMPHATIC AND IMMUNE SYSTEM AND AMELIORATES DISEASE SEVERITY IN A RAT MODEL OF RESPIRATORY INFECTION

Artur Schander, M.S.

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LYMPHATIC PUMP TREATMENT ENHANCES THE LYMPHATIC AND IMMUNE SYSTEM AND AMELIORATES DISEASE SEVERITY IN A RAT MODEL OF RESPIRATORY INFECTION

DISSERTATION

Presented to the Graduate Council of the Graduate School of Biomedical Sciences University of North Texas Health Science Center at Fort Worth in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Artur Schander, M.S.

Fort Worth, Texas

May 2014
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AM</td>
<td>Alveolar Macrophages</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AOP</td>
<td>Arterial Blood Pressure</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>BALT</td>
<td>Bronchus-Associated Lymphoid Tissue</td>
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<tr>
<td>CAM</td>
<td>Complementary and Alternative Medicine</td>
</tr>
<tr>
<td>CAP</td>
<td>Community-Acquired Pneumonia</td>
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<tr>
<td>CC</td>
<td>Cisterna Chili</td>
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<td>CCLT</td>
<td>Conventional Care Plus Light Touch</td>
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<td>CCO</td>
<td>Conventional Care Only</td>
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<tr>
<td>CCOMT</td>
<td>Conventional Care Plus OMT</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
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<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
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<tr>
<td>Cu/Zn-SOD</td>
<td>Cytosolic Superoxide Dismutase</td>
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<tr>
<td>FeSOD</td>
<td>Extracellular Superoxide Dismutase</td>
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<tr>
<td>GALT</td>
<td>Gut-Associated Lymphoid Tissue</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Utilization Committee</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IEC</td>
<td>Intestinal Epithelial Cell</td>
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<td>IFN</td>
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<td>IL</td>
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<tr>
<td>KC</td>
<td>Keratinocyte Chemoattractant</td>
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<tr>
<td>LOS</td>
<td>Hospital Length Of Stay</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina Propria</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LPT</td>
<td>Lymphatic Pump Technique</td>
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<td>LY</td>
<td>Lymphocytes</td>
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<td>M cells</td>
<td>Microfold Cells</td>
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<tr>
<td>mAb</td>
<td>Monoclonal Antibodies</td>
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<tr>
<td>MAdCAM-1</td>
<td>Mucosal Adressin Cell Molecule</td>
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MALT  Mucosa-Associated Lymphoid Tissue
MCP-1  Monocyte Chemotactic Protein-1
MDL  Mesenteric Duct Lymph
MHC  Major Histocompatibility Complex
MIP-1α  Macrophage Inflammatory Protein-1α
MLN  Mesenteric Lymph Nodes
MnSOD  Mitochondrial Superoxide Dismutase
MO  Monocytes
MOPSE  Multicenter Osteopathic Pneumonia Study in the Elderly
NO  Nitric Oxide
NO₂⁻  Nitrite
NT  Nitrotyrosine
OM  Otitis Media
OMM  Osteopathic Manipulative Medicine
OMT  Osteopathic Manipulative Treatments
PP  Peyer’s Patches
RBC  Red Blood Count
RNS  Reactive Nitrogen Species
ROS  Reactive Oxygen Species
SE  Standard Error
SOD  Superoxide Dismutase

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>SP-D</td>
<td>Surfactant Protein-D</td>
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<tr>
<td>sPLA₂</td>
<td>Secretory Phospholipase A₂</td>
</tr>
<tr>
<td>TDL</td>
<td>Thoracic Duct Lymph</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
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CHAPTER I

INTRODUCTION TO THE STUDY

The lymphatic system is vital to the human body due to its numerous responsibilities. It not only returns extravasated fluids and macromolecules back to the circulation, but also transports antigens and immune cells between tissues, lymph nodes, and blood. Decreased or obstructed lymph flow can lead to edema and interstitial accumulation of toxins, exudates, and microbial organisms, which can ultimately lead to decreased immune function and inflammation.1,2 The significance of the lymphatic system in maintaining health has been extensively recognized by the osteopathic medical profession. Osteopathic physicians have developed osteopathic manipulative treatments (OMT), specifically lymphatic pump techniques (LPTs), which are believed to increase lymphatic circulation and improve immunity. Recently, it was validated that LPT in fact does increase lymph flow and leukocyte count in canine thoracic duct lymph (TDL).3,4 Clinically, osteopathic physicians have long used LPTs in order to remove lymphatic obstructions, increase lymph flow, and treat edema and infection. However, direct scientific evidence of their efficacy remains sparse, and few mechanistic studies have been conducted to investigate the benefits and effects of LPT.5-7

Therefore, the purpose of this study is to explore the benefits, effects, and mechanisms of LPT in both a healthy and a diseased animal model, and hence provide scientific rationale for the clinical application of LPT. We will investigate the effectiveness of LPT by evaluating the
modifications in immunomodulatory mechanisms that occur with LPT in a canine animal model during single and multiple treatment interventions. Then, we will develop a LPT small animal rat model and utilize this model to investigate the therapeutic intervention of LPT on disease severity and progression in an acute rat model of respiratory infection.

In this proposal, we will test the hypothesis that the application of abdominal LPT will increase the release and redistribution of inflammatory mediators and leukocytes into lymphatic circulation, and will ameliorate disease severity in a rat model of respiratory infection with *Streptococcus pneumoniae*.

Historically, LPT has been used to treat infections and edema,\(^5\)\(^-\)\(^7\) yet mechanistic studies investigating the efficacy by which LPT enhances immune and lymphatic function are few. While osteopathic practitioners have long claimed LPT as a lymph flow enhancing technique, it was not until 2005 that this claim was validated when Knott, et al. implanted an ultrasonic flow transducer around the thoracic duct and demonstrated the increased lymph flow effects of the abdominal and thoracic pump using real time measurements in canines.\(^3\) Interestingly, this same report also revealed that abdominal LPT increased TDL flow to a similar degree when compared to exercise.\(^3\) This report was followed up by a publication showing that LPT not only increased lymph flow in TDL, but also increased leukocyte count and flux.\(^4\) Specifically, the numbers of macrophages, neutrophils, and total lymphocytes were increased in TDL during LPT.\(^4\) Studies have suggested that gastrointestinal lymphoid tissues (GALT) located in the abdominal viscera (the same area abdominal LPT is applied to) are a major source of thoracic duct leukocytes.\(^8\)\(^-\)\(^12\) Nevertheless, the tissue sources of leukocytes released during LPT intervention are unclear and need to be investigated.
Clinically, LPT intervention has shown efficacy in the treatment of infectious diseases, specifically pulmonary infections;\textsuperscript{13-17} however, the protective mechanism remains enigmatic. Thus, LPT increased lymph flow and circulating leukocytes could improve immune surveillance and response, therefore enhancing protection against infectious diseases. It has previously been reported that leukocytes,\textsuperscript{11,18-25} as well as cytokines and chemokines,\textsuperscript{26-29} can change recirculation patterns, exhibit preferential homing, and re-distribute and exert local effects in organs that are distant to the tissues of their release.

Therefore, we hypothesize that the application of abdominal LPT will mobilize leukocytes and inflammatory mediators from gastrointestinal lymphoid tissues into lymphatic circulation, thereby improving immune surveillance. We further hypothesize that leukocytes and inflammatory mediators released during LPT application will re-distribute to the lung and reduce disease severity in a rat model of respiratory infection with Streptococcus pneumoniae.

LPTs are also used to treat infectious diseases\textsuperscript{6,7} and have clinically been shown to be protective against respiratory infections.\textsuperscript{13-17} Despite the widespread use of these techniques clinically, there have been few randomized controlled trials or animal studies to investigate the clinical efficacy or mechanisms by which LPT enhances the lymphatic and immune system. While we have previously shown that LPT increases lymph flow\textsuperscript{3}, leukocyte count, and flux\textsuperscript{4} in canine TDL, the source of lymph and immune cells released during LPT has yet to be determined. Therefore, it is necessary to further elucidate the source and composition of LPT mobilized lymph, with a focus on leukocytes and inflammatory mediators, which we will accomplish in the proposed experiments. Further, the aforementioned studies only provide indirect evidence as to how LPT may enhance immunity and provide protection seen in respiratory infections. It is likely that an increase in circulating leukocytes may augment
immunological function by increasing immune surveillance and facilitating the interaction of leukocytes and antigens. This in turn could promote earlier and more frequent pathogen recognition, and thereby increase pulmonary clearance of bacteria; however, this remains speculative. As previously mentioned, LPT could also facilitate the re-distribution of leukocytes and inflammatory mediators to the lung and augment local pulmonary immunity by this means. Hence, it is important to directly assess the effects of LPT on immunity, disease progression, and disease resolution in a pneumonia animal model of respiratory infection.

While pneumococcal vaccines have decreased the death toll due to infection, pneumococcus continues to exhibit a high serotype variability and genomic plasticity.\textsuperscript{30,31} This trend has lead to limited treatment options and increased therapeutic failures; therefore, it has become of outmost importance to investigate and develop alternative treatment approaches such as LPT. For these reasons, we will develop a LPT small animal rat model and utilize this model to investigate the therapeutic intervention of LPT on disease resolution in an acute rat model of respiratory infection with \textit{Streptococcus pneumoniae}, and we will identify the immune mechanisms responsible for this protection.

Finally, mechanistic studies and clinical reports in literature discussing the optimal duration, refractory period, and frequencies of LPT application are few and inconsistent. The proposed studies will provide the clinician with scientific rationale to develop effective treatment protocols and provide efficient patient care.

\textbf{The Lymphatic system}

The lymphatic system is recognized for its important roles in transporting lymph, maintaining a healthy immune system, and mounting immune defenses, while simultaneously maintaining body fluid and macromolecular homeostasis and participating in lipid absorption
and transportation.\textsuperscript{1,2,32} This is vital to the immune system of the human body, not only returning extravasated fluids, macromolecules and particulate matter to the blood, but also transporting antigens and immune cells between tissues, lymph nodes, and blood, thus actively participating in immune surveillance and responses, while maintaining self-tolerance. Decreased lymph flow or obstruction in flow can lead to edema and interstitial accumulation of toxins, particulate matter, exudates, microbial organisms, ultimately leading to decreases immune function and impaired immune surveillance, tissue hypoxia, inflammation, and tissue fibrosis.\textsuperscript{1,2,32}

The closed cardiovascular system in humans and other vertebrates exhibits a high mean arterial pressure (~ 100 mmHg), which is necessary in order to achieve adequate perfusion of organ systems. However, it causes the formation of extravascular transudates from the blood into the interstitium.\textsuperscript{33} Despite the fact that this transcapillary tissue fluid plays an essential role in the maintenance of extravascular homeostasis, in part by creating a suitable environment for the stromal and parenchymal cells,\textsuperscript{32} it is vital for the body to gradually remove plasma fluid in order to avoid the formation of local edema and further return parenchymal cell products, immune cells, proteins, apoptotic cells, antigens, and infectious organisms back into circulation.\textsuperscript{32,34} The capillary endothelium participates actively in this transvascular exchange between blood vessels and interstitial space\textsuperscript{35} by permitting the uptake of micro-molecules and fluids back into the blood circulation; however, macro-molecules, like fatty acids, proteins, lipoproteins, and some remaining plasma fluid, must be returned to the blood stream thru the lymphatic system.\textsuperscript{33,35} An increase in interstitial tissue oncotic pressure can result if macro-molecules are not removed at adequate rates, ultimately leading to edema formation and an imbalance in the transcapillary endothelial exchange.\textsuperscript{36,37}
Once the interstitial fluid enters the microscopic lymphatic vessels, it becomes lymph and is transported through an array of vessels and lymph nodes, ultimately draining into either the thoracic duct or the right lymphatic duct.\textsuperscript{33,38} The right lymphatic duct, which runs parallel to the C-2 vertebrae, drains the upper right quadrant of the body and empties into the right subclavian vein.\textsuperscript{33,38} On the other hand, the thoracic duct starts with the \textit{cisterna chyli}, a dilated lymphatic sac in the retrocrural space of the abdomen at the L1-2 vertebral body level, and it continues in a caudocephalic direction joined by further lymphatic tributaries, ultimately emptying at the junction of the left internal jugular vein and left subclavian vein.\textsuperscript{33,39} The cisterna chyli is found to the right of the abdominal aorta and is usually made up of the two lumbar trunks (although different variations do exist), which mainly drain the abdominal wall, kidneys, adrenal glands, pelvic viscera, and the lower limbs, and the intestinal trunk, which mainly drains liver, pancreas, stomach, and intestines.\textsuperscript{37,39,40}

\textbf{Inflammatory mediators}

Inflammatory cytokines, chemokines, and reactive oxygen and nitrogen species are vital for the clearance of infectious diseases, as well as initiating and regulating immune and inflammatory responses.\textsuperscript{1,2,41} Depending on the concentrations secreted, they can induce immune pathology or protective immunity.\textsuperscript{1,2,41} During infection, pathogens come in contact with phagocytes, which in turn leads to the secretion of reactive oxygen and nitrogen species and the initiation of the “acute phase” response.\textsuperscript{41,42} During this response, the pro-inflammatory cytokines and chemokines, interleukin-1β (IL-1β), IL-6, IL-12, IL-18, IL-8, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), macrophage inflammatory protein-1α (MIP-1α), monocyte chemotactic protein-1 (MCP-1), and inducible protein-10 (IP-10) induce leukocyte activation, mediate cell trafficking, and regulate cell-mediated immune responses to the invading
pathogens. In addition, the liver-secreted proteins, C-reactive protein (CRP), secretory phospholipase A\(_2\) (sPLA\(_2\)), and serum amyloid proteins facilitate pathogen opsonization, apoptotic cell clearance, and complement activation.\(^4\)

This system is carefully counter-regulated by anti-inflammatory cytokines IL-10 and transforming growth factor-\(\beta\) (TGF-\(\beta\)), which limit inflammation by suppressing cell-mediated immune responses. In addition, superoxide dismutase (SOD), which participates in anti-inflammatory activity by decreasing ROS and RNS, and therefore protecting tissues from excessive oxidative stress during inflammation.\(^{4,42}\)

**Mucosal Immunity**

The largest collection of lymphoid tissue in the body is found within the mucosal surfaces, which on one hand act as a protective immune system, and on the other hand act as a regulatory mechanism that differentiates between innocuous substances and invading pathogens. Mucosa-associated lymphoid tissue (MALT) is mainly found in areas that are major sites of antigen entry and/or are heavily colonized with microbes.\(^2,44\) For example, the respiratory and gastrointestinal tract have a plethora of well-developed lymphoid tissues, respectively known as bronchus-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT), while only minimal lymphoid aggregates are found in the cervico-vaginal tissues.\(^2,44\) GALT includes the appendix, Peyer’s patches (PP), mesenteric lymph nodes (MLN), and isolated lymphoid follicles, (ILFs) which can be found in multiple sites, such as the lamina propria (LP).\(^2,43,44\)

Antigens are constantly sampled from the gut lumen in various ways. Intestinal epithelial cells (IECs) can take up, process, and present antigens via classical and non-classical MHC pathways, while resident antigen presenting cells (APCs) in the lamina propria can directly
capture antigens. Microfold cells (M cells) in Peyer’s patches obtain lumen antigens by endocytosis and local APCs present these to naïve T cells, which in turn travel to the MLN for classical antigen presentation. Effector T cells expressing CCR9 or CCR10 are recruited to the LP by IEC released chemokines CCL25 and CCL28. Activated lymphocytes expressing the α4β7 integrin are recruited to the mucosal adressin cell molecule (MAdCAM-1) on high endothelial venules, enabling the preferentially homing of these effector cells to PP and the LP. T and B cell lymphocytes reside within the lamina propria among the APCs and B cells produce large amounts of antibodies, mainly secretory IgA (sIgA) and IgM.

Previous reports have demonstrated that lymphocytes of mucosal origin can traffic to other mucosal tissues if they encounter the same antigen at that location. Of interest, some studies have shown that lymphocytes primed in the GALT can redistribute into the lungs where they enhance immunity and provide protection during pulmonary infection. Therefore, if LPT mobilizes GALT specific leukocytes and increases their circulating patterns, it is likely that these immune cells could redistribute to other tissues and provide protection during infection.

**Lymphatic leukocytes and circulation patterns**

Leukocytes, specifically lymphocytes, participate in “immune surveillance” by actively recirculating between tissues, lymph, and blood in order to promote the detection and elimination of foreign pathogens and to provide immunological memory. If leukocytes encounter foreign antigens during this process, they either directly kill the pathogens or induce an antigen-specific immune response via the lymph nodes. The blood compartment mainly receives lymphocytes from the thoracic duct, but also receives lymphatic tributaries from the spleen, head, neck, bone marrow, and thymus, and has been reported to be in equilibrium with lymphocytic reservoirs of
nonlymphoid organs, like the lungs. Consequently, blood is a non-homogenous source of recirculating lymphocytes, and it has been suggested that lymph-derived lymphocytes exhibit more efficient recirculatory patterns when compared to blood-derived lymphocytes.

Early lymphatic studies revealed that the intestinal lymph duct contributes ~95% of leukocytes into the thoracic lymph duct. It was reported, that human TDL mainly contained CD4+ T cells (60%), CD8+ T cells (23%), and 14.5% B cells, while a majority of CD4+CD45RO+ (41.6%) and CD8+CD45RO+ (59.5%) cells expressed the gut homing marker α4β7. In addition, human MDL has been shown to contain 60% naïve T lymphocytes, 25% naïve B lymphocytes, 10% memory T and B lymphocytes, and 2% B cell blasts. Collectively, these data suggest that TDL mainly gathers GALT-derived naïve T and B lymphocytes that have the ability to preferentially home to mucosal tissues.

**Osteopathic Manipulative Medicine**

The osteopathic profession is founded on the principles that the body is a unit of mind, body, and spirit, with self-healing and regulating mechanisms, and a structure and function that are interrelated. Therefore, an osteopathic physician should always consider these principles when choosing an appropriate treatment modality for a patient. Since the inception of osteopathic medicine, osteopathic physicians have acknowledged the importance of the lymphatic system in maintaining good health. The founder of osteopathy, Dr. Andrew Taylor Still, MD, DO, concluded that lymphatics “are undoubtedly the life-giving centers and organs”. He further recognized that “we strike at the source of life and death when we go to the lymphatics”, and thus it is not surprising to find anecdotal descriptions of lymphatic manipulative techniques used by Dr. Still.
Conventional medicine advocates physical movement, compression bandage-centered decongestive lymphatic therapy, and manual lymphatic drainage as the cornerstone of lymphedema treatment.\textsuperscript{57,58} Osteopathic physicians employ such manual lymphatic drainage techniques, which are thought to remove restrictions to the lymphatic vessels and enhance the flow of lymph through the system. \textsuperscript{5-7,59} One such group of techniques, the LPTs, include the thoracic pump, pedal pump, abdominal pump, and splenic pump.\textsuperscript{19} These techniques are believed to aid in the removal of built up metabolic wastes, toxins, exudates, and cellular debris that occur during infection or edema by removing somatic dysfunctions and restrictions, normalizing the sympathetic and parasympathetic innervations and increasing lymph flow.\textsuperscript{3,5-7,59} Furthermore, it is believed that an increase in lymph flow can augment the body’s natural immune defense, enhance vaccine antigen delivery, and redistribute pharmacological agents, such as antibiotics.\textsuperscript{5,6,19,59}

While there are accounts as early as 1889 describing lymph enhancing treatments,\textsuperscript{56} the first true systemic lymphatic pump was invented and perfected by Dr. Miller between 1920-1926.\textsuperscript{6,56} He later coined that pump as the thoracic pump, believing it not only enhanced the lymphatic circulation, but also the arterial and venous circulations.\textsuperscript{60} Dr. Miller suggested that his thoracic pump drained tissues of toxic waste materials, delivered fresh blood and lymph to tissues and increased antigen-antibody interaction and antibody production, while further suggesting that the thoracic pump should be applied at a compression rate of 60-80 times/min for 10 to 30 minutes.\textsuperscript{60}

\textbf{OMM and immune function}

Evidence suggesting that OMM treatment could be successfully applied in order to treat infection and disease first emerged during the 1918 Spanish Influenza Pandemic. R. Kendric
Smith, MD reported that mortality rates due to influenza were estimated to be 5-6% in patients treated with standard medical care, while patients treated by osteopathic physicians, which included OMM treatments, had a reported mortality rate of 0.25%. Likewise, patients with pneumonia receiving standard care during the 1918 pandemic had a reported mortality rate of 33%, in contrast to a mortality rate of 10% in patients treated by osteopathic physicians. Similarly, in a 1919 report to the American Osteopathic Association, Riley corroborated the low rates of mortality and morbidity documented in patients under the care of DOs. It is also noteworthy to add that these reports did not result from a controlled study, and that antibiotics had not been invented at that time; thus, they were not a part of standard care.

The first reported study investigating the effects of LPT on the immune system was performed in 1910 by Dr. Whiting, who studied liver and splenic pumps, and reported that 91% of the patients treated with these techniques had a 15% increase in their phagocytic index. In multiple studies performed in the 1930s, Castlio and Ferris-Swift investigated the effects of splenic manipulations at a rate of 21 compressions for 1.5 to 5 minutes on blood leukocytes in healthy and diseased patients. They found an increase in blood leukocyte count and opsonic index in over 80% of the patients following treatment. Neutrophilia was noted in 76% of the patients. The same group further reported an increase in serum bacteriolytic power in 68% of the cases, and an increase in agglutination, while a decrease in the erythrocyte count in 75% of the cases studied was noted. Noll, et al re-analyzed the raw data of the 1934 published findings by applying contemporary statistical methods, mainly nonparametric statistical analyses. The authors reported a modest post-treatment increase in total leukocyte count, and a statistically significant increase in the immune function tests following the treatment protocol.
A statistically significant increase in immune response to pneumococcal polysaccharide, after vaccination with Pneumovax and daily treatment with thoracic pump for 5 minutes for one week, was reported in healthy individuals when compared to a control group that did not receive post-immunization treatment. In 1986, the same group showed in a double-blind study that LPT in fact could increase total leukocytes, B-cells, and T-cells in peripheral blood. Another study showed an increased protective anti-hepatitis B titers in patients who received a combination of 5 minutes thoracic pump and one minute splenic pump three times a week for two weeks after vaccinations at 0, 5, and 25 weeks when compared to a control group that did not receive post-immunization treatment. In contrast, a study investigating the effects of LPT on influenza vaccine antibody responses did not find a statistically significant difference in serum IgM and IgG antibody titers when pre- and post-vaccination blood samples were compared. Likewise, a clinical study administering thoracic LPT in healthy human subjects did not show a statistically significant difference between pre- and post-treatment serum interferon levels.

Some studies have investigated the clinical benefits of OMM on respiratory infections; however, those were often pilot studies with limited subjects, varying treatment protocols, and limited sample analysis. For example, an early publication by Dr. Facto advocates the thoracic pump at a rate of 120/min for 5-10 minutes in the treatment of lobar Pneumoniae. Kline, et al conducted an investigation on children with respiratory infections, which illustrated that combining OMM treatments with supportive and antibiotic therapy led to a quicker recovery and a decreased length of hospital stay, as compared to OMM or antibiotic therapy alone. Another study investigated the effects of LPTs on lower respiratory infection. Patients in this study either received conventional treatment consisting of antibiotics, expectorants, sedatives, and
fluids as needed, or they received thoracic lymphatic pump for 5 minutes 4 times a day for 4 to 5
days at a rate of 20 compressions/min in addition to conventional treatment. Patients receiving
the later treatment protocol exhibited an increase in sputum production, vital capacity, clearing
of the tracheobronchial tree, and a shorter cough duration as compared to conventional treated
patients. Noll, et al further reported shorter hospital stays and decreased duration of IV
antibiotic treatment in elderly hospitalized patients with acute pneumonia, after performing a
variety of osteopathic treatments for 10-15 minutes twice a day for 7 days including thoracic
pump as an adjunctive therapy to conventional treatment methods.

More recently, the prospective double-blinded, randomized, Controlled Multicenter
Osteopathic Pneumonia Study in the elderly (MOPSE) investigated the effects of 15 minutes of
osteopathic manipulations twice daily as an adjunct therapy in elderly, hospitalized patients with
pneumonia. Amongst other OMM techniques, the thoracic lymphatic pump with activation at a
rate of 120 compressions/min for a duration of 2 minutes, and the pedal pump for a duration of 1
minute were used. In this study, patients were randomly assigned to one of three groups: a)
conventional care only (CCO); b) conventional care plus OMT (CCOMT); and c) conventional
care plus light touch (CCLT); and the following outcomes were evaluated: a) hospital length of
stay (LOS); b) time to clinical stability; c) symptomatic and functional recovery score; d)
duration of intravenous antibiotic treatment; and e) treatment endpoint (included respiratory
failure). The authors found a statistically significant decrease ($P = 0.01$) in median LOS in the
CCOMT group (3.5 days) when compared to the 4.5 days of LOS in the CCO group, but no
difference when compared to the CCLT group (3.9 days). Furthermore, a statistically
significant difference in treatment endpoint and intravenous antibiotic administration was found
between CCOMT and CCO ($P = 0.05$), as well as between CCO and CCLT ($P = 0.006$).
Similarly, patients treated with the CCOMT protocol had decreased rates of respiratory failure and death as compared to the CCO, but not the CCL treatment groups.\textsuperscript{16}

Few studies utilizing animal models to investigate the effects of LPT on immune function exist. One such early investigation was conducted on two rabbits in 1920 by Lane. He found that five minutes of splenic treatments at a rate of 50-60 compressions/min were effective in raising the serum antibody titers against sheep corpuscles, which had been injected one week prior over a period of three days.\textsuperscript{77} Another such study performed by Dery, et al provided indirect evidence that LPTs in fact do increase lymph flow.\textsuperscript{78} These authors injected a fluorescent labeled albumin probe into the interstitial space in the hind limbs of healthy anesthetized laboratory rats and divided the rats into two groups. The control group did not receive treatment, while the experimental group received intermittent pulsation pressure to the ventral thorax for 5 minutes every hour until the rats gained consciousness from anesthesia. A significant increase in the concentration of fluorescent labeled probe in blood samples obtained from tail vein punctures was noted in the treatment group as compared to the control group.\textsuperscript{78}

The first direct evidence that LPT produced significant increases in TDL flow in conscious subjects was published in 2005 by Knott, et al.\textsuperscript{3} The investigators surgically implanted an ultrasonic flow transducer around the thoracic duct of canines and after a recovery period, measured the effects of exercise, pedal pump, abdominal pump, and thoracic pump on the TDL flow. Pedal pump, abdominal pump, and exercise exhibited a significant increase during 30 second interventions as compared to their respective pretreatment baseline measurements. Interestingly, abdominal LPT increased TDL flow to a similar degree as exercise; thus, it is tempting to speculate that LPT could enhance immune functions in a fashion similar to exercise.\textsuperscript{3} Another follow-up study by these same authors demonstrated that exercise and LPT increased
TDL flow for longer than previously reported time periods. In addition, this study showed that extracellular fluid volume expansion enlarged TDL flow and both LPT and exercise further augmented this trend. Our laboratory, along with collaborating investigator Downey, confirmed that LPT will increase lymph flow 4-fold in TDL of anesthetized animals. More importantly, in this study we demonstrated that LPT not only increased lymph flow, but also caused a 2-fold increase in leukocyte numbers, effectively producing an 8-fold net increase in leukocyte flux, without preferentially mobilizing a specific leukocyte population.

Collectively, these reports provide both indirect and direct evidence that OMM, specifically LPTs, can enhance immune function and protect against infections.

**The immune system and *Streptococcus pneumoniae***

Not only is Pneumonia associated with significant mortality, but the clinical burden and economic cost is estimated to be greater than $10 billion dollars annually. In 2005, bacterial pneumoniae and influenza were identified as the eighth leading cause of death in the USA, and the predominant offending pathogen was identified as *Streptococcus pneumoniae*, also known as pneumococcus. Despite advances in treatment and vaccination, antibacterial and vaccine resistance to *Streptococcus pneumoniae* has increased tremendously over the last decade. It continues to increase worldwide, with over 40% of pneumococci displaying multi-drug resistant phenotypes, and with resistance to three or more antibiotics.

*Streptococcus pneumoniae*, a gram positive aerobic bacterium, is part of the human nasopharyngeal normal flora and can be transferred between people via aerosol droplets. Pneumococcus is usually considered a commensal bacterium, but on occasion it can evade the immune system through a variety of virulence factors it possesses, and subsequently cause sinusitis, otitis media, pneumoniae, bacteremia, and meningitis. In order to cause disease,
Pneumococcus has to first colonize the upper respiratory tract, which is usually asymptomatic, and then progress to the lower respiratory tract, where it causes disease.\textsuperscript{84} Pneumococcal colonization of the epithelial cell layer is usually prevented by the respiratory mucociliary escalator and lysozyme secretion.\textsuperscript{85}

During colonization, the innate immune triggers the secretion of CRP, which in turn activates the classical complement pathway, enhances opsonization, and prevents epithelial attachment.\textsuperscript{85,86} In addition, an acute inflammatory response is triggered and includes macrophage cellular responses leading to the secretion of IL-1β, TNF-α, IL-6, and IL-8, and neutrophilic infiltration of paranasal spaces.\textsuperscript{85,86} Further, lactoferrins in nasal secretions exhibit bacteriostatic functions, and bridges the innate and adaptive immune function during colonization.\textsuperscript{87} Adaptive responses during colonization include sIgA secretion, which leads to interference of mucosal binding and opsonization. An increased Th17 response leading to enhanced neutrophil, sIgA, and IgM recruitment into the upper airway lumen has also been reported.\textsuperscript{85,86,88}

Post colonization, pneumococci can reach the alveolar air spaces, where they continue to replicate. An early immune response in the lower respiratory tract consists of alveolar macrophage opsonophagocytosis (a process involving multiple cycles of phagocytosis and killing by resident alveolar macrophages), which is also enhanced by immunoglobulin and complement opsonization.\textsuperscript{86,89,90} Recently, surfactant protein-D has been shown to exhibit a crucial role in the early, innate immune response, as SP-D deficient mice showed increased susceptibility to and decreased clearance of pneumococcal infection, along with earlier onset and higher levels of bacteremia.\textsuperscript{91} While the exact mechanism of SP-D is unknown, it has been
linked to pathogen aggregation, modulation of phagocytosis, phagocytic killing, and most recently to neutrophilic myeloperoxidase activity modulation.  

If bacterial load continues to rise, a late response is initiated by alveolar macrophages. This response includes the secretion of pro-inflammatory cytokines IL-1 and TNF-α, which induce the NFκB transcription factor, which further induce the secretion of a variety of cytokines and chemokines, such as IL-6, IL-8, MCP-1 and consequently lead to heavy neutrophil, and to a lesser extent, a monocyte/macrophage recruitment into alveolar spaces. Interestingly, IL-6 has been reported to induce CRP, delay neutrophil apoptosis, and enhance neutrophile cytotoxic function, such as ROS production.
CHAPTER II

Lymphatic Pump Manipulation Mobilizes Inflammatory Mediators into Lymphatic Circulation

Short Title: LPT and lymph cytokines

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Abstract

Lymph stasis can result in edema and the accumulation of particulate matter, exudates, toxins, and bacteria in tissue interstitial fluid, leading to inflammation, impaired immune cell trafficking, tissue hypoxia, tissue fibrosis, and a variety of diseases. Previously, we demonstrated that osteopathic lymphatic pump techniques (LPT) significantly increased thoracic and intestinal duct lymph flow. The purpose of this study was to determine if LPT would mobilize inflammatory mediators into the lymphatic circulation. Under anaesthesia, thoracic or intestinal lymph of dogs was collected at resting (Pre-LPT), during 4 min of LPT, and for 10 min following LPT (Post-LPT), and the lymphatic concentrations of IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, MCP-1, keratinocyte chemoattractant (KC), superoxide dismutase (SOD), and nitrotyrosine (NT) were measured. LPT significantly increased MCP-1 concentrations in thoracic duct lymph. Further, LPT increased both thoracic and intestinal duct lymph flux of cytokines and chemokines, as compared to their respective Pre-LPT flux. In addition, LPT increased lymphatic flux of SOD and NT. Ten minutes following cessation of LPT, thoracic and intestinal lymph flux of cytokines, chemokines, NT, and SOD were similar to Pre-LPT, demonstrating that their flux was transient and a response to LPT. This redistribution of inflammatory mediators during LPT may provide scientific rationale for the clinical use of LPT to enhance immunity and treat infection.

Keywords

lymph, lymphatic pump technique, cytokines, chemokines, inflammatory mediators, mesenteric duct lymph, thoracic duct lymph, infection, edema, immune system, reactive nitrogen species, reactive oxygen species, immunity, osteopathic manipulative medicine
Introduction

Osteopathic physicians have developed osteopathic manipulations known collectively as lymphatic pump techniques (LPT), which are designed to enhance lymph flow. By increasing lymph flow, LPT is thought to aid in the removal of metabolic wastes, toxins, exudates, and cellular debris that accumulate in the tissue interstitial fluid during infection or edema. Clinically, LPT has been shown to enhance vaccine specific antibodies and reduce the length of hospital stay, and the duration of antibiotic use in elderly patients with pneumonia.

During infection and edema, inflammatory cytokines, chemokines, reactive oxygen species (ROS), such as superoxide dismutase (SOD), and reactive nitrogen species (RNS), such as nitrotyrosine (NT) are generated. The pro-inflammatory cytokines and chemokines, interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), tissue necrosis factor-α, (TNF-α), interferon-γ (IFN-γ), monocyte chemotactic protein-1 (MCP-1), and keratinocyte chemoattractant (KC), induce leukocyte activation, migration, and cell mediated immune responses to pathogens, whereas anti-inflammatory cytokines such as interleukin-10 (IL-10), limit inflammation by suppressing cell-mediated immune responses.

Recent use of animal models has provided insight into the mechanisms by which LPT affects the lymphatic and immune systems. Previously, we reported that LPT enhances thoracic duct lymph (TDL) and mesenteric duct lymph (MDL) flow and leukocyte concentrations in dogs and rats. The purpose of this study was to determine if LPT would mobilize inflammatory mediators into the lymphatic circulation. In addition SOD and NT were measured. The results of this study provide support for the clinical application of LPT to enhance function of the immune system, and may explain, in part, a mechanism by which LPT protects against infection and edema.
Materials and Methods

Animals.

This study was approved by the Institutional Animal Care and Use Committee and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996). Twelve adult mongrel dogs, free of clinically evident signs of disease, were used for this study.

Surgical techniques.

Dogs were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). After endotracheal intubation, the dogs were ventilated with room air supplemented with oxygen to maintain normal arterial blood gases. In addition, arterial blood pressure was monitored via a femoral artery catheter and remained within normal limits throughout the experiment. In six dogs, the chest was opened by a thoracotomy in the left, fourth intercostal space. The thoracic duct was isolated from connective tissue and ligated. Caudal to the ligation, a PE 60 catheter (i.d. 0.76 mm, o.d. 1.22 mm) was inserted into the duct and secured with a ligature. Lymph was drained at atmospheric pressure through a catheter whose outflow tip was positioned 8 cm below heart level to compensate for the hydraulic resistance of the catheter. The outflow tip of the catheter was maintained at this position for all experimental conditions. Approximately 60 min following cannulation of the thoracic duct, thoracic lymph was collected during 4 min pre-LPT, during 4 min of LPT, and for 10 min following cessation of LPT (Post-LPT). Lymph flow rate was computed from the volume of lymph collected during these time intervals.

In separate experiments, mesenteric lymph was collected. Six additional dogs were surgically prepared for experimentation as described above. However, rather than opening the chest, a midline abdominal incision was made to expose a large mesenteric lymph duct. This
duct was isolated, ligated, and a PE 60 catheter was inserted into the duct and secured with a ligature. The catheter was exteriorized through the abdominal incision, which was then closed with 2-0 silk suture. Approximately 60 min after cannulation of the mesenteric lymph duct, mesenteric lymph samples were collected, and lymph flow was measured as described above for TDL.

**Lymphatic pump technique.**

The anesthetized dogs were placed in a right lateral recumbent position. To perform abdominal LPT, the operator contacted the abdomen of the animal with the hands placed bilaterally below the costo-diaphragmatic junction. Pressure was exerted medially and cranially to compress the abdomen until significant resistance was encountered, and then the pressure was released. Abdominal compressions were administered at a rate of approximately 1/s for a total of 4 min of LPT.

**Measurements of thoracic and mesenteric duct lymph.**

A commercially available multiplex assay (Millipore, Billerica, MA, USA) was used to determine the concentrations of cytokines and chemokines in TDL and MDL. Specifically, the cytokines IL-2, IL-4, IL-6, IL-10, IFN-$\gamma$, and TNF-$\alpha$ and the chemokines MCP-1 and KC were measured. A range of standards, provided with the multiplex assay, was used, and the assay was analyzed using the Luminex® 200 System with the xPONENT Software Interface (Millipore, Billerica, MA, USA). The minimum detectable concentrations for IL-2, IL-4, IL-6, IL-8, IL-10, IFN-$\gamma$, TNF-$\alpha$, MCP-1 and KC were 6.4, 28.8, 12.1, 20.3, 1.6, 4.4, 0.4, 8.6, and 1.6 pg/mL, respectively. To compute the cytokine/chemokine flux in TDL and MDL, the respective concentration was multiplied by lymph flow during each minute for each condition, and these values were averaged.
Thoracic lymph concentrations of SOD (Cayman Chemicals, Ann Arbor, MI, USA) and NT (Molecular Probes, Inc. Eugene, OR, USA) were measured using commercially available kits. The SOD assay measures all three forms of SOD by utilizing a tetrazolium salt for the detection of xanthine oxidase and hypoxanthine derived superoxide radicals. One unit of SOD is defined as the amount of enzyme necessary to cause 50% dismutation of the superoxide radical. The SOD minimum detectable concentration for this assay is 0.025 U/mL. NO reacts with superoxide to form peroxynitrite. Subsequently, peroxynitrate reacts with proteins, resulting in measurable NT. The minimum detectable concentration for NT of this assay is 2 nM. SOD and NT were measured only in TDL, since the samples of MDL were not sufficient for both these measurements and the Luminex assays. To compute SOD or NT flux in TDL, the respective concentration was multiplied by lymph flow during each minute for each condition, and these values were averaged.

Statistical analysis.

Data are presented as arithmetic means ± standard error (SE). Values from multiple animals at respective time points were averaged and are shown in either tables or plotted in figures. For statistical evaluation, data were subjected to repeated measures analysis of variance or analysis of variance followed by a Student-Newman-Keuls multiple comparisons test. Analyses were performed with Graphpad Prism version 5.0 for Windows, (GraphPad Software, San Diego, CA, USA). Differences among mean values with at least $P \leq 0.05$ were considered statistically significant.
Results

LPT increased intestinal and thoracic duct lymph flow.

Similar to our previous reports, LPT enhanced TDL and MDL flow. LPT increased TDL flow from 0.90 ± 0.19 ml/min during Pre-LPT to 5.65 ± 0.93 ml/min (P < 0.001) and the flow subsequently decreased to 2.07 ± 0.28 ml/min during Post-LPT (P < 0.01). LPT also increased MDL flow from 0.30 ± 0.03 ml/min during Pre-LPT to 2.71 ± 1.01 ml/min (P < 0.05) and the flow subsequently decreased to 0.32 ± 0.25 ml/min during Post-LPT (P < 0.05).

LPT increased the concentrations of MCP-1 in TDL.

Concentrations of cytokines and chemokines in TDL and in MDL are reported in Table 1. While cytokine and chemokine concentrations in both TDL and MDL moderately increased during LPT compared to Pre and Post-LPT, the only statistically significant increase detected was MCP-1 in TDL (P < 0.05). However, during LPT, differences were detected between MDL and TDL in the concentrations of IL-8 and MCP-1. Specifically, the concentration of IL-8 was greater during LPT in MDL (126%; P < 0.05) compared to TDL.

Of interest, the concentration of MCP-1 was greater in MDL compared to TDL in all samples (Table 1). Specifically, MCP-1 was greater at Pre-LPT (435%; P < 0.01), during LPT (200%; P < 0.01) and Post-LPT (214%; P < 0.01) when compared to respective TDL MCP-1 concentrations.

LPT increased lymphatic cytokine and chemokine flux.

The effect of LPT on flux of cytokines and chemokines in TDL is shown in Figure 1. LPT significantly increased TDL flux of IL-6 (615%; P < 0.05), IL-8 (944%; P < 0.001), IL-10 (917%; P < 0.001), MCP-1 (1505%; P < 0.01) and KC (788%; P < 0.001) compared to Pre-LPT.
Furthermore, these concentrations decreased Post-LPT by 79% in IL-6 (P < 0.05), 55% in IL-8 (P < 0.01), 53% in IL-10 (P < 0.01), 74% in MCP-1 (P < 0.05), and 57% in KC (P < 0.001).

The effect of LPT on flux of cytokines and chemokines in MDL is shown in Figure 2. LPT significantly increased the MDL flux of IL-6 (394%; P < 0.05), IL-8 (741%; P < 0.001), IL-10 (556%; P < 0.05), MCP-1 (651%; P < 0.01), and KC (496%; P < 0.001). As seen in TDL, the flux of cytokines and chemokines in MDL declined after LPT. From LPT to Post-LPT, IL-6 decreased by 67% (P < 0.05), IL-8 by 82% (P < 0.001), IL-10 by 86% (P < 0.05), MCP-1 by 86% (P < 0.01), and KC by 83% (P < 0.001). Cytokines IL-2, IL-4, IFN-\(\gamma\), and TNF-\(\alpha\) were not detectable in TDL or in MDL at any of the time points.

**LPT increased the flux of ROS and RNS in TDL.**

The effect of LPT on the flux of SOD in TDL is shown in Figure 3 and the corresponding effect on NT is shown in Figure 4. Although LPT did not significantly increase the concentrations of SOD and NT in TDL (Table 1), LPT increased SOD flux 367% in TDL from 0.15 ± 0.07 U/min Pre-LPT to 0.7 ± 0.1 U/min during LPT (P < 0.01). Post-LPT, SOD flux decreased 64% to 0.25 ± 0.08 U/min (P < 0.01; Figure 3). LPT increased NT flux in TDL 373% from 5.8 ± mM/min Pre-LPT to 27.4 ± 10.9 mM/min during LPT (P < 0.05). Post-LPT, NT flux decreased 84% to 4.4 ± 1.6 mM/min (P < 0.05; Figure 4).

**IL-6 flux was greater in TDL than in MDL during LPT.**

Flux of cytokines and chemokines in TDL and in MDL during LPT is compared in Figure 5. During LPT, IL-6 flux in TDL increased 318% more than the flux in MDL (P < 0.01).

**Discussion**

This study is the first to report the effects of LPT on the concentration and flux of inflammatory mediators in the lymphatic system. LPT did not significantly increase cytokine,
chemokine, ROS or RNS concentrations in lymph, with the exception of MCP-1; however, LPT increased lymphatic flow, which significantly increased the flux of these inflammatory mediators from tissue to blood via the lymphatic system. Specifically, LPT increased the flux of IL-6, IL-8, IL-10, MCP-1 and KC in thoracic and mesenteric lymph. While we did not measure ROS or RNS in MDL, LPT significantly increased SOD and NT flux in TDL. Collectively, these results suggest that by increasing lymph flow LPT enhances the mobilization of inflammatory mediators into the lymphatic circulation for transport to the blood circulation.

Cytokines, chemokines, ROS and RNS are generated during the innate immune response to pathogens. During infection, the cytokines IL-6, IL-8, MCP-1 and KC induce inflammation by recruiting and activating leukocytes, while IL-10 regulates the inflammatory response.\textsuperscript{1,2,98-100} During acute inflammation, inflammatory cytokines stimulate the formation of edema by accumulating in the interstitial fluid, which initially lowers the interstitial fluid pressure setting the stage for the influx of proteins and plasma fluid.\textsuperscript{101,102} Therefore, LPT may suppress edema by mobilizing inflammatory mediators out of interstitial fluid into the lymphatic circulation, as well as directly increasing lymph flow and removing excessive interstitial fluid.\textsuperscript{3,95}

LPT is used to treat infection,\textsuperscript{14-16,68,70} but the mechanisms by which LPT protect against infectious disease are unclear. LPT may enhance protection against infection by increasing mesenteric-derived inflammatory mediators in circulation, enabling the redistribution of these mediators to other tissues. In support of this notion, lymph has been shown to redistribute mesenteric-derived cytokines and chemokines to distant organs.\textsuperscript{26-28,103} Furthermore, it has been shown \textit{in vitro} that mesenteric lymph can activate neutrophils and increase endothelial cell permeability.\textsuperscript{104} It is not surprising, that LPT would enhance this redistribution and potentially enhance immune function.
Previously, we reported that LPT releases leukocytes from mesenteric lymph nodes into TDL and enhances leukocyte flux in MDL and TDL. Following exposure to microorganisms, phagocytes, such as macrophages and neutrophils, release ROS and RNS which are bactericidal. Thus, by enhancing the lymphatic flux of leukocytes, cytokines, chemokines, ROS and RNS, LPT may facilitate cell-mediated clearance of infection.

It has been hypothesized that following tissue injury, lymph flow quickly increases and provides the earliest signal in the lymphatic system to induce the inflammatory response. It is has been documented that lymphedema impairs immune cell trafficking and increases susceptibility to infection. Recently, transmural flow across lymphatic endothelia was shown to regulate cell and fluid transport functions of lymphatic endothelium. Specifically, transmural flow increased chemokine ligand secretion, influenced dendritic cells migration into lymphatic vessels, increased vessel permeability, and upregulated cell adhesion molecules on lymphatic vessels. The resulting increase in shear stress induces endothelial nitric oxide expression in human lymphatic endothelial cells, so elevated lymph flow causes release of endogenous nitric oxide from lymphatic endothelial cells. Therefore, in addition to releasing leukocytes into lymphatic circulation, by enhancing lymph flow and NT release into lymph, LPT may signal the lymphatic system to increase immune cell trafficking.

We also compared the lymphatic cytokine and chemokine composition between thoracic and mesenteric lymph. The thoracic duct is a large vessel and transports lymph drained from abdominal visceral organs (mainly the liver and intestines), skin, and skeletal muscle. We found the concentrations of cytokines and chemokines were higher in MDL (Table 1), which is consistent with the prior report that most of the lymph and protein in the thoracic duct is derived from the mesenteric lymph. This result suggests that compared to mesenteric lymph, lymph
derived from the liver and other tissues contains low concentrations of inflammatory mediators, and thus dilutes mesenteric-derived cytokines in TDL. It is important to note that these were healthy animals; therefore, during infection or inflammation the concentrations of inflammatory mediators in TDL and MDL may vary.

In conclusion, we have demonstrated that LPT transiently increased the flux of chemokines, cytokines, and reactive oxygen and nitrogen species in lymph. These findings are consistent with our previous reports, which demonstrated that LPT transiently increases thoracic and mesenteric lymph flow and leukocyte concentrations. It is important to note that this study was performed in healthy animals, and the effect of LPT on the lymphatic release of leukocytes and inflammatory mediators may be intensified or altered during infection. Our studies support the hypothesis that LPT may enhances immune response by enhancing the release of leukocytes and inflammatory mediators into lymphatic circulation.
Acknowledgements

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Authors' contributions

AS performed the surgery, animal instrumentation, statistical analysis, data interpretation and provided the LPT and preparation of the manuscript. HFD participated in the study design, data interpretation, and preparation of the manuscript. LMH designed and provided the oversight for the study. In addition, she reviewed and interpreted the data and participated in the preparation of the manuscript.

Competing interests

The authors declare they have no competing interests.
Table 1. LPT significantly altered the concentration of MCP-1, but did not significantly alter other cytokine, chemokine and reactive oxygen species concentrations in lymph.

<table>
<thead>
<tr>
<th></th>
<th>Pre-LPT</th>
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<th>Post-LPT</th>
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<tr>
<td><strong>Thoracic duct lymph (TDL)</strong></td>
<td></td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>193 ± 65</td>
<td>217 ± 74</td>
<td>158 ± 59</td>
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<td>IL-8 (pg/ml)</td>
<td>183 ± 25</td>
<td>240 ± 48</td>
<td>217 ± 42</td>
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<tr>
<td>IL-10 (pg/ml)</td>
<td>24 ± 9</td>
<td>31 ± 5</td>
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<td>MCP-1 (pg/ml)</td>
<td>578 ± 135</td>
<td>1160 ± 304*</td>
<td>958 ± 266</td>
</tr>
<tr>
<td>KC (pg/ml)</td>
<td>1351 ± 165</td>
<td>1501 ± 247</td>
<td>1284 ± 172</td>
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<td>SOD (U/ml)</td>
<td>0.135 ± 0.038</td>
<td>0.176 ± 0.026</td>
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<td><strong>Mesenteric duct lymph (MDL)</strong></td>
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<td></td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>217 ± 43</td>
<td>241 ± 116</td>
<td>333 ± 115</td>
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<td>IL-10 (pg/ml)</td>
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<td>MCP-1 (pg/ml)</td>
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<td>3482 ± 685††</td>
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<td>KC (pg/ml)</td>
<td>2389 ± 554</td>
<td>2522 ± 506</td>
<td>2270 ± 526</td>
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</table>

Data are means ± SE (n=6). *Greater than respective Pre-LPT (p<0.05). †Different from respective TDL value (p<0.05). ††Different from respective TDL value (p<0.01). Repeated Measures ANOVA with Student-Newman-Keuls post test.
Figure 1. LPT increased cytokine and chemokine flux in thoracic duct lymph. Data are means ± SE (n=6). *Greater than respective Pre-LPT and Post-LPT (p<0.05). **Greater than respective Pre-LPT and Post-LPT values (p<0.01). ***Greater than respective Pre-LPT and Post-LPT values (p<0.001). Repeated Measures ANOVA with Student-Newman-Keuls post test.
Figure 2. LPT increased cytokine and chemokine flux in mesenteric duct lymph. Data are means ± SE (n=6). *Greater than respective Pre-LPT and Post-LPT (p<0.05). **Greater than respective Pre-LPT and Post-LPT values (p<0.01). ***Greater than respective Pre-LPT and Post-LPT values (p<0.001). Repeated Measures ANOVA with Student-Newman-Keuls post test.
Figure 3. LPT increased SOD flux in thoracic duct lymph. Data are means ± SE (n=6).

**Greater than respective Pre-LPT and Post-LPT values (p<0.01). Repeated Measures ANOVA with Student-Newman-Keuls post test.
Figure 4. LPT increased NT flux in thoracic duct lymph. Data are means ± SE (n=6).

*Greater than respective Pre-LPT and Post-LPT values (p<0.05). Repeated Measures ANOVA with Student-Newman-Keuls post test.
Figure 5. LPT created a difference in measurable IL-6 in TDL vs. MDL. Data are means ± SE (n=6). **Greater than respective MDL value (p<0.01). ANOVA with Student-Newman-Keuls post test.
CHAPTER III

Title: Lymphatic Pump Treatment Repeatedly Enhances the Lymphatic and Immune Systems

Short Title: Repeated Applications of LPT

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Abstract

Background

Osteopathic practitioners utilize manual therapies called lymphatic pump techniques (LPT) to treat edema and infectious diseases. While previous studies examined the effect of a single LPT treatment on the lymphatic system, the effect of repeated applications of LPT on lymphatic output and immunity has not been investigated. Therefore, the purpose of this study was to measure the effects of repeated LPT on lymphatic flow, lymph leukocyte numbers, and inflammatory mediator concentrations in thoracic duct lymph (TDL).

Methods and Results

The thoracic ducts of five mongrel dogs were cannulated, and lymph samples were collected during pre-LPT, four min of LPT and two hours post-LPT. A second LPT (LPT-2) was applied after a 2 hour rest period. TDL flow was measured, and TDL were analyzed for the concentration of leukocytes and inflammatory mediators. Both LPT treatments significantly increased TDL flow, leukocyte count, total leukocyte flux, and the flux of interleukin-8 (IL-8), keratinocyte-derived chemoattractant (KC), nitrite (NO$_2^-$) and superoxide dismutase (SOD). The concentration of IL-6 increased in lymph over time in all experimental groups; therefore, it was not-LPT dependent.

Conclusion

Clinically, it can be inferred that LPT at a rate of 1 pump per sec for a total of 4 min can be applied every 2 hr, thus providing scientific rationale for the use of LPT to repeatedly enhance the lymphatic and immune system.
Condensed abstract

Osteopathic practitioners utilize manual therapies called lymphatic pump techniques (LPT) to treat edema and infectious diseases. The purpose of this study was to measure the effects of repeated LPT on lymphatic flow, lymph leukocyte numbers and inflammatory mediator concentrations in thoracic duct lymph (TDL) in dogs. Clinically, it can be inferred that LPT at a rate of 1 per sec for a total of 4 min can be applied every 2 hr, thus providing scientific rationale for the use of LPT to repeatedly enhance the lymphatic and immune system.

Keywords:
lymphatic pump technique, manual therapy, manual lymph drainage, thoracic duct lymph, infection, edema, immune system, osteopathic manipulative medicine, immunity, inflammatory mediators, cytokines, chemokines
Introduction

The lymphatic system is essential for interstitial fluid homeostasis and function of the immune system.\textsuperscript{1,2,32} Interstitial fluid homeostasis is maintained by lymphatic absorption of excess interstitial tissue fluid and by transport of this fluid, along with osmotically active proteins, parenchymal cell products, inflammatory mediators, immune cells, proteins, apoptotic cells, antigens, and infectious organisms through the nodes to the circulation.\textsuperscript{32,34} In addition to transporting immunological factors, the lymphatic system actively participates in immune surveillance and the induction of immune responses, while maintaining self-tolerance. Dysfunction of the lymphatic system leads to edema, impaired trafficking of immune cells, accumulation of inflammatory mediators, tissue hypoxia and injury, inflammation, and a variety of diseases.\textsuperscript{6,111-113}

Edema, whether due to lymphatic dysfunction or to other causes, is generally treated by procedures designed to increase lymph flow or prevent the accumulation of fluid into tissue. These procedures include movement or elevation of dependent limbs and tissue compression.\textsuperscript{57,58} When these procedures are inadequate, pharmacological or surgical interventions may be required.\textsuperscript{57,58,114} Osteopathic physicians have developed manual lymphatic pump techniques (LPT) to increase lymph flow.\textsuperscript{6,7} Considering the important role of the lymphatic system in immune function, LPT is also used to treat infections.\textsuperscript{6,7}

Previously, we demonstrated that a single application of LPT increased lymph flow and leukocyte flux in both rats and dogs, and mobilized inflammatory mediators into lymph circulation.\textsuperscript{3,4,79,94-96,115} These findings support the use of LPT to treat edema and to enhance immune function. Importantly, the effects of LPT on the lymphatic system were transient, suggesting this LPT-sensitive lymph reservoir is limited.\textsuperscript{3,4,79,94-96,115} The purpose of this study
was to determine if a second application of LPT could also enhance lymph flow, the number of leukocytes, and the concentration of inflammatory mediators in thoracic duct lymph. Our results demonstrate that LPT can be repeatedly applied to enhance lymph flow, leukocyte numbers, and the flux of inflammatory mediators. The LPT-sensitive reservoir these techniques draw from is largely restored by 2 hr, thus supporting the clinical application of repeated LPT.

**Materials and Methods**

**Animals**

This study was approved by the Institutional Animal Care and Use Committee and was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication no. 85-23, revised 1996). Five adult mongrel dogs, free of clinically evident signs of disease, were used for this study.

**Surgical procedures and experimental protocols**

Prior to surgery, the dogs were fasted overnight and then anesthetized with sodium pentobarbital (30 mg/kg, IV). After endotracheal intubation, the dogs were ventilated with room air and supplemented with oxygen to maintain normal arterial blood gases. Arterial blood pressure (AOP) was monitored via a femoral artery pressure monitoring catheter connected to a pressure transducer (Hewlett–Packard Pressure Monitor, 78354A); AOP remained within normal limits throughout the experiment. Blood samples were periodically collected from the femoral artery catheter and assessed for arterial blood gases and pH (GEM Premier 3000 Blood Gas/Electrolyte Analyzer, Model 5700, Instrumentation Laboratory, Lexington, MA). The chest was opened by a left lateral thoracotomy in the fourth intercostal space. The thoracic duct was isolated from connective tissue and ligated. Caudal to the ligation, a PE 60 catheter (i.d. 0.76 mm, o.d. 1.22 mm) was inserted into the duct and secured with a ligature. Lymph was drained at
atmospheric pressure through a catheter whose outflow tip was positioned 8 cm below heart level to compensate for the hydraulic resistance of the catheter.

**Lymphatic pump technique**

LPT was performed by a medical student (A.S.) trained in osteopathic lymphatic manipulation. During manipulation, the anesthetized dogs were placed in a right lateral recumbent position. To perform abdominal LPT, the operator contacted the ventral side of the animal’s abdomen with the hands placed bilaterally below the costo-diaphragmatic junction. Sufficient pressure was exerted medially and cranially to compress the abdomen until significant resistance was encountered against the diaphragm, and then the pressure was released. Abdominal compressions were administered at a rate of approximately 1 per sec for a total of 4 min.

**Leukocyte enumeration and flux**

Total leukocytes and a differential leukocyte count in lymph samples were enumerated using the Hemavet 950 (Drew Scientific, Waterbury, CT). To compute TDL leukocyte flux, leukocyte concentrations were multiplied by the respective lymph flow rates for each condition. Fluxes of specific leukocyte populations, cytokines, chemokines, nitrite, and SOD were computed in a similar manner.

**Measurement of inflammatory mediators**

A commercially available multiplex assay (Millipore, Billerica, MA, USA) was used to determine the concentrations of cytokines and chemokines in TDL. Specifically, the cytokine IL-6 and chemokines IL-8 and KC were measured. A range of standards provided with the multiplex assay was used, and the assay was analyzed using the Luminex R 200 System with the xPONENT Software Interface (Millipore). The minimum detectable concentrations for IL-6, IL-
8, and KC were 12.1, 20.3, and 1.6 pg/ml, respectively. Cytokine/chemokine fluxes in TDL were computed from the product of respective concentrations and flows, as were fluxes of total leukocytes.

Thoracic lymph concentrations of nitrite (NO$_2^-$) and SOD were measured using commercially available kits (Promega Corporation, Madison, WI, USA, and Cayman Chemicals, Ann Arbor, MI, USA). The Promega Griess Reagent system measures NO$_2^-$, a nonvolatile and stable breakdown product of nitric oxide (NO). The minimum detectable nitrite concentration for this assay is 2.5 μM. The SOD assay measures all three forms of SOD (cytosolic Cu/Zn-SOD, mitochondrial MnSOD, and extracellular FeSOD) by utilizing a tetrazolium salt for the detection of xanthine oxidase and hypoxanthine-derived superoxide radicals. One unit of SOD is defined as the amount of enzyme necessary to cause 50% dismutation of the superoxide radical. The minimum detectable SOD concentration for this assay is 0.025 U/ml. TDL fluxes of these agents were computed similarly to that of other agents, as described above.

**Statistical analysis**

Data are presented as arithmetic means ± standard error (SE). Values from multiple animals at respective time points were averaged, and the mean values are shown in tables or in figures. For evaluation of statistical significance, data were subjected to repeated measures analyses of variance, followed by a Student-Newman-Keuls multiple comparisons post hoc tests. Statistical analyses were performed with GraphPad Prism version 5.04 and GraphPad InStat version 3.06 for Windows, (GraphPad Software, San Diego, CA, USA). Differences among mean values with at least $P \leq 0.05$ were considered statistically significant.
Results

Lymphatic pump treatment repeatedly increases thoracic duct lymph flow

Sample collection points are illustrated in Figure 1. TDL flow values before (pre), during, and after (post) LPT are illustrated in Figure 2. Consistent with our previous reports,\textsuperscript{4,94,115} LPT increased TDL flow from 0.3 ± 0.1 ml/min (pre-LPT) to an average of 6.0 ± 1.2 ml/min ($P < 0.001$) during the 4 min of LPT 1. After cessation of LPT 1, lymph flow decreased to 0.4 ± 0.1 ml/min ($P < 0.001$). TDL flow did not vary during the 2 hr interval (14-124 min) between LPT 1 and LPT 2 (Figure 2). Similar to LPT 1, LPT 2 significantly ($P < 0.001$) increased TDL flow from 0.3 ± 0.1 ml/min during pre-LPT to an average of 5.5 ± 0.5 ml/min during LPT 2 (Figure 2). These LPT-induced increases in lymph flow did not differ significantly ($P > 0.05$). Following LPT 2, lymph flow decreased to 0.3 ± 0.1 ml/min ($P < 0.001$). While lymph flow throughout the 4 min periods of LPT 1 and LPT 2 remained significantly elevated compared to their respective pre-LPT and post-LPT values, both treatments caused the greatest increase in lymph flow during the first minute of LPT (Figure 2).

Lymphatic pump treatment repeatedly increases thoracic duct leukocytes

The effect of LPT on total leukocyte numbers is reported in Figure 3. Leukocyte counts were significantly elevated at 1 min ($P < 0.054$) and continued to elevate at 2, 3, and 4 min of LPT 1, as well as throughout the period of LPT 2. The average pre-LPT 1 baseline leukocyte count was 12.1 ± 3.5 x 10^6 cells/ml, and LPT 1 significantly increased leukocyte numbers to 26.5 ± 3.4 x 10^6 cells/ml ($P < 0.001$). Ten min following LPT 1 (14 min), the leukocyte count in TDL remained elevated at 25.0 ± 2.8 x 10^6 cells/ml ($P < 0.001$). By 90 min, leukocytes returned to baseline (10.5 ± 1.5 x 10^6 cells/ml). LPT 2 significantly increased leukocyte numbers from 7.6 ± 3.1 x 10^6 cells/ml during pre-LPT 2 to 25.6 ± 1.4 x 10^6 cells/ml ($P < 0.001$). As seen following
LPT 1, the leukocyte count remained significantly elevated for 10 min following LPT 2 ($P < 0.01$). There were no significant differences in TDL flow between LPT 1 and LPT 2.

To determine if LPT preferentially increased a specific leukocyte population, we measured the percentage and concentration of neutrophils, monocytes, and lymphocytes in TDL (Table 1). LPT 1 significantly increased the TDL count of neutrophils ($P < 0.001$) by 251%, monocytes by 116% ($P < 0.01$), and of lymphocytes ($P < 0.001$) by 111% compared to pre-LPT 1. Subsequently, the count decreased post-LPT 1 by 73% for neutrophils ($P < 0.001$), 72% ($P < 0.01$) for monocytes, and 56% for lymphocytes ($P < 0.001$). In a similar fashion, LPT 2 significantly increased TDL count of neutrophils by 402% ($P < 0.05$), monocytes by 181% ($P < 0.01$), and of lymphocytes by 240% ($P < 0.05$) compared to pre-LPT 2. Subsequently, the count decreased post-LPT 2 by 36% ($P < 0.05$) for neutrophils, 29% ($P < 0.161$) for monocytes, and 26% ($P < 0.05$) for lymphocytes. No significant ($P > 0.05$) differences in TDL neutrophil, monocyte, and lymphocyte counts were detected between LPT 1 and LPT 2.

**Lymphatic pump treatment repeatedly increases thoracic duct leukocyte flux**

TDL leukocyte flux values are reported in Figure 4. LPT accelerated TDL leukocyte flux from a mean pre-LPT 1 value of $3.9 \pm 1.5 \times 10^6$ cells/min to a peak value of $166 \pm 51 \times 10^6$ cells/min at 3 min of LPT 1 ($P < 0.001$). Throughout 4 min of LPT 1, TDL leukocyte flux remained elevated ($P < 0.001$), averaging $149.3 \pm 34 \times 10^6$ cells/min. The flux at 14 min averaged $8.8 \pm 2.3 \times 10^6$ cells/min ($P < 0.001$) and was $3.0 \pm 0.7 \times 10^6$ cells/min at 90 min, suggesting the effect of LPT on TDL flux is transient. LPT 2 accelerated TDL leukocyte flux from a mean pre-LPT 2 value of $2.3 \pm 1.0 \times 10^6$ cells/min to a peak value of $174 \pm 17 \times 10^6$ cells/min at 2 min. For 4 min of LPT 2, TDL leukocyte flux averaged $136.8 \pm 9 \times 10^6$ cells/min ($P < 0.001$ vs pre-LPT 2). The flux at post-LPT 2 decreased to $5.8 \pm 1.6 \times 10^6$ cells/min ($P < 0.001$ vs pre-LPT 2).
There was no significant ($P > 0.05$) difference in TDL leukocyte flux between LPT 1 and LPT 2.

The TDL flux of neutrophils, monocytes, and lymphocytes is reported in Table 2. LPT 1 significantly increased the flux of neutrophils ($P < 0.001$) by 5500%, monocytes by 3294% ($P < 0.001$), and lymphocytes ($P < 0.001$) by 3646%, compared to pre-LPT 1. The flux decreased post-LPT 1 by 98% for neutrophils ($P < 0.001$), 98% ($P < 0.001$) for monocytes, and 98% for lymphocytes ($P < 0.001$) compared to LPT 1. Similarly, LPT 2 significantly increased the flux of neutrophils by 8114% ($P < 0.001$), monocytes by 4491% ($P < 0.001$), and lymphocytes by 5613% ($P < 0.001$), compared to pre-LPT 2 values. Subsequently, the flux decreased following post-LPT 2 by 96% ($P < 0.001$) for neutrophils, 95% ($P < 0.001$) for monocytes, and 96% ($P < 0.001$) for lymphocytes compared to LPT 2. No statistically significant ($P > 0.05$) differences in TDL neutrophil, monocyte, and lymphocyte counts and flux were detected between LPT 1 and LPT 2.

**Lymphatic pump treatment repeatedly increases the flux of cytokines and chemokines in thoracic duct lymph**

Concentrations of inflammatory mediators in TDL are reported in Table 3. In general, inflammatory mediator concentrations did not significantly change during LPT when compared with pre- and post-LPT, except post-LPT 2 values for IL-6 were statistically greater than pre-LPT 2 (93% increase) and LPT 2 (67% increase). To quantify the effect of LPT on TDL cytokine/chemokine release, we measured three inflammatory mediators previously shown to increase during LPT. LPT 1 significantly increased TDL flux of IL-6 (1653%; $P < 0.001$), IL-8 (7223%; $P < 0.001$), and KC (2052%; $P < 0.001$) when compared with pre-LPT 1 (Figure 5). Subsequently, the flux of these cytokines and chemokines decreased 94% ($P < 0.001$) in IL-6,
IL-8, and KC following LPT 1. Similarly, LPT 2 significantly increased TDL flux of IL-6 (2038%; \( P < 0.001 \)), IL-8 (1575%; \( P < 0.001 \)), and KC (1405%; \( P < 0.001 \)) when compared with pre-LPT 2. As seen in post-LPT 1, the flux of cytokines and chemokines in post-LPT 2 declined after cessation of the intervention; IL-6 decreased by 91% \(( P < 0.001)\), and both IL-8 and KC decreased by 94% \(( P < 0.001)\). The flux of IL-6 was greater during LPT 2 than during LPT 1 \(( P < 0.001)\), a reflection of the increased concentration of IL-6 during LPT 2. On average, LPT 2 released 184 pg/min more IL-6 into the thoracic duct lymph compared to LPT 1.

**Lymphatic pump treatment repeatedly increases the flux of superoxide dismutase and nitrite in thoracic duct lymph**

Neither LPT 1 nor LPT 2 significantly altered the concentrations of nitrite and SOD in TDL (Table 3). The effect of LPT on SOD flux is presented in Figure 6. LPT increased SOD flux in TDL, 2418% from 1.2 ± 0.07 U/min pre-LPT 1 to 30.21 ± 1.53 U/min during LPT 1 \(( P < 0.001)\), and 1848% from 1.41 ± 0.15 U/min pre-LPT 2 to 27.46 ± 0.39 U/min during LPT 2 \(( P < 0.001)\). Post-LPT decreased SOD flux in TDL, 95% to 1.47 ± 0.20 U/min \(( P < 0.001)\) during post-LPT 1 and 95% to 1.40 ± 0.04 U/min \(( P < 0.001)\) during post-LPT 2. During the 4 min period of LPT 2, average SOD flux was 2.75 U/min less than compared to the same period of LPT 1.

The effect of LPT on nitrite flux is presented in Figure 7. LPT 1 increased nitrite flux 1356% in TDL from 2.22 ± 0.27 µM/min pre-LPT 1 to 32.33 ± 2.49 µM/min \(( P < 0.001)\), and LPT 2 increased nitrite flux 1240% in TDL from 1.50 ± 0.35 µM/min pre-LPT 2 to 20.10 ± 2.98 µM/min \(( P < 0.001)\). Nitrite flux decreased 96% to 1.46 ± 0.15 µM/min \(( P < 0.001)\) during post-LPT 1 and 92% to 1.59 ± 0.38 µM/min \(( P < 0.001)\) during post-LPT 2. During 4 min of LPT 2,
average nitrite flux was 12.23 µM/min less than compared to the same period of LPT 1 ($P < 0.001$).

**Discussion**

Animal studies have shown that LPT increased TDL flow in healthy conscious dogs, in conscious dogs with abdominal edema induced by inferior vena cava constriction, and in conscious dogs after extracellular fluid volume expansion. In anesthetized dogs, LPT increased TDL flow, leukocyte count and flux, inflammatory mediators, and mobilized leukocytes from mesenteric lymph nodes. In addition, LPT increased lymph flow and the mobilization of gastrointestinal lymphoid tissue derived leukocyte counts of both dogs and rats. The aim of the present study was to gain new information on replenishment of LPT-sensitive lymph reservoirs by repeating LPT after a 2 hr resting interval. We found both LPT 1 and LPT 2 produced similar increases in TDL flow, TDL leukocyte concentration and the lymphatic flux of leukocytes and immune mediators. In addition, the results confirmed prior findings that LPT mobilizes TDL flow and immune factors in anesthetized dogs.

Our results demonstrate that LPT mobilizes lymph from a reservoir that depletes during 4 min of LPT, but is replenished by 2 hr. At rest, the majority of the TDL is derived from the liver (~30%) and intestines (~70%), with small contributions from the thoracic cavity and lower extremities. Thus, we speculate that during the 2 hr resting interval between LPT 1 and LPT 2, this fluid pool was mainly replenished by lymph formed in the abdominal viscera.

LPT repeatedly enhanced the release of leukocytes into lymphatic circulation, as demonstrated by an increase in leukocytes/ml. This increase in leukocyte numbers was seen at 1 min of LPT, suggesting LPT quickly mobilizes leukocytes into lymphatic circulation. While greatly reduced compared to the leukocytes released during LPT, TDL leukocyte concentrations
remained elevated at 10 min post-LPT, indicating the LPT-sensitive leukocyte reservoir continues to release cells after the cessation of LPT. Since leukocytes directly kill pathogens or induce an antigen-specific immune response via the lymph nodes,\textsuperscript{1,2} the ability to repeatedly mobilize leukocytes and other immune factors by repeated LPT should have important clinical implications.

Although the lymphatic flux of SOD was similar during LPT 1 and LPT 2, the flux of NO\textsubscript{2}-, an index of NO, was reduced during LPT 2. NO in lymph is derived from the lymphatic endothelium, where it regulates phasic contractions and lymph flow.\textsuperscript{110,120} Specifically, increasing lymph flow induces the production and release of NO from lymphatic endothelial cells.\textsuperscript{110} LPT did not increase the concentration of NO in TDL. It is possible that NO was released during LPT, but when the lymph flow increased the NO was diluted; therefore, an increase in NO concentration was not seen. Furthermore, the flux of NO during LPT 2 was significantly reduced compared to LPT 1, suggesting it takes more than 2 hr to fully replenish the NO precursors in the endothelium.

A high lymph flow rate can also inhibit the intrinsic lymph pump, which is predominantly due to the production of NO.\textsuperscript{38,109,110,121} It has been proposed that this flow-dependent inhibition of the intrinsic active lymph pump may have evolved to save energy when the lymphatics do not need to generate lymph flow, because another upstream mechanism, such as elevated interstitial fluid pressure, is generating flow.\textsuperscript{38} Therefore, it is possible that the increase in TDL flow/sheer generated during LPT may relax lymph vessel tone and suppress the intrinsic pump, thus sparing pumping energy within the lymphatic system. This would be particularly important during the management of edema; however, further studies are required to support this hypothesis.
While LPT increased the lymphatic flux of cytokines, KC, SOD, and NO$_2^-$, it is interesting that LPT failed to significantly increase the lymphatic concentration of these inflammatory mediators. Since their increase was flow-dependent, LPT likely mobilizes lymph pools that contain inflammatory mediators. Exceptions were IL-6 and IL-8. The gradual increase in the lymphatic concentration of IL-6 was not LPT-mediated but more likely related to surgical stress. In support, IL-6 is a pro-inflammatory cytokine that has been shown to increase in the plasma following surgery, laparoscopic procedures, and cardiopulmonary bypass. On the other hand, TDL levels of IL-8 were at the minimal detection limit (6.51 pg/ml) and quickly rose (80 ± 9 pg/ml) in response to LPT 1. Furthermore, IL-8 remained increased during the duration of the experiment. This result suggests that LPT 1 was able to stimulate the continuous release of IL-8 into lymphatic circulation and LPT 2 did not further enhance this release; however, the source of this IL-8 and the mechanism responsible for this increase is not clear.

Previous studies measured the effect of lymph flow/shear on lymph vessel function using isolated, pressurized ducts, where transmural pressure and flow were imposed in vitro. While we did not measure the effect of LPT directly on the function of the lymphatic vasculature, we were able to study the effect of enhancing lymph flow on immune cell concentration and inflammatory mediators in vivo. The increase in lymph flow generated during LPT likely affects lymphatic endothelium; however, further studies are needed to confirm this hypothesis. A distinct advantage to our model is that it allows us, and others, to examine the effect of enhancing lymph flow on the development and/or resolution of disease in vivo. For example, in support, recent animal studies demonstrated LPT has a positive effect on infection and edema.
Some limitations of the current study must be recognized. While in general, findings in anesthetized animals have been consistent with our more limited observations in conscious instrumented dogs,\textsuperscript{3,4,79,94,95,115} confirmation of the current findings in a conscious model would be valuable. LPT was repeated only once after a 2 hr resting interval, due to concern about the stability of the anesthetized animal; therefore, more applications of LPT may continue to enhance the lymphatic system. It is also not known if these LPT-sensitive lymph pools replenish before 2 hr. Clearly, a more extensive study with more repetitions of LPT at different intervals, are warranted.

In conclusion, we have demonstrated that LPT can be repeated within a 2 hr period to stimulate the entry of leukocytes into lymph and mobilize lymph pools containing inflammatory mediators into central lymphatic circulation. This finding provides new insight into the kinetics of lymph formation following manual stimulation. Furthermore, clinical guidelines for the duration and frequency of LPT are inconsistent and poorly defined;\textsuperscript{6,7,14,16,17,60,127} therefore, the information gained from this study may encourage a more standardized and aggressive use of LPT during the treatment of infection and edema by osteopathic physicians and other manual medicine practitioners.
Acknowledgements

This study was funded by a grant from the National Institutes of Health, grant R01 AT004361 (LMH). The authors thank the Osteopathic Heritage Foundation for their continued support of the Basic Science Research Chair (LMH). The authors would also like to thank Arthur Williams, Jr., and Linda Howard for assistance in the animal surgery.

Author Disclosure Statement

No competing financial interests exist.
Table 1. LPT increases the concentration of leukocytes in thoracic duct lymph.

<table>
<thead>
<tr>
<th></th>
<th>Pre-LPT 1</th>
<th>LPT 1</th>
<th>Post-LPT 1</th>
<th>Pre-LPT 2</th>
<th>LPT 2</th>
<th>Post-LPT 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte count (x 10^6 cells/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>0.63 ± 0.29</td>
<td>2.21 ± 0.47***</td>
<td>0.59 ± 0.16</td>
<td>0.45 ± 0.26</td>
<td>2.26 ± 0.30*</td>
<td>1.44 ± 0.25</td>
</tr>
<tr>
<td>MO</td>
<td>2.09 ± 0.88</td>
<td>4.52 ± 1.10**</td>
<td>1.25 ± 0.36</td>
<td>1.44 ± 0.83</td>
<td>4.04 ± 0.92†</td>
<td>2.86 ± 0.48</td>
</tr>
<tr>
<td>LY</td>
<td>9.31 ± 2.38</td>
<td>19.6 ± 2.15***</td>
<td>8.64 ± 1.06</td>
<td>5.64 ± 1.97</td>
<td>19.17 ± 0.59*</td>
<td>14.1 ± 1.07</td>
</tr>
<tr>
<td>Percentage of leukocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>4.3 ± 0.9</td>
<td>7.9 ± 0.8**</td>
<td>5.1 ± 0.7</td>
<td>4.7 ± 0.7</td>
<td>8.6 ± 0.8†</td>
<td>7.5 ± 0.9</td>
</tr>
<tr>
<td>MO</td>
<td>14.9 ± 2.7</td>
<td>16.4 ± 2.3</td>
<td>11.2 ± 2.2</td>
<td>15.9 ± 2.3</td>
<td>15.2 ± 2.5</td>
<td>15.3 ± 2.2</td>
</tr>
<tr>
<td>LY</td>
<td>80.8 ± 3.6</td>
<td>75.4 ± 3.1</td>
<td>83.5 ± 2.7</td>
<td>79.0 ± 3.0</td>
<td>75.9 ± 3.2</td>
<td>76.9 ± 2.6</td>
</tr>
</tbody>
</table>

Thoracic duct lymph was collected over ice from five anesthetized dogs and analyzed. LPT, lymphatic pump technique; NO, neutrophils; MO, monocytes; LY, lymphocytes

Data are means ± SE (n = 5). Data were analyzed by repeated measures analysis of variance with Student-Newman-Keuls post-hoc test. *Greater than respective pre-LPT and post-LPT values (P < 0.05). **Greater than respective pre-LPT and post-LPT values (P < 0.01). ***Greater than respective pre-LPT and post-LPT values (P < 0.001). †Greater than respective Pre-LPT (P < 0.01). There were no significant (P>0.05) differences between LPT 1 and LPT 2.
<table>
<thead>
<tr>
<th></th>
<th>Pre-LPT 1</th>
<th>LPT 1</th>
<th>Post-LPT 1</th>
<th>Pre-LPT 2</th>
<th>LPT 2</th>
<th>Post-LPT 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte flux (x 10^6 cells/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>0.20 ± 0.10</td>
<td>11.2 ± 1.86*</td>
<td>0.18 ± 0.07</td>
<td>0.14 ± 0.08</td>
<td>11.5 ± 1.06*</td>
<td>0.46 ± 0.13</td>
</tr>
<tr>
<td>MO</td>
<td>0.66 ± 0.29</td>
<td>22.4 ± 4.10*</td>
<td>0.40 ± 0.16</td>
<td>0.44 ± 0.25</td>
<td>20.2 ± 2.7*</td>
<td>1.00 ± 0.36</td>
</tr>
<tr>
<td>LY</td>
<td>3.07 ± 1.15</td>
<td>115 ± 28.6*</td>
<td>2.45 ± 0.51</td>
<td>1.74 ± 0.62</td>
<td>99.4 ± 11.9*</td>
<td>4.32 ± 1.12</td>
</tr>
</tbody>
</table>

Thoracic duct lymph was collected over ice from five anesthetized dogs and analyzed.

LPT, lymphatic pump technique; NO, neutrophils; MO, monocytes; LY, lymphocytes

Data are means ± SE (n = 5). Data were analyzed by repeated measures analysis of variance with Student-Newman-Keuls post-hoc test. *Greater than respective pre-LPT and post-LPT values (P < 0.001). There were no significant (P>0.05) differences between LPT 1 and LPT 2.
Table 3. Lymphatic pump treatment did not significantly alter the concentration of inflammatory mediators in TDL.

<table>
<thead>
<tr>
<th></th>
<th>Pre-LPT 1</th>
<th>LPT 1</th>
<th>Post-LPT 1</th>
<th>Pre-LPT 2</th>
<th>LPT 2</th>
<th>Post-LPT 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-6 (pg/ml)</strong></td>
<td>48 ± 8</td>
<td>45 ± 5</td>
<td>47 ± 6</td>
<td>71 ± 6</td>
<td>82 ± 5</td>
<td>137 ± 35</td>
</tr>
<tr>
<td><strong>IL-8 (pg/ml)</strong></td>
<td>MDL</td>
<td>80 ± 9</td>
<td>84 ± 8</td>
<td>89 ± 6</td>
<td>81 ± 2</td>
<td>84 ± 14</td>
</tr>
<tr>
<td><strong>KC (pg/ml)</strong></td>
<td>321 ± 47</td>
<td>370 ± 55</td>
<td>401 ± 62</td>
<td>524 ± 65</td>
<td>428 ± 38</td>
<td>454 ± 66</td>
</tr>
<tr>
<td><strong>NO₂⁻ (µM)</strong></td>
<td>6.9 ± 0.8</td>
<td>5.4 ± 0.4</td>
<td>4.4 ± 0.5</td>
<td>5.0 ± 1.2</td>
<td>3.6 ± 0.5</td>
<td>5.3 ± 1.3</td>
</tr>
<tr>
<td><strong>SOD (U/ml)</strong></td>
<td>3.8 ± 0.2</td>
<td>5.0 ± 0.3</td>
<td>4.4 ± 0.6</td>
<td>4.7 ± 0.5</td>
<td>5.0 ± 0.1</td>
<td>4.6 ± 0.1</td>
</tr>
</tbody>
</table>

Thoracic duct lymph was collected over ice from five anesthetized dogs and analyzed.

LPT, lymphatic pump technique; MDL, minimum detection limits (6.51 pg.ml); IL, interleukin; KC, keratinocyte-derived chemoattractant; NO₂⁻, nitrite; SOD, superoxide dismutase. Data are means ± SE (n = 5). ‡Greater than respective LPT 1 (P < 0.001).*Greater than LPT 2 and Pre-LPT 2 (P < 0.001). Repeated measures analysis of variance with Student-Newman-Keuls post-hoc test.
<table>
<thead>
<tr>
<th>Surgery</th>
<th>Pre-LPT</th>
<th>LPT 1</th>
<th>Post-LPT</th>
<th>Rest</th>
<th>Pre-LPT</th>
<th>LPT 2</th>
<th>Post-LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- 4</td>
<td>1 – 4</td>
<td>5-14</td>
<td>90</td>
<td>124</td>
<td>125 - 128</td>
<td>138</td>
</tr>
</tbody>
</table>

**Time (minutes)**

**Figure 1. Experimental design.** Approximately 60 min following cannulation of the thoracic duct, thoracic duct lymph (TDL) was collected during 4 min of baseline (pre-LPT), at 1 min intervals during 4 min of LPT (LPT 1), for 10 min (post-LPT), 90 min (rest), 4 min (pre-LPT 2), at 1 min intervals during 4 min of LPT (LPT 2), and for 10 min after cessation of LPT (post-LPT). Lymph flow rates were calculated from the volume of lymph collected during these intervals.
Figure 2. **Lymphatic pump treatment repeatedly increases thoracic duct lymph flow.** See Figure 1 for protocol details. Data are means ± SE (n = 5). Data were analyzed by repeated measures analysis of variance with Student-Newman-Keuls post-hoc test. *Greater than respective pre-LPT and post-LPT values (P < 0.001). There were no significant (P>0.05) differences between LPT 1 and LPT 2.
Figure 3. Lymphatic pump treatment repeatedly increases thoracic duct leukocytes. See Figure 1 for protocol details. Data are means ± SE (n = 5). Data were analyzed by repeated measures analysis of variance with Student-Newman-Keuls post-hoc test. *Greater than respective pre-LPT and post-LPT values (P < 0.01). **Greater than respective pre-LPT and post-LPT values (P < 0.001). There were no significant (P > 0.05) differences between LPT 1 and LPT 2.
Figure 4. Lymphatic pump treatment repeatedly increases thoracic duct leukocyte flux.

See Figure 1 for protocol details. Data are means ± SE (n = 5). Data were analyzed by repeated measures analysis of variance with Student-Newman-Keuls post-hoc test. *Greater than respective pre-LPT and post-LPT values (P < 0.05). **Greater than respective pre-LPT and post-LPT values (P < 0.01). ***Greater than respective pre-LPT and post-LPT values (P < 0.001). There were no significant (P>0.05) differences between LPT 1 and LPT 2.
Figure 5. Lymphatic pump treatment repeatedly increases thoracic cytokine and chemokine flux. See Figure 1 for protocol details. Data are means ± SE (n = 5). Data were analyzed by repeated measures analysis of variance with Student-Newman-Keuls post-hoc test.

*Greater than respective pre-LPT and post-LPT values (P < 0.001).
Figure 6. Lymphatic pump treatment repeatedly increases thoracic duct SOD flux. See Figure 1 for protocol details. Data are means ± SE (n = 5). Data were analyzed by repeated measures analysis of variance with Student-Newman-Keuls post-hoc test. *Greater than respective pre-LPT and post-LPT values (P < 0.001). There were no significant (P>0.05) differences between LPT 1 and LPT 2.
Figure 7. Lymphatic pump treatment repeatedly increases thoracic duct nitrite flux. See Figure 1 for protocol details. Data are means ± SE (n = 5). Data were analyzed by repeated measures analysis of variance with Student-Newman-Keuls post-hoc test. *Greater than respective pre-LPT and post-LPT values (P < 0.001). ‡Different from LPT 1 (P < 0.001).
CHAPTER IV

Lymphatic Pump Treatment Augments Lymphatic Flux of Lymphocytes in Rats

Running Title: LPT augments lymphocyte flux in rats

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and Lisa M. Hodge, Ph.D.¹,²

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American Osteopathic Association Grant 06-11-547 (LMH).

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Abstract

Background

Lymphatic pump techniques (LPT) are used by osteopathic practitioners for the treatment of edema and infection; however, the mechanisms by which LPT enhances the lymphatic and immune systems are poorly understood.

Methods and Results

To measure the effect of LPT on the rat, the cisterna chyli (CC) of 10 rats were cannulated and lymph was collected during four min of 1) pre-LPT baseline, 2) four min LPT, and 3) 10 min post-LPT recovery. LPT increased significantly (P<0.05) lymph flow from a baseline of 24 ± 5 μl/min to 89 ± 30 μl/min. The baseline CC lymphocyte flux was 0.65 ± 0.21 x 10⁶ lymphocytes/min, and LPT increased CC lymphocyte flux to 6.10 ± 0.99 x 10⁶ lymphocytes/min (P<0.01). LPT had no preferential effect on any lymphocyte population, since total lymphocytes, CD4+ T cells, CD8+ T cells, and B cell numbers were similarly increased. To determine if LPT mobilized gut-associated lymphocytes into the CC lymph, gut-associated lymphocytes in the CC lymph were identified by staining CC lymphocytes for the gut homing receptor integrin α4β7. LPT significantly increased (P<0.01) the flux of α4β7 positive CC lymphocytes from a baseline of 0.70 ± 0.03 x 10⁵ lymphocytes/min to 6.50 ± 0.10 x 10⁵ lymphocytes/min during LPT. Finally, lymphocyte flux during recovery was similar to baseline, indicating the effects of LPT are transient.

Conclusions

Collectively, these results suggest that LPT may enhance immune surveillance by increasing the numbers of lymphocytes released into lymphatic circulation, especially from the
gut associated lymphoid tissue. The rat provides a useful model to further investigate the effect of LPT on the lymphatic and immune systems.
Condensed Abstract

Lymphatic pump techniques (LPT) are used by osteopathic practitioners for the treatment of edema and infection. The cisterna chyli (CC) of rats were cannulated and lymph was collected during four min of 1) pre-LPT, 2) LPT, and 3) post-LPT. LPT significantly increased (P<0.01) the flux CC lymphocytes, including gut-associated lymphoid tissue (GALT) lymphocytes. LPT may enhance immune surveillance by increasing the numbers of lymphocytes released into lymphatic circulation, especially from the GALT. The rat provides a model to further investigate interactions of the lymphatic and immune systems.
Introduction

It is well established that compression of lymph vessels by skeletal muscle contraction, respiration or intestinal peristalsis increases lymph flow.\textsuperscript{7,32,59,128} In addition, cyclic contractions of smooth muscle of the lymph vessels acts as an intrinsic lymph pump.\textsuperscript{129} Failure of intrinsic lymph pumping can result in certain forms of edema.\textsuperscript{7,32,59} Osteopathic manipulative treatments (OMT), identified specifically as lymphatic pump techniques (LPT), were developed by osteopathic physicians to improve the lymphatic system.\textsuperscript{7,128}

Lymphatic pump techniques (LPT) are thought to enhance lymphatic return by increasing gradients for lymph flow and, thus, assisting the return of lymph from the lung, abdomen and other tissues.\textsuperscript{59,128} Theoretically, by improving lymph flow, the interstitial fluid may be drained more efficiently, removing particulate matter, exudates, toxins, and bacteria from tissues.\textsuperscript{32,59} Improved lymph flow can also reduce excessive accumulation of fluid in the interstitial spaces, and thus lessen edema.\textsuperscript{32,59,95,129}

Clinically, LPT has been shown to increase blood leukocyte numbers,\textsuperscript{64} enhance vaccine specific antibodies,\textsuperscript{68,70} enhance bronchial clearance during pulmonary infection\textsuperscript{17,75} reduce the need for antibiotics during infection,\textsuperscript{14} and decrease the length of hospital stay in elderly patients with pneumonia.\textsuperscript{14} In dogs, LPT has been shown to enhance lymph flow and leukocyte numbers in the thoracic\textsuperscript{3,4} and mesenteric lymph ducts.\textsuperscript{94} Furthermore, LPT was shown to mobilize lymphocytes from the mesenteric lymph nodes into thoracic duct lymph.\textsuperscript{94} Collectively, these reports suggest that LPT can enhance function of the lymphatic and immune systems, which may facilitate the clearance of infection; however, the mechanism(s) by which LPT facilitate(s) the clearance of bacteria during infection has not been identified.
While the dog has been used to study the effect of LPT on the lymphatic system, there are several limitations to the use of dogs for the study LPT on infectious and inflammatory diseases. These limitations include animal cost, the availability of reagents for immunological studies. Importantly, rodents are more commonly used for studies of cancer and infectious disease. Therefore, the objective of this study was to develop a rat model to determine if experimental LPT would increase lymph flow and leukocyte concentration as observed in dogs. Our results show LPT increases the flux of lymphocytes in the cisterna chyli (CC) of rats, thus demonstrating that this treatment produces similar effects in two distinct mammalian models. Furthermore, we have developed a small animal model to investigate the effects of LPT on the lymphatic and immune systems.

**Materials and Methods**

**Animals**

This study was approved by the Institutional Animal Care and Use Committee and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication no. 85-23, revised 1996). Ten male Sprague-Dawley rats, weighing 250-300 grams, and free of clinically evident signs of disease were used for this study.

**Cannulation of the thoracic duct**

Thirty to forty-five min preoperatively, 1.5 ml of olive oil was administered via oral gavage to increase lipid content, thus making the lymphatic vessels more visible. At the same time, 3 ml of a 32-36°C, 0.09% sterile saline solution was injected intraperitoneally to compensate for fluid loss during surgery. Anesthesia was induced in a chamber with a combination of 1-1 oxygen and 5% isoflurane. The rat was then transferred onto a heating pad to maintain a body temperature between 36-37°C. A nose cone with a 1-1 oxygen and 2%
isoflurane mixture was used to maintain anesthesia throughout the procedure. The rat was placed in a right lateral recumbent position with its feet secured to the heating pad. The hair on the left side was clipped, and a 70% isopropyl alcohol solution was generously applied to the clipped area.

The abdomen was opened through a left subcostal, mediolateral incision spanning two-thirds of the length of the abdomen. The intestines, left kidney, adrenal, stomach and spleen were separated from connective tissue and fat with cotton swabs wrapped in warm, saline soaked gauze, which was then retracted to the side. Excessive tension on the renal circulation was avoided. Multiple retractors were used to further visualize the aorta, and the parietal peritoneum was incised just below the diaphragm. Cotton swabs were used to clear fat and connective tissue, thus freeing the aorta from surrounding tissues and exposing the CC containing milky lymph. A silk suture was placed under the aorta, so that the aorta could be retracted during the cannulation procedure. Caution was taken to not damage the thoracic duct or other ducts converging on the CC during this procedure. A 23G hypodermic needle, bent at ~100° angle, was used to puncture the CC for introduction of MicroRenathane® implantation tubing (MRE-033; 0.84 mm O.D. x 0.36 mm I.D.) filled with hepranized saline. The insertion site was sealed with 1 or 2 drops of surgical glue, and lymph was allowed to drain by gravity into collection tubes. CC lymph was collected during four min pre-LPT, four min LPT, and 10 min post-LPT. Lymph flow rates were measured as the volume of lymph collected during each minute, and these values were averaged for each condition.

**Lymphatic pump treatment**

LPT was performed by a medical student trained in osteopathic lymphatic manipulation. During manipulation, the rat was in a right lateral recumbent position. The retractors were
removed from the incision, allowing only the implantation tubing to exit the incision side. To perform LPT, the operator contacted the abdomen of rat with the thumb on one side and index finger and middle finger on the other side of the medial sagittal plane. The fingers were placed bilaterally caudal to the ribs, and attention was paid not to contact the left subcostal, mediolateral incision. Sufficient pressure was exerted medially and cranially to compress the abdomen until significant resistance was met against the diaphragm, then the pressure was released. Compressions were administered at approximately one/sec for the duration of the 4-min treatment.

**Leukocyte enumeration**

Leukocytes in samples of CC lymph were enumerated using the Hemavet 950 (Drew Scientific). To compute leukocyte flux, the lymphocyte concentration was multiplied by the volume of lymph during each minute for each condition, and these values were averaged.

**Flow cytometry**

Two-color immunofluorescent staining was performed to identify specific lymphocyte populations using FITC or PE-labeled goat anti-rat isotype control IgG2b, FITC-anti-rat CD3, PE-anti-rat B cell, PE-anti-rat CD4, PE-anti-rat CD8, FITC-anti-rat CD61 (β7), or PE-anti-rat CD49 (α4) monoclonal antibodies (mAb) (Serotech, Raleigh, NC). Between 2x10^5 and 1x10^6 cells were incubated with the mAb as recommended by the manufacturer. The cells were washed in staining buffer consisting of Mg^{2+}-free, Ca^{2+}-free phosphate buffered saline supplemented with 2% fetal bovine serum (HyClone Laboratories, Logan, UT) and fixed with 0.05% paraformaldehyde until analyzed.

Fluorescently labeled lymphocytes were analyzed using a Cytomix FC 500 flow cytometer (Beckman Coulter, Fullerton, CA). Lymphocyte gates and detector voltages were set
using isotype control stained cells, and stained cell populations were seen as distinct peaks or clusters of cells. The proportion of each cell population was expressed as the percentage of the number of stained cells. To determine the total number of a specific lymphocyte population in a milliliter of lymph, their percentage was multiplied by the total number of cells.

**Statistical analyses**

Data were subjected to analysis of variance followed by a Tukey-Kramer multiple comparisons post test. Analyses were performed with Graphpad Prism version 5.0 for Windows, (GraphPad Software, San Diego, CA). Differences among mean values with $P \leq 0.05$ were considered statistically significant. Data are presented as arithmetic mean ± standard error (SE).

**Results**

**LPT increases CC leukocytes**

The baseline leukocyte count was $0.30 \pm 0.09 \times 10^5$ cells/µl of lymph, and LPT significantly ($P< 0.05$) increased leukocytes to $1.24 \pm 0.41 \times 10^5$ cells/µl. Furthermore, LPT increased significantly ($P< 0.05$) lymph flow from a baseline of $24 \pm 5$ µl/min to $89 \pm 30$ µl/min. Figure 1 summarizes the effect of LPT on CC leukocyte flux. The baseline CC lymphocyte flux was $0.65 \pm 0.21 \times 10^6$ lymphocytes/min, and LPT increased CC lymphocyte flux to $6.10 \pm 0.99 \times 10^6$ lymphocytes/min ($P<0.01$ versus Pre- and Post-LPT). Approximately 97% of the white blood cells in the CC lymph were lymphocytes, and LPT had no preferential effect on any lymphocyte populations, since total lymphocytes, CD4+ T cells, CD8+ T cells, B cell numbers were similarly increased (Table1). Finally, the leukocyte flux during recovery was similar to pre-LPT, indicating that LPT transiently increases CC lymph flow and leukocyte numbers.
LPT mobilizes gut associated lymphocytes into CC lymph.

Previously, we demonstrated that LPT mobilizes gut associated lymphoid tissue (GALT) derived lymphocytes into the thoracic duct lymph of anesthetized dogs.94 Therefore, to determine if LPT mobilizes GALT derived lymphocytes into the CC of rats, we stained lymphocytes collected from the CC for the gut homing leukointegrin α4β7.130 Approximately 40% of the lymphocytes in the CC stained positive for α4β7 (data not shown). LPT did not increase the percentage of α4β7 positive lymphocytes; however, LPT significantly increased (P<0.01) the flux of α4β7 positive lymphocytes from a baseline of 0.70 ± 0.03 x 10^5 leukocytes/min to 6.50 ± 0.10 x 10^5 leukocytes/min during LPT (Figure 2). Finally, following LPT, the α4β7 positive lymphocyte flux was similar to that observed pre-LPT, indicating LPT has a transient effect on the mobilization of α4β7 positive lymphocytes. This result demonstrates that the GALT is a tissue source of the lymphocytes mobilized during LPT in rats.

Discussion

This investigation examined for the first time in rats responses to simulated osteopathic manipulation, a therapy advocated to enhance lymph flow and immune function.7,128 The most important findings of this investigation are 1) LPT augmented thoracic duct lymph flow, 2) LPT increased leukocyte concentration in thoracic duct lymph, 3) since both lymph flow and leukocyte count were increased during LPT, leukocyte flux from lymphoid reservoirs was markedly elevated, 4) a significant number of these leukocytes originated from GALT. While these findings are consistent with our previous studies in dogs,3,4,94 they demonstrate that the rat can be a useful model for future research on mechanisms by which LPT enhances immune function.
Osteopathic philosophy and education suggest that OMT enhances immune function by removing restrictions to blood and lymph flow, by optimizing respiratory mechanics, and by restoring balance between the sympathetic and parasympathetic nervous systems.\textsuperscript{7,128} Anecdotal reports support the belief that LPT stimulates the immune system and accelerates clearance of infection;\textsuperscript{14,17,64,68,70,75} however, the mechanisms responsible for this protection have not been identified. The current finding that LPT greatly increases leukocyte flux in CC lymph provides experimental support for clinical applications of LPT. Furthermore, the increase in lymph flow generated by LPT would enhance distribution of antigens or antibiotics, and thus improve infection control.

It is established that mucosal homing of GALT derived lymphocytes is due to the molecular interaction between integrin $\alpha 4\beta 7$ on lymphocytes and the mucosal vascular addressin, MAdCAM-1.\textsuperscript{130} The GALT is a major inductive site, and lymphocytes primed in the GALT can migrate into many different effector mucosal tissues, suggesting the existence of a common mucosal immune system.\textsuperscript{19} Specifically, antigen specific IgA producing B cells and T cells have been shown to migrate from GALT to other mucosal associated lymphoid tissues, including the bronchus associated lymphoid tissue (BALT).\textsuperscript{18,19,130,131} Of clinical importance, intestinal immunization enhances protective immunity in the lower respiratory tract compared to parenteral immunization.\textsuperscript{18,19,49,52} These studies suggest that GALT primed memory lymphocytes can either recirculate through or reside in the lung; however, the involvement of $\alpha 4\beta 7$ positive lymphocytes in clearance of lower respiratory tract infection is still unclear.

Consistent with our findings in the dog,\textsuperscript{4} LPT increased the numbers of $\alpha 4\beta 7$ positive (GALT) derived lymphocytes in the thoracic duct lymph of rats. Mobilization of primed leukocytes from the GALT by LPT should be beneficial during infection. In addition, the
increased numbers of circulating leukocytes produced by LPT might improve immune surveillance, which in turn would further boost protection against infectious disease.

It must be recognized that further research is required to determine the migration kinetics of the leukocytes mobilized by LPT, and especially those from GALT. Such research would be most informative if LPT were applied to an animal model with concurrent disease. The present results suggest that the rat would be a useful and practical model for such research.

The result of LPT on lymph flow has been reported in dogs, but the effect of LPT on lymph flow has not been determined in rodents or humans. In this study, LPT treatment was applied to the rat to simulate, as nearly as possible, how cyclic applications of manual pressure to the abdomen are applied to humans. It is important to note that there are obvious differences between the application of LPT in humans, dogs and rats. This is primarily due to the size, anatomy, and positioning of the animals during treatment as compared to humans. This is an inherent flaw in using animal models to study the mechanisms of human manual medicine treatments, which cannot be overcome at the current state of technology. In spite of the concerns with the use of animal models, they still provide the opportunity to study the simulated effects of LPT on humans.

While LPT was developed to increase lymph flow, it is possible that such maneuvers would mobilize cells directly from lymphoid tissue to the blood independent of the lymphatic circulation. Thus, studies that evaluate effects of LPT only on lymph flow and composition may underestimate the clinical implications of this alternative and complementary therapy for infection.
Acknowledgements

This research was supported by National Institutes of Health Grant R01 AT004361 (LMH), American Osteopathic Association Grant 06-11-547 (LMH). Furthermore, the expert assistance of Kim Winterrowd during the animal surgery is gratefully acknowledged. The authors thank the Osteopathic Heritage Foundation for their continued support of the Basic Science Research Chair (LMH).

Author Disclosure Statement

No competing financial interests exist for any author.
**Figure 1. Leukocyte Flux.** Cisterna chyli lymph was collected during 1) 4 min pre-LPT, 2) 4 min LPT, and 3) 10 min post-LPT. Data are means x 10^6 total leukocytes/min ± SE from 10 animals. **Greater than pre-LPT and post-LPT (P< 0.01).**
Figure 2. $\alpha^+\beta^+$ Lymphocyte flux. Cisterna chyli lymph was collected during 1) pre-LPT, 2) four min LPT, and 3) four min post-LPT. Data are means x $10^6$ total of $\alpha\beta$ positive lymphocytes/min ± SE from 10 animals. *Greater than pre-LPT and post-LPT ($P<0.05$).
Table 1. Lymphatic Pump Treatment Increases Lymphocyte Flux in Cisterna Chyli Lymph.

<table>
<thead>
<tr>
<th>Lymphocyte Flux (x 10^6 cells/min)</th>
<th>Pre-LPT</th>
<th>LPT</th>
<th>Post-LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lymphocytes^a</td>
<td>0.63 ± 0.20</td>
<td>5.90 ± 0.99**</td>
<td>0.52 ± 0.13</td>
</tr>
<tr>
<td>CD4^+ T cells^b</td>
<td>0.37 ± 0.17</td>
<td>3.60 ± 0.54**</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>CD8^+ T cells</td>
<td>0.11 ± 0.05</td>
<td>1.00 ± 0.28**</td>
<td>0.09 ± 0.06</td>
</tr>
<tr>
<td>B cells</td>
<td>0.14 ± 0.01</td>
<td>0.70 ± 0.50*</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

Lymph was collected during four min pre-LPT, four min LPT and four min post-LPT. Values are means ± SE or means x 10^6 lymphocytes/min from 10 experiments. ^aConcentration of total lymphocytes. ^bConcentration of total lymphocytes that were T or B cells. ** P<0.01, compared to pre-LPT and post-LPT. *P<0.05, compared to pre-LPT and post-LPT.
CHAPTER V

LYMPHATIC PUMP TECHNIQUE ENHANCES PULMONARY IMMUNITY AND FACILITATES THE CLEARANCE OF RESPIRATORY INFECTION WITH STREPTOCOCCUS PNEUMONIAE

Short Title: LPT inhibits pneumonia

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In preparation for publication
Abstract

*Streptococcus pneumoniae* is the leading pathogen of community-acquired pneumonia and remains a major cause of morbidity and mortality worldwide, despite advancements in vaccines and antimicrobial therapy. Due to the projected growth of the elderly population, who have the highest rates of pneumococcal disease, as well as an increasing incidence of antibiotic resistance seen with this pathogen, there is an urgent need to explore alternative treatments and increase our knowledge of the mechanisms involved in the pathogenicity and host defense that influence the clearance of the bacteria. Clinically, osteopathic physicians have shown that manual treatments such as lymphatic pump techniques shorten the duration of intravenous antibiotic therapy and hospital stay in patients with pneumonia. More recently, we developed a rat model and showed that LPT reduces *S. pneumoniae* colony forming units (CFU) in the lungs of rats with acute pneumonia; however, the mechanism by which LPT protects against pneumonia has not been previously studied. Alveolar macrophages (AM) are the major resident phagocytic cells of the lung and have been shown to play a major role in the pulmonary host defense and the clearance of *S. pneumoniae*. We tested the hypothesis that LPT would increase immune cell and inflammatory mediators trafficking to pulmonary tissues, enhance the function of AM, and in turn facilitate cell-mediated clearance during pneumococcal infection. LPT significantly decreased the number of *S. pneumoniae* CFU in the lungs of rats at day 4 post-infection. Further, LPT significantly increased total pulmonary leukocytes with a preferential increase of macrophages and neutrophils; however, LPT did not alter BALF, splenic, and blood leukocytes at day 4 post-infection. Further, LPT increased the concentrations of SP-D, IL-6, IL-12p70, and IL-17 in BALF and enhanced the release of NO\(_2^–\) and IL-6 by AM 4 days post-infection. In conclusion, our data suggests LPT protects the lung during acute pneumococcal
pneumonia by increasing macrophages, neutrophils, anti-microbial products, inflammatory mediators, and AM function in the lungs of rats. Our findings are clinically relevant as they provide for the first time scientific rationale for the use of LPT to treat pneumonia and propose a mechanism by which LPT decreases bacterial pulmonary burden.

**Keywords**

lymph, lymphatic pump technique, pneumococcus, *Streptococcus pneumoniae*, community-acquired pneumonia, cytokines, chemokines, inflammatory mediators, infection, edema, immune system, immunity, osteopathic manipulative medicine, alveolar macrophages, BALF, SP-D, IL-6, IL-12p70, and IL-17, NO$_2^-$
Introduction

*Streptococcus pneumonia* is a common bacterial pathogen and can cause localized disease such as sinusitis and otitis media (OM), as well as life-threatening diseases such as pneumonia, septicemia, and meningitis.\(^9\)\(^{13}\) This extracellular human pathogen infects the host predominantly through the respiratory tract and is the culprit of serious infection and disease in immunocompromised patients, infants, and elderly over the age of 60 years.\(^{13}\)

Community-acquired pneumonia (CAP), which is most often caused by *S. pneumonia*, is a common and serious infection, which is associated with high health care cost, morbidity, and mortality, despite the availability of potent new antimicrobials and vaccines.\(^{13}\) It is estimated that pneumococcal disease in the U.S. is responsible for 4 million illness episodes and $3.5 billion in annual direct medical cost, with pneumonia consuming 72% of the total pneumococcal costs.\(^{13}\)

Further, it is projected that pneumococcal disease and more specifically pneumococcal pneumonia will substantially increase the clinical and economic burden in the U.S., due to a significant increase in frequency of organisms that are resistant to vaccines and antimicrobials, and a substantial growth in the elderly population, who have been shown to have the highest rates of pneumococcal disease.\(^{13}\)\(^{13}\)\(^{5}\)\(^{13}\)\(^{6}\) It is estimated that hospitalizations due to pneumococcal pneumonia will increase by 96% between 2004 and 2040, leading to a doubling of the total annual direct cost associated with pneumococcal pneumonia.\(^{13}\)\(^{6}\) Therefore, there is a need to explore the benefits of complementary and alternative medicine (CAM) procedures that may aid in the treatment and prevention of pneumococcal pneumonia and elucidate the mechanisms involved the protection.
Osteopathic physicians have long used lymphatic pump techniques (LPT), a form of manual medicine, to enhance immunity and improve lymphatic flow.⁵,⁷,⁵⁹ Clinical utilization of LPT has shown to decrease the duration of intravenous antibiotic therapy and hospital stay in patients with pneumonia,¹⁶ decrease the duration of cough in patients with lower respiratory tract disease,¹⁷ and improve antibody response to bacterial vaccines.⁶⁸,⁷⁰ In previous animal experiments, it was demonstrated that single and multiple applications of LPT increased lymph flow and leukocyte flux, and mobilized inflammatory mediators into lymph circulation.³,⁴,⁹⁴,⁹⁶,¹¹⁵,¹³⁷ In addition, it was shown that application of LPT decreased the bacterial burden in lungs of rats;¹²⁶,¹³⁸ however, the mechanism by which LPT protects against pneumonia has not been studied previously.

Therefore, in this study, we explored the mechanisms by which LPT provides protection during acute pneumococcal pneumonia.

Materials and Methods

Animals

Immune competent male inbred Fischer 344 (F344) rats (Charles Rivers Laboratories, Wilmington, MA) with jugular vein catheters, weighing 250-300 grams, and free of clinically evident signs of disease were used for this study. Catheterized rats were used in order to avoid repeated administration of intraperitoneal anesthesia, minimizing pain and distress, as well as decreasing a stress response during treatment. A minimum of five rats were used per group of control, sham, and lymphatic pump treatment during each experiment. Rats were housed and fed according to the Institutional Animal Care and Utilization Committee (IACUC) of the University of North Texas Health Science Center in the barrier facility. This study was approved by the
IACUC and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication no. 85-23, revised 1996).

**Preparation of *Streptococcus pneumoniae***

Frozen stock of *Streptococcus pneumoniae* (ATCC 6301) was streaked on fresh tryptic soy agar (TSA) with 5% sheep blood-agar plates and grown overnight at 37°C, 5% CO\textsubscript{2}. Bacterial cells were scraped from the plate in endotoxin-free PBS and the concentration of bacteria was calculated by measuring absorbance at 600 nm. Standard values based on known colony forming units (CFU) previously established in our laboratory were used to calculate an inoculum concentration of 1 x 10\textsuperscript{8} CFU/ml. Final concentration was confirmed by quantitative culture of the inoculum in serial 10-fold dilutions on TSA blood-agar.

**Infection procedure and experimental protocol***

Rats were anesthetized intraperitoneally (IP) with ketamine (30 mg/kg) and xylazine (5 mg/kg) and intranasally inoculated with 100µl of 1x10\textsuperscript{8} *Streptococcus pneumoniae* CFU/ml. Twenty-four hours after inoculation, rats were divided into control, sham or LPT treatment groups (Figure 1). Rats received either: 1) a daily sham treatment consisting of intravenous administration of 10 mg/kg propofol anesthesia (Hospira Inc., Lake Forest, IL) followed by 4 min of light touch (Sham); 2) 4 min of LPT daily under anesthesia (LPT); or 3) no treatment or anesthesia (Control). Weights and clinical signs of disease were monitored daily.

In order to assess the three groups for bacterial dissemination and measure the effects of LPT on the clearance of lung CFU after various treatment frequencies, rats were treated either for one, two or three consecutive days, and then sacrificed the following day. Rats were anesthetized with a lethal dose of a combination of ketamine and xylazine by i.p. injection, cardiac blood was collected, and the animal was exsanguinated by sectioning of the abdominal
aorta. Lungs and spleen were removed aseptically and homogenized, and then evaluated for bacterial burden.

In separate experiments, lung and spleen leukocytes in rats infected with *S. pneumoniae* were evaluated. Rats were treated as described above; however, rather than homogenizing the lungs and spleens, four days following infection, lungs and spleen were collected and the total number of leukocytes and the numbers of CD4+ T cells, CD8+ T cells, B cells, macrophages, and neutrophils were measured by flow cytometric analysis.

In another set of experiments, bronchoalveolar lavage fluid (BALF) was collected at day four and evaluated for inflammatory mediators. Also, in order to determine inflammatory cell counts in total lung lavage, BALF was analyzed for total numbers of viable cells using a hemocytometer with trypan blue (Sigma-Aldrich, St. Louis, MO) exclusion. In addition, cytospins were prepared from BAL cells and counted to determine differential cell types, including macrophages, neutrophils, and lymphocytes.

In order to determine the metabolic activity of AM, in yet another set of experiments, AM were isolated from BALF, stimulated in vitro, and their supernatants were evaluated for IL-6 and nitrite production.

**Lymphatic pump technique**

LPT was performed by a medical student (A.S.) trained in osteopathic lymphatic manipulation. During manipulation, the anesthetized rats were placed in a right lateral recumbent position. To perform abdominal LPT, the operator contacted the ventral side of the animal’s abdomen with the thumb on one side and index finger and middle finger on the other side of the medial sagittal plane below the costo-diaphragmatic junction. Sufficient pressure was exerted medially and cranially to compress the abdomen until significant resistance was
encountered against the diaphragm, and then the pressure was released. Abdominal compressions were administered at a rate of approximately 1 per sec for a total of 4 min.

**Bacteriology**

Lungs and spleen were removed aseptically at selected time points after intranasal challenge, placed in 5 mL sterile PBS and minced with sterile scissors. The tissues were homogenized (OMNI international 5100) under aseptic conditions. Ten-fold (1:10 to 1:1,000,000) serial dilutions were made in a 96 well plate. Eight microliters of each dilution was spot plated on TSA with 5% sheep blood-agar plates. Due to the plating technique utilized, the detection limit was 562.5 CFU/ml of homogenate. The plates were grown overnight at 37°C in a humidified atmosphere containing 5% CO₂, and CFU were counted after 18 hours.

**Leukocyte enumeration**

Total leukocytes and a differential leukocyte count in blood were determined using the Hemavet 950 (Drew Scientific). Total leukocyte count in lung and spleen homogenate was determined using a hemacytometer. Differential cell counts were determined by flow cytometric analysis in lung and spleen homogenate.

**Isolation of leukocyte from lung and spleen**

At day 4 post-infection, the lungs and spleens of rats were removed aseptically. For the preparation of lung single-cell suspensions, lung tissue was placed in RPMI wash media (5% FBS, 1X antibiotics/antimycotics, 10mM Hepes) and finely minced for subsequent tissue dissociation in a gentleMACS (MACS Miltenyi Biotec). Next, lung cell suspensions were passed through a nylon mesh filter to remove non-dissociated tissue. After lung cell suspensions were washed twice with RPMI wash media, they were gently layered over a Lympholyte Rat gradient (Cedarlane Laboratories Limited) to purify leukocytes by density gradient centrifugation.
Samples were centrifuged at 1200g for 20 min, 25°C, slow break. The leukocytes separated on the gradient were collected for enumeration using a hemacytometer and flow cytometric analysis of leukocyte subsets.

Single-cell suspensions of spleen cells were prepared by mashing spleens through a nylon mesh filter. In order to remove red blood cells, ACK lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA) was used. Spleen cells were collected and washed twice in RPMI wash media (5% FBS, 1X antibiotics/antimycotics, 10mM Hepes). The spleen leukocytes were used for Hemavet total cell count and flow cytometric analysis of leukocyte subsets.

**Flow cytometry**

Two-color immunofluorescent staining of spleen and lung tissue was performed to identify specific lymphocyte populations using FITC or PE-labeled goat anti-rat isotype controls, FITC-anti-rat CD3, PE-anti-rat B cell, PE-anti-rat CD4, PE-anti-rat CD8, PE-anti-rat granulocytes, FITC-anti-rat CD161, PE-anti-rat ED-2 monoclonal antibodies (mAb) (BioLegend; San Diego, CA). For each stain, 1 x 10⁶/100µL of lung or spleen cells were incubated with the optimal concentration of monoclonal antibody as recommended by the manufacturer for 30 min in the dark at room temperature. One lung and spleen sample were left unstained to serve as a negative control. The cells were washed two times with 2mL of staining buffer consisting of Mg²⁺-free, Ca²⁺-free phosphate buffered saline supplemented with 2% fetal bovine serum (HyClone Laboratories, Logan, UT) (PBS with 2% fetal bovine serum) at 400g, 5 min, 4°C. Following removal of supernatant, lung and spleen cells were fixed with 0.5% paraformaldehyde (Sigma) in PBS and stored at 4°C until flow cytometry analysis. Fluorescently labeled leukocytes were analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA). Leukocyte gates and detector voltages were set using isotype control cells, and
stained cell populations were detected from histogram peaks or dot plot cell clusters. Each cell population was expressed as the percentage of the number of stained cells. To calculate the total number of a specific lymphocyte population, their percentage was multiplied by the total number of cells in lung or spleen samples as determined by hemacytometer or hemavet count.

**Bronchoalveolar lavage**

To obtain BALF from isolated rat lung, the lung was lavaged 6 times with 5 ml aliquots of ice-cold, sterile PBS immediately after euthanasia and exsanguinations. Aliquots were slowly infused in the lungs through a polyethylene tube cannulated into the trachea then gently withdrawn. The collected lavage fluid was stored in a 50-ml conical tube on ice. Lavage fluid was examined through a light microscope for the presence of red blood cell contamination. It was determined that none of the samples required RBC lysis during processing. Collected lavage fluids were centrifuged at 800g for 10 min to pellet the cells. The cell pellet was resuspended in HBSS and either prepared for total leukocyte counts and differentials according to the cytospin protocol, or for *in vitro* stimulation. The supernatant was poured into a Centriprep centrifugal filter units with YM-3 3000 MW filters (EMD Millipore, Billerica, Massachusetts) and concentrated 20-fold according to manufacturer’s instructions. The concentrated supernatants were stored at -80°C for subsequent protein measurements.

**Cytospin protocol**

BAL cells were re-suspended in HBSS containing 10% FBS. Cells were loaded on a hemacytometer in trypan blue to determine total cell count and viability. Subsequently, cells were adjusted to a concentration of $5 \times 10^5$ cells/ml and 200µl aliquots of each cell suspension were placed in chambers fitted with clean microscope slides. Slides were spun at 800 rpm for five minutes, using a Cytospin centrifuge (Shandon Inc., Pittsburgh, Pa.), and were allowed to air
dry. The slides were treated with the Hema-3 staining kit (Fisher Scientific). Differential cell counts were determined by counting 100 cells/slide and were expressed as a percentage of the total number of cells. Proportions of lymphocytes, macrophages, and neutrophils were assessed

**In vitro stimulation of alveolar macrophages**

In order to obtain rat AM, the cell pellet obtained in the Bronchoalveolar lavage procedure was resuspended in RPMI and the number of living cells was determined by hemacytometer. BAL cells from similarly treated rats were then combined to adjust AM to a concentration of $1 \times 10^6$ Cells/ml. AMs were incubated for 1 hr at a final concentration of $5 \times 10^5$ cells/ml in 48-well cell culture plates (Costar, Corning, NY) at a total volume of 500 µl. In order to remove nonadherent cells 1 hr after the initial culture period, the media was aspirated and the adhered macrophages were gently washed twice with 500 µl RPMI. Culture media with or without stimuli of a combinations of lipopolysaccharide (LPS) and interferon (IFN)–γ in a total volume of 500 µl was added to the cell culture plates at 37°C. IFN-γ was added at the beginning of the incubation process and LPS after a 12 hr period. Supernatants were then collected and stored at −20°C for subsequent analysis of IL-6 and NO₂⁻.

**Measurement of inflammatory mediators**

A commercially available multiplex assay (Millipore, Billerica, MA, USA) was used to determine the concentrations of cytokines and chemokines in BALF. Specifically, the cytokine IL-1β, IL-6, IL-12p70, IL-17, TNF-α, and the chemokine MCP-1 were measured. A range of standards, provided with the multiplex assay, was used, and the assay was analyzed using the Luminex R 200 System with the xPONENT Software Interface (Millipore). The minimum detectable concentrations for IL-1β, IL-6, IL-12p70, IL-17, MCP-1, and TNF-α were 2.32, 9.8, 4.13, 1.61, 3.81, and 4.44 pg/ml, respectively. To determine IL-6 concentrations in stimulated
alveolar macrophage supernatants, a rat IL-6 ELISA Kit (BD Bioscience, San Diego, CA, USA) was used and plates were immediately read at an absorbance of 450nm using a MRX Microplate Reader (Dynex Technologies, Inc, Chantilly, VA).

Concentrations of nitrite (NO₂⁻) and SP-D were measured using commercially available kits (Promega Corporation, Madison, WI, USA, and USCNK Life Science Inc., Wuhan, China). The Promega Griess Reagent system measures NO₂⁻, a nonvolatile and stable breakdown product of nitric oxide (NO).¹¹⁶,¹¹⁷ The minimum detectable nitrite concentration for this assay is 2.5 μM. The minimum detectable SP-D concentration for this assay is 31.2 pg/mL.

**Statistical analyses**

Data are presented as arithmetic means ± standard error (SE). Values from multiple animals at respective treatment conditions were averaged, and the mean values are shown in tables or in figures. For evaluation of statistical significance, data were subjected analyses of variance followed by a Tukey-Kramer multiple comparisons post hoc tests.

Statistical analyses were performed with GraphPad Prism version 5.04 and GraphPad InStat version 3.06 for Windows, (GraphPad Software, San Diego, CA, USA). Differences among mean values with at least \( P \leq 0.05 \) were considered statistically significant.

**Results**

**LPT protected against bacterial pneumoniae after 3 consecutive treatments.**

Previously, we demonstrated that four once daily applications of LPT protect against *S. pneumoniae* infection.¹³⁸ In order to determine the exact number of LPT treatments necessary to protect against *S. pneumoniae* infection, rats were treated for either one, two or three consecutive days, and then sacrificed the following day (Figure 1). Infection of *S. pneumoniae* in the lungs was confirmed by measuring CFU from homogenized lung tissue after each rat harvest.
Although pulmonary CFU were lower following one or two LPT, no significant changes were noted between groups. However, LPT significantly \((P < 0.05)\) reduced pulmonary bacteria compared to control and sham treatment, suggesting that 3 treatments of LPT are necessary to enhance the clearance of \(S.\ pneumoniae\) (Figure 2). Further, \(S.\ pneumoniae\) CFU were measured in the spleens of rats to rule out LPT induced bacterial dissemination. At day 4, CFU were isolated from spleens in \(~ 10\%\) of rat from each treatment group (data not shown).

**LPT significantly altered lung, but not BALF or spleen leukocyte populations at day 4.**

In order to determine if LPT protected against pneumococcus by enhancing the entrance of leukocytes into the lung, lung homogenates were evaluated for total and differential leukocyte concentrations at day 4 post-infection. LPT significantly \((P > 0.05)\) increased total pulmonary leukocytes compared to Sham and Control animals (Table 1). To determine if LPT preferentially increased a specific leukocyte population, we measured the percentage and concentration of neutrophils, macrophages, NK cells, and lymphocytes in lung homogenates (Table 1). LPT did not increase the percentages of a specific leukocyte population; however, LPT significantly \((P > 0.05)\) increased the number of pulmonary macrophages and neutrophils compared to Sham and Control animals. Further, both Sham and LPT significantly \((P > 0.05)\) increased the number of pulmonary \(\text{CD}4^+\) T cells, \(\text{CD}8^+\) T cells, and B cells, but not NK cells.

There were no significant \((P > 0.05)\) differences in total and differential BALF (Table 3) and Spleen (Table 2) leukocytes between the treatment groups.

In order to monitor systemic infection and leukocyte trafficking in the blood, a blood leukocyte count was obtained via cardiac puncture at the time animals were sacrificed. There was no significant difference in absolute blood leukocyte counts at day 4 (Table 4).
LPT significantly increased IL-6, IL-12p70, IL-17, and surfactant protein D (SP-D), but not MCP-1, IL-1β, and TNF-α in BALF.

In order to determine if LPT increased chemo-attractant and anti-pneumococcal factors, BALF was obtained at day 4 and evaluated for inflammatory mediators via multiplex assay and ELISA. Concentrations of inflammatory mediators in BALF are reported in Table 5. LPT significantly ($P < 0.05$) increased the concentrations of IL-6, IL-12p70, and IL-17 in BALF. LPT also significantly ($P < 0.05$) increased SP-D in BALF when compared to Control, but not Sham animals. Further, the results indicate that LPT slightly increased MCP-1; however, the increase was not significant ($P = 0.15$). Application of LPT did not have any effect on IL-1β concentrations and no TNF-α levels were obtained, as all samples were below the minimum detection limits.

**LPT enhanced alveolar macrophage function.**

AM are important during the early stages of pneumococcal pneumonia clearance. In order to determine if LPT has an effect on AM function and secretion of soluble factors, we isolated and performed *in vitro* stimulation of AM. Our data shows that activation of BALF AM by culture with IFN-γ and LPS *in vitro* significantly enhanced the release of IL-6 (Figure 3) and NO$_2^-$ (Table 6) in LPT-treated rats.

**Discussion**

Previous animal studies and reports by clinicians have shown a protective effect of manipulative medicine against *S. pneumoniae* pneumonia.$^{14-16,63,74,76,126,138}$ This study is the first to explore the mechanism by which LPT protects against pneumonia and describe the effects of LPT on the pulmonary environment and AM. LPT significantly decreased the number of *S. pneumoniae* CFU in the lungs of rats at day 4 post-infection (Figure 2). Further, LPT
significantly increased total pulmonary leukocytes and preferentially mobilized macrophages and neutrophils into the lungs of rats (Table 1); however, we did not find a significant difference in BALF (Table 3), splenic (Table 2), and blood (Table 4) leukocytes at day 4 post-infection. Further, an increase in antibacterial products and inflammatory mediators in BALF was noted. Specifically, LPT increased the concentrations of SP-D, IL-6, IL-12p70, and IL-17 in BALF (Table 5) and enhanced the release of NO$_2^-$ (Table 6) and IL-6 (Figure 3) by AM 4 days post-infection.

In earlier studies, we reported that three and seven daily applications of LPT significantly reduced *S. pneumoniae* bacteria in the lungs of rats. Consistent with these findings, our results in this report indicate that a minimum of three daily LPT are necessary to significantly reduce CFU in the lungs of rats (Figure 2). Further, at day 4 post-infection, bacteria were isolated from spleens in ~10% of rats from each group and lower numbers of CFU were found in the spleens of LPT animals, although the difference was not significant (data not shown). These data suggest that LPT protects against pneumococcal pneumonia by inhibiting bacterial growth in the lungs and does not promote hematogenous spreading to the spleen.

Previous studies have not explored the mechanism responsible for the protection seen in rats with pneumococcal pneumonia; however, application of LPT in healthy animals increased lymph flow and leukocyte concentrations in both rats and dogs, and enhanced the flux of inflammatory mediators into lymph circulation. Therefore, LPT may protect against infectious disease by redistributing these leukocytes and inflammatory mediators to other tissues. We hypothesized that LPT would increase immune cell and inflammatory mediators trafficking to pulmonary tissues and facilitate cell-mediated clearance during pneumococcal infection. To elucidate the mechanism responsible for the enhanced clearance of pulmonary bacteria in rats,
the pulmonary environment was evaluated for the presence of leukocyte concentrations, inflammatory mediators, and anti-pneumococcal factors. In addition, the function of AM was investigated, as they have been shown to play a key role in initiating the innate immune response during the early stages of pneumococcal pneumonia clearance.\textsuperscript{91,139}

Consistent with previous studies, we saw an increase of pulmonary leukocytes in response to \textit{S. pneumoniae} infection.\textsuperscript{83,140-142} We found a significant difference between treatment groups in total and differential leukocyte numbers in lung homogenates (Table 1), but not in the spleen (Table 2) and BALF (Table 3) at day 4 post-infection. Collectively, these results suggest LPT promotes macrophage and neutrophil trafficking to the lungs at day 4, but does not alter absolute leukocyte concentrations in the spleen and blood.

To determine the effect of LPT on the pulmonary microenvironment, we next evaluated BALF for anti-pneumococcal and inflammatory mediators.

We found that both sham and LPT increased surfactant protein D (SP-D) in BALF (Table 5), suggesting propofol anesthesia enhances surfactant production. SP-D is produced by alveolar type II cells and is known to bind to oligosaccharides on the surface of many microbial pathogens.\textsuperscript{143} While our results suggest that propofol might augment SP-D production, it is important to note that LPT increased SP-D compared to sham. Recently, SP-D has been shown to exhibit a crucial role in the early, innate immune response, as SP-D deficient mice showed increased susceptibility to and decreased clearance of pneumococcal infection, along with earlier onset and higher levels of bacteremia.\textsuperscript{91} While the exact mechanism of SP-D is unknown, it has been linked to pathogen aggregation, modulation of phagocytosis, phagocytic killing, and most recently to neutrophilic myeloperoxidase activity modulation.\textsuperscript{91} Our results indicate that LPT
induced SP-D production might be one mechanism responsible for the clearance of S. pneumonias.

LPT slightly increased MCP-1 (Table 5) in BALF at day 4; however, the increase was not statistically significant. Of interest, we have previously shown that LPT significantly increased MCP-1 concentrations in thoracic duct lymph (TDL). MCP-1 is produced by a variety of cells, but mainly macrophages, in order to recruit monocytes/macrophages to areas of inflammation. Studies have shown that neutralization of MCP-1 or mice deficient in CCR-2 (the MCP-1 receptor) lead to decreased monocyte recruitment and increased severity during pulmonary infection. Therefore, LPT increased concentrations of MCP-1, which may explain the increase of macrophages in lung homogenate found at day 4 (Table 1), and may be, in part, responsible for the protection seen in our model; however, further studies are warranted to explore such a mechanism, and MCP-1 concentrations in BALF should be measured at earlier time points, and immediately after LPT is performed.

LPT significantly \((P < 0.05)\) increased the concentrations of IL-17, IL-12p70, and IL-6 in BALF (Table 5). IL-6 is important for the early clearance of pneumococcal pneumonia. Specifically, IL-6 mobilizes neutrophils, delays neutrophil apoptosis, and enhances neutrophil cytotoxic function. Recently, IL-17 was found to promote the local release of IL-6 and MIP-2 from non-lymphocytic, non-macrophage cells, which promoted neutrophil recruitments into the airways. Additionally, exogenous IL-12 has been shown to protect against S. pneumonias in mice by enhancing IFN-\(\gamma\) mediated recruitment of neutrophils in the lungs. Therefore, by increasing IL-6, IL-17, and IL12p70 in BALF, LPT may enhance phagocytosis of S. pneumonias. LPT enhanced neutrophil traffic to the lung at day 4. Further studies should explore
if neutrophils traffic to the lungs at an earlier time point. In addition, further studies should also measure apoptosis and neutrophil function and the effect of LPT on them.

AM are important during the early stages of pneumococcal pneumonia clearance. Specifically, they phagocytose bacteria and release soluble factors, such as nitric oxide (NO) and IL-6, which recruit monocytes and neutrophils and sustain neutrophil function in the lung. In addition, NO has a direct antimicrobial functions. In vitro activation of BALF macrophages by culture with IFN-γ and LPS enhanced the release of IL-6 (Figure 3) and NO2⁻ (Table 6) in LPT-treated rats. Collectively, these data demonstrate that LPT enhanced the release of NO2⁻ and IL-6 from BALF macrophages, which in turn, may enhance neutrophil function and promote the killing of extracellular bacteria. Further, it suggests that AM, at least in part, are the source of pro-inflammatory mediators found in the lung BALF milieu.

Previously, we have shown that LPT transiently increased the flux of chemokines, cytokines, and reactive oxygen and nitrogen species. Specifically, application of LPT significantly increased TDL and MDL (mesenteric duct lymph) flux of IL-6, IL-8, IL-10, MCP-1, KC, SOD, NT, and NO2⁻, a nonvolatile and stable breakdown product of nitric oxide. Therefore, we speculate that LPT may have enhanced the protection against S. pneumoniae by redistributing some these inflammatory mediators from the mesentery to the lungs, where they might: a) enhance recruitment of macrophages and neutrophils; b) participate in direct killing of the pneumococcus; and c) act locally on neutrophils and AM to enhance their function. In support of this notion, multiple studies have shown a close interaction amongst lymphatic drainage between the thorax and abdomen (reviewed elsewhere), as well as lymph mediated redistribution of cytokines and chemokines to distant organs. More specifically, mesenteric derived inflammatory mediators have been shown to redistribute to lungs. In
addition, *in vitro* studies have shown that mesenteric lymph can activate and prime neutrophils for an increased respiratory burst.\textsuperscript{156-158}

In conclusion, we have demonstrated that daily application of LPT for 3 consecutive days reduces *S. pneumoniae* bacteria in the lungs during acute infection. We have previously shown that LPT mobilizes leukocytes into thoracic and mesenteric duct lymph. This study, for the first time, also indicates that LPT can mobilize leukocytes, more specifically macrophages and neutrophils into lung tissues during pulmonary infection with *S. pneumoniae* at day 4. In addition, we found that LPT enhances the pulmonary immune response during *S. pneumoniae* respiratory infection by increasing anti-microbial products and inflammatory mediators. AM are heavily implicated in the clearing of respiratory infections, and our results indicate that LPT treated animals express AM that are functionally superior when compared to control or sham.\textsuperscript{92,144,148}
Acknowledgements

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Authors' contributions

AS performed the experiments, statistical analysis, data interpretation and provided the LPT and preparation of the manuscript. LMH designed and provided the oversight for the study. In addition, she reviewed and interpreted the data and participated in the preparation of the manuscript.

Competing interests

The authors declare they have no competing interests.
Figure 1. Experimental protocol. *In separate experiments, pulmonary CFU were determined on days 2, 3, and 4 post-infection.
Figure 2. LPT exhibited protection against *Streptococcus pneumoniae*. On day 0, rats were intranasally infected with $1 \times 10^7$ *Streptococcus pneumoniae* CFU. For days 1-3, the control group received no treatment or anesthesia, the sham group received 4 min of light touch under anesthesia, and the LPT group received 4 min of LPT under anesthesia. At days 2, 3 and 4 post-infection, rats were euthanized, lungs were collected and homogenized, and *S. pneumoniae* CFU were quantified. Data are means ± SE. N= 16 or 17 rats per treatment group. *Greater than control and sham at respective time point (P < 0.05). ANOVA with Tukey’s multiple comparisons post-test.*
Table 1. LPT significantly increased lung macrophages and neutrophils at day 4.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sham</th>
<th>LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute Count (x10^5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Leukocytes</td>
<td>42 ± 3</td>
<td>56 ± 6</td>
<td>97 ± 19**</td>
</tr>
<tr>
<td>Macrophages</td>
<td>16 ± 3</td>
<td>20 ± 2</td>
<td>35 ± 3**</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>12 ± 2</td>
<td>16 ± 2</td>
<td>25 ± 3**</td>
</tr>
<tr>
<td>NK cells</td>
<td>1.9 ± 0.2</td>
<td>4.4 ± 0.6*</td>
<td>4.6 ± 0.4*</td>
</tr>
<tr>
<td>B Cells</td>
<td>2.8 ± 0.2</td>
<td>4.3 ± 0.4*</td>
<td>6.0 ± 0.6*</td>
</tr>
<tr>
<td>CD3^+CD4^+</td>
<td>1.4 ± 0.3</td>
<td>3.2 ± 0.3*</td>
<td>4.1 ± 0.4*</td>
</tr>
<tr>
<td>CD3^+CD8^+</td>
<td>2.0 ± 0.3</td>
<td>3.1 ± 0.3*</td>
<td>4.3 ± 0.8*</td>
</tr>
<tr>
<td>Relative Leukocyte Percentage (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>36.1 ± 1.9</td>
<td>35.6 ± 5.6</td>
<td>35.4 ± 4.7</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>28.8 ± 3.3</td>
<td>28.6 ± 4.4</td>
<td>25.0 ± 5.2</td>
</tr>
<tr>
<td>NK cells</td>
<td>4.4 ± 0.5</td>
<td>7.7 ± 1.9</td>
<td>4.7 ± 1.3</td>
</tr>
<tr>
<td>B Cells</td>
<td>6.6 ± 0.7</td>
<td>7.6 ± 1.1</td>
<td>6.3 ± 1.0</td>
</tr>
<tr>
<td>CD3^+CD4^+</td>
<td>3.6 ± 0.8</td>
<td>5.5 ± 0.5</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>CD3^+CD8^+</td>
<td>5.4 ± 1.4</td>
<td>5.4 ± 1.2</td>
<td>4.5 ± 0.8</td>
</tr>
</tbody>
</table>

On day 0, rats were intranasally (IN) infected with 1 x 10^7 *Streptococcus pneumoniae* CFU. For days 1-3, the control group received no treatment or anesthesia, the sham group received 4 min of light touch under anesthesia, and the LPT group received 4 min of LPT under anesthesia. At day 4 post-infection, rats were euthanized and the lungs were collected and measured for the total number of pulmonary leukocytes via flow cytometry. Data are means ± SEM. N=13 rats per group. *Greater than control (P < 0.05). **Greater than control and sham (P < 0.05). ANOVA with Tukey’s multiple comparisons post-test.
Table 2. LPT did not significantly alter leukocytes in the spleen at day 4.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sham</th>
<th>LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absolute Count</strong> (x10^7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Leukocytes</td>
<td>39.1 ± 7.9</td>
<td>44.9 ± 5.8</td>
<td>47.8 ± 9.2</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.9 ± 0.9</td>
<td>1.9 ± 0.5</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Macrophages</td>
<td>3.9 ± 1.3</td>
<td>4.8 ± 0.9</td>
<td>5.7 ± 1.7</td>
</tr>
<tr>
<td>NK cells</td>
<td>2.4 ± 0.8</td>
<td>2.4 ± 0.5</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>B Cells</td>
<td>10.9 ± 4.3</td>
<td>12.4 ± 3.0</td>
<td>15.0 ± 4.5</td>
</tr>
<tr>
<td>CD3^+ CD4^+</td>
<td>3.6 ± 1.5</td>
<td>4.9 ± 1.4</td>
<td>5.7 ± 1.6</td>
</tr>
<tr>
<td>CD3^+ CD8^+</td>
<td>2.5 ± 1.0</td>
<td>3.9 ± 1.2</td>
<td>4.5 ± 1.3</td>
</tr>
<tr>
<td><strong>Relative Leukocyte count (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4.2 ± 0.9</td>
<td>4.1 ± 0.6</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>Macrophages</td>
<td>10.0 ± 1.3</td>
<td>12.1 ± 1.1</td>
<td>11.7 ± 1.7</td>
</tr>
<tr>
<td>NK cells</td>
<td>6.0 ± 0.8</td>
<td>6.4 ± 0.6</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td>B Cells</td>
<td>25.0 ± 5.6</td>
<td>29.2 ± 4.7</td>
<td>29.2 ± 5.0</td>
</tr>
<tr>
<td>CD3^+ CD4^+</td>
<td>7.9 ± 2.0</td>
<td>11.0 ± 1.9</td>
<td>12.1 ± 1.7</td>
</tr>
<tr>
<td>CD3^+ CD8^+</td>
<td>5.6 ± 1.6</td>
<td>8.3 ± 1.6</td>
<td>9.2 ± 1.4</td>
</tr>
</tbody>
</table>

On day 0, rats were intranasally (IN) infected with 1 x 10^7 *Streptococcus pneumoniae* CFU. At day 4 post-infection, rats were euthanized and the spleens were collected and measured for the total number of pulmonary leukocytes via flow cytometry. Data are means ± SEM. N=8-10 rats per group. ANOVA with Tukey’s multiple comparisons post-test.
Table 3. LPT did not significantly alter BALF leukocytes.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sham</th>
<th>LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absolute Count</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Leukocytes</td>
<td>71.3 ± 3.1</td>
<td>74.5 ± 7.7</td>
<td>71.4 ± 8.0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>64.1 ± 3.3</td>
<td>66.9 ± 6.9</td>
<td>65.6 ± 7.5</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2.6 ± 0.4</td>
<td>3.0 ± 0.6</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>4.6 ± 0.4</td>
<td>4.4 ± 1.0</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td><strong>Relative Leukocyte count</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>89.8 ± 1.0</td>
<td>89.6 ± 1.2</td>
<td>91.8 ± 1.6</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.6 ± 0.7</td>
<td>4.6 ± 0.8</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6.6 ± 0.7</td>
<td>5.8 ± 0.7</td>
<td>5.0 ± 0.8</td>
</tr>
</tbody>
</table>

On day 0, rats were intranasally (IN) infected with 1 x 10^7 *Streptococcus pneumoniae* CFU. At day 4 post-infection, rats were euthanized and BALF was collected and measured for the total number of leukocytes via hemocytometer. Differential was obtained via cytopsin. Data are means ± SEM. N=8-10 rats per group. ANOVA with Tukey’s multiple comparisons post-test.
Table 4. LPT did not significantly alter blood leukocytes at day 4.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sham</th>
<th>LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absolute Leukocyte count (x10^6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Leukocytes</td>
<td>4.6 ± 0.4</td>
<td>5.1 ± 0.4</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2.0 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.24 ± 0.03</td>
<td>0.23 ± 0.05</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.4 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

Blood was collected via cardiac puncture in: 1) LPT; 2) Sham; and 3) Control rats after the administration of each treatment protocol for 3 days. All data was calculated utilizing a Hemavet. Data are means ± SEM. N= 17-19 rats per group. ANOVA with Tukey’s multiple comparisons post-test were performed.
Table 5. LPT enhances the concentration of inflammatory mediators in BALF.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sham</th>
<th>LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>167 ± 29</td>
<td>148 ± 41</td>
<td>975 ± 122***</td>
</tr>
<tr>
<td>IL-12</td>
<td>15 ± 1</td>
<td>15 ± 5</td>
<td>88 ± 23**</td>
</tr>
<tr>
<td>IL-17</td>
<td>15 ± 4</td>
<td>19 ± 2</td>
<td>98 ± 28**</td>
</tr>
<tr>
<td>MCP-1</td>
<td>142 ± 25</td>
<td>113 ± 26</td>
<td>227 ± 56</td>
</tr>
<tr>
<td>SP-D</td>
<td>115 ± 33</td>
<td>296 ± 66</td>
<td>438 ± 85*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>35 ± 4</td>
<td>38 ± 5</td>
<td>35 ± 4</td>
</tr>
</tbody>
</table>

On day 0, rats were intranasally infected with $1 \times 10^7$ *Streptococcus pneumoniae* CFU. For days 1-3, the control group received no treatment or anesthesia, the sham group received 4 min of light touch under anesthesia, and the LPT group received 4 min of LPT under anesthesia. At day 4 post-infection, rats were euthanized, BALF from isolated rat lungs was obtained and measured for inflammatory mediators. Data are means ± SE pg/mL from 3 separate experiments. N= 5-10 rats per treatment group. *Greater than control ($P < 0.05$). **Greater than control and sham ($P < 0.01$). ***Greater than control and sham ($P < 0.001$). ANOVA with Tukey’s multiple comparisons post-test.
Figure 3. LPT increased *in vitro* IL-6 release by LPS + IFN-γ stimulated alveolar macrophages at day 4. On day 0, rats were intranasally infected with $1 \times 10^7$ *Streptococcus pneumoniae* CFU. For days 1-3, the control group received no treatment or anesthesia, the sham group received 4 min of light touch under anesthesia, and the LPT group received 4 min of LPT under anesthesia. At day 4 post-infection, rats were euthanized and leukocytes were obtained from BALF. AM were then isolated, adjusted to a concentration of $5 \times 10^5$ cells/well, and stimulated with LPS+IFN-γ. Cell culture supernatants were analyzed via IL-6 ELISA. Data are means ± SE. N= 10-15 rats per treatment group. **Greater than control and sham ($P < 0.01$). ANOVA with Tukey’s multiple comparisons post-test.
Table 6. LPT increased \textit{in vitro} nitrite release from stimulated alveolar macrophages.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Control</th>
<th>Sham</th>
<th>LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 % FBS</td>
<td>3.6 ± 1.9</td>
<td>3.7 ± 2.1</td>
<td>3.9 ± 2.5</td>
</tr>
<tr>
<td>INF-γ</td>
<td>7.4 ± 0.7</td>
<td>7.9 ± 1.1</td>
<td>18.2 ± 3.0*</td>
</tr>
<tr>
<td>LPS</td>
<td>6.8 ± 0.8</td>
<td>11.6 ± 2.1</td>
<td>21.1 ± 3.3**</td>
</tr>
<tr>
<td>LPS + INF-γ</td>
<td>7.9 ± 0.5</td>
<td>9.5 ± 1.6</td>
<td>17 ± 1.6**</td>
</tr>
</tbody>
</table>

On day 0, rats were intranasally infected with $1 \times 10^7$ \textit{Streptococcus pneumoniae} CFU. For days 1-3, the control group received no treatment or anesthesia, the sham group received 4 min of light touch under anesthesia, and the LPT group received 4 min of LPT under anesthesia. At day 4 post-infection, rats were euthanized and leukocytes were obtained from BALF. AM were then isolated, adjusted to a concentration of $5 \times 10^5$ cells/well, and either not stimulated (10% FBS) or stimulated under various conditions. Cell culture supernatants were analyzed for nitrite production via Griess assay. Data are means ± SE. N= 10-15 rats per treatment group. **Greater than control ($P < 0.01$). ANOVA with Tukey’s multiple comparisons post-test.
The lymphatic system provides a one-way transport system, which collects excess fluid, proteins, and waste products from the interstitial space and returns it back to the vascular system. In addition, the lymphatic system is a major transport route for immune cells and lymph. Immune cells continuously pass through lymph nodes, which provide an area for filtration, antigen processing, and lymphocyte activation and proliferation. Continuous lymph movement is imperative for health, and impaired lymph flow resulting from conditions such as infection, burns, radiation, surgery, tumor metastasis, tissue grafting, and tissue transplantation can result in decreased lymphocyte recirculation and tissue fluid imbalance, which can then lead to edema. Edema is a serious medical problem, and conservative treatment options such as physical therapy and tissue compression therapy often yield limited and only transient results. Surgical approaches such as lympho-venous, lympho-venous-lymphatic bypass anastomosis, lympho-lymphatic segmental interposition, and free lymph node transplantation are costly, rarely performed, and produce limited results. Drug therapy treatment with Coumarin, a member of the benzoprone family, has shown limited success and is currently not approved in the U.S.

Partially due to the limited availability of treatment approaches, osteopathic physicians have created and utilized OMT. Lymph enhancing techniques specifically have not only been utilized to treat edema, but also to increase lymph flow and immune surveillance in order to
effectively treat infections and inflammatory conditions. Osteopathic physicians have successfully used LPT to treat infectious diseases\textsuperscript{6,7} and respiratory infections such as influenza and pneumonia;\textsuperscript{13-17} however, the mechanisms by which LPT enhances the lymphatic and immune systems are unknown.

Previously, our lab demonstrated that LPT increases lymph flow\textsuperscript{3}, leukocyte count, and flux\textsuperscript{4} in TDL of healthy canine under anesthesia. Novel findings in this dissertation demonstrate that in anesthetized canines: 1) LPT mobilizes leukocytes from the GALT into lymphatic circulation; 2) LPT mobilizes inflammatory mediators into lymphatic circulation; and 3) repeated application of LPT increases lymph flow, concentration of leukocytes, and flux of inflammatory mediators into lymphatic circulation. In addition, this dissertation for the first time demonstrates: 1) the development of a novel lymph enhancing rodent model, in which LPT increases leukocyte flux in the cisterna chili, predominantly from the GALT; and 2) that LPT facilitates the clearance of pneumococcal respiratory infection and suggests a mechanism by which LPT might facilitate the clearance of pneumococcal pneumonia.

Studies have suggested that gastrointestinal lymphoid tissues (GALT) located in the abdominal viscera (the same area that abdominal LPT is applied to) are a major source of thoracic duct leukocytes.\textsuperscript{8-12} In addition, antigen-specific lymphocytes can home to various effector sites in the common mucosal immune system. Therefore, we speculated that leukocytes released during LPT intervention are released from the GALT, would redistribute to the lung, and would then reduce disease severity during respiratory infection with pneumococcus. The studies in Appendix I showed that LPT in canine, in fact, transiently mobilized leukocytes from the mesenteric lymph nodes into MDL, and ultimately into TDL. Further, studies in Chapter III revealed that LPT increased TDL leukocytes repeatedly after a 2 hour resting interval. In Chapter
IV, we developed a rat LPT model in order to study the effects of LPT during infection. In addition, we showed that LPT in rats also mobilized GALT derived leukocytes. As expected, and as shown in previous studies, the majority of cells mobilized in both the canine and rat model were lymphocytes, specifically CD4+ T cells, CD8+ T cells, and B cells, which is in accordance with previous studies. In addition, studies have shown that GALT derived lymphocytes can traffic into the lungs where they enhance immunity and provide protection during pulmonary infection. We hypothesized that LPT would mobilize GALT derived lymphocytes into the lung and provide protection during pneumococcal infection; however, our studies did not show an LPT induced increase in pulmonary lymphocytes. This might be due either to the fact that our harvest was performed during the innate immune response, and adoptive immune response did not have sufficient time to develop, or due to the possibility that there may be considerable more compartmentalization in the common immune system (intestinal vs. nonintestinal) than previously thought.

While LPT enhanced the clearance of *S. pneumoniae* after 3 consecutive daily treatments as shown in Chapter V, this protection was most likely not mediated by an increase in total lymphocytes, but rather by an increase in macrophages and neutrophils. Interestingly, in LPT treated animals we found an increase of macrophages and neutrophils in total lung homogenate, but not BALF, suggesting that the additional macrophages and neutrophils were ‘recruited’ by LPT to the lung interstitium or lung vascular bed, rather than the bronchoalveolar space.

Since the functional capacity of leukocytes, as well as the subset composition and activation of lymphocytes, can differ depending on the lung compartment they are found in, future studies should focus on leukocytes from the separate compartments individually, rather than as a whole. These studies would shed light as to how LPT affects: 1) the leukocyte subset
composition in the different compartments; 2) leukocyte recruitment from the blood and intrapulmonary migration patterns; 3) functional capacity of leukocytes in each compartment; 4) drug/antibiotic delivery to compartments; 5) disease resolution during pulmonary infection; and 6) cytokine/chemokine secretions of leukocytes in the different compartments.

We also hypothesized that LPT would mobilize inflammatory mediators from GALT into lymphatic circulation. Studies presented in Chapter II showed that LPT significantly increased the concentrations of MCP-1 in TDL and the flux of IL-6, IL-8, IL-10, MCP-1, and KC in TDL and MDL. In addition, LPT significantly increased SOD and NT (a marker of NO production) in TDL; however, no concentrations of IL-2, IL-4, IFN-γ, and TNF-α were detected. Further, in Chapter III we showed that LPT repeatedly increased the flux of IL-6, IL-8, KC, NO₂⁻ (nitrite), and SOD in TDL. Of note, the data in Chapters II and III were obtained from healthy, anesthetized dogs, and the cytokines/chemokines mobilized during an infection would most likely vary.

Since we also hypothesized that LPT would redistribute GALT derived inflammatory mediators to the lung where they would participate in reducing the severity of pulmonary disease, we measured inflammatory mediators in BALF from rats infected with Streptococcus pneumoniae. LPT significantly increased the concentrations of SP-D, IL-6, IL-12p70, IL-17, and moderately increased MCP-1 in BALF at day 4; however, LPT did not increase IL-1β, and we were unable to measure concentrations of TNF-α. Previous experiments in mice infected with pneumococcus have shown that concentrations of TNF-α and IL-1β in BALF peak at 12 hours post-infection and continue to decline within the first 48 hours post-infection; therefore, future experiments should measure these cytokines at an earlier time point.140
Also, it has to be noted that we only measured inflammatory mediators in lymph from healthy, anesthetized dogs, and BALF from rats infected with Streptococcus pneumoniae. Since there is the possibility that inflammatory mediators can exhibit interspecies variations in healthy animals, future studies should determine which cytokines/chemokines are present in TDL and BALF of healthy rats. In addition, it is likely that the lymphatic and BALF inflammatory mediator milieu changes during respiratory infection, and therefore future studies should also examine the concentrations and presence of various cytokines/chemokines in TDL from rats infected with pneumococcus.

This information is important as we speculate that LPT can redistribute inflammatory mediators from the mesentery to the lungs, where they may enhance the protection against pneumococcal infection by: 1) enhancing recruitment of macrophages and neutrophils; 2) participating in the direct killing of the pneumococcus; and 3) acting locally on neutrophils and AM to enhance their function. In support of this notion, multiple studies have shown a close interaction amongst lymphatic drainage between the thorax and abdomen (reviewed elsewhere), as well as lymph mediated redistribution of cytokines and chemokines to distant organs. More specifically, mesenteric derived inflammatory mediators such as IL-1β, IL-6, IL-10, and TNF-α, have been shown to redistribute to lungs in various disease rat models. In our experiments, we found a significant increase in the concentrations of MCP-1 in TDL, as well as a significant increase in IL-6 flux in TDL and MDL in anesthetized dogs. Interestingly, both IL-6 and MCP-1 were present in BALF of rats infected with pneumococcus, and LPT significantly increased IL-6 and moderately increased MCP-1 concentrations compared to Sham and Control animals, which supports our notion that LPT may increase cytokine/chemokine redistribution from the mesentery to the lung. In addition, we also
demonstrated *in vitro* that alveolar macrophages from LPT treated animals showed a significantly higher release of IL-6 and NO$_2$ when compared to Control and Sham. This suggests that LPT enhanced the local release of IL-6 and NO$_2$ from alveolar macrophages and most likely skewed pulmonary alveolar macrophages towards an M1 phenotype. In support of this, LPT significantly enhanced the concentrations of IL-6 and IL-12 and moderately increased MCP-1 in BALF of infected rats, all of which are secreted by the M1 alveolar macrophage phenotype.$^{169,170}$

Alveolar macrophages are the resident phagocytes in the alveolar space and play an essential role in the protection against bacterial pneumonia.$^{171}$ In addition to phagocytosis, they also secrete pro-inflammatory cytokines and chemokines to recruit and activate neutrophils and monocytes as well as directly kill bacteria via NO.$^{46,47}$ Macrophage polarization is driven by the types of cytokines present in the pulmonary milieu. Classical, or M1, polarization can be induced by LPS and IFN-γ and leads to enhanced phagocytosis and a pro-inflammatory immune response via secretion of IL-1β, IL-6, IL-8, IL-12, TNF-α, MCP-1 (CCL2), RANTES (CCL5), ROS, and NO production. Alternative, or M2, polarization is induced by IL-4 and IL-13 and leads to an anti-inflammatory response via IL-10 secretion and resolution of inflammation as well as tissue remodeling and repair.$^{46,47}$

The majority of AM originate from monocytes which are recruited from the blood; however, the replication of AM and a parenchymal intermediate-stage macrophages also contribute to the total AM population.$^{171}$ In the steady state, AM are long-lived cells (up to 8 months) with little local replication and small turnover ratios; however, during infection there is an acceleration of monocytes recruited from the blood, as well as an increase in parenchymal intermediate-stage macrophages recruitment and differentiation into the alveolar space in order to control the infection.$^{171}$ Therefore, it is possible that LPT influences the macrophage turnover
kinetics of AM by increasing blood derived monocyte recruitment to the lung, as well as accelerating the recruitment and differentiation of the parenchymal intermediate-stage macrophages into AM. While we are just now starting to understand the importance of the turnover kinetics during infection, studies with Streptococcus Pneumoniae have shown that an increase in AM apoptosis and replacement of AM play a critical role in the clearance of pneumococcus.\textsuperscript{148,172-175} It is thought that once the killing capacity of AM is exhausted, cathepsin D is activated and leads to ‘apoptosis-associated killing’ of pneumococcus, followed by recruitment of new AM.\textsuperscript{176} Recruitment of AM during pneumococcal infection is strongly dependent on the CCL2-CCR2 axis.\textsuperscript{177} While we only have shown a moderate elevation in MCP-1 in BALF of infected rats on day 4, further studies should investigate the effects of LPT on turnover kinetics (measure apoptosis) and MCP-1 levels prior to day 4, especially since studies have shown that \~60\% of infection-induced macrophage turnover in BALF occurs by 24 hours post infection and \~81\% by 7 days post-infection.\textsuperscript{178}

If AM are unable to resolve a pneumococcal infection via ROS, RNS, phagocytosis, and apoptosis-associated killing, neutrophils are recruited via: 1) IL-6 release by M1 macrophages; 2) IL-1\(\beta\) mediated release of IL-8 by macrophages and epithelial cells; 3) IL-17 mediated release of IL-6 from non-lymphocytic, non-macrophage cells; or 4) IL-12 mediated INF-\(\gamma\) release.\textsuperscript{146,147,179,180} In Chapter V, LPT treated animals not only showed an increase in macrophages, but also an increase in neutrophils. Therefore, LPT could enhance the recruitment of neutrophils to the lung through any of the above mentioned mechanisms, but probably mainly via the increase of IL-8 and IL-6.\textsuperscript{181-186} In support of this notion, we showed in Chapter V that \textit{in vitro} activation of LPT derived BALF macrophages enhanced the release of IL-6, suggesting that some of the IL-6 found in BALF originated locally from M1 AM. In addition, we found in
Chapters II and III that LPT increased the flux of IL-6, IL-8, and KC into lymphatic circulation, and we found increased levels of IL-6 in BALF. While we did not measure IL-8 and KC in BALF, our data does suggest that at least some of the IL-6 could have originated from the mesentery. In addition, we found an increased concentration of IL-17 and IL-12 in BALF of LPT treated animals, further explaining the increase in neutrophils found in lungs from LPT animals. Specifically, IL-17 has been shown to recruit neutrophils, induce antimicrobial peptides, induce cell-mediated immunity, and inhibit recolonisation.\textsuperscript{187-189} In order to determine if LPT enhanced IL-8 and KC redistribution from the mesentery to lungs, as well as to determine if IL-12 and IL-17 are produced locally in the alveolar space or derive from the mesentery, future studies should ascertain the presence of these cytokines and chemokines in lymphatic circulation and BALF of infected rats, as well as the effects of LPT on their concentrations and flux. In addition, future studies depleting AM with liposomes containing clodronate would not only shed light on the proposed protective effect of LPT on AM, but would also help clarify if, and how much, of the inflammatory mediators found in BALF are produced locally by AM and if they are redistributed from the mesentery.

We have shown that a single application of LPT significantly increased the flux of IL-6 (Chapter II) in TDL and MDL and LPT repeatedly increased IL-6 flux after a 2 hour period (Chapter III). In addition, we found increased concentrations of IL-6 in BALF of LPT treated animals and demonstrated \textit{in vitro} that alveolar macrophages from LPT treated animals showed a significantly higher release of IL-6 (Chapter V). Previous studies have shown that intratracheal instillation of IL-6 resulted in increased neutrophil recruitment into alveoli and lung interstitium, and IL-6 gene deficient mice showed increased pneumococci in their lungs and had reduced survival times.\textsuperscript{181,190} In addition, IL-6 prolonged neutrophil life span in vitro and enhanced the
bacterial killing by increasing the release of granules containing myeloperoxidase and lactoferrin from neutrophils\textsuperscript{191-193} Since IL-6 plays a significant role in the host defense against pneumococcal pneumonia, we reason that increased concentrations of IL-6 in BALF of LPT treated animals plays a majority role in the protection seen with LPT treatment. Thus, further studies should investigate the source of neutrophil and macrophage recruitment, their life spans and apoptosis, and their functional state. Since myelopoiesis is the major source of macrophage and neutrophil recruitment during infection and inflammation, the effects of LPT on concentrations of haematopoietic cytokines such as granulocyte colony stimulating factor (G-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF) should be investigated. This would reveal whether or not bone marrow is the source of increased pulmonary tissue macrophages and neutrophils found in LPT treated animals, especially since our studies suggest the splenic reservoir not to be the source.\textsuperscript{194} Of interest, recent studies suggest that low levels of circulating microbial molecules (TLR agonists) can travel from peripheral infected tissues and promote inflammatory cell emigration from bone marrow into blood circulation without effecting splenic reservoirs.\textsuperscript{194,195} Therefore, LPT could enhance TLR agonist delivery to the bone marrow, thus providing an alternative mechanism of inflammatory cell recruitment.

On a separate note, we found that LPT increased the flux of NT and NO\textsubscript{2} (both surrogate markers for NO) into lymphatic circulation (Chapters II and III), and increased the release of NO\textsubscript{2} from AM \textit{in vitro} (Chapter V). Given the dual role of NO as a signaling molecule and antimicrobial agent as well as its short half-life, we believe that NO is not redistributed from the mesentery to the lungs, but rather is produced locally.\textsuperscript{116,196} Previous publications and our data suggest that NO found in lymph originates from lymphatic endothelial cells and plays an important role in modulating the lymphatic pump activity by dilating lymphatic vessels.\textsuperscript{197}
Chapter III, we hypothesized that an increase in TDL flow/sheer generated during LPT inhibited the intrinsic lymph pump via production of NO as an energy saving measurement. However, sustained dilation of lymph vessels could also result in the formation of edema.\textsuperscript{197} Hence, an alternative explanation is that LPT not only increases lymph flow via elevation of interstitial fluid pressure, but also increases intrinsic lymph pump frequency by removing NO; however, further studies evaluating contraction frequency \textit{in vivo} are necessary to test this theory. In contrast, we have shown that LPT increased NO production from AM in the lung and thus we hypothesize that NO acts as an antimicrobial agent in the pulmonary environment.\textsuperscript{198,199} Previous publications support this notion, as inducible NO has been shown to recruit cells during pneumococcal pneumonia, exert a direct antibacterial effect against \textit{Streptococcus pneumoniae}, and NO-deficient mice showed increased bacterial loads when infected with \textit{S. pneumoniae}.\textsuperscript{140,200}

Interestingly, the anesthetic propofol has been shown to inhibit inducible NO production and spontaneous lymphatic vessel activity in rats, as well as reduce the production of pro-inflammatory mediators, inhibit neutrophil function, and increase the incidence of pneumonia in ventilated patients.\textsuperscript{201-205} In Chapter V, propofol was used during Sham and LPT treatments to avoid inducing a stress response; however, it is likely that daily application of propofol inadvertently decreased the beneficial effects of LPT induced NO production by AM, decreased lymphatic flow, and decreased pro-inflammatory mediators. On the contrary, propofol has been shown to increase basal (constitutive) NO production by neutrophils in a concentration-dependent manner, thus it is possible that the administration of propofol enhanced anti-pneumococcal activity. However, our data does not support this notion and future studies are needed to investigate if prolonged propofol administration benefits or negates the effects of
LPT. In addition, future studies should ascertain if LPT increases propofol concentrations in the pulmonary environment, thus possibly altering the pulmonary environment. In support of this, our data in Chapter V showed an increase in lung NK cells, B cells, CD4+, and CD8+ in both Sham and LPT treated animals, suggesting an anesthesia-mediated increase in pulmonary lymphocytes. Furthermore, previous studies have shown that propofol administration enhances cytotoxic T lymphocytes, and thus it is speculated that propofol might enhance antitumor immunity.

Finally, future studies should focus on establishing a mouse model of LPT due to increased commercial availability of experimental reagents and lower costs. In addition, we could take advantage of a broader variety of mice strains/phenotypes (transgenic and knockout strains) and pneumococcal serotypes, as well as utilize well-established mice models to investigate the effects of LPT on pharmacokinetics and antibiotic efficacy. For example, the phenotypes nm1054 and bg (ciliary dyskinesia) could be utilized to determine the effects of LPT on the mucociliary escalatory and associated clearance of pneumococcus.

In conclusion, our experiments have shown that LPT has the potential to act as an immunostimulatory therapy. This is of significant clinical importance as lymphostimulating and lymphotropic agents are sparse. Specifically, certain antibiotics and diuretics commonly used in pulmonary infection and lung pathologies have been shown to inhibit the lymphatic system (decrease lymph circulation velocity and inhibit lymphatic vessel contractility) thus adversely effecting management of pulmonary pathologies and limiting treatment approaches. LPT thus should be explored as a potential adjunctive therapy to antibiotics during pulmonary infection, as well as a potential targeted delivery system for antibiotics, immunomodulators, and chemotherapeutics.
Figure 1. Proposed model of LPT enhanced clearance of S. pneumoniae.
APPENDIX I

Lymphatic Pump Treatment Mobilizes Leukocytes from the Gut Associated Lymphoid Tissue into Lymph

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Abstract

Lymphatic pump techniques (LPT) are used clinically by osteopathic practitioners for the treatment of edema and infection; however, the mechanisms by which LPT enhances lymphatic circulation and provides protection during infection are not understood. Rhythmic compressions on the abdomen during LPT compress the abdominal area, including the gastrointestinal lymphoid tissues (GALT), which may facilitate the release of leukocytes from these tissues into lymphatic circulation. The purpose of this study was to determine if LPT would increase the mobilization of leukocytes from the GALT into the lymphatic circulation. To determine the acute effect of LPT on thoracic and intestinal lymph, a catheter was inserted into the either the thoracic or the large intestinal lymph ducts of dogs. In addition, to determine if LPT enhanced the release of leukocytes from the mesenteric lymph nodes (MLN) into thoracic duct lymph, the MLN were fluorescently labeled in situ. Lymph samples were collected during 4 min of baseline, 4 min of LPT, and 10 min following cessation of LPT (recovery). The application of LPT significantly increased lymph flow and leukocytes in both intestinal and thoracic duct lymph. LPT had no preferential effect on any specific leukocyte population, since neutrophil, monocyte, T cell, and B cell numbers were similarly increased. In addition, LPT significantly increased the mobilization of leukocytes from the MLN into thoracic duct lymph. Finally, recovery values were similar to baseline values, indicating that the effects of LPT are transient. Thus, the hypothesis that LPT facilitates mobilization of leukocytes from the GALT into the lymphatic circulation is supported. The enhanced release of leukocytes during LPT may provide scientific rationale for the clinical use of LPT to enhance immunity and treat infection. In addition, this new information has expanded our basic understanding of the lymphatic system and its role in immunological function.
Key words:
lymphatic pump treatment, osteopathic manipulation, lymphatic circulation, thoracic duct lymph, leukocytes, edema, infection, gastrointestinal lymphoid tissue, mesenteric lymph nodes.
Introduction

The lymphatic system collects proteins and excess interstitial fluid into afferent lymphatic vessels. During infection, this lymph also carries antigens and antigen-bearing cells from infected tissues to lymph nodes, where antigen-specific immune responses are initiated. The resulting primed lymphocytes then exit the lymph nodes via efferent lymphatic vessels and re-enter the lymphatic circulation. The thoracic duct is the largest lymphatic vessel, and it transports lymph from most body tissues, excluding the right arm, the right side of the head, neck and chest and the right lung and lower left lung lobe, which are drained by the right lymphatic duct. Lymph from the thoracic duct enters the blood circulation at the left subclavian vein, allowing primed lymphocytes to enter general blood circulation. This lymphocyte recirculation facilitates interactions of lymphocytes with foreign antigens in blood and tissue, and is an important component of the immune system.

Unlike circulating blood, the movement of lymph through lymphatic vessels is not maintained by the pumping of the heart. Instead, lymphatic circulation is maintained through the rhythmic, phasic contraction of lymph vessel walls and external compression of the lymph vessels. A series of one-way valves along the vessels ensures unidirectional lymph flow toward their junction with the blood circulation. Forces external to the lymph vessels such as respiration, intestinal peristalsis, and muscle contraction facilitate lymph flow. In addition, activities such as exercise, passive limb movement, and body-based manipulative medicine techniques have been shown to increase thoracic duct lymph flow.

Diseases that result in congestion of the lymphatic circulation, such as infection and lymphedema, can inhibit leukocyte recirculation and exacerbate the disease process. Therefore, interventions that relieve lymphedema and enhance lymph-tissue recirculation of
immune cells, immune products, or pharmaceuticals should aid in the treatment of infectious disease. Limb elevation and compression garments reduce filtration of fluid from vascular capillaries and accelerate removal of excess interstitial fluid by the lymphatic drainage. This lymphatic drainage can be further stimulated by intermittent, external pneumatic compression.

Manual therapies used by osteopathic practitioners, physical therapists and massage therapists reduce lymphedema. Specifically, osteopathic physicians believe that removing obstructions to tissue blood and lymph flow is one of the most effective ways to promote and restore health. A group of osteopathic manipulations known collectively as the lymphatic pump techniques (LPT) are designed to enhance lymph return from specific areas of the body. These techniques include the thoracic, liver, splenic, and pancreatic pumps, the pedal pump, and the abdominal lymphatic pump. The increased lymph flow that results from these treatments accelerates removal of cellular wastes, toxins, and bacteria from the interstitial fluid in addition to reducing edema. These lymphatic pump techniques are also report to enhance immune function.

In clinical studies, LPT increased vaccine-specific antibodies, reduced antibiotic use during infection, and reduced the duration of hospital stay in elderly patients with pneumonia. Collectively, these studies suggest that LPT stimulates immune responses, which may accelerate the clearance of infection. However, the mechanisms by which LPT enhances immunity and provides protection during infection are still poorly understood.

Previously, we demonstrated that abdominal LPT increased leukocyte counts in thoracic lymph; however, the tissue source of these mobilized leukocytes was unknown. Studies to identify the source of thoracic duct lymphocytes indicate that the majority of thoracic duct lymphocytes are derived from the gastrointestinal lymphoid tissue (GALT). Rhythmic
compressions of the abdomen during LPT most likely compress regional lymphoid tissues, which release pooled leukocytes into the lymph circulation. Specifically, by compressing the abdominal area, including the gastrointestinal mucosa, the abdominal LPT should promote the release of leukocytes from this tissue and accelerate their transit through the lymphatic circulation. The purpose of this study was to determine if, in fact, abdominal LPT increases leukocytes in both thoracic and intestinal duct lymph, and determine if the mesenteric lymph nodes are a source of these leukocytes.

**Material and Methods**

**Animals.**

This study was approved by the Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996). Seventeen adult mongrel dogs, free of clinically evident signs of disease were used for this study.

**Surgical Procedures.**

To demonstrate that abdominal LPT increases leukocytes in both thoracic and intestinal duct lymph, and to determine if the mesenteric lymph nodes are a source of these leukocytes, 17 dogs were anesthetized with sodium pentobarbital (30 mg/kg, i.v.) and placed in a right, lateral, recumbent position. After intubation, the dogs were ventilated with room air supplemented with O2 to maintain normal arterial blood gases. Arterial blood pressure was monitored through a catheter inserted into a femoral artery. Supplementary anesthetic and fluids were administered through a catheter inserted into a femoral vein.

In 6 dogs, the chest was opened by a thoracotomy in the left, fourth intercostal space. The thoracic duct was isolated from connective tissue and ligated. Caudal to the ligation, a PE
60 catheter (i.d 0.76 mm, o.d. 1.22 mm) was inserted into the duct and secured with a ligature. Thoracic lymph was collected at 1 min intervals during 4 min baseline and 4 min LPT conditions, and at 2-5 min intervals for 10 min following cessation of LPT (recovery condition); lymph flow rate was computed from the volume of lymph collected during these time intervals.

For collection and analysis of intestinal lymph, 6 dogs were surgically prepared for experimentation as described above, except that instead of opening the chest, a midline abdominal incision was made to expose a large mesenteric lymph duct. This duct was isolated, ligated, and a PE60 catheter was inserted into the duct and secured with a ligature. This catheter was exteriorized through the abdominal incision, which was then closed with 2-0 silk suture. Mesenteric lymph samples were collected and flow measured as described above for thoracic duct lymph.

For labeling mesenteric lymph nodes in situ, 5 dogs were surgically prepared for experimentation as described above, with incisions in both the left chest and in the midline abdomen. After the abdominal cavity was opened, readily visible mesenteric lymph nodes (MLN) were labeled as previously described. Briefly, 12 mg of lyophilized 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) were dissolved in 5 ml Dimethyl sulfoxide (Sigma, St Louis, MO). Then, each MLN was directly injected with 100-200 μl of CFSE. The abdominal cavity was closed with 2-0 silk suture. The thoracic lymph duct was catheterized for collection of lymph and analysis of CFSE. These lymph samples were collected at 1, 10, 20, 30, 45, and 60 min after labeling the MLN with CFSE. When CFSE in thoracic duct lymph had reached a steady state, additional samples of thoracic duct lymph were collected during 4 min of resting (baseline), 4 min of LPT and 10 min following treatment (recovery).
Lymphatic Pump Technique.

The anesthetized animal was placed in a supine position. To perform abdominal LPT, the operator contacted the animal with the hands placed bilaterally at the costo-diaphragmatic junction. Pressure was exerted medially and cranially sufficient to compress the lower ribs until significant resistance was encountered, and then the pressure was released. Rib compressions were administered at a rate of approximately one per second for a total of 4 min of LPT.

Leukocyte Enumeration.

Leukocytes were enumerated using the Hemavet 950 (Drew Scientific). To compute the leukocyte flux, the total number of a specific leukocytes population was multiplied by the lymph flow per minute.

Determination of Total Fluorescence and Fluorescing Leukocytes in Lymph Samples.

Two-color immunofluorescent staining was performed to identify lymphocyte populations using FITC or PE-labeled mouse anti-canine isotype control IgG2b, FITC-anti-canine CD3, PE-anti-canine B cell, PE-anti-canine CD4, AlexaFluor 647-anti-canine CD8, FITC-anti-canine IgA, or FITC-anti-canine IgG monoclonal antibodies (mAb) (Serotech, Raleigh, NC). A total of $10^6$ cells were incubated with the mAb as described by the manufacturer. The cells were washed in staining buffer consisting of Mg$^{2+}$-free, Ca$^{2+}$-free phosphate buffered saline supplemented with 2% fetal bovine serum (HyClone Laboratories, Logan, UT) and fixed with 0.05% paraformaldehyde until analyzed.

Following intra-nodal labeling of the MLN, thoracic duct lymph supernatants were directly measured for CFSE using the Varian, Cary Eclipse spectrofluorometer attached with a suitable high-throughput plate reader developed by the same manufacturer. The wavelength 480 nm was used for excitation, and the emission signal was collected from 500 nm-570 nm. Both
excitation and emission slits were set to 5nm, 670 V were applied on the photomultiplier tube detector and spectral scan was performed at 120 nm/min speed. Each sample was scanned three times and a mean intensity was generated by the software. The samples were read in 96 well plates. The adjacent wells of each sample were left empty to avoid any loss of signal. Fluorescence intensity spectra of each sample-well were scanned. The spectra of the scan were checked to ensure they represented signals from CFSE. To determine background, readings were taken from blank wells. The average peak intensity from the spectra was plotted against the time of sample collection.

The fluorescently labeled cells were analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA). Lymphocyte gates and detector voltages were set using isotype control stained cells, and stained cell populations were seen as distinct peaks or clusters of cells. The proportion of each cell population was expressed as the percentage of the number of stained cells. To determine the total number of a specific lymphocyte population in a milliliter of lymph, their percentage was multiplied by the total number of cells. To determine the flux of a leukocyte population (cells/min), the cell population number was multiplied by the thoracic duct lymph flow.

**Statistical Analysis.**

Data were logarithmically transformed and analyzed by repeated measure analysis of variance followed by a Tukey-Kramer multiple comparisons post test. Analyses were performed using Graphpad Prism version 5.0 for Windows, GraphPad Software (San Diego, CA). Differences among mean values with P < 0.05 were considered statistically significant. Data is presented as arithmetic means ± standard error (SE).
Results

Abdominal LPT Increases Leukocytes in Thoracic Duct Lymph.

During the baseline, pre-LPT condition, thoracic duct lymph contained $12.5 \pm 2.2 \times 10^6$ leukocytes/ml, a value consistent with prior measurements in dogs,\textsuperscript{4} rats,\textsuperscript{8,224} and humans.\textsuperscript{11} As illustrated in Figure 1, LPT quickly increased leukocyte count in thoracic duct lymph to a peak value $34.0 \pm 15.0 \times 10^6$ cells/ml at 2 min of treatment ($P < 0.05$, vs baseline). For 4 min of LPT, the lymph leukocyte count averaged $32.2 \pm 9.4 \times 10^6$ cells/ml ($P < 0.01$, vs baseline). By 10 min post-LPT, the lymph leukocyte count had returned to baseline ($P > 0.05$).

Leukocyte subpopulations of the thoracic duct leukocyte pool are reported in Table 1. LPT had no preferential effect on any of these components, since neutrophils, monocytes, total lymphocytes, CD4+ T cells, CD8+ T cells, and IgA+ and IgG+ B cell numbers were similarly increased. By 10 min post-LPT, numbers of each of these cell types had decreased, near to baseline concentrations.

The thoracic duct lymph flow at baseline was $0.62 \pm 0.12$ ml/min and during the 4 min of LPT, the lymph flow averaged $4.2 \pm 0.39$ ml/min ($P < 0.01$, vs baseline). Additionally, by 10 min post-LPT, thoracic duct lymph flow was $2.0 \pm 0.77$ ml/min ($P < 0.05$, vs baseline), demonstrating that thoracic duct lymph flow remained elevated for 10 min following LPT treatment.

Leukocyte flux was computed from the product of thoracic duct lymph flow and cell count. Figure 2 illustrates that LPT markedly accelerated leukocyte flux in the thoracic duct from a baseline of $8.88 \pm 2.0 \times 10^6$ to a peak value of $153 \pm 67 \times 10^6$ cells/min ($P < 0.01$, vs baseline). For 4 min of LPT, the leukocyte flux averaged $138 \pm 43 \times 10^6$ cells/ml ($P < 0.01$, vs baseline). By 10 min post-LPT, the leukocyte flux was similar to baseline. Similar increases
were observed for fluxes of CD4+ T cells, CD8+ T cells, and IgA+ B cells and IgG+ B cells during LPT (Table 2).

**Abdominal LPT Mobilizes Leukocytes into Intestinal Duct Lymph.**

The average baseline, pre-LPT leukocyte count in intestinal lymph was 6.3 ± 0.67 x 10^6 cells/ml, consistent with values previously reported for sheep^{225,226} and rats.^8 As illustrated in Figure 3, LPT quickly increased leukocyte count in intestinal lymph to a peak value 18.0 ± 4.11 x 10^6 cells/ml (P < 0.01, vs baseline). For 4 min of LPT, the lymph leukocyte count averaged 16.7 ± 3.8 x 10^6 cells/ml (P < 0.01, vs baseline). By 10 min post-LPT, the lymph leukocyte count had returned to near baseline (P > 0.05, vs baseline). CD4+ T cells, CD8+ T cells, and IgA+ and IgG+ B cells in intestinal lymph were similarly increased during LPT (Table 3), and these numbers returned to baseline by 10 min post-LPT.

The average baseline, pre-LPT, intestinal duct lymph flow was 0.35 ± 0.07 ml/min. During the first 2 min of LPT, the intestinal duct lymph flow increased to an average of 1.4 ± 0.42 ml/min (P < 0.05, vs baseline). However, during the last 2 min of LPT, the intestinal duct lymph flow average was 0.87 ± 0.14 ml/min. By 10 min post-LPT, the intestinal lymph flow was similar to baseline. This finding suggests that LPT mobilizes intestinal lymph from a fluid pool that becomes depleted during 4 min of LPT treatment.

As illustrated in Figure 4, LPT markedly increased flux of leukocytes in intestinal lymph. This flux increased from an average baseline value of 2.1 ± 0.25 cells/min to a peak of 25.0 ± 6.2 cells/min at 2 min LPT (P < 0.01, vs baseline). During the first 2 min LPT, intestinal leukocyte flux averaged 25 ± 7.3 cell/min (P < 0.01, vs baseline). During the later 2 min of LPT, intestinal leukocyte flux subsided due to less of an increment in lymph flow. LPT also increased flux of CD4+ T cells, CD8+ T cells, and IgA+ and IgG+ B cells in intestinal lymph (Table 4).
Abdominal LPT Mobilizes Leukocytes from the Mesenteric Lymph Nodes into Thoracic Duct Lymph.

Immediately following intra-nodal labeling of the MLN with CFSE, 80-90% of leukocytes in samples of thoracic duct lymph were labeled with CFSE. This percentage then gradually fell until 45-60 min when the percentage of labeled leukocytes in thoracic duct lymph was nearly constant at approximately 16%. Thus, in this steady state, baseline condition, about 16% of the thoracic duct leukocytes originated from labeled MLN. The initial greater percentage of labeled leukocytes in thoracic duct lymph was most likely due to extra-nodal labeling of circulating leukocytes with free, unbound CFSE that had escaped from the injection sites, since initially unbound CFSE was detected in thoracic duct lymph. However, at 45-60 minutes following intra-nodal injections of CFSE, the amount of unbound CFSE in thoracic duct lymph was not elevated (P > 0.05). Thus, by 60 min post-injection, the amount of CFSE in thoracic duct lymph provided a stable index of the rate of release of leukocytes by MLN.

Sixty minutes after labeling the MLN in situ with CFSE, thoracic duct lymph samples were collected during 4 min of the baseline, pre-LPT condition, during 4 min of LPT, and for 10 min following cessation of LPT. The percentage of CFSE labeled leukocytes in the thoracic duct samples was approximately 16% pre-LPT, 19% during LPT, and 16% after LPT. In addition, thoracic duct lymph flow was increased, approximately 4-fold. While LPT did not significantly increase the percentage of CFSE labeled leukocytes in the thoracic duct lymph, LPT did significantly (P < 0.05) increase the flux of CFSE labeled leukocytes (Fig. 5). These data demonstrate that 4 min of LPT produced an incremental mobilization of $27 \times 10^6$ leukocytes from MLN relative to the baseline release rate. Importantly, if the MLN were not a source of the leukocytes released into lymphatic circulation during LPT, we would expect a decrease in the
percentage of CFSE labeled leukocytes. Thus, the hypothesis that LPT facilitates mobilization of leukocytes from the MLN into the lymphatic circulation is supported.

**Discussion**

We have demonstrated that LPT increases leukocyte numbers and lymph flow in both the thoracic and intestinal lymph ducts. Consistent with our previous report, we found that LPT did not preferentially release any specific leukocyte population, but increased neutrophils, monocytes, CD3+CD4+ T cells, and CD3+CD8+ T cells. In addition, LPT increased the numbers of IgA and IgG surface bearing B cells into intestinal and thoracic duct lymph, suggesting that LPT is able to mobilize mature, antigen-specific lymphocytes into lymphatic circulation.

In addition, we found that approximately 16% of the leukocytes in the thoracic duct lymph came from labeled mesenteric lymph nodes, and LPT increased the number of these leukocytes in thoracic duct lymph. However, it is important to note that only 25-50% of the MLN were labeled; therefore, the actual percentage of thoracic duct leukocytes that originate from the MLN would be much greater than our data indicate. The greatest increase in leukocyte concentrations was observed during 2-4 minutes of LPT. This finding suggests that it takes approximately 1-2 minutes for leukocytes from the MLN to migrate into the thoracic duct.

While the release of leukocytes into lymph during LPT was transient, LPT produced a net increase of $6 \times 10^8$ leukocytes in lymphatic circulation. Although this increase of leukocytes transported into circulation via lymph during LPT may seem relatively minor compared to the total leukocytes found in blood, increased mobilization of leukocytes could enhance immune surveillance and promote earlier responses to pathogens. Mature lymphocytes bearing antigen-specific receptors recirculate continually from the bloodstream through the peripheral or
secondary lymphoid organs, and then return to the bloodstream via the lymphatic vessels. Most adaptive immune responses are initiated when these recirculating lymphocytes recognize specific antigens on the surface of an activated professional antigen presenting cell. If this circulation of lymphocytes is restricted in any way, there could be a delay in the immune response to a pathogen, which could compromise the health of an individual. Therefore, LPT may be an approach to not only to improve lymph flow but, more importantly may also increase the release of lymphocytes into circulation, resulting in earlier and more frequent encounters and responses to a pathogen.

The mucous membranes, which line the respiratory, digestive, and urogenital tracts, are a major site of antigen entry. Studies have shown that antigen specific lymphocytes primed in the gastrointestinal tract can migrate into the lungs and provide protection during pulmonary infection. Furthermore, leukocytes from the GALT comprise a different array of immune specificities and responses than other tissues due to the antigens that they encounter. Thus, LPT may not only enhance circulation of leukocytes, but it may also facilitate the trafficking of local immune responses to other tissues, such as the lungs, that may benefit from their presence during infection.

In conclusion, clinical studies suggest that osteopathic manipulative treatments, including LPT, can stimulate immune responses, which may accelerate the clearance of infection. In support of this notion, we have demonstrated that LPT facilitates the release of leukocytes from the GALT into lymphatic circulation. Leukocytes from the GALT comprise a different array of immune specificities and responses than leukocytes from other tissues, due to the antigens that they encounter. Thus, LPT may not only enhance circulation of leukocytes, but it may also facilitate the trafficking of local immune responses to other tissues, such as the
lungs, that may benefit from their presence during infection. While this LPT-induced leukocyte release is impressive, and it is likely that LPT increases the numbers of leukocytes that can traffic into tissue and provide protection during infection, the clinical impact of this leukocyte increase in lymph circulation merits further investigation. The information gained from this study provides a scientific basis and rationale for the use of LPT to enhance immunity and treat infection. In addition, this new information has expanded our basic understanding of the lymphatic system and its role in immunological function.
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</table>

\(^a\) Data are means x 10\(^6\) leukocytes/ml ± SE from 6 animals.

\(^b\) Greater than baseline and recovery (P < 0.05).

\(^c\) Greater than baseline and recovery (P < 0.01).

\(^d\) Greater than baseline (P < 0.01) and recovery (P<0.05).
Table 2. Abdominal LPT Increases Leukocyte Flux in Thoracic Duct Lymph

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>LPT</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>0.27 ± 0.12</td>
<td>3.67 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75 ± 0.23</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.34 ± 0.14</td>
<td>4.24 ± 1.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.91 ± 0.25</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>10.32 ± 4.53</td>
<td>81.1 ± 22.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.3 ± 6.62</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>3.25 ± 0.62</td>
<td>43.7 ± 5.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.4 ± 4.74</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>1.24 ± 0.37</td>
<td>16.3 ± 4.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.31 ± 2.00</td>
</tr>
<tr>
<td>IgA&lt;sup&gt;+&lt;/sup&gt;B cells</td>
<td>0.65 ± 0.18</td>
<td>9.02 ± 0.86&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.48 ± 0.53</td>
</tr>
<tr>
<td>IgG&lt;sup&gt;+&lt;/sup&gt;B cells</td>
<td>1.06 ± 0.21</td>
<td>13.4 ± 4.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.95 ± 0.45</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are means x 10^6 leukocytes/min ± SE from 6 animals.

<sup>b</sup>Greater than baseline and recovery (P < 0.01).

<sup>c</sup>Greater than baseline (P < 0.001) and recovery (P < 0.01).

<sup>d</sup>Greater than baseline (P < 0.01) and recovery (P < 0.05).
Table 3. Abdominal LPT Increases Leukocytes in Intestinal Duct Lymph $^a$

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>LPT</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>0.44 ± 0.11</td>
<td>2.30 ± 0.29  $^a$</td>
<td>0.90 ± 0.22</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.31 ± 0.06</td>
<td>1.30 ± 0.18  $^c$</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>5.94 ± 0.65</td>
<td>15.5 ± 3.32  $^c$</td>
<td>8.57 ± 1.58</td>
</tr>
<tr>
<td>CD4$^+$ T cells</td>
<td>2.76 ± 0.32</td>
<td>6.60 ± 1.80  $^b$</td>
<td>3.69 ± 0.87</td>
</tr>
<tr>
<td>CD8$^+$ T cells</td>
<td>0.56 ± 0.07</td>
<td>1.42 ± 0.43  $^b$</td>
<td>0.82 ± 0.19</td>
</tr>
<tr>
<td>IgA$^+$ B cells</td>
<td>0.37 ± 0.15</td>
<td>1.02 ± 0.56  $^b$</td>
<td>0.62 ± 0.28</td>
</tr>
<tr>
<td>IgG$^+$ B cells</td>
<td>0.27 ± 0.07</td>
<td>0.83 ± 0.20  $^b$</td>
<td>0.46 ± 0.25</td>
</tr>
</tbody>
</table>

$^a$ Data are means x 10^6 leukocytes/ml ± SE from 6 animals.

$^b$ Greater than baseline (P < 0.05).

$^c$ Greater than baseline and recovery (P < 0.05).

$^d$ Greater than baseline (P<0.01).
Table 4. Abdominal LPT Increases Leukocyte Flux in Intestinal Duct Lymph

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>LPT</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>0.12 ± 0.03</td>
<td>1.79 ± 0.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.44 ± 0.15</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.10 ± 0.02</td>
<td>1.10 ± 0.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.32 ± 0.12</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.85 ± 0.23</td>
<td>13.1 ± 4.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.71 ± 3.50</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>1.36 ± 0.36</td>
<td>9.00 ± 3.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.10 ± 1.15</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>0.27 ± 0.08</td>
<td>1.84 ± 0.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.72 ± 0.03</td>
</tr>
<tr>
<td>IgA&lt;sup&gt;+&lt;/sup&gt;B cells</td>
<td>0.28 ± 0.13</td>
<td>4.10 ± 3.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70 ± 0.48</td>
</tr>
<tr>
<td>IgG&lt;sup&gt;+&lt;/sup&gt;B cells</td>
<td>0.14 ± 0.06</td>
<td>2.00 ± 1.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.52 ± 0.39</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are means x 10<sup>6</sup> leukocytes/min ± SE from 6 animals.

<sup>b</sup>Greater than baseline (P < 0.05).

<sup>c</sup>Greater than baseline (P<0.01) and recovery (P < 0.05).
Figure 1. Abdominal LPT increases thoracic duct lymph leukocyte numbers. Thoracic duct lymph was collected under 1) resting (baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery). Data are means x 10^6 total leukocytes/ml ± SE from 6 animals. * P < 0.05 compared to baseline and recovery values.
Figure 2. Abdominal LPT increases thoracic duct lymph leukocyte flux. Thoracic duct lymph was collected under 1) resting (baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery). Data are means x 10^6 total leukocytes/minute ± SE from 6 animals. ** P < 0.01 compared to baseline and recovery values.
Figure 3. Abdominal LPT increases intestinal lymph leukocyte numbers. Intestinal duct lymph was collected under 1) resting (baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery). Data are means x 10^6 total leukocytes/ml ± SE from 6 animals. ** P < 0.01 compared to respective baseline and recovery values.
Figure 4. *Abdominal LPT increases intestinal lymph leukocyte flux.* Intestinal duct lymph was collected under 1) resting (baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery). Data are means x 10^6 total leukocytes/minute ± SE from 6 animals. ** denotes statistical increases ($P < 0.01$) compared to respective baseline and recovery values. * denotes statistical increases ($P < 0.05$) compared to respective baseline and recovery values.
Figure 5. Abdominal LPT increases mesenteric derived leukocyte flux in thoracic duct lymph. Sixty minutes after labeling the MLN in situ with CFSE, thoracic duct lymph was collected under 1) resting (baseline) conditions, 2) during 4 min LPT, and 3) during 10 minutes following LPT (Recovery). Data are means x 10⁶ of CFSE labeled leukocytes/minute ± SE from 5 animals. * P < 0.05 compared to respective baseline values.
APPENDIX II

The Development of a Novel Osteopathic Animal Modality of Inflammatory Bowel Disease: Preliminary Findings and Clinical Implications.

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1Department of Cell Biology and Immunology, 2Texas College of Osteopathic Medicine, 3Osteopathic Research Center, University of North Texas Health Science Center. 5 AOA Research fellow, American Osteopathic Association.

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In preparation for publication
Abstract

Context:

A substantial amount of IBD patients utilize complementary and alternative treatments, including Osteopathic Manipulative Treatments (OMT) in order to alleviate symptoms such as pain. Osteopathic clinicians have reported some success in the treatment of IBD patients; however, objective evidence-based research of OMT in IBD patients is severely lacking, in part due to the lack of an osteopathic IBD animal model.

Objective:

To establish an experimental colitis rat model of IBD induced by dextran sodium sulfate (DSS) polymers in their drinking water, and to assess if application of osteopathic abdominal lymphatic pump treatments (LPT) will reduce signs and severity in such a model. This information will be used to determine the safety and efficacy of LPT, and to create a scientific justification for the clinical use of LPT to treat patients with IBD.

Methods:

Colitis in male, Wistar rats was induced by replacing normal drinking water with water containing 3.5% DSS for 10 days (days 0-9). LPT or Sham treatment was performed daily on days 3-8 under 2-5% isoflurane gas anesthesia. Experiments included an LPT group (DSS+ISO+LPT), a Sham group (DSS+ISO+light touch), a disease control group: DSS-C (DSS only), and a healthy control group: H2O-C (no treatment and no DSS). Weights and clinical signs of disease were monitored daily. On day 9, rats were sacrificed and their colons were evaluated using macroscopic score parameters.

Results:

Daily treatment with DSS significantly decreased body weight in DSS-C (days 7-9), Sham (days 5-9), and LPT (days 6-9) groups compared to the Control group. Body weight in the
DSS-C was also significantly different from Sham group at days 8 and 9. The Daily Disease Activity Index (DAI) score increased over time, with a significant difference between H2O-C and all other groups at days 4-9. LPT significantly decreased DAI as compared to Sham and DSS-C at days 8 and 9. Mucosal damage was assessed using the macroscopic inflammatory scoring system. LPT decreased colonic tissue damage and normalized stool consistency at day 9 compared to DSS-C and Sham.

**Conclusion:**

The data presented in this manuscript confirm that the osteopathic DSS colitis model does in fact present a safe and efficacious disease model with clinical measurable variables such as weight changes, macroscopic colonic scores, and DAI. Further, these data suggest that once daily LPT significantly decreased disease reverse clinical symptoms of IBD while leading to a healthier colon, most likely by influencing colonic motility, colonic bleeding, and effecting stool consistency. Therefore, applying this treatment in a clinical setting could significantly alleviate the symptoms experienced by IBD patients.

**Keywords:**

lymphatic pump technique, lymphatic pump treatment, manual therapy, manual lymph drainage, IBD, inflammatory bowel disease, ulcerative colitis, Crohn’s disease, inflammation, edema, immune system, osteopathic manipulative medicine, immunity, complementary and alternative medicine, CAM
Introduction

Inflammatory Bowel Disease (IBD) is the leading chronic gastrointestinal disorder in the U.S., affecting 1.5 million Americans and 4 million individuals worldwide. The two main clinicopathological subtypes of IBD are ulcerative colitis (UC), which primarily affects the mucosal lining of the colon and rectum, and Crohn’s (CD) disease, which can involve any segment of the gastrointestinal tract. Healthcare costs related to the treatment of IBD in the U.S is estimated to total $6.3 billion in direct medical expenditures, and $5.5 billion annually in indirect costs, such as employment related opportunity losses. While early onset IBD can result in delayed puberty, growth retardation, depression, and anxiety, all IBD patients suffer from chronic pain and fatigue as a result of the inflammatory symptoms and diarrhea, leading to mental and emotional distress in patients and a severe decline in quality of life during disease exacerbation. Some IBD patients are also afflicted by bloody stools, abdominal cramps, ulcerations, and fever, along with an increased risk for developing colorectal cancer (CRC). In addition, extraintestinal manifestations like arthritis, uveitis, iritis, erythema nodosum, aphthous stomatitis, and pyoderma gangrenosum are also reported by some IBD patients. IBD is often progressive and ultimately 20%-30% of UC patients will receive a colectomy, while 70%-80% of CD patients will receive some form of intestinal surgery, of which 30% will require follow-up surgeries. Therefore, there is a need to investigate new therapies that may reduce disease progression and enhance the quality of life for these patients.

Conventional therapies for IBD include methotrexate, aminosalicylates, thiopurines, corticosteroids, and anti-TNF agents and are efficacious if prescribed appropriately by clinicians (reviewed elsewhere), but they are also associated with clinical limitations and have a multitude of side effects. New therapeutic strategies can lead to activation of alternative inflammatory pathways, persistent inflammation, loss of the response to drugs with time, and can
even have fatal side effects.\textsuperscript{235-240} Thus, there is an urgent clinical need to explore alternative therapies. Due to the problems with current therapies, afflicted patients are increasingly using complementary and alternative medicine (CAM), including manipulative therapies.\textsuperscript{241,242} It has been reported that up to three-quarters of IBD patients have used CAM therapy at some point during their illness, while up to fifty percent are considered to be consistent or prolonged CAM therapy users.\textsuperscript{235,241,243,244} Hence, there is an urgent clinical need to explore the safety and efficacy of alternative therapies, such as osteopathic manipulative medicine.

Osteopathic physicians have long advocated and used adjunctive osteopathic manipulative treatments (OMT), including lymphatic system manipulations, for the management of a variety of gastrointestinal pathologies, including IBD.\textsuperscript{245-248} The belief in the osteopathic profession that OMT influences intestinal metabolism and gut immune responses, and therefore can be used to successfully treat intestinal disorders, such as IBD, is based on clinical experience and anecdotal evidence; however, objective evidence-based medical studies producing laboratory or imaging evidence is severely lacking.\textsuperscript{249,246,250} Specifically, to our knowledge, no studies have investigated the mechanisms and effects of lymphatic system manipulations in IBD patient management. In their clinical practice, osteopathic physicians apply a variety of lymphatic system manipulations, most often lymph enhancing techniques, such as the thoracic pump, abdominal pump, and pedal pump, which are clinically used to enhance immunity to fight infections, remove lymphatic obstructions, enhance metabolic waste removal, and improve lymphatic circulation.\textsuperscript{7}

The pathogenesis of IBD remains enigmatic, but is believed to be multifactorial and involve an aberrant immune response.\textsuperscript{231} While IBD pathogenesis involves complex interactions of multiple factors, there is growing evidence that lymphatic vessels are a key player in the
inflammatory response, and both UC and CD are known to exhibit lymphatic disturbances, despite different causes.\textsuperscript{251-258} Interestingly, lymphatic dilation, edema, and obstructions of mesenteric lymphatics have often been reported by some researchers as the culprit of IBD disease.\textsuperscript{254,257,259-262} Hence, the mesenteric lymphatics may be a potential therapeutic target during IBD. While there are no published reports measuring the effects of LPT on the lymphatic system of humans, we have previously demonstrated that LPT significantly increased lymph flow and the lymphatic concentration of leukocytes in dogs and rats, and also significantly enhanced the lymphatic flux of inflammatory cytokines, chemokines, and reactive oxygen and nitrogen species in both thoracic and mesenteric lymph.\textsuperscript{3,4,79,94,96,115} These studies suggest that LPT can enhance the lymphatic and immune systems and remove inflammatory mediators from the gastrointestinal tissue in healthy animals; therefore, it is likely that LPT will ameliorate disease severity during IBD by improving mesenteric lymphatic outflow from inflamed tissues, consequently removing inflammatory mediators and leukocytes from affected areas.

**Methods**

**Animals**

This study was approved by the Institutional Animal Care and Use Committee and was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication no. 85-23, revised 1996). Male Wistar rats, weighing 275-300 grams, and free of clinically evident signs of disease, were purchased from Harlan Laboratories Inc. and used for this study. Rats were housed and fed according to the IACUC of UNTHSC. Rats were acclimatized for 3 days after which they were weighed and randomly distributed in the different experimental groups. Three separate experiments were performed.
**Experimental protocols and DSS induction**

Experimental protocols were derived with some modifications from previously described publications.\(^{263-266}\) After a 3-day acclimatization period, colitis in rats was induced by replacing normal drinking water with water containing 3.5% DSS (w/v) for 10 days (days 0-9). DSS was prepared daily. Treatment was performed daily on days 3-8, which allowed for 3 days of IBD induction by DSS. To minimize stress to both animals and animal handlers, 2-5% isoflurane gas was administered to LPT or Sham rats prior to and during treatment. This was done daily for 6 days (days 3-8). Experiments included a LPT group (DSS+ISO+LPT), a Sham group (DSS+ISO+light touch), a disease control group: DSS-C (DSS only), and a healthy control group: H\(_2\)O-C (no treatment and no DSS). Weights and clinical signs of disease were monitored daily. In every experiment, all rats were weighed and assessed for Disease Activity Index (DAI) daily. On harvest day 9, animals were euthanized with an overdose of ketamine (180 mg/kg)/xylazine (25 mg/kg) given IM, followed by cardiac puncture to collect blood.

**Surgical Procedure & Processing**

The abdomen was opened along the median line; the entire colon was then rapidly excised from the top of the cecum to 1 cm above the anus and then straightened as previously described.\(^{267}\) The colon was then weighed containing fecal contents, opened longitudinally, rinsed carefully with an iced phosphate buffered saline solution, blotted dry, and re-weighed without the fecal contents.\(^{267}\) During the removal of the luminal contents, the colon was observed for stool consistency, bleeding, and colon damage, which was used to calculate a total macroscopic score based on various parameters (Table 1). The macroscopic score parameters were adopted with slight modifications from previous publications.\(^{268,269}\) The medial longitudinal length of each colon was then measured.
Sham and Lymphatic pump treatment

LPT was performed as previously described\textsuperscript{96} by a medical student (A.S.) trained in osteopathic lymphatic manipulation. During manipulation, the anesthetized rats were placed in a right lateral recumbent position. To perform abdominal LPT, the operator contacted the ventral side of the animal’s abdomen with the hands placed bilaterally below the costo-diaphragmatic junction. Sufficient pressure was exerted medially and cranially to compress the abdomen until significant resistance was encountered against the diaphragm, and then the pressure was released. Abdominal compressions were administered at a rate of approximately one per sec for a total of four min. Anesthetized Sham animals were placed in the same position and held for the same amount of time as the treatment animals so as not to bias data.

Leukocyte enumeration

Total leukocytes and a differential leukocyte count in cardiac blood samples were enumerated using the Hemavet 950 (Drew Scientific, Waterbury, CT).

Disease Activity Index (DAI)

Animal body weight loss, stool consistency, and the presence of occult or gross blood per rectum were recorded daily for each rat by a blinded researcher and scored as described in Table 2. The sum of scores for each category was used to calculate a final daily DAI score. The scoring table has previously been described in detail and was adopted with modifications.\textsuperscript{269-271} The presence of blood in the stools was assessed by the guaiac paper test and the presence of gross blood. Fluid consumption was also recorded daily.

Leukocyte enumeration

Total leukocytes and a differential leukocyte count will be determined in blood samples using a Hemavet 950 Cell Analyzer.
Statistics

Data are presented as arithmetic means ± standard error (SE), except in figures 1, 2. Values from multiple animals at respective time points were averaged, and the mean values are shown in the figures below. For evaluation of statistical significance, data were subjected to analyses of variance followed by a Tukey’s multiple comparisons post hoc tests. Differences between Weight changes and DAI were subjected to repeated measures analyses of variance followed by Bonferroni multiple comparisons post hoc tests. Statistical analyses were performed with GraphPad Prism version 5.04 and GraphPad InStat version 3.06 for Windows, (GraphPad Software, San Diego, CA, USA). Differences among mean values with at least $P \leq 0.05$ were considered statistically significant.

Results/Comments

Weight loss is used in animal studies as a parameter to monitor disease severity as well as IBD progression. To determine if DSS induction lead to weight loss, daily body weights of the animals were monitored and weight change over time was calculated by dividing body weight on specific day by body weight at day 0. The administration of DSS significantly decreased body weight gain in DSS-C (days 7-9), Sham (days 5-9), and LPT (days 6-9) groups compared to healthy rats (Fig 1). These data are consistent with previous studies and have shown that weight loss in experimental ulcerative colitis models induced by DSS occurs within 6-10 days post ingestion. Of interest, daily treatment with DSS significantly decreased body weights in the Sham group at days 8 ($P < 0.05$) and 9 ($P < 0.01$), but not the time matched LPT group, compared to DSS-C. To explore if the weight change was due to changes in fluid status, we monitored and reported daily fluid consumption. No statistical significant difference in daily fluid consumption was found between time matched groups, except for day 9 (Fig 2). On day 9,
animals from the disease control group (DSS-C) consumed significantly less fluids compared to healthy animals (H20-C; Fig 2).

In order to follow disease progression over time and compare amongst groups, acute clinical symptoms were recorded daily and the Disease Activity Index was calculated (Table 1). The DAI score provides a daily clinical assessment of general health and inflammation in IBD. The administration of DSS increased DAI scores over time in all treatment groups, and was significantly different ($P < 0.01$) at days 4-9 compared to healthy animals (H20-C; Fig 3). Further, LPT significantly decreased ($P < 0.01$) DAI scores at days 8 and 9 compared to Sham and DSS-C (Fig 3). These data suggest that 4 once daily LPT are necessary to achieve protection during an acute IBD flare.

To assess inflammation and mucosal health on harvest day, a variety of parameters were recorded and a total macroscopic score was calculated (Table 2). The administration of DSS significantly increased ($P < 0.001$) the macroscopic score in all treatment groups compared to healthy animals (Fig 4). Of interest, animals from the LPT group had a significantly decreased ($P < 0.05$) total macroscopic score at day 9 compared to DSS-C and Sham animals, suggesting LPT attenuates mucosal damage and protects the intestinal mucosa.

In order to assess for diarrhea on harvest day, the stool consistency of all animals was observed following the opening of the colon, and a stool consistency score was calculated (Table 2). The administration of DSS significantly increased stool consistency scores in DSS-C ($P < 0.001$), Sham ($P < 0.001$), and LPT groups ($P < 0.05$) compared to healthy rats (Fig 5). Of interest, LPT significantly decreased stool consistency scores compared to Sham ($P < 0.05$) and DSS-C ($P < 0.001$). An increased score in stool consistency is associated with a more severe disease process and increased diarrhea, which was attenuated by LPT. These data are of immense
clinical significance, as patients’ main complaints during an acute flare of IBD are diarrhea and pain. These data suggest that LPT decreases diarrhea and leads to more solid-formed stool.

An additional indicator of colonic health are colonic fecal contents, as previous studies have shown that healthy animals have significantly increased fecal contents in their colon compared to animals with severe colitis. Therefore, we measured colonic fecal weight at day 9. Fecal weight was significantly decreased ($P < 0.01$) in all DSS treated animals compared to healthy control animals (Fig 6). Additionally, LPT treated animal had a significantly higher fecal weight compared to DSS-C ($P < 0.05$), and slightly higher compared to Sham; however, this difference was not significant ($P = 0.12$; Fig 6). This is of significance because increases in filled colon weights are indicative of decreased colonic hypermotility, and therefore colons from severely afflicted IBD animals are found to be nearly devoid of fecal contents. These data suggest that LPT in IBD increases fecal contents and restores colonic health, most likely by decreasing colonic hypermotility. Additionally, LPT decreased colonic bleeding slightly at day 9; however, this change was not significantly different from DSS-C and Sham groups ($P = 0.08$; Fig 7).

Next, in order to determine if the protective effect seen with LPT was caused by a change in systemic immune responses, blood was collected and evaluated for leukocyte concentrations. Slight increases in total leukocytes and neutrophils were observed in animals treated with DSS, but no significant differences between groups were found, most likely due to the high variability in SD (Table 3). Of note, these data only represent the systemic immune response at one point in time and other possible changes could have occurred either during the previous days, or during or after the application of LPT. Additionally, systemic immune responses often do not adequately reflect local immune responses (i.e. at the colon); therefore, no definitive conclusions
can be drawn from the lack of leukocyte alterations. Further studies are needed to evaluate the mucosal areas for regional changes in leukocyte and inflammatory mediator concentrations during LPT.

In summary, our data suggests during an acute flare of IBD, the application of four once daily LPT decreased disease severity and protected the bowel by reducing diarrhea and protecting the integrity of intestinal mucosa. The information gained from these data creates a scientific justification for the clinical use of LPT to treat patients with IBD, and suggests that LPT could potentially alleviate some of the symptoms experienced by IBD patients. This in turn could lead to a reduction in current drug usage, decreasing the development of resistance and severe side effects, which is often seen with current treatments.

Future studies should focus on elucidating the protective mechanisms seen with LPT, exploring a variety of treatment regimens and guidelines, and testing LPT as an adjunct therapy with currently used pharmacological interventions. In addition, it should be tested if LPT delays the onset, or even decreases the occurrence of colorectal cancer (CRC) in IBD. This is of interest, as previous publications have shown that IBD patients are at a higher risk for colorectal cancer,\textsuperscript{273-275} which rises with increased IBD severity\textsuperscript{276-279} and stagnant lymphatics.\textsuperscript{255}
### Table 1. Disease Activity Index score parameters

<table>
<thead>
<tr>
<th>DAI Score</th>
<th>Stool Consistency</th>
<th>Bleeding</th>
<th>Weight Loss</th>
<th>Maximum Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Well-formed pellets</td>
<td>Normal color stool</td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>Mixture of formed and soft</td>
<td>Slight blood on guanic paper (faintly blue)</td>
<td>1%-5%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mildly soft</td>
<td>Sever blood on guanic paper (dark blue)</td>
<td>6%-10%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Very soft</td>
<td>Bloody stool visible to eye</td>
<td>11%-15%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Diarrhea (watery)</td>
<td>Gross ANAL bleeding</td>
<td>&gt;15%</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Macroscopic Score Parameters

<table>
<thead>
<tr>
<th>Stool Consistency</th>
<th>Bleeding</th>
<th>Colon damage score</th>
<th>Colon weight score</th>
<th>Maximum score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = formed</td>
<td>0 = absent</td>
<td>0 = no inflammation</td>
<td>0 = &gt; 5000 mg</td>
<td>11</td>
</tr>
<tr>
<td>1 = loose</td>
<td>1 = present</td>
<td>1 = hyperemia</td>
<td>1 = 4001-5000 mg</td>
<td></td>
</tr>
<tr>
<td>2 = liquid</td>
<td>2 = severe</td>
<td>2 = slight erosion</td>
<td>2 = 3001-4000 mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 = extensive erosion/ulceration</td>
<td>3 = 2000-3000 mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 = &lt; 2000 mg</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. LPT did not significantly alter leukocytes in the blood at harvest day 9.

<table>
<thead>
<tr>
<th></th>
<th>H₂O-C</th>
<th>DSS-C</th>
<th>Sham</th>
<th>LPT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukocytes (x10⁵)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>40.0 ± 14.1</td>
<td>44.3 ± 19.3</td>
<td>50.2 ± 21.5</td>
<td>45.2 ± 21.8</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>9.7 ± 8.2</td>
<td>16.7 ± 12.5</td>
<td>15.5 ± 8.5</td>
<td>12.9 ± 7.9</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1.8 ± 1.4</td>
<td>1.9 ± 1.3</td>
<td>2.9 ± 1.7</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>28.2 ± 8.5</td>
<td>25.6 ± 11.6</td>
<td>29.0 ± 12.5</td>
<td>29.9 ± 15.3</td>
</tr>
<tr>
<td><strong>Percentage Leukocytes (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>22.6 ± 13.4</td>
<td>37.0 ± 17.8</td>
<td>29.6 ± 10.8</td>
<td>27.3 ± 11.2</td>
</tr>
<tr>
<td>Macrophages</td>
<td>4.5 ± 2.2</td>
<td>4.1 ± 2.2</td>
<td>5.4 ± 1.9</td>
<td>5.1 ± 1.3</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>72.4 ± 13.3</td>
<td>58.6 ± 16.6</td>
<td>64.7 ± 11.0</td>
<td>67.2 ± 11.1</td>
</tr>
</tbody>
</table>

Data are means ± SD; n = 14 rats per group from three different experiments. Data were analyzed by ANOVA followed by a Tukey’s multiple comparisons post-test.
Figure 1. DSS administration inhibits weight gain. On day 0, colitis in the DSS-C, Sham, and LPT groups was induced for 10 days (days 0-9) by replacing drinking water with water containing 3.5% DSS. Isoflurane administration was performed daily on days 3-8 in the Sham and LPT groups. Weights were recorded daily and % weight change was calculated by dividing body weight on specific day by body weight at day 0. Data are shown as means, n = 14 rats per group from three separate experiments; *Different from Sham at day 8 (P < 0.05). **Different from Sham at day 9 (P < 0.01). 1Greater than a) DSS-C at days 7 (P < 0.05), 8 (P < 0.01), 9 (P < 0.001) b) Sham at days 5 (P < 0.05), 6-9 (P < 0.001) c) LPT at days 6,7 (P < 0.01) and 8,9 (P < 0.001). Data were analyzed by ANOVA followed by a Tukey’s multiple comparisons post-test.
Figure 2. Fluid consumption did not significantly differ between groups. On day 0, colitis in DSS-C, Sham, and LPT groups was induced for 10 days (days 0-9) by replacing drinking water with water containing 3.5% DSS. Sham and LPT treatment was performed daily for 6 days (days 3-8) under 2-5% isoflurane gas. Fluid consumption was recorded daily. Data are shown as means; \( n = 14 \) rats per group from three separate experiments; *Different from DSS-C values at Day 9 (\( P < 0.05 \)). Data were analyzed by repeated measure ANOVA followed by Bonferroni multiple comparisons post-test.
Figure 3. Administration of LPT decreases daily Disease Activity Index score. On day 0, colitis in DSS-C, Sham, and LPT groups was induced for 10 days (days 0-9) by replacing drinking water with water containing 3.5% DSS. Sham and LPT treatment was performed daily for 6 days (days 3-8) under 2-5% isoflurane gas. Weight loss, stool consistency, and presence of blood in stool were recorded daily and were used to calculate daily DAI. Data are means ± SE; \( n = 14 \) rats per group from three separate experiments; **LPT is different from Sham and DSS-C values at Days 8 and 9 \((P < 0.01)\). \(^1\)Different from all other groups at Days 4-9 \((P < 0.01)\). Data were analyzed by Repeated Measures ANOVA followed by Bonferroni multiple comparisons post-test.
Figure 4. **LPT decreases total macroscopic score at Day 9.** On day 0, colitis in DSS-C, Sham, and LPT groups was induced for 10 days (days 0-9) by replacing drinking water with water containing 3.5% DSS. Sham and LPT treatment was performed daily for 6 days (days 3-8) under 2-5% isoflurane gas. Rats were harvested at Day 9 and their colons were evaluated for macroscopic damage. Data are means ± SE; n = 14 rats per group from three separate experiments; *Different from DSS-C and Sham values (P < 0.05). †Different from DSS-C, Sham, LPT values (P < 0.001). Data were analyzed by ANOVA followed by Tukey’s multiple comparisons post-test.
Figure 5. Stool consistency in LPT treated animals significantly differed from all other groups. On day 0, colitis in DSS-C, Sham, and LPT groups was induced for 10 days (days 0-9) by replacing drinking water with water containing 3.5% DSS. Sham and LPT treatment was performed daily for 6 days (days 3-8) under 2-5% isoflurane gas. Rats were harvested at Day 9 and evaluated for colonic stool consistency. Data are means ± SE; \( n = 14 \) rats per group from three separate experiments; \( * (P < 0.05), ***(P < 0.001) \). †Different from DSS-C, and Sham values \( (P < 0.001) \). Data were analyzed by ANOVA followed by Tukey’s multiple comparisons post-test.
Figure 6. Colonic fecal weight in LPT treated animals was increased at day 9. On day 0, colitis in DSS-C, Sham, and LPT groups was induced for 10 days (days 0-9) by replacing drinking water with water containing 3.5% DSS. Sham and LPT treatment was performed daily for 6 days (days 3-8) under 2-5% isoflurane gas. Rats were harvested at Day 9 and evaluated for fecal weight in their colon. Data are means ± SE; n = 14 rats per group from three separate experiments; *Different from DSS-C values ($P < 0.05$). **Different from DSS-C, Sham, and LPT values ($P < 0.01$); Data were analyzed by ANOVA followed by Tukey’s multiple comparisons post-test.
Figure 7. LPT slightly decreased colon bleeding at Day 9. On day 0, colitis in DSS-C, Sham, and LPT groups was induced for 10 days (days 0-9) by replacing drinking water with water containing 3.5% DSS. Sham and LPT treatment was performed daily for 6 days (days 3-8) under 2-5% isoflurane gas. Rats were harvested at Day 9 and evaluated for colonic bleeding. Data are means ± SE; n = 14 rats per group from three different experiments; While LPT slightly decreased colonic bleeding at day 9, this was not statistically significant from DSS-C (P = 0.08) and Sham (P = 0.08). ***Different from DSS-C, Sham, and LPT values (P < 0.001). Data were analyzed by ANOVA followed by Tukey’s multiple comparisons post-test.
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Author Disclosure Statement

No competing financial interests exist.


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