OSTEOPATHIC LYMPHATIC PUMP TREATMENT

AS AN ADJUNCTIVE THERAPY TO PROTECT

AGAINST INFECTION AND INFLAMMATION

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OSTEOPATHIC LYMPHATIC PUMP TREATMENT AS AN ADJUNCTIVE THERAPY TO PROTECT AGAINST INFECTION AND INFLAMMATION

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CHAPTER I

INTRODUCTION

Osteopathic medicine

The osteopathic medical profession recognizes the importance of the lymphatic system during disease and has focused on techniques to influence lymphatic flow and promote the movement of lymph. Osteopathic physicians have designed a set of body-based manipulative therapies, called osteopathic manipulative techniques (OMT), that include movement and stretching of muscles, fascia and joints, gentle pressure and manual pumping techniques [50, 51]. Anecdotal studies support the use of OMT for the treatment of pneumonia, inflammatory bowel disease, fibromyalgia, and low back pain [52-56].

Lymphatic pump techniques (LPT) are a subset of OMT that target the musculoskeletal system and are designed to target the lymphatic system [50, 51]. When applied to the thoracic cage, LPT is thought to transiently increase the range of motion of the thorax and increase the expiratory recoil of the lungs thereby reducing pressure within the intra-thoracic pressure and promote lymph flow. Abdominal LPT is thought to transiently increase abdominal pressure thereby increasing the thoraco-abdominal pressure gradient similar to thoracic LPT. When applied to the feet and legs LPT is thought to enhance lymphatic and venous drainage. The splenic pump is applied to areas of the spleen is designed to enhance the immune response [51, 57].

Clinical studies support the use of LPT as an adjunctive therapy for the treatment of pneumonia, influenza, and pulmonary disease [52, 53, 55]. However, little is known about the mechanism of protection offered by LPT. The multicenter osteopathic pneumonia study in the elderly (MOPSE) supports the use of OMT, which included LPT, as an adjunctive therapy for pneumonia. The MOPSE study was a registered, double-blinded, randomized, controlled trial involving 7 hospitals and conducted on 406 elderly patients with CAP. All patients were treated with conventional care, including intravenous antibiotics, and placed into three groups: control (conventional care only), sham (received light touch), OMT. The OMT group received 15 minutes of treatment, twice daily beginning with 24 hours of admission until patients were discharged. Treatment included 8 standardized OMT techniques, among them thoracic lymphatic pump and pedal lymphatic pump [53, 55]. The study concluded that OMT reduced the length of stay, duration of intravenous antibiotics and death or respiratory failure compared to the control group [55]. These results suggested that OMT acts as an adjunctive therapy to antibiotic treatment for the treatment of acute pneumonia.

Large animal studies demonstrated that movement, compression, massage or manipulative therapy increases the flow of lymph and enhances the uptake of materials into the lymphatic system. Passive movement enhanced lymph flow in unconscious dogs [60, 61] and rabbits [62]. Manual compression enhanced lymph flow in unconscious sheep [15] and enhanced the transport of material into the initial lymphatics of rabbits when applied to the injection site [4]. Knott *et al* (2005) demonstrated that thoracic LPT, abdominal LPT or exercise increased lymph flow in the thoracic duct of conscious dogs [60].

Previous studies have demonstrated that 4 minutes of abdominal LPT increased lymph flow, the concentration of leukocytes and the flux of cytokines, chemokine and reactive oxygen,

and nitrogen species in the thoracic and mesenteric ducts of anesthetized dogs [63-66]. In these studies, the dogs were anesthetized and the chest was opened by a left lateral thoracotomy and the thoracic duct was isolated from the connective tissue. The thoracic duct was ligated and a catheter inserted into the duct. Approximately 60 minutes after securing the catheter with a ligature, thoracic lymph was collected at 1 minute intervals for 4 minutes before LPT, 4 minutes during LPT, and for 10 minutes after the cessation of LPT. Abdominal LPT was performed as previously described [64-66]. The anesthetized dogs were placed in a right lateral recumbent position. To perform LPT, the operator contacted the ventral side of the animal's abdomen bilaterally below the costo-diaphragmatic junction. Pressure was applied and released medially and cranially to compress the abdomen until resistance was encountered against the diaphragm. Compressions to the abdomen were administered at a rate of approximately 1 pump per sec for a total of 4 min. LPT transiently increased thoracic duct lymph flow, the concentration of thoracic duct leukocytes, and thoracic duct leukocyte flux compared to baseline and recovery leukocyte flux [64].

In a separate set of experiments, dogs were surgically prepared and midline abdominal incision was made to isolate a large mesenteric lymph duct. The duct was ligated and a PE60 catheter was inserted into the duct and secured with a ligature. The catheter was exteriorized through the incision and the incision was closed with a suture. As above, lymph was collected approximately 60 minutes after the cannulation. Abdominal LPT was performed as previously described. LPT transiently increased mesenteric duct lymph flow, the concentration of mesenteric duct leukocytes, and mesenteric duct leukocyte flux compared to baseline and recovery leukocyte flux [64].

Furthermore, using the same experimental approach, Schander *et al* (2012) demonstrated that LPT transiently increased the flux of cytokines and chemokines, in both mesenteric and thoracic ducts of dogs. Similarly, LPT increased the flux of the inflammatory mediators, superoxide dismutase and nitrotyrosine, in the thoracic and mesenteric duct lymph. Of interest, LPT did not increase the concentration of these inflammatory mediators in thoracic or mesenteric duct except for monocyte chemotactic protein-1 (MCP-1) in thoracic duct lymph [65]. These results suggest LPT redistributes a pool of lymph containing these mediators.

While the studies in dogs demonstrate that LPT enhances the lymphatic and immune systems, rodents models are used for the study of infectious disease. Therefore, a rat model to study the effect of LPT on the lymphatic system was developed. A previous study in rats reported that a lymph-enhancing technique increased the delivery of an injected fluorescent protein probe into blood circulation. In this study, rats were anesthetized and injected in the hind leg with a fluorescent probe. One group of rats received lymph flow enhancing treatment that consisted of an intermittent pressure applied to the ventral thorax for five minutes every hour until rats recovered from anesthesia. Rats from their control group were left lying prone. Tail blood was assayed for the fluorescent probe over 15 hours. The appearance of the probe was greater during the first nine hours in the treatment group compared to the control group [67].

To measure the effect of abdominal LPT on the rat, the cisterna chyli of rats were cannulated through a left, subcostal, mediolateral incision and lymph collected before (baseline), during (LPT) or after (recovery) LPT performed under anesthesia. The retractors were moved from the incision and the tubing implanted into the cisterna chyli remained in place. To perform LPT, compressions were administered by contacting the abdomen with thumb on one side and the index and middle finger on the other side of the medial sagittal plane without contacting the

incision. Pressure was applied medially and cranially against the diaphragm until resistance is met at a rate of 1 compression per second for a total of 4 minutes. Lymph flow was significantly higher in rats that received LPT compared to baseline. Furthermore, the flux of leukocytes and gut-associated leukocytes was significantly higher in lymph collected during LPT compared to baseline [68].

In a follow up study, the combination of LPT and levofloxacin reduced the concentration of *S. pneumoniae* CFU in the lungs of rats at 96 hours post infection compared to levofloxacin alone. Rats were intranasally infected with *S. pneumoniae*, and received control (no treatment), sham (anesthesia only) or LPT under anesthesia as previously described [58]. Rats then received injection of levofloxacin or saline once daily for 3 consecutive days. Rats that received levofloxacin had significantly lower concentration of bacteria in the lungs at 96 hours post infection compared to PBS group. Interestingly, the combination of LPT and levofloxacin significantly lowered the concentration of bacteria in the lungs compared to LPT or levofloxacin alone suggesting LPT is an adjunctive therapy to levofloxacin treatment. Furthermore, LPT and levofloxacin increased the percentage of rats that had no measurable CFU of *S. pneumoniae* [59].

Although OMT is used clinically and has been reported to be beneficial [52, 53, 55], the mechanism is unknown; therefore, there is a need for basic science research to elucidate the protection provided by LPT. Understanding the mechanism of these therapies will provide scientific rationale for the use of OMT and guide osteopathic practitioners to optimally apply LPT on patients. Previous literature suggests lymph-enhancing techniques, such as LPT, may promote the movement of lymph-borne factors and pharmaceuticals through the thoracic duct into circulation and protect against pneumonia [53, 55, 58, 59, 69]. Therefore, one purpose of

these studies was to determine how the use of abdominal LPT to enhance lymph flow could act as an adjunctive therapy for the treatment of pneumonia.

The lymphatic system

The cardiovascular system delivers blood and plasma fluid to the tissues. Blood capillaries reabsorb the majority of this plasma fluid. However, the excess plasma fluid in the interstitial space enters the lymphatic system and is known as lymph. The lymphatic system, responsible for tissue fluid homeostasis, consists of linear networks of capillaries, collecting vessels, lymph nodes, trunks, and ducts that return lymph into circulation. Lymphatic capillaries consist of lymphatic endothelial cells that lack smooth muscle and are anchored to the extracellular matrix by anchoring filaments. These capillaries are usually found collapsed during a resting state but open in response to high interstitial pressure or stretch of the extracellular matrix driving the formation of lymph [1, 2].

The lack of basement membrane and tight cell-to-cell junctions are unique to lymphatic capillaries and contribute to their permeability and the lack of exclusion of interstitial fluid. The single layers of lymphatic endothelial cells are connected by specialized intercellular junctions [3]. Folds in lymphatic endothelium form gaps that can reach several micrometers in width allow the initial lymphatics to uptake large molecules [4]. As a result, interstitial fluid protein concentration is similar to lymphatic protein concentration but can vary due to the source of lymph, changes in tissue hydrostatic pressure and lymph filtration by lymph nodes [5-9]. Recently, proteomic studies have identified proteins unique to lymph or found at higher concentrations in lymph compared to serum. Dzieciatkowska *et al* 2014 determined that approximately 28% of all proteins found in human mesenteric lymph were unique to lymph [10].

Among those proteins are low molecular weight proteins suggested to be fragments of self-peptides as a result of catabolic processing in the interstitium [11, 12]. Furthermore, studies suggested lymph and serum share many proteins including interstitial proteins, extracellular proteins, complement, transporters, metabolic regulators and protease inhibitors [3]. However, the abundance of certain proteins classes, protease inhibitors, and proteins related to innate immunity, are higher in lymph compared to serum [13].

Lymphatic capillaries drain lymph into the collecting lymphatic vessels. These capillaries consist of a single layer of endothelial cells that lack muscle and are anchored to the extracellular matrix by anchoring filaments. The discontinuous intercellular junctions that adhere lymphatic endothelium together are largely responsible for the high permeability of the initial lymphatics. The structure of these endothelial cells form primary valves that prevent retrograde flow of interstitial fluid from the lymphatics into the interstitium. In contrast, collecting lymphatic vessels are typically not anchored to the extracellular matrix but have a basement membrane and continuous endothelial junctions that are lined with smooth muscle cells. Similarly, these lymphatic vessels contain bicuspid luminal valves that maintain a one-way flow of lymph and prevent retrograde flow [2, 14].

The heart is responsible for the propulsion of blood through the cardiovascular system; however, the lymphatic system lacks a central pump and relies on extrinsic factors and the contraction of the smooth muscle lining lymphatic vessels [2]. Lymph formation and propulsion are inversely related and drive the net flow rate of lymph through the lymphatic system [4]. Lymph propulsion is regulated intrinsically by the rhythmic spontaneous contraction of the smooth muscle lining lymphatic vessels. Similarly, the lymphatic pump resembles the cardiovascular system in diastolic and systolic phases. Contracting segments between valves of

the lymphatic system are known as lymphangions and drive lymph propulsion in one direction with the aid of funnel-shaped valves to prevent retrograde flow [1].

Lymph propulsion in lymphatic vessels is also influenced by extrinsic factors such as the force of lymph formation, cardiac and arterial pulsations, peristalsis, respiration, blood pressure, contraction of skeletal muscle, massage, and physiologically active molecules such as neuromodulators [1, 4, 9, 15]. Substance P is a neuromodulator that can vasoconstrict lymphatic vessels. Phenylephrine and norepinephrine can also increase contractility of lymphatic vessels. In contrast, endothelin 1, nitric oxide, prostaglandin, and prostacyclin are potent vasodilators that can decrease the frequency of the lymphatic pump. The regulation of lymphatic function and flow by nitric oxide has been studied extensively. Endothelial nitric oxide synthase in lymphatic endothelial cells produce nitric oxide (NO) in response to shear stress or flow resulting in the relaxation of the lymphatic vessels [2, 14].

Stretch-dependent regulation of lymph propulsion is an important feature of the lymphatic system. The contraction of lymphatic vessels can be influenced by the stretch of the lymphatic wall within a lymphangion during the filling phase. An increase in pressure within a lymphangion can increase both the frequency and the strength of contraction thus enhancing lymphatic output. Low-pressure gradients within the lymphatic vessels are overcome by valves between lymphangions that allow for pressure build up. These intrinsic factors result in a change of fluid pressure, which drives the propulsion of lymph through vessels [2, 14].

Lymph also travels through lymph nodes that act as filters to trap antigens before lymph enters large lymphatic trunks. Within the lymph nodes, immune cells encounter pathogens and sample tissue antigens contributing to immune surveillance. Therefore, the lymphatic system plays a vital role in immunological processes such as immune tolerance, autoimmunity, and

inflammation. Lymphocytes circulating in the blood can enter lymph nodes through high endothelial venules with the aid of adhesion molecules and chemokines and recognize antigen-presenting cells. Lymph drained from the intestinal, hepatic, and lumbar regions of the body drain through the cisterna chyli before entering the thoracic duct. Lymph re-enters venous circulation through the subclavian vein where it is delivered to the first major vascular bed in the lung [2, 14, 16, 17].

The gut-lung axis

The lymphatic vasculature that connects the gastrointestinal region to the lungs has been of interest due to the association of respiratory disease such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, respiratory viral infections and intestinal disease [1, 30, 31]. Collectively, this literature suggests lymph may play a role during pulmonary inflammation. The common mucosal immune system also reveals a connection between these of two organs. Immunological cross-talk has been highlighted in several studies between the mucosal sites [17]. Importantly, this gut to lung axis, also known as the gut-lung hypothesis, remains a poorly defined mechanism that could impact the etiology and treatment of respiratory disease.

The association of pulmonary disease and inflammatory bowel disease was first observed in patients in 1960s and 1970s. Recently, chronic lung disease, such as asthma, was associated with food allergies and other gastrointestinal disorders [17]. The intestinal lymphatics have been studied recently during ARDS and MODS. The gut lymphatic vessels redistribute a large pool of lymph rich in immune cells, inflammatory mediators, and lipids into circulation. Factors in mesenteric lymph can activate pulmonary leukocytes, increase endothelial cell permeability, increase the expression of cell adhesion molecules on endothelial cells and lung tissue,

redistribute leukocytes, cytokines, and chemokines to the lung and enhance cell signaling [31]. Furthermore, intestinal ischemia and reperfusion in animals induced lung injury, suggesting factors released from the gut during gastrointestinal ischemia can contribute to respiratory distress. The ligation of the thoracic or mesenteric lymph ducts prevents lung injury associated with intestinal ischemia or severe inflammation [16, 30, 32-35]. Additionally, intestinal lymph infused into naïve animals resulted in lung injury, endothelium injury, or systemic inflammation [36, 37]. However, factors in lymph leading to lung injury are unknown. Collectively, these studies suggest soluble factors in lymph are responsible for pulmonary-intestinal cross-talk and support the gut-lung hypothesis.

The innate immune system

Innate immunity is responsible for the initiating the host response against pathogens. The innate immune response is triggered upon recognition of pathogen-associated molecular patterns by soluble pattern recognition receptors and includes a collaborative effort from macrophages, neutrophils, and other leukocytes that target and eliminate pathogens while priming the adaptive immune response [18, 19].

Macrophages are unique phagocytic cells which are part of the innate immune system that are capable of phagocytosing bacteria, apoptotic cells and particulates, secreting proinflammatory and anti-inflammatory mediators, and presenting antigen to help initiate the adaptive immune response. Most macrophages are derived from monocytes that developed from myeloid bone marrow progenitors. Monocytes leave from the bone marrow and circulate through the bloodstream with short half-lives. When monocytes cross the walls of blood capillaries into tissue, they undergo differentiation that increases their size and phagocytic capacity and their

development into macrophages [20]. Among these macrophages are tissue-resident macrophages with longer half-lives that undergo local expansion during development and self-renew during adulthood [21]. Tissue-resident macrophages serve their tissue-specific functions at several sites such as the brain, liver, lungs, spleen, gastrointestinal tract, and skin [22].

Pattern recognition receptors such as toll-like receptors (TLR) on macrophages, initiate intracellular signaling cascades with the aid of adaptor molecules that lead to the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1, interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-12 [23]. In addition to cytokines, macrophages release chemokines, leukotrienes, prostaglandins and anti-bacterial factors such as NO. Locally, these inflammatory mediators increase vascular permeability and recruit neutrophils and monocytes to sites of infection while systemically inducing fevers and stimulating the production of acute phase proteins. However, these inflammatory mediators have also been associated with immunopathology; therefore, modulating their production is crucial to resolution and returning to homeostasis [18, 24].

Macrophages also secrete anti-inflammatory cytokines such as interleukin-10 (IL-10) and tumor growth factor-beta that target macrophages and various other target cells. The absence of anti-inflammatory cytokines contributes to systemic inflammation and significantly impacts survival in animal models [20]. Specifically, macrophages have been shown to contribute to acute respiratory distress syndrome (ARDS), acute lung injury, and multiple organ dysfunction syndrome (MODS) through their uncontrolled release of proinflammatory cytokines and activation of neutrophils, platelets and endothelial cells [25].

The mucosal immune system

The common mucosal immune system is a collaborative effort between distant mucosal sites. Mucosa-associated lymphoid tissues are priming sites for mucosal sites. Gut-associated lymphoid tissues such as Peyer's patches, mesenteric lymph nodes and isolated lymphoid follicles serve as priming sites for the gastrointestinal tract. Similarly, nasopharyngeal-associated lymphoid tissues such as tonsils, bronchus-associated lymphoid tissue, cervical lymph and hilar lymph nodes are priming sites for the lungs, bronchus, and nasal passages. Antigens in the lumen of the gastrointestinal and respiratory tracts are sampled when dendritic cells uptake antigens using their intraepithelial cellular extensions or when microfold cells uptake antigens and transport them to dendritic cells or other antigen-presenting cells underlying the epithelium. Dendritic cells travel through the lymphatic system after processing antigen to induction sites to prime T and B cells. Mucosal dendritic cells induce the expression of mucosal homing markers on T cells that gain access to mucosal effector sites through the lymphatics and bloodstream. With the aid of CD4 T cells and cytokines interleukin-4, transforming growth factor-beta, IL-5, IL-6, and IL-10, immunoglobulin A positive B cells and IgA producing plasma cells are generated at effector sites such as the lamina propia of the gastrointestinal and respiratory tracts. The adaptive mucosal immune response continues to promote the generation of memory CD4 T cells, B cells, and plasma cells [17, 26].

In the gastrointestinal and respiratory tracts, the first line of defense is the epithelium that is separated from microbes and stimuli by mucus. In the airways, mucus traps and removes antigenic factors and pathogens from the airways with the help of type I alveolar epithelial cells a coordinated beating of cilia on their surface. Mucus produced by goblet cells and surfactants produced by type II alveolar cells also help opsonize pathogens for removal by alveolar

macrophages. However, due to their location in the lung, alveolar macrophages sample antigens and pathogens on the luminal side not filtered by mucus in the rest of the airways. At both sites, the epithelium layer also separates these stimulatory factors from immune cells underneath the epithelium layer. Tight junctions contribute to the physical barriers by preventing easy transport of pathogens. The exception are the extensions of dendritic cells that extend through the epithelium to sample antigens in the lumen [27, 28].

In the lungs, alveolar macrophages reside in the alveolar space and serve as another line of defense to respiratory pathogens and particulate antigens. Upon the recognition and phagocytosis of a pathogen, alveolar macrophages secrete inflammatory cytokines and chemokines that activate pulmonary epithelium and recruit neutrophils and monocytes to the lung [24]. Alveolar macrophages typically have less phagocytic activity and respiratory burst than traditional inflammatory macrophages. This allows immunosuppression and communication with alveolar epithelium to modulate the immune response to maintain tissue homeostasis during aseptic conditions and prevent tissue damage and edema in the alveolar space during infection. As a result, alveolar macrophages promote tolerance to innocuous antigens and promote the differentiation of T regulatory cells by the release of transforming growth factor beta-1 and prostaglandins. Furthermore, alveolar macrophages are hypo-responsive to bacterial toxins after the resolution of infection to promote tissue repair [29].

Under homeostatic conditions, the gut and the lung microenvironment is mainly suppressive largely in part to these epithelial cells. Hyporesponsiveness to TLR2 and TLR4 agonists has been reported and are likely due to lack of adaptor molecules required for optimal TLR signaling. It has also been suggested that surfactant in the respiratory tract may have anti-inflammatory properties by binding to TLRs on epithelial cells. Interaction of epithelial cells

with alveolar macrophages is also thought to dampen the immune response by dampening alveolar macrophage activation. The activation of several alveolar macrophages in coordination epithelial cells is required to release large amounts of inflammatory mediators, recruit leukocytes and mount an immune response to a respiratory pathogen. Mucosal epithelial cells act as a barrier that can sense pathogens and modulate the immune response similar to the mechanism found in the gastrointestinal tract [17, 26-28].

Streptococcus pneumoniae

Host defense mechanisms are vital for the clearance of *Streptococcus pneumoniae*, the most common bacterial cause of community-acquired pneumonia (CAP) leading to morbidity and mortality in young, elderly, and immunocompromised individuals. Pneumococcal disease results in 600,000 hospitalizations with a mortality rate of 5-7% and medical costs of $10.6 billion annually [38-40]. Pneumococci are gram-positive bacteria that are part of the commensal microbiome in the human nasopharynx. Pneumococcal disease occurs when asymptomatic nasopharyngeal colonization transitions to local, distant, and systemic infections such as otitis media, sinusitis, invasive pneumococcal disease, meningitis, pneumonia, and bacteremia/sepsis [41].

The pulmonary epithelium and mucociliary escalator prevent adherence and colonization of *S. pneumoniae*. Respiratory pathogens are neutralized by mucus and removed by cilia on the surface of pulmonary epithelium. The innate immune response to *S. pneumoniae* has been well described in the mouse and has been broadly divided into five stages [18, 42, 43].

Initially, the first cells to respond to *S. pneumoniae* in the airways are the resident alveolar macrophages. During the first stage of infection (0-4 hours), alveolar macrophages

poorly phagocytose bacteria and secrete TNF-α, IL-1, IL-6, and NO, which can be measured in the bronchoalveolar lavage fluid, lung tissue, and serum. The polysaccharide capsule prevents effective phagocytosis and bacterial killing during this stage; however, the cytokines secreted by alveolar macrophages recruit neutrophils to the site of infection. During the second stage of infection (4-24 hours), bacterial load increases in the alveolar space while neutrophils rapidly phagocytose and kill bacteria through the formation of phagolysosomes and the release antimicrobial factors such as myeloperoxidase. The release of interferon-gamma (IFN-γ) also promotes the entry of additional monocytes and lymphocytes from the blood. Simultaneously, complement and antibody aid in the opsonization and killing of pneumococci. Neutrophils can be detected in the bronchoalveolar lavage fluid as early as 4 hours post-infection and their numbers continue to rise at 4-72 hours post-infection. TNF-α, IL-6, and IL-1 also increase in bronchoalveolar lavage fluid and lung tissue. During the third stage of infection (24-48 hours), alveolar injury, and interstitial edema is seen as bacteria invade the lung interstitium and leukocyte extravasation continues from the blood. During the fourth stage (48-72 hours), monocytes and lymphocytes enter the BALF from the lung tissue and blood with an increase in NO release in the BALF and the lung. The effects of pneumococcal bacteremia can also be seen during this stage. During the fifth stage of infection (72-96 hours), uncontrolled bacterial proliferation leads to high NO release, severe air-space disorganization, and high mortality [18, 24].

If *S. pneumoniae* is cleared from the lung, alveolar macrophages and the recruited macrophages in the lung promote resolution of pulmonary inflammation and tissue repair through the release of anti-inflammatory cytokines and growth factors [29]. The recognition of apoptotic cells by macrophages also promotes resolution, which inhibits the release of

inflammatory mediators [44, 45]. Therefore, promoting resolution during pneumonia infection may aid in improving clinical outcomes. However, uncontrolled bacterial proliferation and inflammation results in severe pulmonary tissue damage and mortality [18]. The recruitment of neutrophils and monocytes to the lungs contribute to the clinical features of pneumonia [24]. Persistent macrophage activity is correlated with mortality in murine models of pneumonia [46]. Furthermore, the lack of IFN-γ in a murine model of pneumonia lowered the recruitment of neutrophils and the release inflammatory mediators but reduced bacterial load in the lungs of mice infected with *S. pneumoniae* [47, 48]. Furthermore, lack of TNF-α may enhance survival from a disseminated lethal infection of pneumonia [49]. These studies suggest that the continuous release of inflammatory mediators during pneumonia is detrimental.

Global hypothesis and specific aims

OMT has been reported to be beneficial for respiratory disease and pneumonia [52, 53, 55], although the mechanism is unknown. Furthermore, previous studies demonstrated that LPT enhances the lymphatic and immune systems of both rats and dogs and further protected rats against *S. pneumoniae* [53, 55, 58, 59, 69]. Therefore, the purpose of these studies was to identify the mechanism(s) by which LPT protects the lung during infection. **We hypothesized that by enhancing lymph flow, lymphatic pump treatment mobilizes lymph-borne factors and pharmaceuticals into circulation that protect against infectious and inflammatory disease.**

The purpose of the first aim was to determine if LPT facilitates the delivery of antibiotics to the lung. Specifically, we hypothesized that LPT would enhance the delivery of levofloxacin from the blood to the lung, which may accelerate the clearance of pulmonary bacteria. In

previous studies, LPT protected rats against pneumonia without the need of antibiotics, suggesting there is another mechanism of protection offered by LPT. LPT increased the number of leukocytes and increased the flux of inflammatory mediators in lymphatic circulation [63, 64, 65, 66, 68]. LPT also increased the concentration of MCP-1, a potent chemokine for monocytes, in thoracic duct lymph suggesting the composition of lymph may change during LPT.

The purpose of the second aim was to determine the effect of thoracic duct lymph collected during LPT on macrophage activity *in vitro*. Previous studies establishing a gut-to-lung axis provide evidence that LPT may influence the pulmonary immune response by mobilizing lymph-borne factors to the lung [30]. The unique role of macrophages during infection and inflammation provides a potential target of lymph-borne factors [25, 29]. Furthermore, no studies have been published describing the effect of thoracic duct lymph on macrophages.. Therefore, we hypothesized that lymph mobilized during LPT would suppress macrophage activation *in vitro* in response to lipopolysaccharide.

The purpose of the third aim was to determine if thoracic duct lymph can inhibit macrophage activation *in vitro* by lipoteichoic acid and interferon-gamma, a bacterial toxin and cytokine associated with *S. pneumoniae* infection. Lung immunopathology caused by *S. pneumoniae* is associated with morbidity and mortality during pneumonia. Therefore, by mobilizing protective factors into circulation, LPT may protect the lung suppressing macrophage activity. We hypothesized that thoracic duct lymph would suppress macrophage activity *in vitro* in response to lipoteichoic acid and interferon-gamma.

These aims will determine if LPT aids during the treatment of pneumonia by facilitating the delivery of antibiotics the lung. These aims will also determine the effect of thoracic duct lymph mobilized during LPT on macrophages and demonstrate for the first time, the biological

activity of thoracic duct lymph on macrophages and fill a gap of knowledge in lymphatic biology. Furthermore, these studies will provide further evidence for the protective effect of lymph and a potential mechanism of LPT during respiratory infection and disease.

CHAPTER II

LYMPHATIC PUMP TREATMENT DOES NOT ALTER THE CONCENTRATION OF LEVOFLOXACIN IN THE LUNG

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia. Despite the success of vaccines and antibiotics, the treatment of pneumonia remains a challenge. Lymphatic pump techniques (LPT) are a subset of osteopathic manipulative techniques that are designed to enhance the flow of lymph and are supported clinically as an adjunctive therapy for the treatment of pneumonia. Recent studies have demonstrated that LPT can reduce the concentration of bacteria in the lungs of rats infected with *S. pneumoniae*. Furthermore, the combination of LPT and antibiotic further reduced the concentration of bacteria compared to antibiotics alone. However, the mechanism of protection offered by LPT is unknown. The purpose of this study was to determine if LPT would facilitate the delivery of antibiotic to the lung. Specifically, we hypothesized that LPT would enhance the concentration of levofloxacin in blood and lung epithelial lining fluid (ELF) compared to a sham treatment. To test this hypothesis, naïve rats were subcutaneously injected with levofloxacin into the hind leg and randomly divided into sham or LPT groups. The sham group received 4 minutes of anesthesia while the LPT group received 4 minutes of abdominal LPT. Plasma (from cardiac blood) and epithelial lining fluid (from a bronchoalveolar lavage) was collected at 10, 30, 60, and 120 minutes post injection. LPT increased the concentration of levofloxacin in epithelium lining fluid

over time. These results suggest LPT facilitates the delivery of antibiotics to the lung and supports the adjunctive use of LPT for the treatment of pneumonia.

Introduction

Community acquired pneumonia accounts for over 1,000,000 hospital admissions each year. *Streptococcus pneumoniae* is the most common cause of community-acquired pneumonia leading to hospitalization with a mortality rate of 5-7 % and medical costs of $10.6 billion annually [38-40]. *S. pneumoniae* infection can be sudden and the bacteria can disseminate into the bloodstream, resulting in bacteremia and in some cases leads to meningitis [41]. While vaccines and antibiotics are generally effective for the prevention and treatment of infection, the emergence of resistant strains of *S. pneumoniae* threatens their success [38, 70, 71]. Therefore, there is a need to investigate alternative therapies for the treatment of pneumococcal pneumonia.

The osteopathic medical profession utilizes a set of body-based manipulative medicine techniques called lymphatic pump techniques (LPT) that were designed to enhance the flow of lymph and are thought to remove excess fluid proteins, exudates, toxins, and bacteria from the interstitial fluid space. It is proposed that by enhancing lymph flow, LPT removes stagnant fluid that may adversely affect cellular activity and predispose tissue to dysfunction and disease [51].

Clinical studies support the use of LPT to enhance immunity and act as an adjunctive therapy for the treatment of pneumonia [52, 53, 55]. The multi-center osteopathic pneumonia study in the elderly (MOPSE) conducted a double-blinded, randomized, controlled trial on hospitalized elderly patients to measure the efficacy of osteopathic manipulative treatment (OMT) as an adjunctive therapy for pneumonia [53, 55]. Within 24 hours of admission, subjects were randomized into conventional care (including antibiotics), conventional care and light-

touch, or conventional care and OMT (which included LPT). Intention-to-treat analysis found no significant difference between the groups for any outcome; however, per-protocol analysis found OMT and conventional care significantly reduced the length of hospital stay, the duration of intravenous antibiotic use, and the incidence of respiratory failure or death compared to the conventional care group. Conventional care and light touch offered intermediate benefits [53, 55]. These results suggest that OMT enhances protection against pneumonia compared to antibiotics and supportive care alone; however, the mechanism(s) responsible for this protection has not been identified.

Recent studies using a rat model of *S. pneumoniae*-mediated pneumonia have provided insight into the mechanisms of protection offered by LPT. Specifically, LPT reduced the concentration of bacteria in the lungs of rats compared to a sham treatment [58, 69]. Furthermore, the combination of antibiotic and LPT significantly reduced the concentration of pulmonary *S. pneumoniae* compared to the use of antibiotics alone [59]. These results suggest that LPT may protect against bacterial pneumonia by inhibiting bacterial growth in the lung; however, the mechanism responsible for this clearance is still under investigation.

Enhancing lymph output using therapies such as LPT might facilitate the delivery of antibiotics to the lung and provide additional protection against pneumonia. The purpose of this study was to determine if LPT would facilitate the delivery of antibiotic to the lung. Specifically, we hypothesized that LPT would enhance the concentration of levofloxacin in blood and lung epithelial lining fluid (ELF) compared to a sham treatment. A better understanding of the physiological effects of osteopathic manipulative therapies, such as LPT, will guide osteopathic practitioners to optimally apply LPT on patients with pneumonia.

Methodology

Animals

Male inbred Fischer 344 rats (Envigo, Livermore, CA) weighing between 150-175 grams, free of any specific pathogens, were used in these studies and housed and fed according to the Institutional Animal Care and Utilization Committee (IACUC) of UNTHSC in the barrier facility [84].

Treatment

Rats were anesthetized intramuscularly in the right hind leg with ketamine (30 mg/kg) and xylazine (5 mg/kg) (Miller Veterinary Supply, Fort Worth, TX) and kept in a right lateral recumbent position. Under anesthesia, rats were subcutaneously injected with 50 of mg/kg of levofloxacin (Sigma-Aldrich, St. Louis, MO) in 1 mL of sterile phosphate-buffered saline (PBS, Hyclone, Logan, UT) into the left hind leg. This dose was chosen based on a previous study [59]. Rats were then randomly divided into sham or LPT groups. Sham or LPT were applied as previously described [58, 59]. To perform LPT, compressions were administered by contacting the abdomen with the thumb on one side and the index and middle finger on the other side of the medial sagittal plane. Pressure was applied medially and cranially against the diaphragm until resistance was met at a rate of 1 compression per second for a total of 4 minutes. During sham therapy, rats were anesthetized and kept in a right lateral recumbent position for 4 minutes. Ten additional rats received a subcutaneous injection of sterile PBS into the left hind leg and served as a background control group for the levofloxacin bioassay.

Data collection

At 10, 30, 60, and 120 minutes after levofloxacin injection, rats were euthanized using a lethal dose of ketamine (100mg/kg) and xylazine (15mg/kg) and cardiac blood and epithelial lining fluid (ELF) were collected. Blood was centrifuged at 1200G for 15 minutes at 4° C in EDTA coated vacutainer blood tubes (BD Biosciences, San Jose, CA) to collect plasma. The trachea was cannulated with polyethylene tubes and 10 mL of sterile PBS was used to lavage the lung. The recovered ELF was centrifuged at 800G for 10 minutes at 4 C to collect cell-free supernatant. Plasma and ELF were stored at -80° C.

Measurement of levofloxacin in plasma and pulmonary epithelial lining fluid

The concentration of levofloxacin was determined using microbial bioassays. *Klebsiella pneumoniae* (ATCC-10031) was grown in Tryptic Soy Broth (Fisher), shaking overnight at 37° C. Muller Hinton II Broth (Fisher) plus 1.1% noble agar (Sigma) was inoculated with *K. pneumoniae* at 1% v/v and poured into the bioassay plate until the agar solidified. Holes were generated into the agar and filled with 50 ul of plasma or recovered ELF or the internal standard (levofloxacin). Bioassay plates were incubated for 2 hours at 4° C then incubated overnight at 37° C. Zones of inhibition were measured and levofloxacin concentration interpolated using the standard curve generated from the internal standard controls. The concentration of urea was measured in plasma and recovered ELF samples using a urea colorimetric assay (BioVision, Milpitas, CA). The ratio of urea concentration in plasma to urea concentration in the recovered ELF was used to determine the dilution factor and calculate the adjusted concentration of levofloxacin in ELF. To calculate penetration of levofloxacin into ELF, the ratio of ELF levofloxacin concentration to plasma levofloxacin was calculated for each animal.

Statistical analysis

A power analysis using means and standard deviations of ELF penetration revealed a minimum of 12 animals per group to detect differences between means with a statistical power of 0.90. Data are presented as log-transformed arithmetic means ± standard error (SE). The concentrations of levofloxacin in plasma and ELF were log-transformed and analyzed by one-way ANOVA followed by a Tukey's multiple comparisons test to compare mean values over time in the control group. To compare sham and LPT groups over time, log transformed data were analyzed by two-way ANOVA followed by a Sidak's multiple-comparisons test. GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA). Differences among mean values with $P<0.05$ were considered statistically significant.

Results

Levofloxacin concentration increased in plasma and ELF over time

The concentration of levofloxacin in plasma was measured at 10, 30, 60, and 120 minutes after the subcutaneous injection of levofloxacin in rats that did not receive anesthesia or LPT. The highest log-transformed concentration of levofloxacin in plasma (3.84 ± 0.06 ng/mL, $N=10$) was measured 30 minutes post injection and was significantly higher ($P<0.01$) than 10 (3.6 ± 0.03 ng/mL, $N=8$) and 120 (3.4 ± 0.07 ng/mL, $N=9$) but not 60 (3.4 ± 0.07 ng/mL, $N=4$) minutes post injection. The log-transformed concentration of levofloxacin in plasma at 60 minutes was significantly higher ($P<0.05$) than the concentration measured at 120 minutes (Figure 2.1).

The highest log-transformed concentration of levofloxacin in ELF (3.7 ± 0.07 ng/mL, *N*=4) was measured 60 minutes post injection and was significantly higher ($P<0.05$) compared to 10 minutes post injection (3.18± 0.2 ug/mL, *N*=8) but not 30 (3.5 ± 0.06 ng/mL, *N*=10) or 120 (3.4 ± 0.05 ug/mL, *N*=9) post injection (Figure 2.2). The calculated levofloxacin penetration into ELF did not significantly ($P>0.05$) increase over time (Figure 2.3).

LPT did not alter the concentration of levofloxacin in plasma

In the sham group, the highest log-transformed concentrations of levofloxacin in plasma of the sham group was measured at 30 minutes (*N*=10) and 60 minutes (*N*=11) and were significantly higher ($P<0.05$) than 10 minutes (*N*=9). Similarly, in the LPT group, the highest log-transformed concentration of levofloxacin in plasma were measured at 60 minutes (*N*=10) and was significantly higher ($P<0.05$) than 10 minutes (*N*=10). There were no significant differences between sham and LPT at any time point (see Table 2.1).

LPT did not alter the concentration of levofloxacin in ELF

In the sham group, the highest log-transformed concentration of levofloxacin in ELF was measured at 120 minutes (*N*=10) but was not significantly higher than 10 minutes (*N*=9), 30 minutes (*N*=10), or 60 minutes (*N*=11) post injection. However, in the LPT group, levofloxacin significantly increased ($P<0.05$) in the ELF at 30 minutes (*N*=10), 60 (*N*=10), and 120 minutes (*N*=10) compared to 10 minutes (*N*=10). There were no significant differences between sham and LPT at any time point (see Table 2.2).

LPT did not increase levofloxacin penetration in ELF

To determine if LPT enhanced the penetration of levofloxacin into the ELF, the ratio of log-transformed levofloxacin concentration in ELF to levofloxacin concentration in plasma was calculated for each animal and averaged. In the sham group, the highest ELF penetration occurred at 120 (N=10) minutes but was not significantly higher (P>0.05) than the penetration calculated at 10 minutes, 30 minutes, or 60 minutes. Similarly, levofloxacin penetration in the ELF did not significantly increase (P>0.05) over time in the LPT group. As above, there were no significant differences between sham and LPT at any time point (see Table 2.3).

Discussion

In this study the concentration of levofloxacin in ELF increased over time in the LPT group, suggesting LPT may have facilitated the entry of levofloxacin in the lung. Specifically, we discovered increased the concentration of levofloxacin in the ELF significantly increased over time in the LPT group but it did increase over time in the sham group. This is a significant finding as it supports the use of LPT promotes the delivery of pharmaceuticals in the lung. Importantly, the multicenter osteopathic study in the elderly (MOPSE) supports the use of OMT, including LPT, as an adjunctive therapy for pneumonia, however, the mechanism of protection is not clear [53, 55]. The results from this study support the findings from the MOPSE study and provide insight into the mechanisms by which LPT acts as an adjunctive therapy for the treatment of pneumonia.

The structure of lymphatic vessels near the subcutaneous space facilitates the uptake of molecules into the lymphatic system [72-74]. Of interest, a lymph flow enhancing technique was reported to enhance the uptake of protein from the interstitial fluid space and its transport to the blood of rats [67]. Therefore, by enhancing lymph flow, LPT may have enhanced the uptake of

levofloxacin from the subcutaneous space and its delivery to the blood and ELF. Levofloxacin passively diffuses through the blood-bronchus barrier to reach the EFL; therefore, the concentration of levofloxacin rises in the ELF as the concentration of levofloxacin rises in the blood [75]. However, LPT did not increase the concentration of levofloxacin in the blood suggesting that levofloxacin may have diffused into the lung tissue before reaching the ELF. However, antibiotic concentration was not measured in the lung tissue. Therefore, further studies are needed to test this hypothesis.

It important to note that a power analysis of this data revealed more animals per group are necessary to detect differences between sham and LPT. Therefore, future studies should include more animals per group and identify if LPT increases antibiotic concentrations in the blood and lung compared to sham.

In these studies rats were not infected with *S. pneumoniae* which is another limitation of this study. It is unknown whether *S. pneumoniae* pulmonary infection would have altered the diffusion of levofloxacin into the lung. Previous literature has proposed that antibiotic penetration into the lung is lower in patients with bronchitis compared to healthy subjects suggesting that the diffusion of antibiotic is impaired during pulmonary infection [76]. Future studies using a similar approach as this study in *S. pneumoniae* infected rats would be more translational and provide more insight into the mechanisms of protection offered by LPT.

The results from this study suggest that LPY may protect against pneumonia by an alternate mechanism. As reported in previous studies, LPT protected rats against pneumonia without the use of antibiotics [58, 69]. LPT may have improved mucociliary clearance of *S. pneumoniae* from the lungs and sinuses of infected rats. Indeed, OMT has been proposed to enhance mucociliary transport from the sinuses [51]. Factors in mesenteric lymph can activate

pulmonary leukocytes, increase endothelial cell permeability, increase the expression of cell adhesion molecules on endothelial cells and lung tissue [31]; therefore, LPT may have mobilized these factors to the lung to promote leukocyte trafficking. Inflammatory cytokines are among factors mobilized by LPT into lymphatic circulation [65]. One possible mechanism of LPT is that mobilizing lymph delivers factors to the lung that enhance the innate immune response to *S. pneumoniae* by promoting leukocyte trafficking, stimulating phagocytes, and promoting bacterial clearance. It is also possible, lymph-borne factors alter the response of macrophages. Therefore, studies in Chapters III-V will test this hypothesis. In addition to inflammatory cytokines, LPT may also have mobilized complement from lymph and enhanced phagocytosis of *S. pneumoniae* [77].

The results from this study suggest that LPT does not alter the concentration of antibiotics in the lung compared but may protect against pneumonia by facilitating the delivery of antibiotics to the lung. Achieving adequate drug concentrations at the site of infection is an important consideration for antibiotic dosage and efficacy [78]. Therefore, LPT may promote the entry of antibiotics to the lung suggesting osteopathic manipulative treatment, such as LPT, work in synergy with antibiotics to protect against pneumonia. Understanding the physiologic effects of osteopathic manipulative treatment on antibiotic distribution may guide osteopathic physicians in treatment of patients with pneumonia to reduce the length of hospital stay, the duration of antibiotic use, and the incidence of respiratory failure.

Table 2.1. LPT did not alter the concentration of levofloxacin in plasma

Time	Sham (log levo. ng/mL)	LPT (log levo. ng/mL)
10 min	3.4 ± 0.1	3.5 ± 0.1
30 min	3.8 ± 0.1[a]	3.7 ± 0.03
60 min	3.7 ± 0.02[a]	3.9 ± 0.05
120 min	3.6 ± 0.1	3.7 ± 0.03

Data are means ± SE of log transformed levofloxacin concentration (ng/mL) in plasma. *N*=9-11 animals per group. Data were analyzed by two-way ANOVA followed by a Sidak's multiple comparisons test. [a]$P<0.05$ significantly higher compared to 10 minutes within the group.

Table 2.2. LPT did not alter the concentration of levofloxacin in ELF

Time	Sham (log levo. ng/mL)	LPT (log levo. ng/mL)
10 min	3.0 ± 0.1	2.8 ± 0.2
30 min	3.3 ± 0.1	3.6 ± 0.1[a]
60 min	3.5 ± 0.1	3.4 ± 0.05[a]
120 min	3.6 ± 0.1	3.6 ± 0.1[a]

Data are means ± SE of log transformed levofloxacin concentration (ng/mL) in epithelial lining fluid (ELF). *N*=5-11. Data were analyzed by two-way ANOVA followed by a Sidak's multiple comparisons test. [a]$P<0.05$ significantly higher compared to 10 minutes within the group.

Table 2.3. LPT did not alter levofloxacin penetration in ELF

Time	Sham (ELF:plasma)	LPT (ELF:plasma)
10 min	0.9 ± 0.02	0.8 ± 0.06
30 min	0.9 ± 0.04	1.0 ± 0.25
60 min	0.9 ± 0.04	0.9 ± 0.2
120 min	0.99 ± 0.02	0.98 ± 0.03

Data are mean ratios ± SE of log transformed epithelial lining fluid (ELF) levofloxacin to plasma levofloxacin. *N*=5-11 animals per group. Data were analyzed by two-way ANOVA followed by a Sidak's multiple comparisons test.

Figure 2.1. Levofloxacin concentration increased in plasma at 30 and 60 minutes post injection. Data are means ± SE of log transformed levofloxacin concentration (ng/mL) in plasma. *N*=9-16 animals per group. Data were analyzed by ANOVA followed by a Tukey's multiple comparisons test. $^{a}P<0.01$ compared to 10 and 120 minutes. $^{b}P<0.05$ compared to 120 minutes.

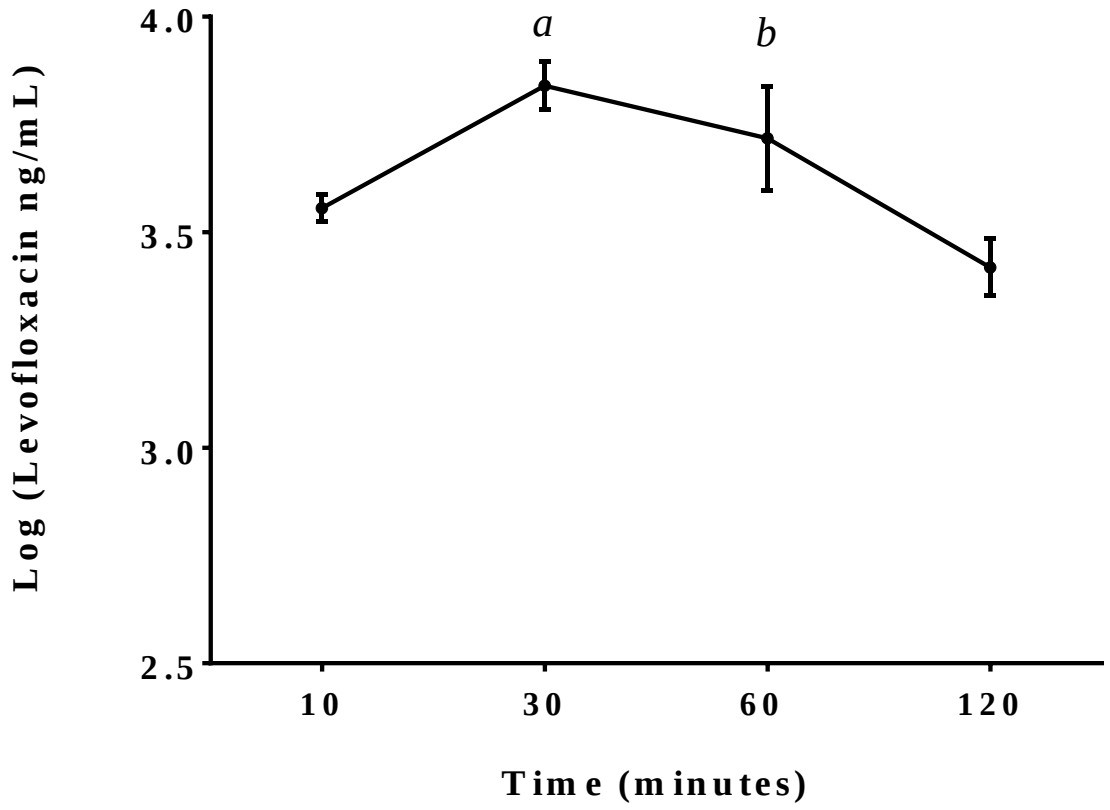

4.0
3.5
3.0
2.5
Log (Levofloxacin ng/mL)
a
b
10
30
60
120
Time (minutes)

Figure 2.2. Levofloxacin concentration increased in ELF at 60 minutes post injection

Data are means ± SE of log transformed levofloxacin concentration (ng/mL) in ELF. *N*=4-10 animals per group. Data were analyzed by ANOVA followed by a Tukey's multiple comparisons test. $^{c}P<0.05$ compared to 10 minutes.

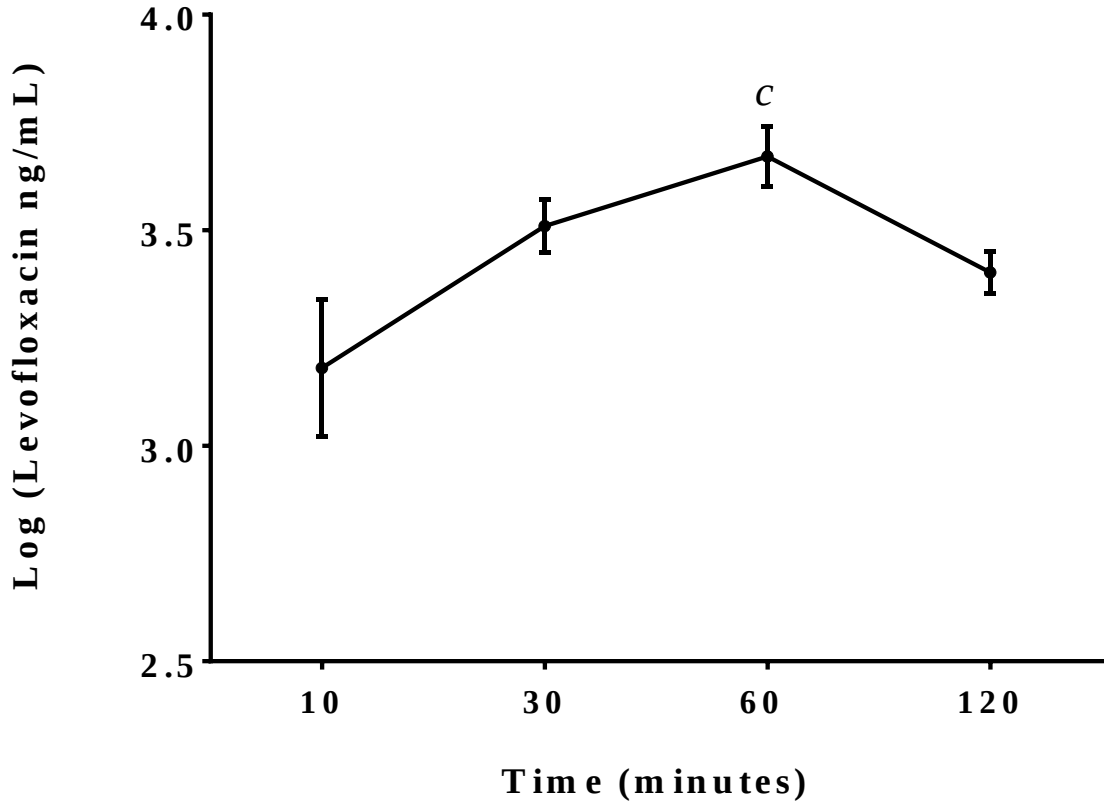
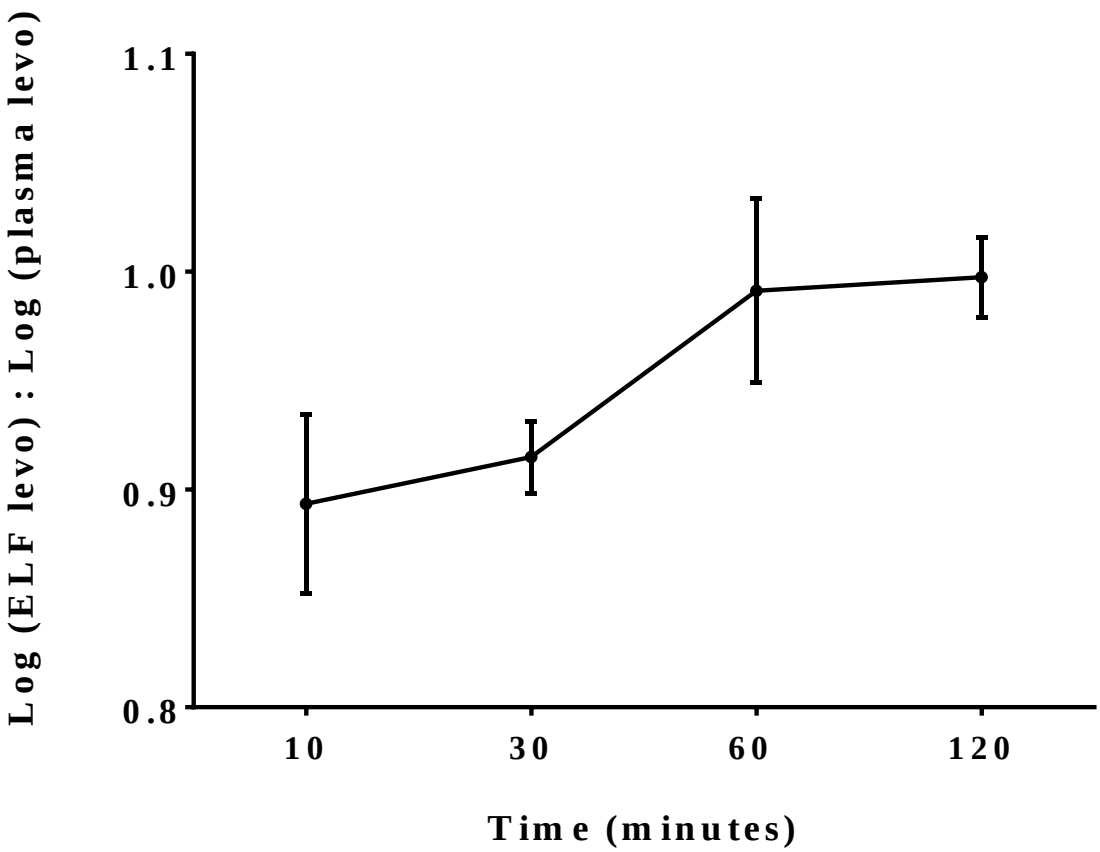

4.0
3.5
3.0
2.5
Log (Levofloxacin ng/mL)
C
10
30
60
120
Time (minutes)

Figure 2.3. Levofloxacin penetration in ELF did not increase over time

Data are mean ratios ± SE of ELF levofloxacin to plasma levofloxacin. Data were analyzed by ANOVA. *N*=4-10 animals per group.


Log (ELF levo) : Log (plasma levo)
1.1
1.0
0.9
0.8
10
30
60
120
Time (minutes)

CHAPTER III

LYMPHATIC PUMP TREATMENT MOBILIZES BIOACTIVE LYMPH THAT SUPPRESSES MACROPHAGE ACTIVITY IN VITRO

Rudy Castillo, BS; Artur Schander, MS, DO, PhD; Lisa M. Hodge, PhD

Context: By promoting the recirculation of tissue fluid, the lymphatic system preserves tissue health, aids in the absorption of gastrointestinal lipids, and supports immune surveillance. Failure of the lymphatic system has been implicated in the pathogenesis of several infectious and inflammatory diseases. Thus, interventions that enhance lymphatic circulation, such as osteopathic lymphatic pump treatment (LPT), should aid in the management of these diseases.

Objective: To determine whether thoracic duct lymph (TDL) mobilized during LPT would alter the function of macrophages in vitro.

Methods: The thoracic ducts of 6 mongrel dogs were cannulated, and TDL samples were collected before (baseline), during, and 10 minutes after LPT. Thoracic duct lymph flow was measured, and TDL samples were analyzed for protein concentration. To measure the effect of TDL on macrophage activity, RAW 264.7 macrophages were cultured for 1 hour to acclimate. After 1 hour, cell-free TDL collected at baseline, during LPT, and after TDL was added at 5%

total volume per well and co-cultured with or without 500 ng per well of lipopolysaccharide (LPS) for 24 hours. As a control for the addition of 5% TDL, macrophages were cultured with phosphate-buffered saline (PBS) at 5% total volume per well and co-cultured with or without 500 ng per well of LPS for 24 hours. After culture, cell-free supernatants were assayed for nitrite (NO_2^-), tumor necrosis factor α (TNF-α) and interleukin 10 (IL-10). Macrophage viability was measured using flow cytometry. Results: Lymphatic pump treatment significantly increased TDL flow and the flux of protein in TDL (P<.001). After culture, macrophage viability was approximately 90%. During activation with LPS, baseline TDL, TDL during LPT, and TDL after LPT significantly decreased the production of NO_2^-, TNF-α, and IL-10 by macrophages (P<.05). However, no significant differences were found in viability or the production of NO_2^-, TNF-α, or IL-10 between macrophages cultured with LPS plus TDL taken before, during, and after LPT (P>.05).

Conclusion: The redistribution of protective lymph during LPT may provide scientific rationale for the clinical use of LPT to reduce inflammation and manage edema.

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The lymphatic system maintains tissue fluid homeostasis by returning excess interstitial fluid to the blood circulation. Lymph can contain apoptotic or necrotic cells, immune cells, soluble antigens, microbes, toxins, proteins, and lipids. By promoting the recirculation of lymph, the lymphatic system preserves tissue health, aids in the absorption of gastrointestinal lipids, and promotes immune surveillance. Failure of the lymphatic system has been implicated in the pathogenesis of cardiovascular disease, inflammation, and edema [1, 2]. Therefore, interventions that promote lymphatic circulation should promote tissue health and aid in the management of infectious and inflammatory diseases.

Since its inception, the osteopathic medical profession has emphasized the importance of the lymphatic system in maintaining health. Andrew Taylor Still, MD, DO, asserted that stimulating lymph flow would facilitate the removal of blood cells, particulate matter, exudates, toxins, and bacteria that may adversely affect cellular activity and predispose tissue to dysfunction and diseas [79]. Many osteopathic manipulative treatment techniques were designed to promote lymph circulation [50, 51, 80]. Lymphatic pump treatment (LPT) is used to manage congestive heart failure, upper and lower gastrointestinal tract dysfunction, respiratory tract disease, infection, and edema [51]. Although the mechanisms of action of LPT are still under investigation, it has been proposed that LPT can improve health by promoting circulation, stimulating immunity, and enhancing the efficacy of vaccines and medications [50].

Previous studies [63-66] have demonstrated that abdominal LPT can significantly enhance thoracic and mesenteric lymphatic flow and the concentration of leukocytes in lymph in dogs. Additionally, abdominal LPT can significantly increase the lymphatic flux of inflammatory cytokines, chemokines, and reactive oxygen and nitrogen species in thoracic and mesenteric lymph [65, 66]. Collectively, results of these studies [63-66] suggest that abdominal LPT can

enhance the lymphatic redistribution of cells and inflammatory mediators, which may protect against a variety of infectious and inflammatory diseases. Lymph has been reported to suppress inflammation [37, 81, 82], blunt the pulmonary inflammatory response to endotoxins, inhibit neutrophil apoptosis in vitro, [81, 82] increase endothelial cell permeability, [30, 83] and redistribute leukocytes, cytokines, and chemokines to distant organs [16]. However, the effect of lymph on macrophage function has not been described. Macrophages reside in the tissue and alert the immune system to tissue injury, infection, and inflammation. During infection, macrophages phagocytose microorganisms; release antibacterial factors, such as nitric oxide; and produce inflammatory cytokines and chemokines, such as tumor necrosis factor α (TNF-α), which recruit monocytes and neutrophils to the site of infection [20]. Macrophages also aid wound healing and tissue repair by producing anti-inflammatory cytokines and growth factors, such as interleukin 10 (IL-10), that dampen leukocyte activity and prevent immunopathologic reactions [20].

The purpose of the current study was to determine whether the lymph mobilized during abdominal LPT would suppress macrophage activity in vitro. Specifically, we hypothesized that thoracic duct lymph (TDL) collected during LPT would suppress macrophage activation in vitro. If distant organs are inflamed, promoting lymph output using treatments such as LPT may redistribute lymph-borne factors to the affected tissue and enhance protection against disease.

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 [84]. Six

adult mongrel dogs free of clinically evident signs of disease were used. At the completion of the study, the dogs were euthanized in accordance with the American Veterinary Medical Association guidelines [85].

Surgical Procedure

The dogs were fasted overnight and anesthetized with intravenous sodium pentobarbital (30 mg/kg) before the surgical procedure. After endotracheal intubation, the dogs were ventilated with room air and supplemented with oxygen to maintain normal arterial blood gases. Arterial blood pressure was monitored via a femoral artery pressure monitoring catheter connected to a pressure transducer (Hewlett-Packard Pressure Monitor, 78354A) to ensure that arterial blood pressure remained within normal limits. The chest was opened by a left lateral thoracotomy in the fourth intercostal space, and the thoracic duct was isolated from connective tissue and ligated. Caudal to the ligation, a PE60 catheter (0.76-mm inner diameter; 1.22-mm outer diameter), whose outflow tip was positioned 8 cm below heart level to compensate for the hydraulic resistance of the catheter, was inserted into the duct and secured with a ligature. The TDL was drained at atmospheric pressure through the catheter. Thoracic duct lymph was continuously collected for 4 minutes before LPT (baseline), for 4 minutes during LPT, and for 10 minutes after the cessation of LPT.

Lymphatic Pump Treatment

The anesthetized dogs were placed in a right lateral recumbent position. The operator (A.S.) contacted the dog's abdomen with his hands placed bilaterally below the costodiaphragmatic junction. Pressure was exerted medially and cranially to compress the

abdomen until resistance was encountered against the diaphragm before being released. Abdominal compressions were administered at a rate of approximately 1 pump per second for a total of 4 minutes.

TDL Protein Concentration and Flux

Thoracic duct lymph was centrifuged to remove the cellular component and stored at −80°C until use. Total protein concentration in TDL was determined using the Bradford Method and Coomassie Brilliant Blue G-250 dye (Bio-Rad Laboratories) to measure the presence of all proteins in the TDL. To compute TDL protein flux, protein concentrations were multiplied by the respective TDL flow rates for each TDL sample.

Cell Culture

The murine macrophage cell line RAW 264.7 was used to measure the effect of TDL on macrophage function. Cells were grown in high-glucose Dulbecco modified eagle medium with 10% fetal bovine serum at 37°C under 5% carbon dioxide and passaged 5 to 10 times before cell stimulations. One hundred thousand cells per well were cultured for 1 hour to allow the cells to acclimate. After 1 hour, cell-free TDL taken at baseline, during LPT, and after LPT was added at 5% total volume per well and cocultured with and without 500ng per well of lipopolysaccharide (LPS) for 24 hours.12,15 As a control, RAW 264.7 macrophages were cultured for 1 hour to allow cells to acclimate, then phosphate-buffered saline (PBS) was added at 5% total volume per well with and without 500 ng per well of LPS for 24 hours. After 24 hours of culture, cell-free supernatants were collected and stored at −80°C.

Cell Viability

After cell stimulations, cells were collected by centrifugation and scraping. To determine cell viability, immunofluorescent staining was performed using annexin V and propidium iodide. Cells were subjected to a BD LSR II flow cytometer, and data were analyzed using FlowJO software (TreeStar Inc). The percentages of live cells were determined by gating intact cells that were not positive for annexin V or propidium iodide.

Inflammatory Mediators

The concentrations of nitrite (NO_2^-), TNF-α, and IL-10 in cell-free supernatants were measured using commercially available kits. The Promega Griess Reagent system (Promega Corporation) measures NO_2^-, a nonvolatile and stable breakdown product of nitric oxide. The minimum detectable NO_2^- concentration for this assay is 2.5 μM. The minimum detectable concentrations for TNF-α and IL-10 are 15.5 pg/mL and 31.3 pg/mL, respectively.

Statistical Analysis

Data are presented as arithmetic mean (SE). Values from all dogs at respective time points were averaged. For evaluation of statistical significance, data were subjected to analyses of variance followed by a Tukey multiple comparisons test. Statistical analyses were performed with GraphPad Prism version 7. $P \leq .05$ was considered statistically significant.

Results

LPT Increases Total Protein Flux in TDL

The effect of LPT on TDL flow, protein concentration, and protein flux are shown in Table 3.1. Four minutes of LPT increased TDL flow approximately 10-fold (P<.001). The effect of LPT on TDL flow was transient, as TDL flow decreased during the 10 minutes after LPT. Lymphatic pump treatment did not significantly increase the concentration of total protein in TDL (P>.05). Additionally, the flux of protein significantly increased during LPT (P<.05), but protein flux after LPT was similar to baseline.

TDL Suppresses Macrophage Activity In Vitro

After 24 hours of culture, RAW 264.7 macrophage viability was approximately 90%. The addition of 5% TDL taken at baseline, during LPT, and after LPT to RAW 264.7 macrophages did not alter cell viability (data not shown) compared with RAW 264.7 macrophages cultured with 5% PBS. Culture with LPS, LPS and baseline TDL, LPS and TDL taken during LPT, and LPS and TDL taken after LPT did not alter viability compared with RAW 264.7 macrophages cultured with 5% PBS (Figure 3.1).

In the absence of LPS, the production of NO_2^- was below the assay limit of detection. The addition of LPS significantly increased the production of NO_2^- by RAW 264.7 macrophages (16 [0.2] μM; P<.001). Furthermore, the addition of 5% baseline TDL, TDL taken during LPT, and TDL taken after LPT significantly decreased the production of NO_2^- by RAW 264.7 macrophages (9.1 [0.6] μM, 8.2 [1.0] μM, and 7.8 [0.4] μM, respectively; P<.001). No significant differences were found in the production of NO_2^- by RAW 264.7 macrophages between baseline TDL, TDL during LPT, and TDL after LPT (P>.05) (Figure 3.2).

Similarly, in the absence of LPS, the production of TNF-α was below the assay limit of detection. The addition of LPS significantly increased the production of TNF-α by RAW 264.7 macrophages (3625 [800] pg/mL; *P*<.001). The addition of 5% baseline TDL, TDL taken during LPT, and TDL taken after LPT significantly decreased the production of TNF-α by RAW 264.7 macrophages (222 [79] pg/mL, 772 [154] pg/mL, and 307 [85] pg/mL, respectively; P<.001). Also, there were no significant differences in the production of TNF-α by RAW 264.7 macrophages in baseline TDL, TDL during LPT, and TDL after LPT (P>.05) (Figure 3.3).

Additionally, in the absence of LPS, the production of IL-10 was below the assay limit of detection. The addition of LPS significantly increased the production of IL-10 by RAW 264.7 (3513 [239] pg/mL; P<.001). Furthermore, the addition of 5% baseline TDL, TDL taken during LPT, and TDL taken after LPT significantly decreased the production of IL 10 by RAW 264.7 macrophages (1168 [98] pg/mL, 1047 [167] pg/mL, and 677 [166] pg/mL, respectively; *P*<.0001). No significant differences were found in the production of IL-10 by RAW 264.7 macrophages and baseline TDL, TDL during LPT, and TDL after LPT (P>.05) (Figure 3.4).

Discussion

To our knowledge, this is the first study to report that normal TDL suppresses macrophage activity in vitro, suggesting that TDL has an anti-inflammatory effect. In support of this finding, normal mesenteric lymph has been reported to decrease the expression of cell adhesion molecule 1 on primary human microvascular pulmonary endothelial cells that were activated with LPS in vitro [82]. Furthermore, the transfusion of normal mesenteric lymph into rats alleviated LPS-induced lung injury in vivo [37]. Results of the current study showed that TDL did not reduce macrophage viability, demonstrating that TDL does not injure macrophages.

Collectively, results of these studies [37, 82] and the current study suggest that normal TDL contains bioactive mediators that are able to block the macrophage response to LPS.

Thoracic duct lymph collected before LPT, during LPT, and after LPT equally suppressed macrophage activity approximately 3-fold, suggesting that LPT did not release additional bioactive mediators into TDL. This result was not surprising because LPT did not increase the concentration of protein per milliliter of TDL. Furthermore, in previous studies, [65, 66] LPT did not increase the concentration of specific proteins, such as cytokines and chemokines. However, consistent with previous studies [63-66] LPT increased TDL flow and the flux of protein in TDL approximately 10-fold in the current study. By increasing the TDL flux, LPT may increase the concentration of lymph-borne factors in blood circulation. Miller [86], who asserted that the normal circulation of body fluids is essential to normal body function, developed the thoracic pump technique in 1920 to relieve venous and lymphatic stasis and restore normal circulation. Consistent with this philosophy, by enhancing TDL flow, LPT may redistribute a large pool of bioactive lymph into circulation. Once in circulation, this LPT-mobilized lymph may protect against disease by removing inflammatory mediators from diseased tissue or by transporting lymph that is rich in bioactive mediators to sites of inflammation.

In the current study, we did not identify the bioactive mediators in TDL that suppressed macrophage activity, which is a limitation. Thoracic duct lymph collects cells, proteins, lipids, and lipoproteins from the tissues and intestines and transports them into blood circulation. It has been proposed that during inflammation, the lymphatic transport of proteins, such as cytokines and chemokines, augment the inflammatory response at distant tissues, such as the lung [16]. However, Davidson *et al* [33] found that the bioactive mediator in lymph from rats subjected to

hemorrhagic shock was not a cytokine. Other reports [16, 81, 82] suggested that the bioactive mediator in lymph is within the cell-free lipid/lipoprotein fraction. In the present study, macrophage activity was suppressed during culture with cell free TDL from healthy donors, supporting the hypothesis that the suppressive factor(s) in lymph are soluble. Additional experiments are necessary to confirm whether the bioactive mediator in normal TDL is a protein, lipid, or lipoprotein. Another limitation is that the tissue environment differs in vivo, but this study did not examine the effect of TDL on tissues isolated from healthy or diseased animals. Also, canine TDL suppressed the function of a murine macrophage cell line, suggesting that the biological factor in TDL is not species specific. Species-specific factors may exist in lymph that were not discovered in this study.

Conclusion

Results from this study demonstrate that soluble factors in TDL have an anti-inflammatory effect. The lymphatic system is vital for the drainage and delivery of tissue fluids to blood circulation, and failure of the lymphatic transport of this fluid has been associated with edema and diseases associated with inflammation. A better understanding of the physiologic effects of osteopathic manipulative treatment techniques, such as LPT, on the lymphatic system will allow us to expand translational and clinical research and guide osteopathic physicians in their clinical practice.

Table 3.1. Thoracic duct lymph flow, protein concentration, and protein flux measurements before, during, and after lymphatic pump treatment in Dogs (*N*=6)[a]

TDL Measurements	Before LPT	During LPT	After LPT
Flow, mL/min	0.62 (0.11)	6.8 (0.39)[b]	0.38 (0.05)
Protein concentration, mg/mL	33 (5)	25 (7)	28 (7)
Protein flux, pg/min	19 (4)	170 (44)[c]	11 (3)

[a] Data are given as mean (SE).
[b] $P<.001$.
[c] $P<.05$.

Abbreviations: TDL, thoracic duct lymph; LPT, lymphatic pump treatment.

Figure 3.1. TDL did not alter RAW 264.7 macrophage viability in the presence of LPS. The viability percentage of RAW 264.7 macrophage cultured with 5% phosphate-buffered saline (PBS) and cell-free thoracic duct lymph (TDL) taken before, during, and after lymphatic pump treatment (LPT) and cocultured with 500 ng per well of lipopolysaccharide.

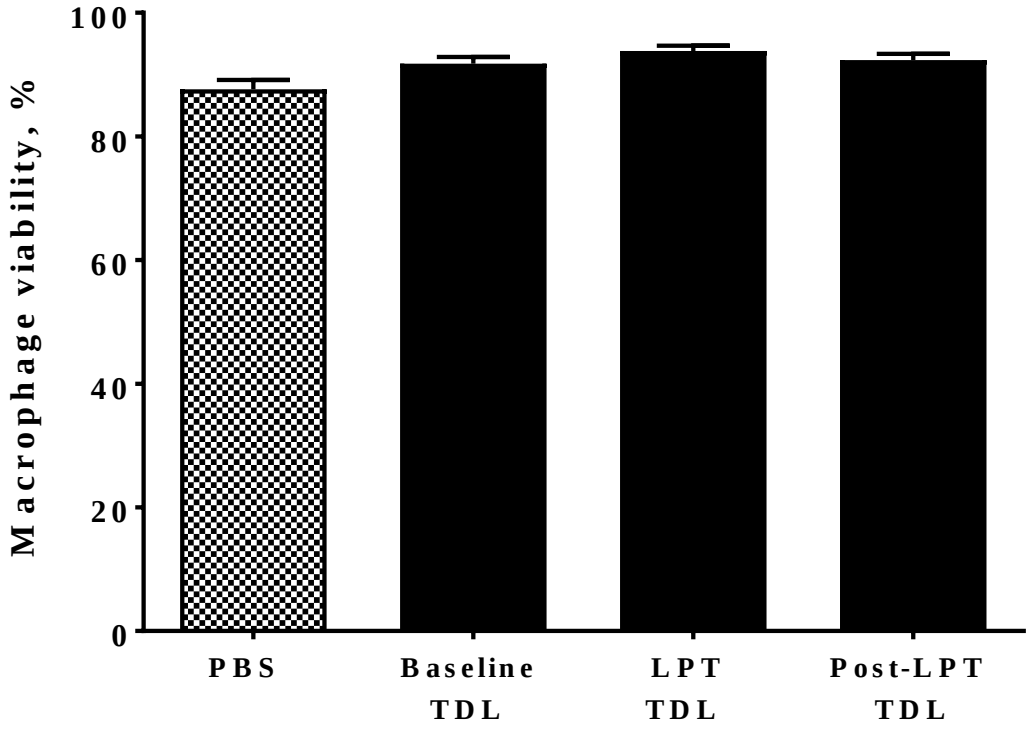

Macrophage viability, %
100
80
60
40
20
0
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 3.2. TDL suppressed the production of nitrite by RAW 264.7 macrophages activated with LPS. Production of nitrite by RAW 264.7 macrophages cultured with 5% phosphate-buffered saline (PBS) and cell-free thoracic duct lymph (TDL) taken before, during, and after lymphatic pump treatment (LPT) and cocultured with 500 ng per well of lipopolysaccharide.

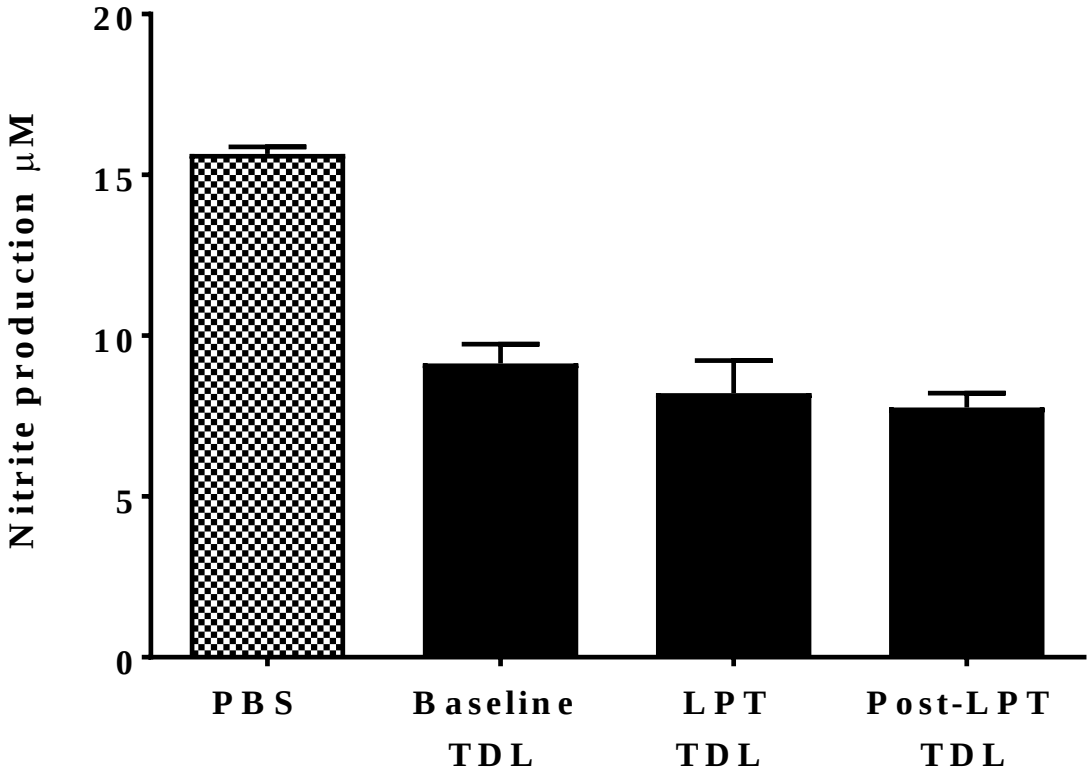

Nitrite production μM
20
15
10
5
0
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 3.3. TDL suppressed the production of TNF-α by RAW 264.7 macrophages activated with LPS. Production of tumor necrosis factor α (TNF-α) by RAW 264.7 macrophages cultured with 5% phosphate-buffered saline (PBS) and cell-free thoracic duct lymph (TDL) taken before, during, and after lymphatic pump treatment (LPT) and cocultured with 500 ng per well of lipopolysaccharide.

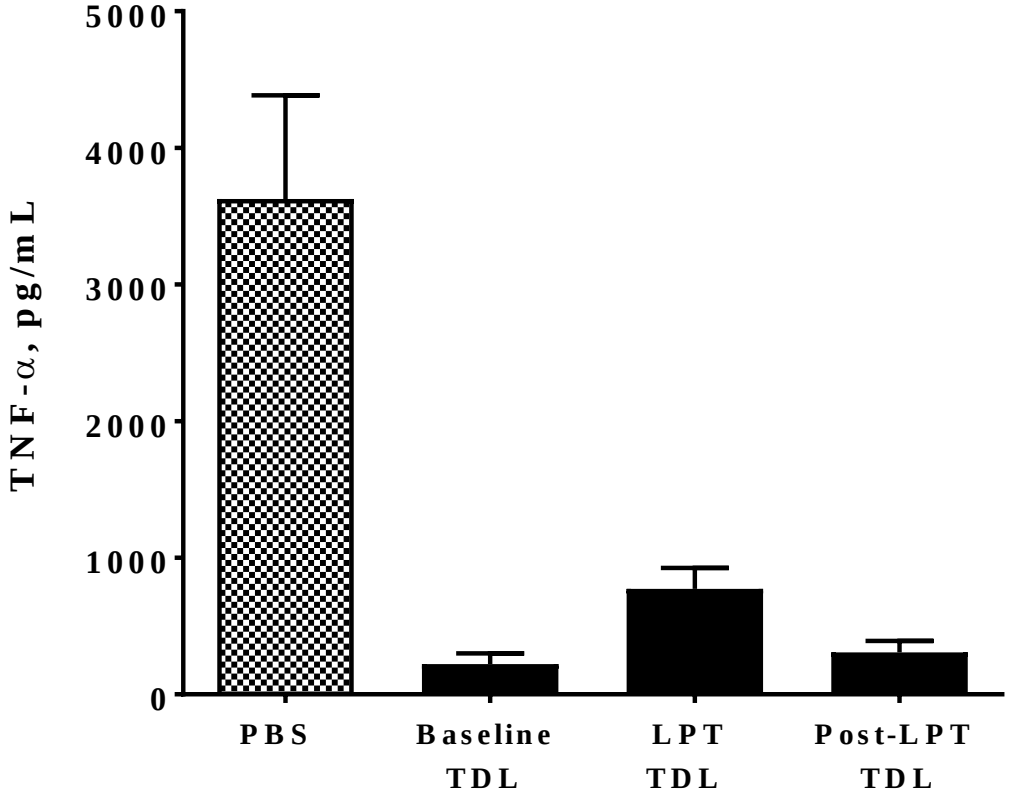
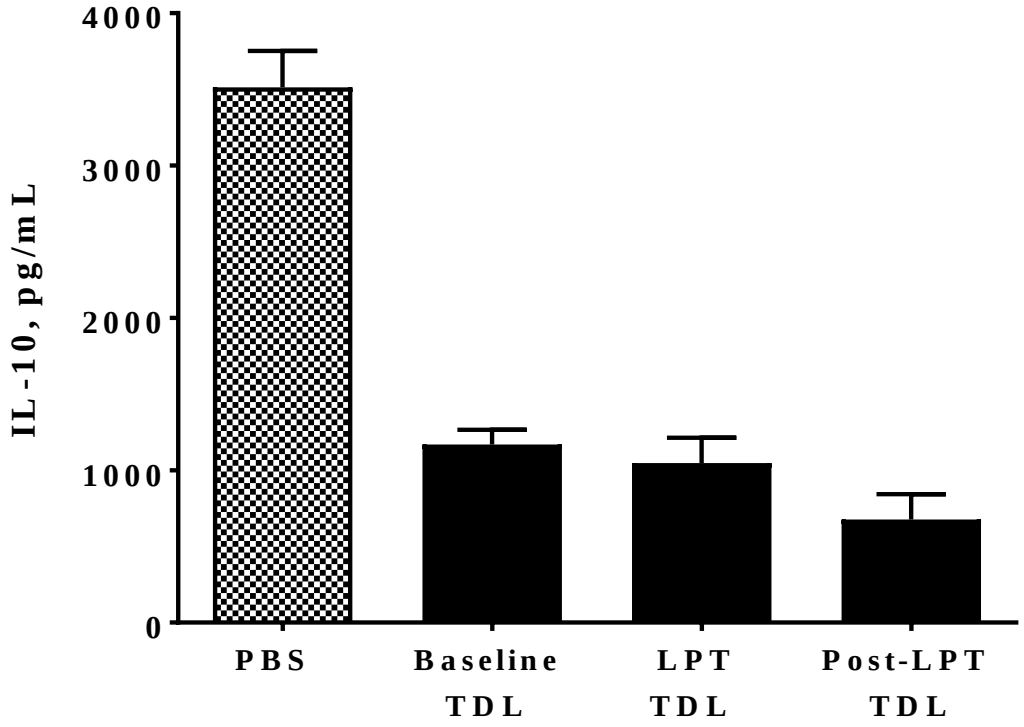

5000
4000
3000
2000
1000
0
TNF-α, pg/mL
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 3.4. TDL suppressed the production of IL-10 by RAW 264.7 macrophages activated with LPS. The production of interleukin 10 (IL-10) by RAW 264.7 macrophages cultured with 5% phosphate-buffered saline (PBS) and cell-free thoracic duct lymph (TDL) taken before, during, and after lymphatic pump treatment (LPT) and cocultured with 500 ng per well of lipopolysaccharide.


4000
3000
2000
1000
0
IL-10, pg/mL
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

CHAPTER IV

THORACIC DUCT LYMPH SUPPRESSES ALVEOLAR MACROPHAGE ACTIVITY *IN VITRO*

The lymphatic system transports lymph from the tissue into circulation to maintain tissue fluid homeostasis and aid in immune surveillance. Lymph enters venous circulation through the subclavian vein where it is delivered to the lung. Previous studies have proposed that lymph-borne factors travel through the lymphatic system to the lung where they modulate pulmonary inflammation. Osteopathic physicians have designed a set of manipulative therapies that may promote the movement of lymph. Lymphatic pump treatment (LPT) is a subset of osteopathic manipulative techniques that have been used clinically for the treatment of pneumonia. Recent studies have demonstrated that LPT can reduce the concentration of bacteria in the lungs of rats infected with *S. pneumoniae*. Furthermore, LPT mobilized bioactive lymph that suppressed macrophage activity *in vitro*. However, it is unknown if thoracic duct lymph (TDL) suppresses alveolar macrophage activity. The purpose of this study was to determine if thoracic duct lymph mobilized during LPT would suppress alveolar macrophage activity *in vitro*. Specifically, we hypothesized that thoracic duct lymph collected during LPT would suppress alveolar macrophage activity *in vitro*. To test this hypothesis, the murine alveolar macrophage cell line, MH-S, was cultured with 5% baseline TDL, 5% LPT TDL, or 5% post-LPT TDL and co-cultured with or without lipopolysaccharide (LPS). As a control, MH-S macrophages were

cultured with PBS and co-cultured with or without LPS. After 24 hour culture, supernatant was collected to measure nitrite, tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) production. Macrophage viability was measured by flow cytometry using markers, annexin V and propidium iodide. During activation with LPS, baseline, LPT, or post-LPT TDL significantly decreased the production of nitrite and TNF-α. Baseline, LPT, or post-LPT TDL and did not significantly alter the production of IL-10 or alter macrophage viability. Furthermore, there were no differences in TNF-α and IL-10 production by MH-S macrophages cultured with baseline, LPT, or post-LPT TDL with or without LPS. By mobilizing lymph into circulation, LPT may mobilize protective factors to the lung to reduce inflammation, thereby protecting from respiratory disease

Introduction

The lymphatic system is responsible for interstitial fluid homeostasis, immune surveillance, and the absorption of fat. This interstitial fluid, known as lymph, may contain dead cells, immune cells, soluble antigens, microbes, toxins, proteins and lipids that are returned into systemic circulation through the lymphatic system [1, 2]. Lymph enters venous circulation through the subclavian vein where it is delivered to the first major vascular bed in the lung [30]. The lymphatic vasculature that connects the gastrointestinal region to the lungs has been of interest in the study of respiratory diseases that have been associated with intestinal inflammation [32, 36, 37]. Collectively, this literature suggests lymph may play a role during pulmonary inflammation.

Osteopathic physicians recognize the importance of the lymphatic system during disease and have designed techniques to promote lymphatic flow. Osteopathic manipulative techniques

(OMT) include the movement and stretching of muscles, fascia and joints, gentle pressure and manual pumping techniques. LPT is a subset of OMT that target the musculoskeletal system and designed to enhance the flow of lymph [50, 51]. Clinical studies support the use of LPT as an adjunctive therapy for the treatment of pneumonia, influenza, and pulmonary disease [52, 53, 55]. However, little is known about the mechanism of protection offered by LPT.

LPT has been previously shown to enhance lymph flow, the concentration of leukocytes and the flux of cytokines, chemokine and reactive oxygen and nitrogen species in the thoracic duct of animals [63-66]. Furthermore, LPT protected rats against *S. pneumoniae* infection [58, 69]. Collectively, these studies suggest that lymph-enhancing techniques may enhance pulmonary immunity.

As the first line of defense, resident alveolar macrophages respond to airway pathogens by engulfing bacteria and secreting antimicrobials such as nitric oxide and inflammatory mediators such as TNF-α [29]. The release of cytokines and chemokines recruit neutrophils and monocytes to the lung tissue. Alveolar macrophages can also protect from tissue damage by the release of anti-inflammatory mediators such as IL-10 promote tissue repair and maintain homeostasis in the airways [29].

Alveolar macrophages have less phagocytic activity and respiratory burst than interstitial macrophages and monocytes recruited to the lung. Furthermore, alveolar macrophages have a higher expression of regulatory receptors, poorly present antigens, and are hyporesponsive to toll-like receptor ligands. This allows immunosuppression to limit inflammatory responses to innocuous antigens but contribute to initiating the immune response [29]. In contrast, infiltrating macrophages from the blood and interstitium, have higher phagocytic activity and ability to present antigens, and contribute to sustaining the inflammatory response, bacterial clearance, and

the clearance of apoptotic macrophages and neutrophils. Similar to alveolar macrophages, recruited macrophages can also promote resolution and tissue repair through the release of anti-inflammatory mediators and growth factors. However, alveolar macrophages, interstitial macrophages, and monocytes contribute to pulmonary inflammation resulting in immunopathology and lung injury [20, 21, 22, 29]

Lymph has been reported to reduce inflammation [34, 35, 87], blunt the inflammatory response of pulmonary endothelium to endotoxin [82], and reduce neutrophil apoptosis *in vitro* [81]. Recently, thoracic duct lymph was reported to suppress macrophage activation by lipopolysaccharide *in vitro* (Chapter III). However, the effect of lymph on alveolar macrophage activity is unknown. The purpose of this study was to determine if thoracic duct lymph mobilized during LPT would suppress alveolar macrophage activity *in vitro*. If distant organs such as the lungs are inflamed, promoting lymph output using therapies may redistribute lymph-borne factors to the lungs and enhance protection against pneumonia.

Methods

Animals

The animals were housed as previously described (Chapter III). 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* [84]. Six adult mongrel dogs, free of clinically evident signs of disease, were used for this study.

Surgical procedure

The surgical procedure was performed as previously described (Chapter III). Before surgery, the dogs were fasted overnight and then anesthetized with sodium pentobarbital. Under anesthesia, the dogs were intubated and ventilated with room air supplemented with O_2 to maintain normal arterial blood gases. A pressure transducer connected to a femoral artery catheter was used to monitor arterial blood pressure, arterial blood gases, and used to administer additional anesthetic. The chest was opened by a left lateral thoracotomy in the left fourth intercostal space and the thoracic duct was isolated from the connective tissue. The thoracic duct was ligated and a PE 60 catheter inserted into the duct, caudal to the ligation, and the outflow tip positioned eight centimeters below the heart level to compensate for the hydraulic resistance of the catheter. Approximately sixty minutes after securing the catheter with a ligature, thoracic lymph was collected at one-minute intervals for 4 minutes before LPT, 4 minutes during LPT, and 10 minutes after the cessation of LPT.

Lymphatic pump technique

Abdominal LPT was performed as previously described (Chapter III). The anesthetized dogs were placed in a right lateral recumbent position. To perform LPT, the operator contacted the ventral side of the animal's abdomen bilaterally below the costo-diaphragmatic junction. Pressure was applied and released medially and cranially to compress the abdomen until resistance was met against the diaphragm. Compressions to the abdomen were administered at a rate of approximately 1 pump per sec for a total of 4 minutes.

Cell culture

The murine alveolar macrophage cell line, MH-S (ATCC CRL-2019), was used to determine the effect of thoracic duct lymph on macrophage activation. MH-S macrophages were grown and maintained in Roswell Park Memorial Institute 1640 medium (ThermoFisher, Waltham, MA) with 10% fetal bovine serum (Hyclone, Logan, UT) and 0.05mM of 2-mercaptoethanol (ThermoFisher) at 37°C under 5% CO_2. MH-S cells were passaged 5-15 times by collecting both suspended and adherent cells using 0.25% trypsin (Hyclone).

Twenty-four hours after passage, MH-S macrophages were counted using trypan blue (Corning, Manassas, VA) exclusion and $1x10^5$ cells per well were cultured for 1 hour to allow the cells to acclimate. TDL from six dog donors were pooled into groups of two (*N*=3) with equal volumes of TDL from each dog. After one hour, cell-free baseline, LPT, or post-LPT TDL was added at 5% and co-cultured with or without 1000 ng per well of lipopolysaccharide (LPS) (Sigma) for 24 hours. To determine the optimal concentration of LPS to use in this study, MH-S cells were cultured with 0, 50, 250, 500, 1000, or 2500ng of LPS. Culture with 1000ng of LPS elicited the strongest inflammatory response without altering cell viability and was therefore chosen for this study (data not shown). Also, this dose of TDL is consistent with a previous study (Chapter III). As a control, MH-S macrophages were cultured for one hour to allow cells to acclimate then sterile 1X PBS (Hyclone) at 5% total volume per well was added with or without 1000 ng per well of lipopolysaccharide (LPS) for 24 hours. Each condition was done in triplicate and replicate supernatants and cells assayed separately. After 24 hours of culture, cell-free supernatants were collected by centrifugation at 130G for 10 minutes at 4°C and stored at -80°C.

Cell viability

After the collection of cell-free supernatants, 10% FBS in PBS was added to each well and cells collected by scraping. Cells were transferred to a 96-well plate and centrifuged at 130G for 10 minutes at 4°C. Cells were resuspended in a 5% annexin V and 5% propidium iodide solution with 1X annexin V binding buffer (BD Biosciences, San Jose, CA) for 15 minutes at room temperature. Cells were subjected to a BD LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJO software (TreeStar Inc). The percentages of live cells were determined by gating on intact cells not positive for annexin V or propidium iodide. There were no significant differences in the percentage of cell debris or dead cells when cells were cultured with PBS, baseline, LPT, or post-LPT TDL with or without LPS.

Measurement of inflammatory mediators in culture supernatants

The concentration of nitrite, TNF-α and IL-10 in cell free supernatants were measured using commercially available kits and their recommended protocols (Promega Corporation, Madison, WI, and BD Biosciences, San Jose, CA). The Promega Griess Reagent system measures nitrite, a nonvolatile and stable breakdown product of nitric oxide. The minimum detectable nitrite concentration for this assay is 2.5 μM. The minimum detectable concentrations for TNF-α and IL-10 were 15.5 pg/mL and 31.3 pg/mL, respectively.

Statistical analysis

A power analysis using means and standard deviations of TNF-α production by RAW 264.7 macrophages stimulated with LPS, revealed a minimum of 3 animals per group to detect differences between PBS and baseline TDL, LPT TDL, or post-LPT TDL with a statistical power

of 0.90. Using another power analysis using means and standard deviations of TNF-α production by RAW 264.7 macrophages stimulated with LPS, revealed a minimum of 16 animals per group to detect differences between baseline TDL, LPT TDL, and post-LPT TDL with a statistical power of 0.90. Data are presented as arithmetic means ± standard error (SE). Values from triplicate wells from each condition and 1 experiment were averaged and shown in the figures. For evaluation of statistical significance, data were subjected to analyses of variance followed by a Tukey's multiple comparisons test. Statistical analyses were performed with GraphPad Prism version 7 (GraphPad Software). Differences among mean values with $P \leq 0.05$ were considered statistically significant.

Results

Thoracic lymph does not alter alveolar macrophage viability

The effect of LPT on TDL flow, protein concentration, and protein flux are summarized in Chapter III (Table 3.1). To determine the optimal dose of TDL to use in this study, cell-free baseline TDL was added at 0.5%, 1%, 2%, 5%, or 10% total volume per well from six dog donors (N=6) and co-cultured with or without LPS for 24 hours. Increasing the concentration of baseline TDL decreased the production of nitrite by MH-S. Specifically, 5% baseline TDL (9.5 ± 1.1 μM) or 10% (10 ± 0.1 μM) baseline TDL significantly (P<0.05) decreased the production of nitrite compared to 10% PBS (14 ± 0.1 μM) (Figure 4.1).

To identify the effect of TDL on alveolar macrophage viability, 5% PBS, 5% baseline TDL, 5% LPT TDL or 5% post-LPT TDL was cultured with the murine macrophage cell line, MH-S. After 24 hour culture, the viability of MH-S macrophages cultured with 5% PBS was

94%. The addition of 5% baseline TDL (94%), 5% LPT TDL (95%), or 5% post-LPT TDL (95%) to MH-S macrophages did not alter cell viability compared to 5% PBS (94%) (Figure 4.2).

Culture with LPS plus 5% PBS (91%), LPS plus 5% baseline TDL (90%), LPS plus 5% LPT TDL (89%), or LPS plus 5% post-LPT TDL (90%) did not alter viability compared to MH-S macrophages cultured with LPS plus 5% PBS (Figure 4.3).

Thoracic duct lymph suppressed MH-S macrophage activity in vitro

In the absence of LPS, the production of nitrite (0.4 ± 0.1 μM) by MH-S macrophages was below the assay limit of detection (data not shown). The addition of LPS significantly ($P<0.0001$) increased the production of nitrite by MH-S macrophages (10.1 ± 0.1 μM) compared to 5% PBS (0.4 ± 0.1 μM). During activation with LPS, the addition of 5% baseline TDL (7.4 ± 0.8 μM), 5% LPT TDL (8.0 ± 0.8 μM), or 5% post-LPT TDL (7.6 ± 0.8 μM) significantly ($P<0.001$) decreased the production of nitrite by MH-S macrophages compared to 5% PBS (10.1 ± 0.1 μM). There were no significant differences ($P>0.05$) between the production of nitrite by MH-S macrophages cultured in 5% baseline TDL, 5% LPT TDL, or 5% post-LPT TDL (Figure 4.4).

Similarly, in the absence of LPS the production of TNF-α (0.0 ± 0.0) was below the assay limit of detection (data not shown). The addition of LPS significantly ($P<0.0001$) increased the production of TNF-α by MH-S macrophages (692 ± 110 pg/mL) compared to 5% PBS (0.0 ± 0.0). During activation with LPS, the addition of 5% baseline TDL (170 ± 74), 5% TDL LPT (155 ± 142), or 5% post-LPT TDL (91 ± 81) significantly ($P<0.0001$) decreased the production of TNF-α by MH-S macrophages compared to 5% PBS (692 ± 110 pg/mL). Similarly, there were

no significant differences (P>0.05) between the production of TNF-α by MH-S macrophages between 5% baseline TDL, 5% LPT TDL, or 5% post-LPT TDL (Figure 4.5).

Finally, as above, in the absence of LPS the production of IL-10 (11.4 ± 16.1 pg/mL) by MH-S macrophages cultured with 5% PBS was below the assay limit of detection (data not shown). The addition of LPS significantly (P<0.01) increased the production of IL-10 by MH-S (314 ± 5.8 pg/mL) compared to 5% PBS (11.4 ± 16.1 pg/mL). During activation with LPS, the addition of 5% baseline TDL (273 ± 11.3 pg/mL), 5% LPT TDL (317 ± 9 pg/mL), or 5% post-LPT TDL (326.5 ± 20.3 pg/mL) did not significantly (P>0.05) alter the production of IL-10 compared to 5% PBS (314 ± 5.8 pg/mL) (Figure 4.6).

Discussion

In this study, TDL suppressed the release of nitrite and TNF-α by alveolar macrophages *in vitro*, suggesting that lymph contains inhibitory mediators. Furthermore, TDL did not alter the viability of alveolar macrophages suggesting the reduction of inflammatory mediators was not due to a decrease in viability. This is consistent with results reported in Chapter III using the RAW 264.7 macrophages. Other studies also reported that normal mesenteric lymph suppresses the response of microvascular pulmonary endothelial cells to endotoxin *in vitro* [82]. Collectively, these studies suggest that normal lymph contains anti-inflammatory mediators that inhibit the alveolar macrophage response to LPS.

TDL collected during baseline, LPT, or post-LPT equally suppressed the release of TNF-α by approximately 4-fold when MH-S macrophages were activated with LPS. TNF-α is potent inflammatory cytokine that induces local and systemic inflammation, but can also play a role in immunopathology. During *S. pneumoniae* infection, TNF-α can decrease survival in murine

model [49]. Therefore, limiting the production TNF-α by alveolar macrophages may decrease immunopathology during pneumonia [29].

An important difference to note between RAW 264.7 and MH-S macrophages, is the concentration of TNF-α released in response to LPS. Production of TNF-α by RAW 264.7 macrophages was approximately 5-fold higher than the production of TNF-α by MH-S macrophages. Of interest, alveolar macrophages have a lower expression of CD14, a co-receptor for TLR-4 and TLR-2, than peritoneal macrophages and interstitial macrophages [29, 88], which may explain the reduced activity of MH-S macrophages in response to LPS.

Interestingly, TDL did not inhibit the release of IL-10, an anti-inflammatory mediator, by MH-S macrophages in response to LPS. This is in contrast to the results reported in Chapter III suggesting lymph suppresses the release of IL-10 in response to LPS in RAW 264.7 macrophages. The differential effect of TDL on RAW 264.7 and MH-S macrophages may provide some insight to the possible factor(s) in lymph responsible for suppression. TDL may have suppressed IL-10 production in RAW 264.7 macrophages through a receptor not present in MH-S macrophages. Therefore, comparing the expression of receptors on RAW 264.7 and MH-S macrophages associated with IL-10 production may provide some rationale for this hypothesis.

Nonetheless the suppressive effect of baseline, LPT, and post-LPT TDL on the production of inflammatory mediators did not differ in this study. LPT increased lymph flow and lymphatic protein flux (Chapter III). Therefore, LPT may increase lymph-borne factors in circulation that travel to the lung and reduce pulmonary inflammation during pneumonia. In support, increasing the concentration of TDL suppressed the production of nitrite by RAW 264.7 macrophages in response to LPS (Figure AI.1). Also, the optimal dose of TDL used in this study has been proposed to be physiologically relevant [30]; therefore, it is likely that LPT could

increase the amount of thoracic duct lymph in circulation which may suppress alveolar macrophages *in vivo*. Persistently high levels of inflammatory cytokines have been associated with disease severity and mortality in patients with pneumonia [18]. Lastly, prolonged activation of macrophages is correlated with mortality in murine models of pneumonia [46]. Therefore, promoting resolution during *S. pneumoniae* infection may be beneficial for patients with pneumonia.

This study also adds to the current knowledge of lymphatic biology. Previous studies have shown the biologic effect of lymph on activated pulmonary endothelial cells [82], neutrophils [81], and macrophages (Chapter III). This study suggests that lymph suppresses the inflammatory response of alveolar macrophages in response to LPS. In support, normal rat mesenteric lymph blunted the response of human pulmonary endothelial cells to endotoxin *in vitro* suggesting lymph contains soluble protective factors [82]. Similarly, normal canine mesenteric lymph infused into rats with endotoxin-mediated lung injury, reduced cell-adhesion molecules, myeloperoxidase, and lung injury *in vivo* [37]. Furthermore, normal canine mesenteric lymph reduced endotoxin-mediated hepatic tissue damage and kidney injury *in vivo* suggesting lymph infused intravenously could protect systemically as well [35, 87]. Furthermore, the current study further supports the hypothesis that lymph-borne factors modulate pulmonary inflammation

The novel results from this study demonstrate that thoracic duct lymph suppresses the activation of alveolar macrophages. During respiratory infection, alveolar macrophages protect the lungs from tissue damage and maintain airway homeostasis [29]. Enhancing lymph flow using therapies such as LPT may mobilize protective lymph into circulation and the lung to promote resolution to improve clinical outcomes during respiratory infections. Understanding the

mechanism(s) of LPT will support the use of osteopathic medicine for respiratory infection and help guide osteopathic physicians in clinical practice.

Figure 4.1. Increasing TDL suppressed the production of nitrite by MH-S macrophages activated with LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.05$) compared to 10% PBS, 0.5% TDL, 1% TDL, and 2% TDL. N=3 dogs for baseline TDL from 1 experiment.

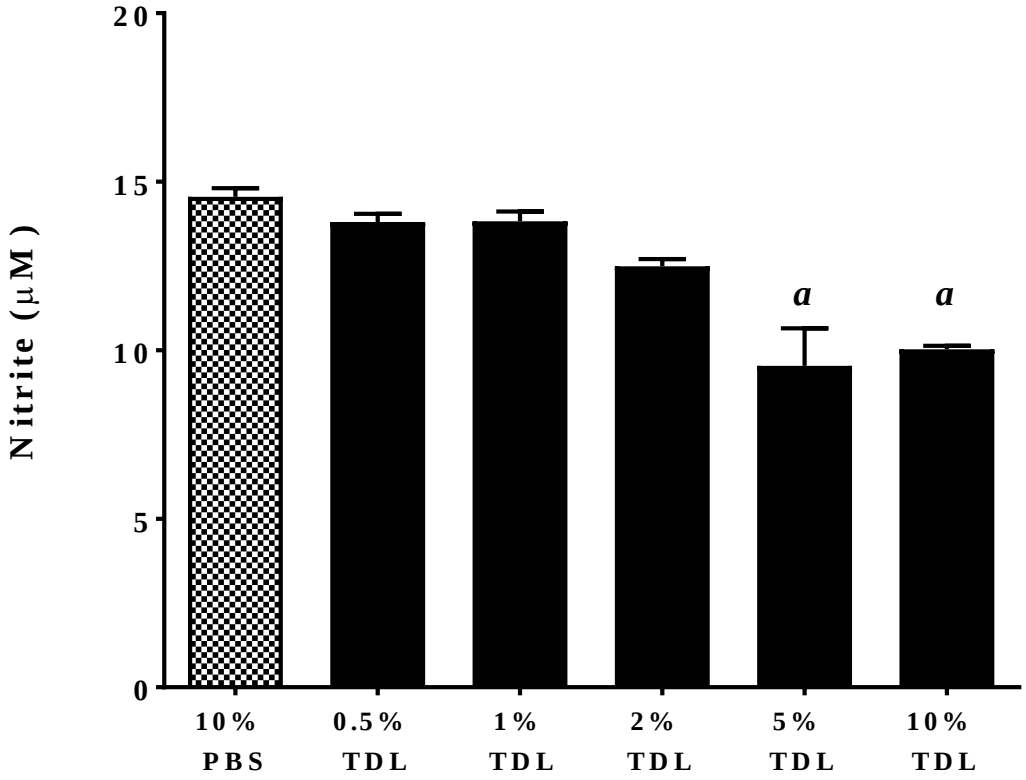

Nitrite (μM)
20
15
10
5
0
a
a
10% PBS
0.5% TDL
1% TDL
2% TDL
5% TDL
10% TDL

Figure 4.2. TDL did not alter MH-S macrophage viability in the absence of LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

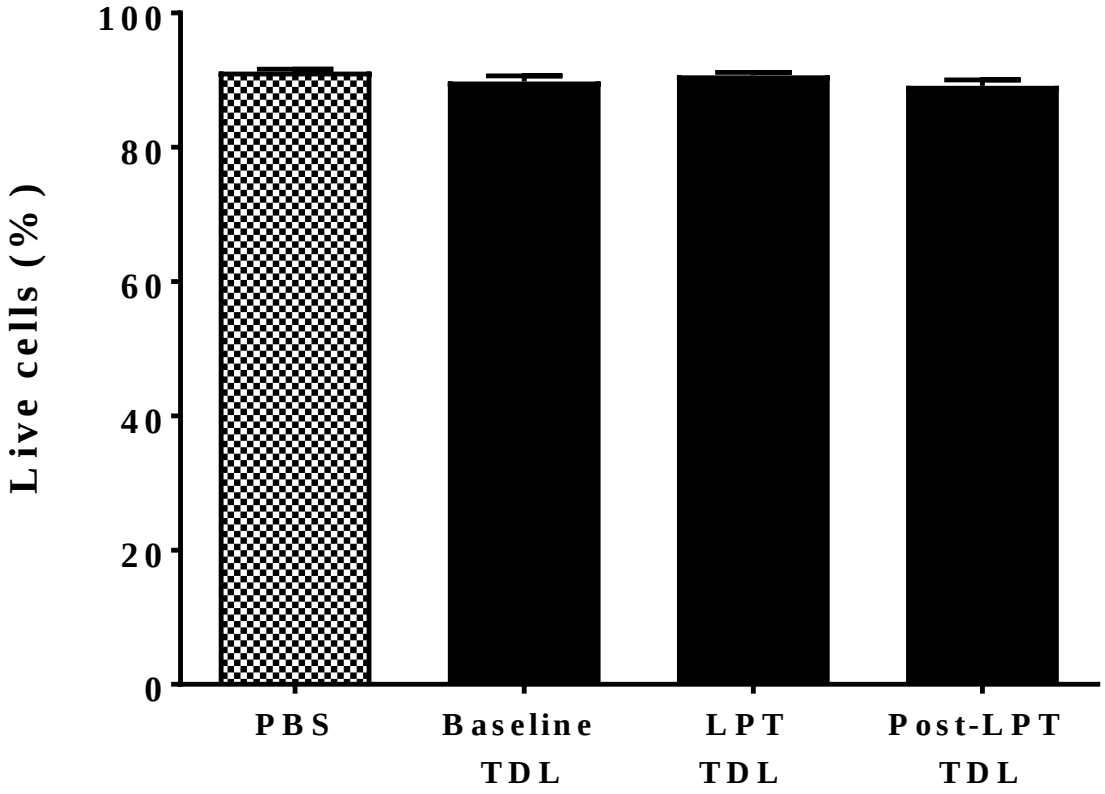

100
80
60
40
20
0
Live cells (%)
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 4.3. TDL did not alter MH-S macrophage viability in the presence of LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

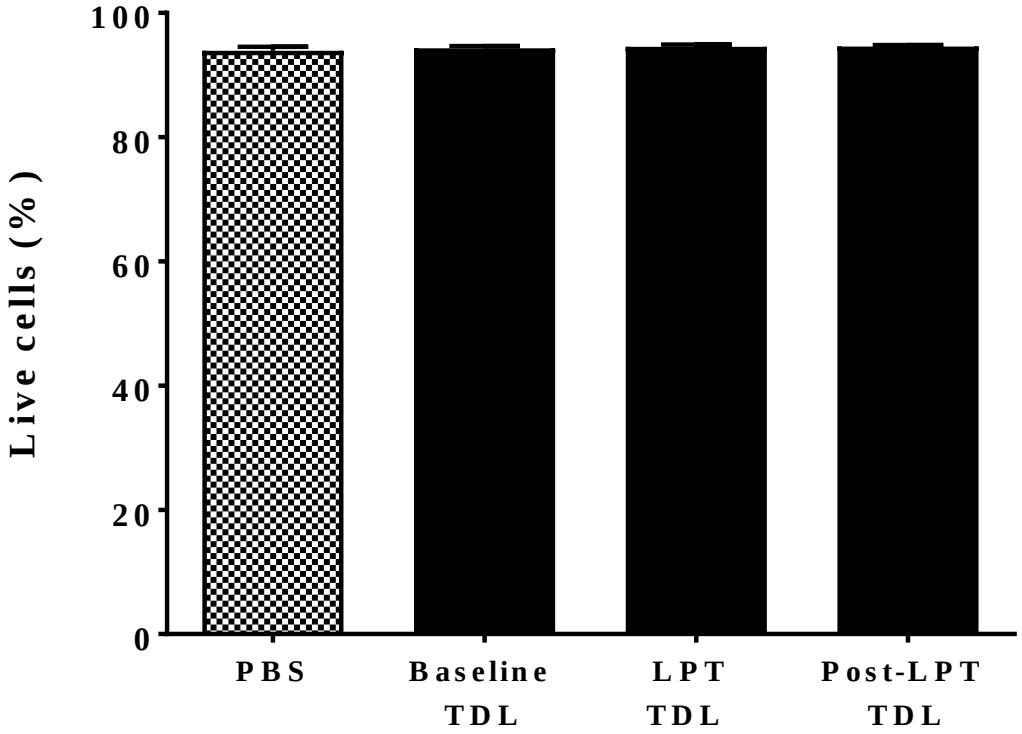

100
80
60
40
20
0
Live cells (%)
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 4.4. TDL suppressed the production of nitrite by MH-S macrophages activated with LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.001$) compared to PBS. N=3 dogs for baseline, LPT, and post-LPT lymph from 1 experiment.

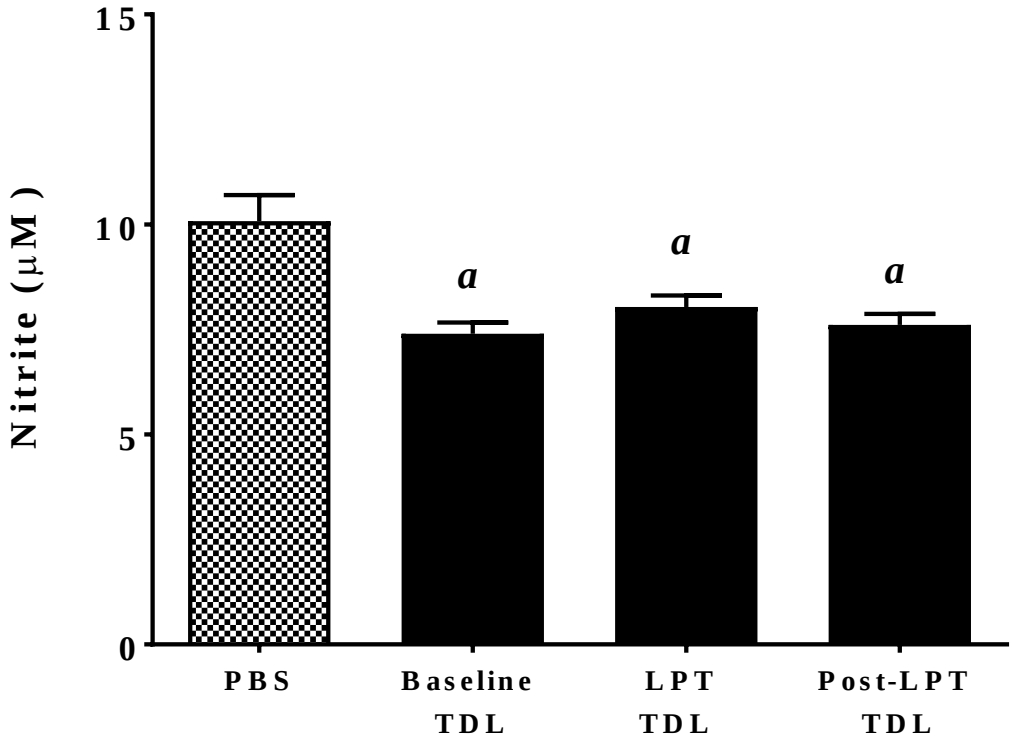

15
10
5
0
Nitrite (μM)
a
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 4.5. TDL suppressed the production of TNF-α by MH-S macrophages activated with LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. [a] denotes statistical decrease ($P < 0.0001$) compared to PBS. *N=3* dogs for baseline, LPT, and post-LPT lymph from 1 experiment.

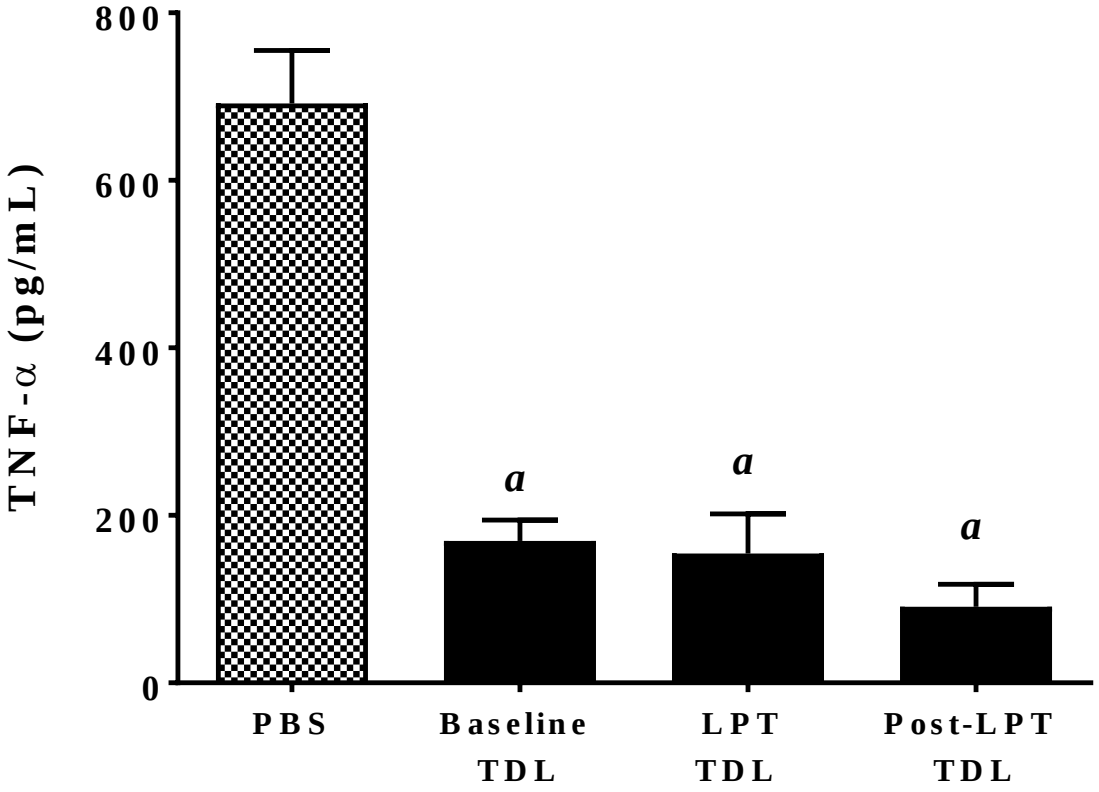
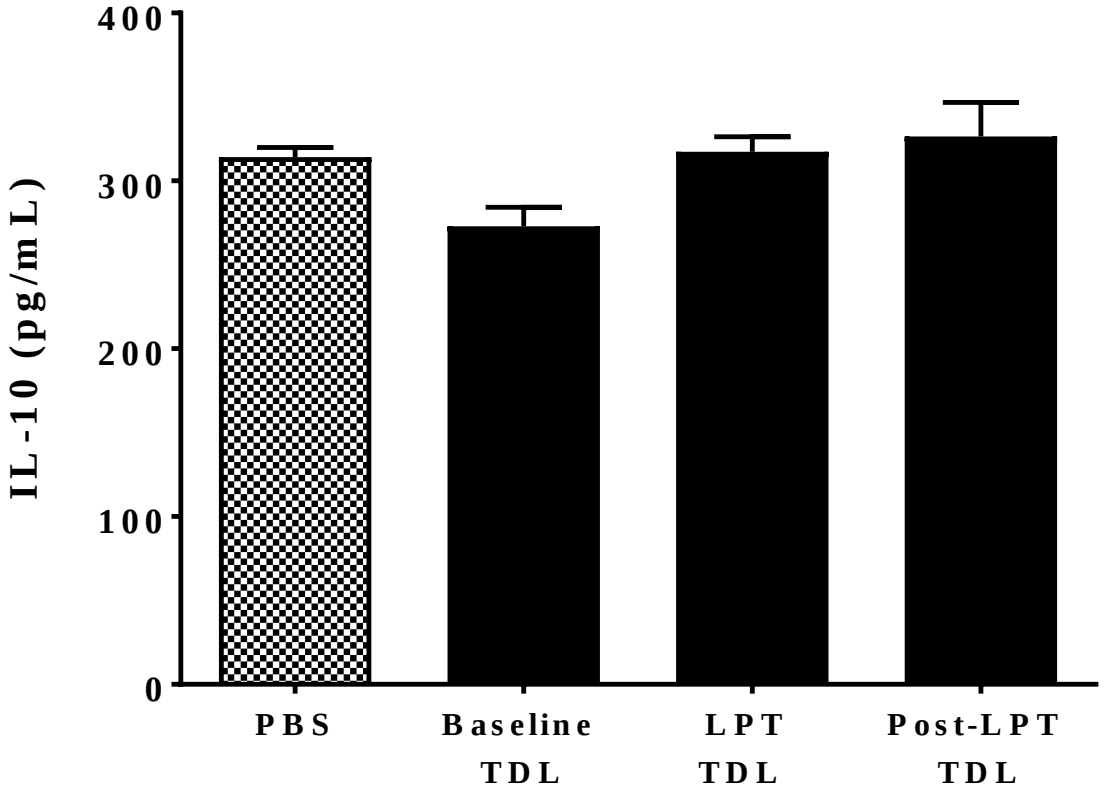

800
600
400
200
0
TNF-α (pg/mL)
a
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 4.6. TDL did not suppress the production of IL-10 by MH-S macrophages activated with LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT lymph from 1 experiment.


400
300
200
100
0
IL-10 (pg/mL)
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

CHAPTER V

THORACIC DUCT LYMPH SUPPRESSES MACROPHAGE ACTIVATION BY LIPOTEICHOIC ACID OR INTERFERON-GAMMA

The lymphatic system transports lymph from the interstitial space into circulation where it is delivered to the lung. Lymph-borne factors have been proposed to travel to the lung where they can modulate pulmonary inflammation. Lymphatic pump treatment (LPT) is a subset of osteopathic manipulative techniques that have been used clinically for the treatment of pneumonia. Recent studies have demonstrated that LPT can reduce the concentration of bacteria in the lungs of rats infected with *S. pneumoniae*. Furthermore, LPT mobilized bioactive lymph that suppressed macrophage activity *in vitro*. However, the effect of thoracic duct lymph (TDL) on macrophage activity in response to lipoteichoic acid (LTA) or interferon-gamma (IFN-γ) is unknown. LTA is a bacterial toxin present in *S. pneumoniae*, and IFN-γ is a cytokine important for host defense during pneumonia. Therefore, the purpose of this study was to determine if thoracic duct lymph mobilized during LPT would suppress macrophage activity in response to LTA and IFN-γ *in vitro*. Specifically, we hypothesized that thoracic duct lymph would suppress macrophage activity *in vitro*. To test this hypothesis, the murine macrophage cell line, RAW 264.7, or the murine alveolar macrophage cell line, MH-S, was cultured with baseline, LPT, or post-LPT TDL and co-cultured with or without LTA. RAW 264.7 or MH-S macrophages were also cultured with baseline, LPT, or post-LPT TDL and co-cultured with or without IFN-γ. As a

control, RAW 264.7 or MH-S macrophages were cultured with PBS and co-cultured with or without LTA or IFN-γ. After 24 hour culture, supernatant was collected to measure nitrite, tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) production. Macrophage viability was measured by flow cytometry using markers, annexin V and propidium iodide. During activation with LTA or IFN-γ, baseline, LPT, and post-LPT TDL significantly decreased the production of nitrite, TNF-α and IL-10 by RAW 264.7 macrophages. During activation with LTA or IFN-γ, baseline, LPT, and post-LPT TDL significantly decreased the production of nitrite, and TNF-α but not IL-10 by MH-S macrophages. Although LPT may not change the suppressive effect of TDL overall, LPT does increase lymph flow and lymphatic flux; therefore, LPT may mobilize protective lymph into circulation and the lung to promote resolution, prevent immunopathology, and improve clinical outcomes during pneumonia.

Introduction

The lymphatic system is responsible for interstitial fluid homeostasis, immune surveillance, and the absorption of fat. This interstitial fluid, known as lymph, may contain dead cells, immune cells, soluble antigens, microbes, toxins, proteins and lipids that are returned into systemic circulation through the lymphatic system [1, 2]. Lymph enters venous circulation through the subclavian vein where it is delivered to the first major vascular bed in the lung [30] . The lymphatic vasculature that connects the gastrointestinal region to the lungs has been of interest in the study of respiratory diseases that have been associated with intestinal inflammation [32, 36, 37]. Previous studies reported that lymph suppressed the inflammatory response of pulmonary endothelial cells [82], macrophages (Chapter III) and alveolar macrophages (Chapter IV) *in vitro*. Collectively, this literature suggests lymph can suppress

inflammation and may protect the lung by reducing the pulmonary inflammatory response during pulmonary disease.

LPT is a subset of osteopathic manipulative techniques that target the musculoskeletal system and designed to enhance the flow of lymph [50, 51]. Clinical studies support the use of LPT as an adjunctive therapy for the treatment of pneumonia, influenza, and pulmonary disease [52, 53, 55]. However, little is known about the mechanism of protection offered by LPT. LPT has been previously shown to enhance lymph flow, the concentration of leukocytes and the flux of cytokines, chemokine and reactive oxygen and nitrogen species in the thoracic duct of animals [63-66]. Furthermore, LPT protected rats against *S. pneumoniae* infection [58, 69]. Collectively, these studies suggest that lymph-enhancing techniques may enhance pulmonary immunity.

The recognition of lipoteichoic acid on the surface of *S. pneumoniae* by alveolar macrophaages results in phagocytosis and the release antibacterial factors and inflammatory cytokines [24]. During the earlier stages of infection, neutrophils and monocytes are recruited to the lung to aid in bacterial clearance. Cytokines, such as TNF-α, IL-1, IL-6, and IFN-γ, and anti-bacterial factors, such as NO and myeloperoxidase, are quickly released in the lung [18]. During the later stages of infection, macrophages aid in tissue repair by releasing anti-inflammatory cytokines such as IL-10 [29]. However, uncontrolled bacterial proliferation and inflammation results in severe pulmonary tissue damage and mortality [18, 24].

IFN-γ is an important cytokine for host defense that can prime neutrophils and macrophages and is associated with enhanced clearance of *S. pneumoniae* [48, 89]. However, IFN-γ can also contribute to persistent inflammation and immunopathology [47, 48]. Furthermore, the recruitment of neutrophils and monocytes to the lungs contribute to the clinical

features of pneumonia [24]. Therefore, promoting resolution during pneumonia infection may aid in improving clinical outcomes.

Lymph has been reported to inhibit the response of pulmonary endothelial cells to endotoxins [82]. Furthermore, TDL has been reported to inhibit macrophage activation to lipopolysaccharide. Specifically, TDL collected before, during, and after LPT inhibited macrophage activation (Chapter IV). It is possible that lymph-enhancing techniques, such as LPT, aid in mobilizing lymph-borne factors into the circulation and the lungs. However, it is unknown if TDL inhibits macrophage activation by bacterial toxins and cytokines associated with *S. pneumoniae* infection.

The purpose of this study was to determine whether the lymph mobilized during LPT would suppress macrophage activation by LTA or IFN-γ *in vitro*. Specifically, we hypothesized that TDL collected before, during, or after LPT would suppress macrophage activation *in vitro*. The use of LPT to mobilize suppressive factors in lymph may redistribute lymph-borne factors to the lung to reduce inflammation in response to pneumonia.

Methods

Animals

The animals were housed as previously described (Chapter III). 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). Six adult mongrel dogs, free of clinically evident signs of disease, were used for this study.

Surgical procedure

The surgical procedure was performed as previously described (Chapter III). Before surgery, the dogs were fasted overnight and then anesthetized with sodium pentobarbital. Under anesthesia, the dogs were intubated and ventilated with room air supplemented with O_2 to maintain normal arterial blood gases. A pressure transducer connected to a femoral artery catheter was used to monitor arterial blood pressure, arterial blood gases, and used to administer additional anesthetic. The chest was opened by a left lateral thoracotomy in the left fourth intercostal space and the thoracic duct was isolated from the connective tissue. The thoracic duct was ligated and a PE 60 catheter inserted into the duct, caudal to the ligation, and the outflow tip positioned eight centimeters below the heart level to compensate for the hydraulic resistance of the catheter. Approximately sixty minutes after securing the catheter with a ligature, thoracic lymph was collected at one-minute intervals for 4 minutes before LPT, 4 minutes during LPT, and 10 minutes after the cessation of LPT.

Lymphatic pump technique

Abdominal LPT was performed as previously described (Chapter III). The anesthetized dogs were placed in a right lateral recumbent position. To perform LPT, the operator contacted the the ventral side of the animal's abdomen bilaterally below the costo-diaphragmatic junction. Pressure was applied and released medially and cranially to compress the abdomen until resistance was met against the diaphragm. Compressions to the abdomen were administered at a rate of approximately 1 pump per sec for a total of 4 min.

Cell culture

The murine monocyte/macrophage cell line, RAW 264.7 (ATCC TIB-71) and the murine alveolar macrophage cell line, MH-S (ATCC CRL-2019), were used to determine the inhibitory

effect of thoracic duct lymph on macrophage activation. RAW 264.7 macrophages were grown in Dulbecco's modified Eagle high glucose medium with 10% fetal bovine serum (Hyclone) at 37°C under 5% CO_2. RAW 264.7 macrophages were passaged 5-15 times by scraping adherent cells. MH-S macrophages were grown and maintained in Roswell Park Memorial Institute 1640 medium (ThermoFisher) with 10% fetal bovine serum (Hyclone) and 0.05mM of 2-mercaptoethanol (ThermoFisher) at 37°C under 5% CO_2. MH-S cells were passaged 5-15 times by collecting both suspended and adherent cells using 0.25% trypsin (Hyclone). One day after passage, RAW 264.7 or MH-S macrophages were counted using trypan blue (Corning, Manassas, VA) exclusion and $1x10^5$ cells per well were cultured for one hour to allow the cells to acclimate. The optimal dose of LTA was determined by measuring the production of nitrite, TNF-α and viability of RAW 264.7 or MH-S macrophages (data not shown). TDL from six dog donors were pooled into groups of two (*N*=3) with equal volumes of TDL from each dog. After one hour, cell-free baseline, LPT, or post-LPT TDL was added at 5% total volume per well and co-cultured with or without 100 ug (RAW 264.7) or 200 ug (MH-S) per well of LTA (Invivogen, San Diego, CA) for 24 hours. As a control, RAW 264.7 or MH-S macrophages were cultured for one hour to allow cells to acclimate then sterile 1X PBS (Hyclone) at 5% total volume per well was added with or without 100 ug (RAW 264.7) or 200 ug (MH-S) per well of LTA for 24 hours. Each condition was repeated in triplicate and replicate supernatants and cells treated assayed separately.. After 24 hours of culture, cell-free supernatants were collected by centrifugation at 400G for 5 minutes at 4°C (RAW 264.7) or 130G for 10 minutes at 4°C (MH-S) and stored at -80°C.

In a separate set of experiments, $1x10^5$ RAW 264.7 or MH-S macrophages were cultured for 1 hour to allow the cells to acclimate. The optimal dose of IFN-γ was determined by

measuring the production of nitrite, TNF-α and viability of RAW 264.7 or MH-S macrophages (data not shown). After one hour, cell-free baseline, LPT, or post-LPT TDL was added at 5% total volume per well from six dog donors (N=6) and co-cultured with or without 80ng per well of IFN-γ (R&D systems, Minneapolis, MN) in triplicate for 24 hours. As a control, RAW 264.7 or MH-S macrophages were cultured for one hour to allow cells to acclimate then sterile 1X PBS (Hyclone) at 5% total volume per well added with or without 80 ng per well of IFN-γ in triplicate for 24 hours. After 24 hours of culture, cell-free supernatants were collected by centrifugation at 400G for 5 minutes at 4°C (RAW 264.7) or 130G for 10 minutes at 4°C (MH-S) and stored at -80°C.

Cell viability

After the collection of cell-free supernatants, 10% FBS in PBS was added to each well and cells collected by scraping. Cells were transferred to a 96-well plate and centrifuged at 400G for 5 minutes at 4°C (RAW 264.7) or 130G for 10 minutes at 4°C (MH-S). Cells were resuspended in a 5% annexin V and 5% propidium iodide solution with 1X annexin V binding buffer (BD Biosciences) for 15 minutes at room temperature. Cells were subjected to a BD LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJO software. The percentages of live cells were determined by gating on intact cells not positive for annexin V or propidium iodide. There were no significant differences in the percentage of cell debris or dead cells when cells were cultured with 5% PBS, 5% baseline TDL, 5% LPT TDL, or 5% post-LPT TDL with or without LTA or IFN-γ.

Measurement of inflammatory mediators in culture supernatants

The concentration of nitrite, TNF-α, and IL-10 in cell free supernatants were measured using commercially available kits and their recommended protocols (Promega Corporation, and BD Biosciences). The Promega Griess Reagent system measures nitrite, a nonvolatile and stable breakdown product of nitric oxide. The minimum detectable nitrite concentration for this assay is 2.5 μM. The minimum detectable concentrations for TNF-α and IL-10 were, 15.5 pg/mL and 31.3 pg/mL, respectively.

Statistical analysis

A power analysis using means and standard deviations of TNF-α production by RAW 264.7 macrophages stimulated with LPS, revealed a minimum of 3 animals per group to detect differences between PBS and baseline TDL, LPT TDL, or post-LPT TDL with a statistical power of 0.90. Using another power analysis using means and standard deviations of TNF-α production by RAW 264.7 macrophages stimulated with LPS, revealed a minimum of 16 animals per group to detect differences between baseline TDL, LPT TDL, and post-LPT TDL with a statistical power of 0.90. Data are presented as arithmetic means ± standard error (SE). Values from triplicate wells from each condition and 1 experiment were averaged and shown in the figures. For evaluation of statistical significance, data were subjected to analyses of variance followed by a Tukey's multiple comparisons test. Statistical analyses were performed with GraphPad Prism version 7 (GraphPad Software). Differences among mean values with $P \leq 0.05$ were considered statistically significant.

Results

TDL suppresses RAW 264.7 macrophage activation by LTA

The effect of LPT on TDL flow, protein concentration, and protein flux are summarized in Chapter III (Table 1). The dose of TDL is consistent with previous studies in Chapters III and IV. After 24 hour culture, the viability of RAW 264.7 macrophages cultured with 5% PBS was 96%. The addition of 5% baseline TDL (97%), 5% LPT TDL (96%), or 5% post-LPT TDL (96%) to RAW 264.7 macrophages did not alter cell viability compared to 5% PBS (96%) (Figure 5.1).

In the absence of LTA, the production of nitrite (2.1 ± 0.02 μM), TNF-α (4.5 ± 4.5 pg/mL), and IL-10 (0 ± 0 pg/mL) by RAW 264.7 macrophages (data not shown) were below the assay limits of detection.. To determine if thoracic duct lymph suppresses macrophage activation by LTA, RAW 264.7 macrophages were cultured with 5% baseline TDL, 5% LPT TDL or 5% post-LPT TDL for 24 hours.

Culture with LTA plus 5% PBS (98%), LTA plus 5% baseline TDL (98%), LTA plus 5% LPT TDL (95%), or LTA plus 5% post-LPT TDL (97%) did not alter viability compared to RAW 264.7 macrophages cultured with LTA plus 5% PBS (98%) (Figure 5.2).

The addition of LTA significantly ($P<0.0001$) increased the production of nitrite (22.4 ± 0.9 μM) by RAW 264.7 macrophages compared to 5% PBS (2.1 ± 0.02 μM). During activation with LTA, the addition of 5% baseline TDL (14 ± 0.9 μM), 5% LPT TDL (15.2 ± 0.8 μM), or 5% post-LPT TDL (15.9 ± 0.5 μM) significantly ($P<0.001$) decreased the production of nitrite compared to 5% PBS (22.4 ± 0.9 μM). However, there were no significant differences ($P>0.05$)

between the production of nitrite by RAW 264.7 macrophages cultured with 5% baseline TDL, 5% LPT TDL, or 5% post-LPT TDL (Figure 5.3).

The addition of LTA significantly (P<0.001) increased the production of TNF-α (1977 ± 29 pg/mL) by RAW 264.7 macrophages compared to 5% PBS (4.5 ± 4.5 pg/mL). During activation with LTA, the addition of 5% baseline TDL (1271 ± 130 pg/mL), significantly (P<0.01) decreased the production of TNF-α by RAW 264.7 macrophages compared to 5% PBS (1977 ± 29 pg/mL). However, during activation with LTA, the addition of 5% LPT TDL (1511 ± 62 pg/mL) or 5% post-LPT TDL (1612 ± 76 pg/mL) did not significantly (P>0.05) alter the production of TNF-α by RAW 264.7 macrophages compared to 5% PBS (1977 ± 29 pg/mL) (Figure 5.4).

The addition of LTA significantly (P<0.0001) increased the production of IL-10 (2274 ± 38 pg/mL) by RAW 264.7 macrophages compared to 5% PBS (0 ± 0 pg/mL). During activation with LTA, the addition of 5% baseline TDL (1235 ± 195 pg/mL) significantly (P<0.01) decreased the production of IL-10 by RAW 264.7 macrophages compared to 5% PBS (2274 ± 38 pg/mL). However, during activation with LTA, the addition of 5% LPT TDL(1659 ± 130 pg/mL) or 5% post-LPT TDL(1813 ± 70 pg/mL) did not significantly (P>0.05) alter the production of IL-10 by RAW 264.7 macrophages compared to 5% PBS (2274 ± 38 pg/mL) (Figure 5.5).

TDL suppresses MH-S macrophage activation by LTA

After 24 hour culture, the viability of MH-S macrophages cultured with 5% PBS was 94%. The addition of 5% baseline TDL (94%), 5% LPT TDL (95%), or 5% post-LPT TDL (95%) to MH-S macrophages did not alter cell viability compared to 5% PBS (94%) (Figure 5.6).

In the absence of LTA, the production of nitrite, TNF-α, and IL-10 by MH-S macrophages were 0.8 ± 0.3 μM, 43.8 ± 11 pg/mL, and 0 ± 0 pg/mL respectively (data not shown). To determine if TDL suppresses alveolar macrophage activation by LTA, MH-S macrophages were cultured with 5% baseline TDL, 5% LPT TDL or 5% post-LPT TDL for 24 hours.

Culture with LTA plus 5% PBS (90%), LTA plus 5% baseline TDL (93%), LTA plus 5% LPT TDL (95%), or LTA plus 5% post-LPT TDL (92%) did not alter viability compared to MH-S macrophages cultured with LTA plus 5% PBS (90%) (Figure 5.7).

The addition of LTA significantly (P<0.0001) increased the production of nitrite (12.6 ± 0.2 μM) by MH-S macrophages compared to 5% PBS (0.8 ± 0.3 μM). During activation with LTA, the addition of 5% baseline TDL (9.1 ± 0.1 μM), 5% LPT TDL (10 ± 0.2 μM), or 5% post-LPT TDL (9.8 ± 0.2 μM) significantly (P<0.0001) decreased the production of nitrite compared to 5% PBS (12.6 ± 0.2 μM). The production of nitrite by MH-S macrophages cultured with LTA plus 5% LPT TDL (10 ± 0.2 μM) was significantly higher (P<0.05) than MH-S macrophages cultured with LTA plus 5% baseline lymph (9.1 ± 0.1 μM) (Figure 5.8).

The addition of LTA significantly (P<0.0001) increased the production of TNF-α (740 ± 22 pg/mL) by MH-S macrophages compared to 5% PBS (43.8 ± 11 pg/mL). During activation with LTA, the addition of 5% baseline TDL (316 ± 23 pg/mL), 5% LPT TDL (331 ± 12 pg/mL), or 5% post-LPT TDL (298 ± 8 pg/mL), significantly (P<0.01) decreased the production of TNF-α by MH-S macrophages compared to 5% PBS (740 ± 22 pg/mL) Furthermore, there were no significant differences (P>0.05) between the production of TNF-α by MH-S macrophages cultured with LTA plus 5% baseline TDL, LTA plus 5% LPT TDL, or LTA plus 5% post-LPT TDL (Figure 5.9).

The addition of LTA significantly (P<0.0001) increased the production of IL-10 (279 ± 8 pg/mL) by MH-S macrophages compared to 5% PBS (0 ± 0 pg/mL). Interestingly, the addition of 5% baseline TDL (295 ± 10 pg/mL), 5% LPT TDL (282 ± 9 pg/mL), or 5% post-LPT TDL (298 ± 15 pg/mL) did not significantly (P>0.05) alter the production of IL-10 by MH-S macrophages compared to 5% PBS (279 ± 8 pg/mL). Furthermore, there were no significant differences (P>0.05) between the production of IL-10 by MH-S macrophages cultured with LTA plus 5% baseline TDL, LTA plus 5% LPT TDL, or LTA plus 5% post-LPT TDL (Figure 5.10).

TDL suppresses RAW 264.7 macrophage activation by IFN-γ

In the absence of IFN-γ, the production of nitrite (2.1 ± 0.02 μM), TNF-α (4.5 ± 4.5 pg/mL), and IL-10 (0 ± 0 pg/mL) by RAW 264.7 macrophages (data not shown) were below the assay limits of detection. To determine if TDL suppresses macrophage activation by IFN-γ, RAW 264.7 macrophages were cultured with 5% baseline TDL, 5% LPT TDL or 5% post-LPT TDL for 24 hours.

Culture with IFN-γ plus 5% PBS (92%), IFN-γ plus 5% baseline TDL (89%), IFN-γ plus 5% LPT TDL (91%), or IFN-γ plus 5% post-LPT TDL (92%) did not alter viability compared to RAW 264.7 macrophages cultured with IFN-γ plus 5% PBS (92%) (Figure 5.11).

The addition of IFN-γ significantly (P<0.0001) increased the production of nitrite (27.6 ± 0.7 μM) by RAW 264.7 macrophages compared to 5% PBS (2.1 ± 0.02 μM). During activation with IFN-γ, the addition of 5% baseline TDL (18.1 ± 0.4 μM) or 5% LPT TDL (19.7 ± 0.6 μM), significantly (P<0.05) decreased the production of nitrite compared to 5% PBS (27.6 ± 0.7 μM). However, during activation with IFN-γ, the addition of 5% post-LPT TDL (22 ± 0.4 μM) did not significantly (P>0.05) alter the production of nitrite compared to 5% PBS (27.6 ± 0.7 μM).

Furthermore, there were no significant differences (P>0.05) between the production of nitrite by RAW 264.7 macrophages cultured in 5% baseline TDL or 5% LPT TDL (Figure 5.12).

The addition of IFN-γ significantly (P<0.001) increased the production of TNF-α (484 ± 54 pg/mL) by RAW 264.7 macrophages compared to 5% PBS (4.5 ± 4.5 pg/mL). During activation with IFN-γ, the addition of 5% baseline TDL (84 ± 26 pg/mL), 5% LPT TDL (101 ± 39 pg/mL), or 5% post-LPT TDL (218 ± 50 pg/mL), significantly (P<0.05) decreased the production of TNF-α by RAW 264.7 macrophages compared to 5% PBS (484 ± 54 pg/mL) (Figure 5.13).

The addition of IFN-γ significantly (P<0.0001) increased the production of IL-10 (1574 ± 132 pg/mL) by RAW 264.7 macrophages compared to 5% PBS (0 ± 0 pg/mL). During activation with IFN-γ, the addition of 5% baseline TDL (327 ± 53 pg/mL), 5% LPT TDL (539 ± 111 pg/mL), or 5% post-LPT TDL (638 ± 76 pg/mL), significantly (P<0.01) decreased the production of IL-10 by RAW 264.7 macrophages compared to 5% PBS (2274 ± 38 pg/mL) (Figure 5.14).

TDL suppresses MH-S macrophage activation by IFN-γ

In the absence of IFN-γ, the production of nitrite, TNF-α and IL-10 by MH-S macrophages were 0.8 ± 0.3 μM, 43.8 ± 11 pg/mL, and 0 ± 0 pg/mL respectively (data not shown). To determine if TDL suppresses alveolar macrophage activation by IFN-γ, MH-S macrophages were cultured with 5% baseline TDL, 5% LPT TDL or 5% post-LPT TDL for 24 hours.

Culture with IFN-γ plus 5% PBS (82%), IFN-γ plus 5% baseline TDL (88%), IFN-γ plus 5% LPT TDL (90%), or IFN-γ plus 5% post-LPT TDL (84%) did not alter viability compared to MH-S macrophages cultured with IFN-γ plus 5% PBS (82%) (Figure 5.15).

The addition of IFN-γ significantly (P<0.0001) increased the production of nitrite (20.1 ± 0.2 μM) by MH-S macrophages compared to 5% PBS (0.8 ± 0.3 μM). During activation with IFN-γ, the addition of 5% baseline TDL (17.5 ± 0.4 μM), 5% LPT TDL (15.2 ± 0.4 μM), or 5% post-LPT TDL (15.5 ± 0.2 μM) significantly (P<0.01) decreased the production of nitrite compared to 5% PBS (20.1 ± 0.2 μM). The production of nitrite by MH-S macrophages cultured with IFN-γ plus 5% baseline TDL (17.5 ± 0.4 μM) was significantly higher (P<0.01) than MH-S macrophages cultured with IFN-γ plus 5% LPT TDL (15.2 ± 0.4 μM) or IFN-γ plus 5% post-LPT TDL (15.5 ± 0.2 μM) (Figure 5.16).

The addition of IFN-γ significantly (P<0.0001) increased the production of TNF-α (226 ± 13 pg/mL) by MH-S macrophages compared to 5% PBS (43.8 ± 11 pg/mL). During activation with IFN-γ, the addition of 5% baseline TDL (15 ± 12.3 pg/mL), 5% LPT TDL (5.4 ± 3.2 pg/mL), or 5% post-LPT TDL (11.9 ± 11.6 pg/mL), significantly (P<0.0001) decreased the production of TNF-α by MH-S macrophages compared to 5% PBS (226 ± 13 pg/mL) Furthermore, there were no significant differences (P>0.05) between the production of TNF-α by MH-S macrophages cultured in IFN-γ plus 5% baseline TDL, IFN-γ plus 5% LPT TDL, or IFN-γ plus 5% post-LPT TDL (Figure 5.17).

The addition of IFN-γ significantly (P<0.0001) increased the production of IL-10 (96 ± 7 pg/mL) by MH-S macrophages compared to 5% PBS (0 ± 0 pg/mL). Interestingly, the addition of 5% baseline TDL (79.2 ± 2.8 pg/mL), 5% LPT TDL (89.1 ± 7.3 pg/mL), or 5% post-LPT

TDL (86.4 ± 4 pg/mL) did not significantly (P>0.05) alter the production of IL-10 by MH-S macrophages compared to 5% PBS (96 ± 7 pg/mL). Furthermore, there were no significant differences (P>0.05) between the production of IL-10 by MH-S macrophages cultured in IFN-γ plus 5% baseline TDL, IFN-γ plus 5% LPT TDL, or IFN-γ plus 5% post-LPT TDL (Figure 5.18).

Discussion

TDL suppressed the release of inflammatory mediators from RAW 264.7 and MH-S macrophages in response to LTA or IFN-γ without altering cell viability. These results are consistent with previous studies where TDL suppressed the activity of macrophages cultured with LPS (Chapters III-IV). Collectively, these results suggest TDL contains an inhibitory factor(s) that is not restricted to LPS, LTA, or IFN-γ.

Importantly, this study is the first to report that lymph suppresses macrophage activity in response to LTA or IFN-γ suggesting the suppressive factor(s) in TDL is not restricted to toll-like receptor or IFN-γ receptor signaling. TLR ligands and IFN-γ bind to target cells through independent receptors and adapter molecules to initiate signaling cascades [19] suggesting the effect of lymph may be intracellular. The activation of NF-κB results from downstream toll-like receptor signaling and indirectly results from IFN-γ receptor signaling [90]. One possible molecule is nitric oxide, which has been identified as an inhibitor of NF-κB [91] and measured in the lymph samples used in Chapter III-V.

In some conditions, TDL mobilized during or after LPT was less inhibitory to RAW 264.7 macrophages during LPS, LTA, or IFN-γ activation compared to baseline. Similarly, TDL mobilized during or after LPT was more suppressive to MH-S macrophages. These differences

between baseline, LPT, and post-LPT TDL suggested that LPT may have changed the composition of lymph. A power analysis revealed more animals per group are necessary to detect differences between baseline, LPT, and post-LPT TDL; therefore in future studies more animals per group should be considered. However, it is important to note that although these differences in nitrite and TNF-α were statistically significant, these changes were moderate; therefore, it is unclear whether these differences would be significant *in vivo*. Total protein concentration in TDL did not change between baseline TDL, LPT TDL, and post-LPT TDL. However, LPT increases lymph flow and lymphatic protein flux approximately 10-fold; therefore, the more profound effect of LPT on TDL is likely through flow and flux of proteins. (Chapter III).

Lymph enters venous circulation through the subclavian vein where it is delivered to the first major vascular bed in the lung [30]. By increasing TDL flow, LPT likely redistributes a large pool of lymph to the lung. During pneumonia, this LPT-mobilized lymph may protect the lung by further suppressing pulmonary inflammation. In support, increasing the concentration of TDL suppressed the release of nitrite from RAW 264.7 macrophages (Figure AI.1) and MH-S macrophages (Chapter IV) in response to LPS *in vitro*, suggesting TDL reduced macrophage activation in a dose-dependent manner. Therefore, LPT may be enhancing the flux of lymph-borne factors into circulation and the lung to reduce pulmonary inflammation.

This study is the first to demonstrate that TDL suppresses the activation of macrophages. Furthermore, TDL suppressed macrophage activity in response to LTA or IFN-γ, which have not been explored in the context of lymph biology. Enhancing lymph flow using therapies such as LPT may mobilize protective lymph into circulation and the lung to promote resolution by suppressing alveolar macrophage activity and the activity of recruited monocytes in the lung thereby improving clinical outcomes during respiratory infection. Understanding the

mechanism(s) of LPT will support the use of osteopathic medicine for respiratory infection and help guide osteopathic physicians in clinical practice.

Figure 5.1. TDL did not alter RAW 264.7 macrophage viability in the absence of LTA or IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

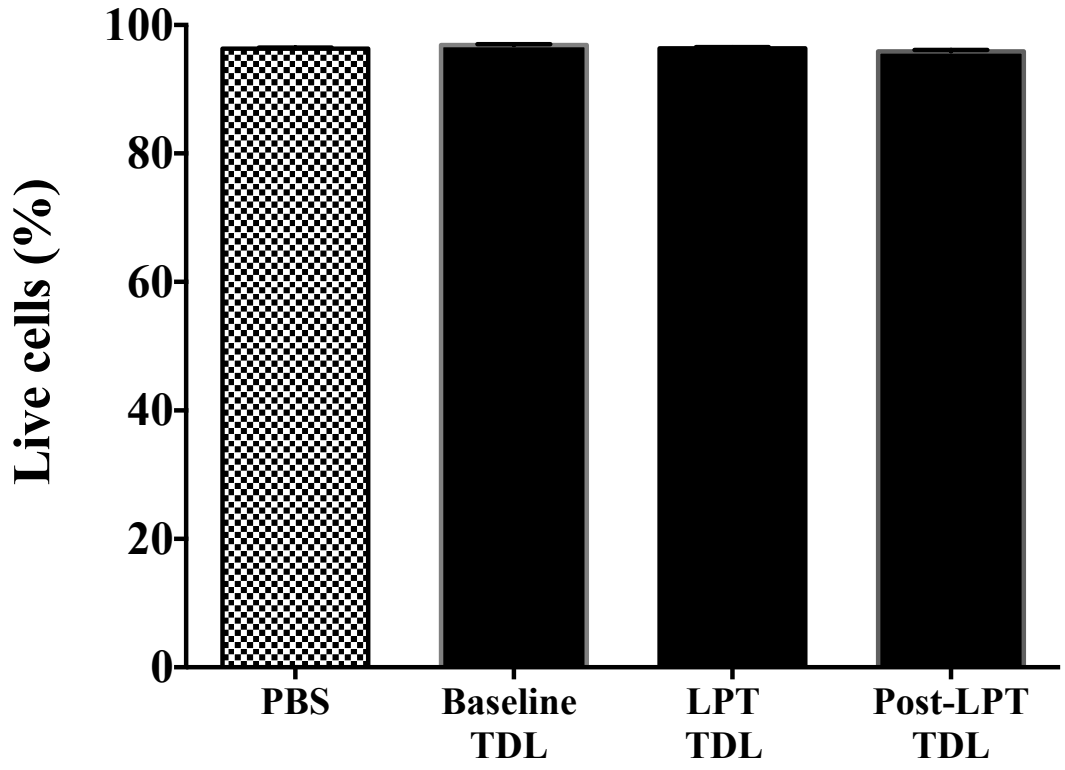

Live cells (%)
100
80
60
40
20
0
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.2. TDL did not alter RAW 264.7 macrophage viability in the presence of LTA. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

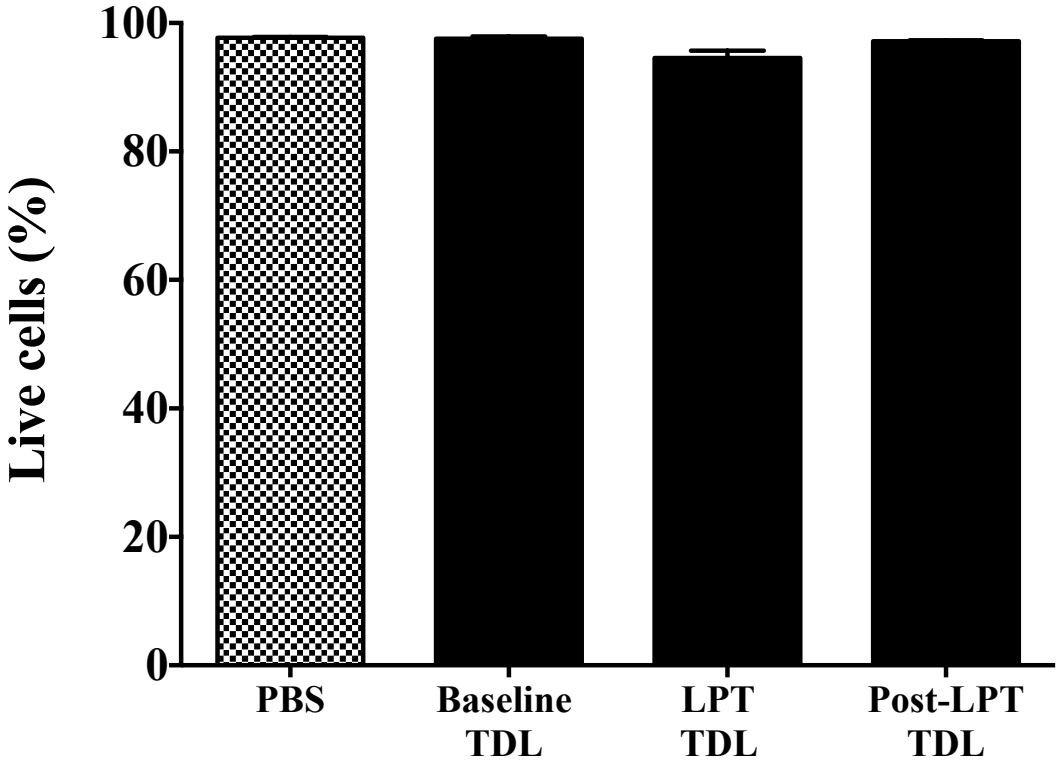

Live cells (%)
100
80
60
40
20
0
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.3. TDL suppressed the production of nitrite by RAW 264.7 macrophages activated with LTA. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.001$) compared to PBS. *N=3* dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

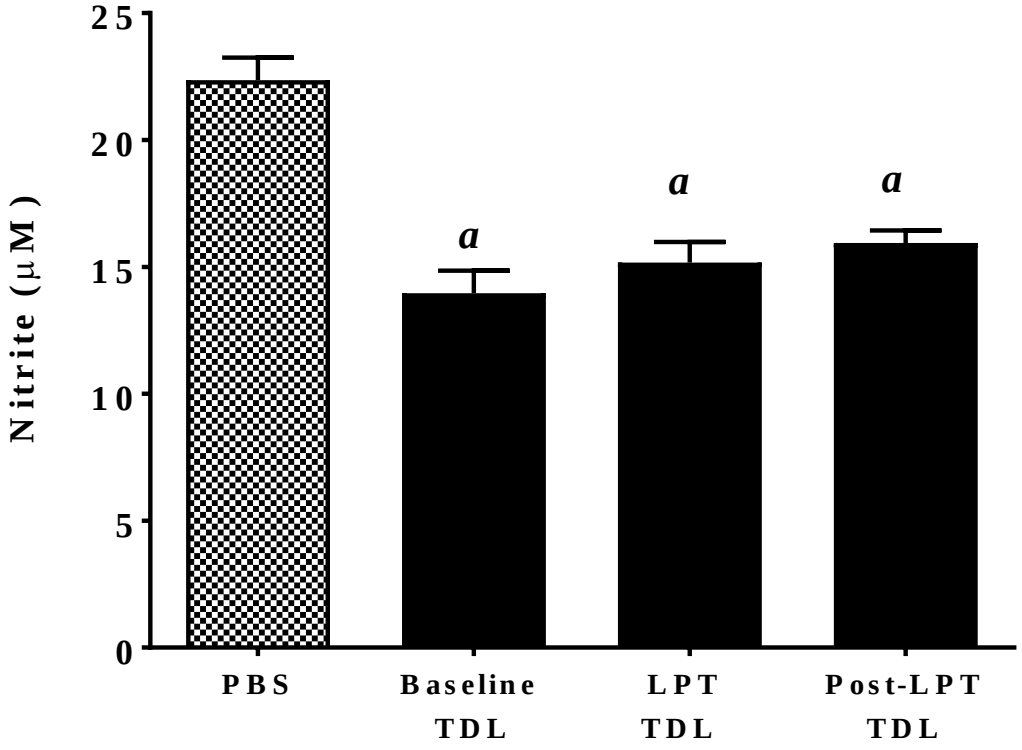

Nitrite (μM)
25
20
15
10
5
0
a
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.4. TDL suppressed the production of TNF-α by RAW 264.7 macrophages activated with LTA. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.01$) compared to PBS. *N=3* dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

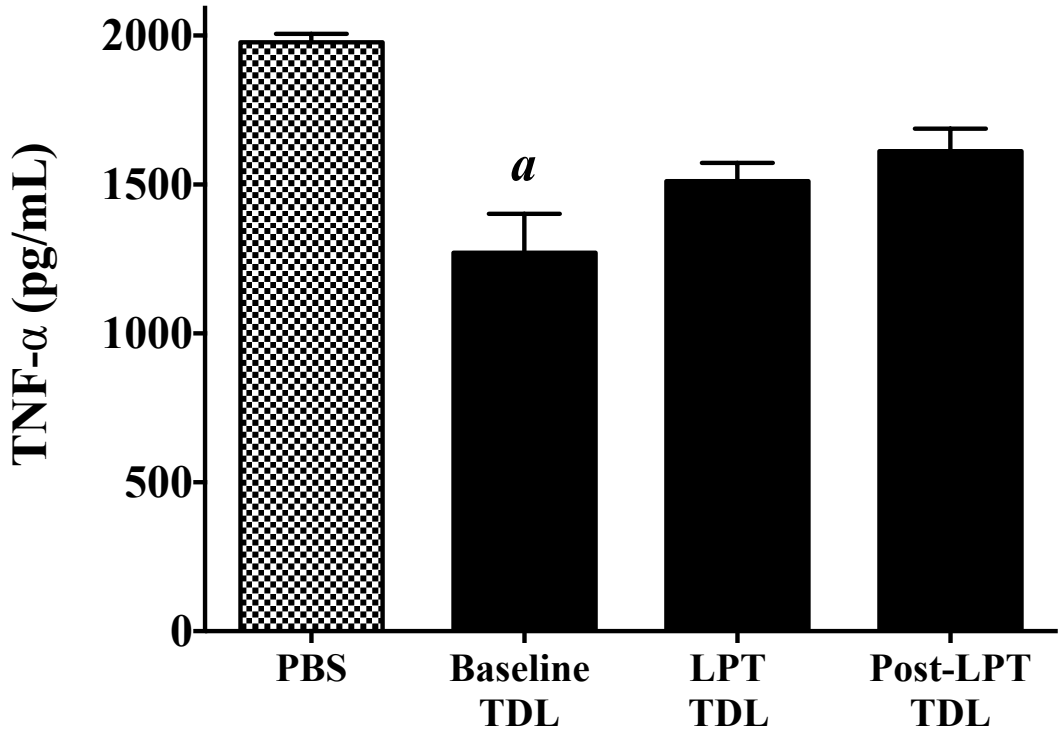

TNF-α (pg/mL)
2000
1500
1000
500
0
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.5. TDL suppressed the production of IL-10 by RAW 264.7 macrophages activated with LTA. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.01$) compared to PBS. N=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

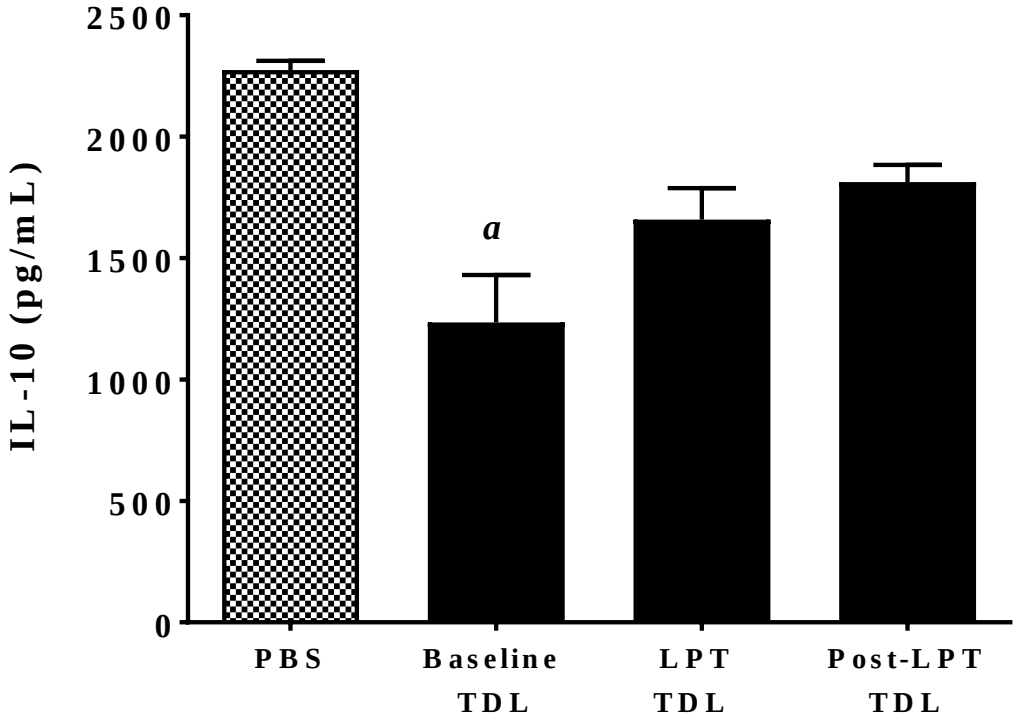

2500
2000
1500
1000
500
0
IL-10 (pg/mL)
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.6. TDL did not alter MH-S macrophage viability in the absence of LTA or IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

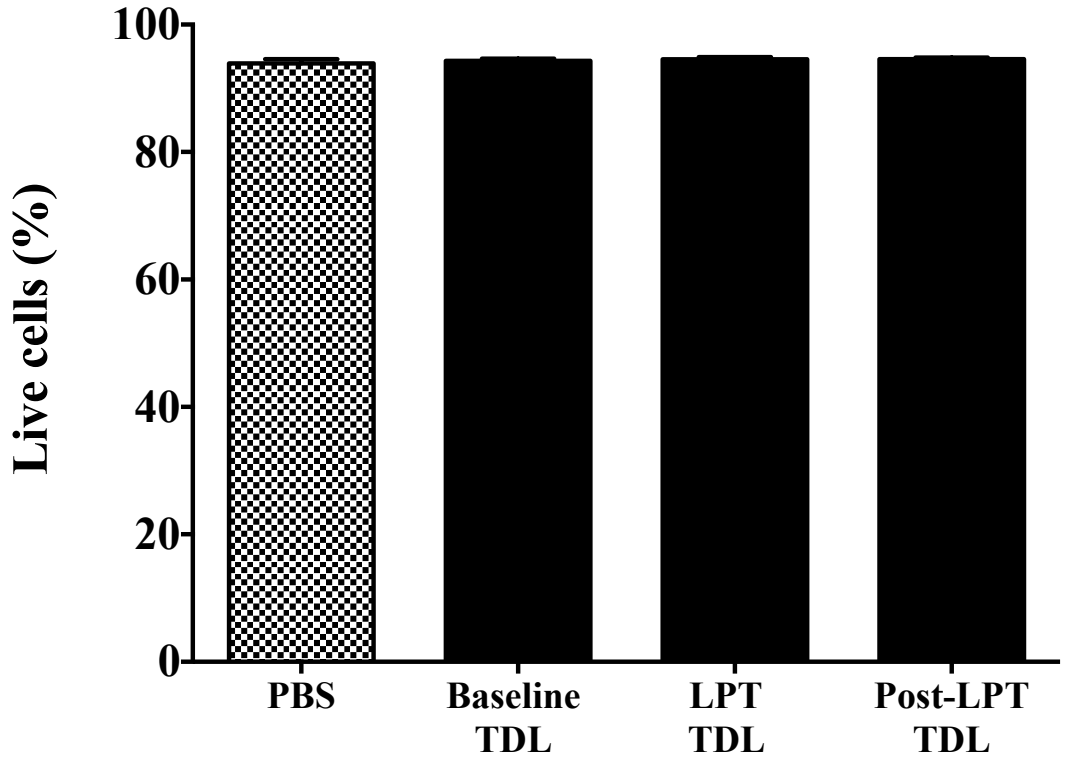

Live cells (%)
100
80
60
40
20
0
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.7. TDL did not alter MH-S macrophage viability in the presence of LTA. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

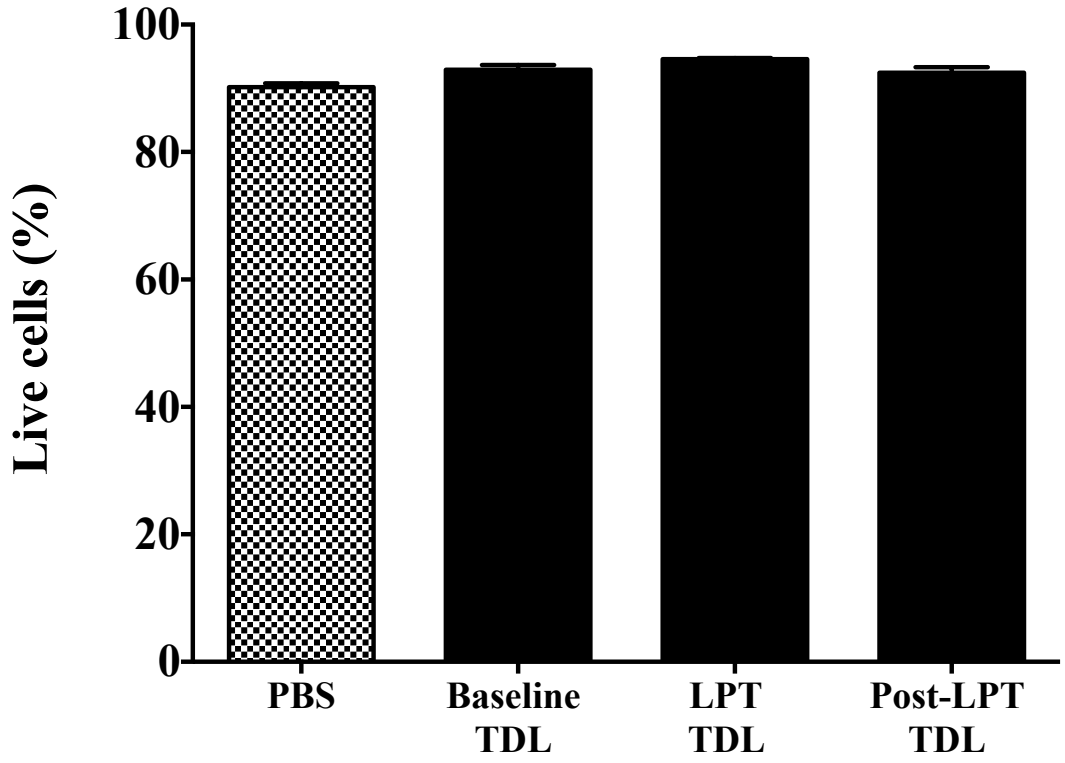

Live cells (%)
100
80
60
40
20
0
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.8. TDL suppressed the production of nitrite by MH-S macrophages activated with LTA. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.0001$) compared to PBS. N=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

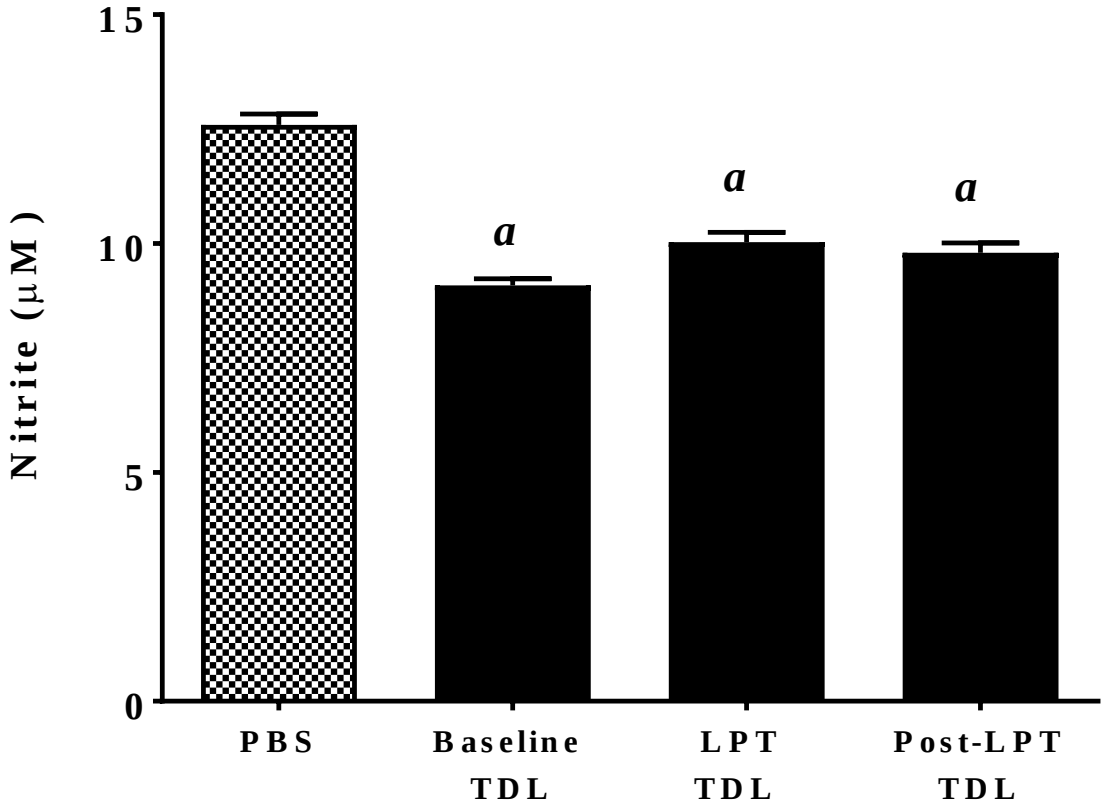

15
10
5
0
Nitrite (μM)
a
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.9. TDL suppressed the production of TNF-α by MH-S macrophages activated with LTA. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. [a] denotes statistical decrease ($P < 0.0001$) compared to PBS. *N=3* dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

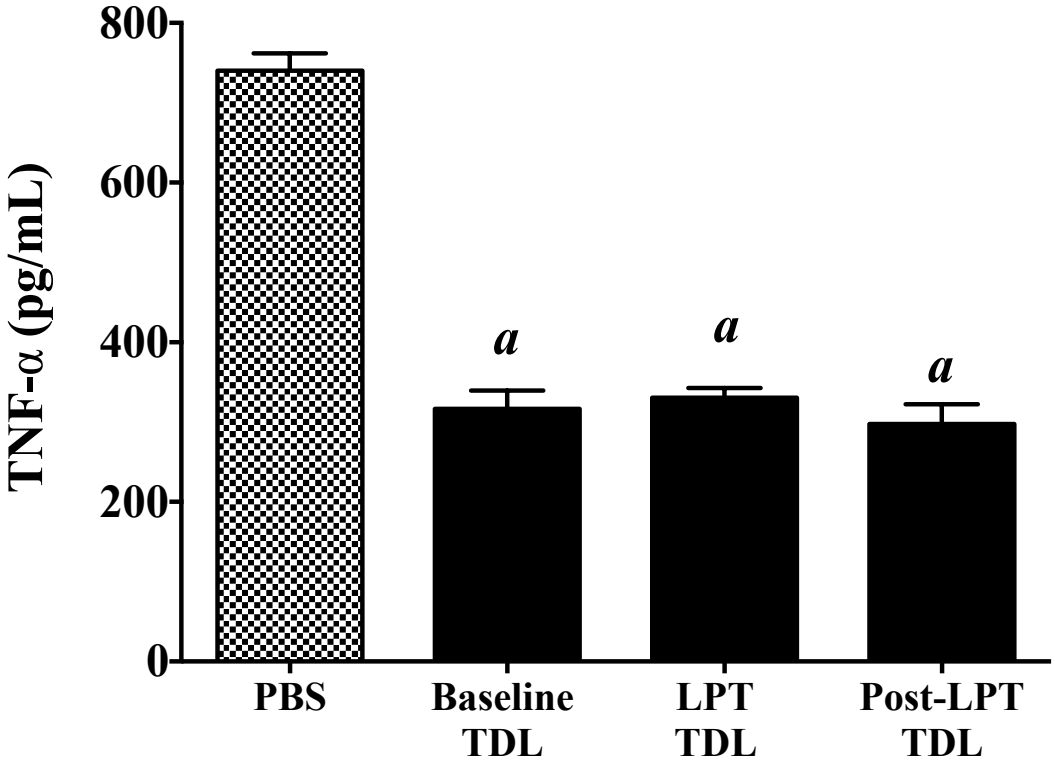

800
600
400
200
0
TNF-α (pg/mL)
a
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.10. TDL did not suppress the production of IL-10 by MH-S macrophages activated with LTA. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

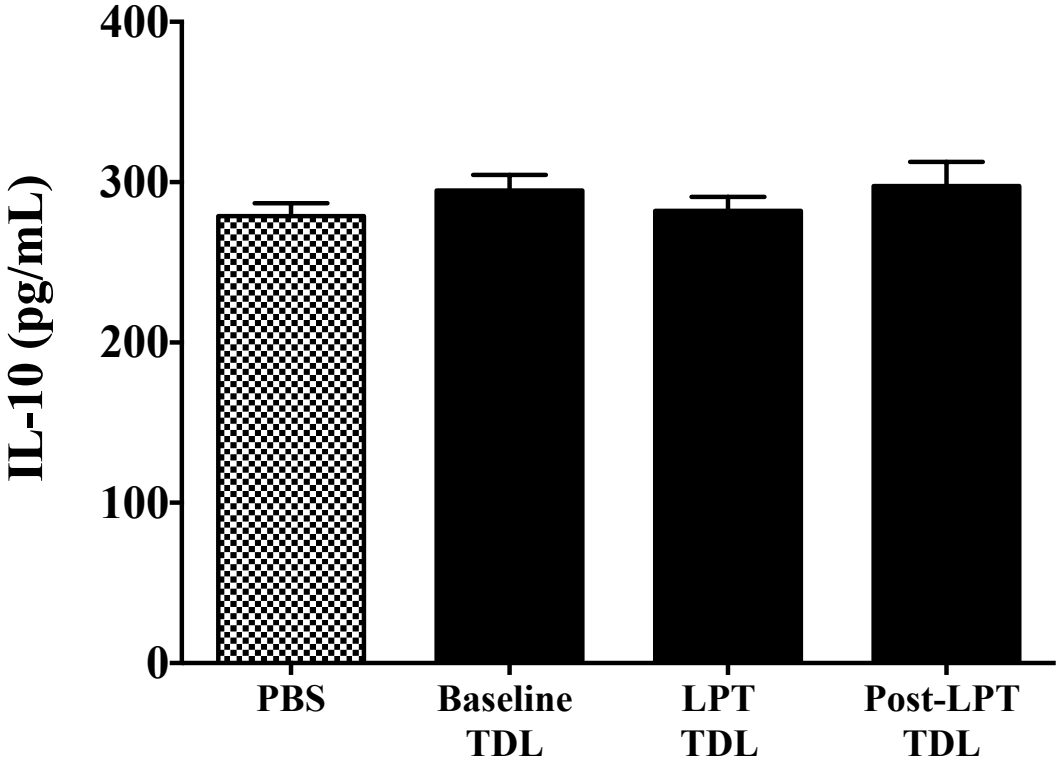

400
300
200
100
0
IL-10 (pg/mL)
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.11. TDL did not alter RAW 264.7 macrophage viability in the presence of IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SD. *N=3* dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

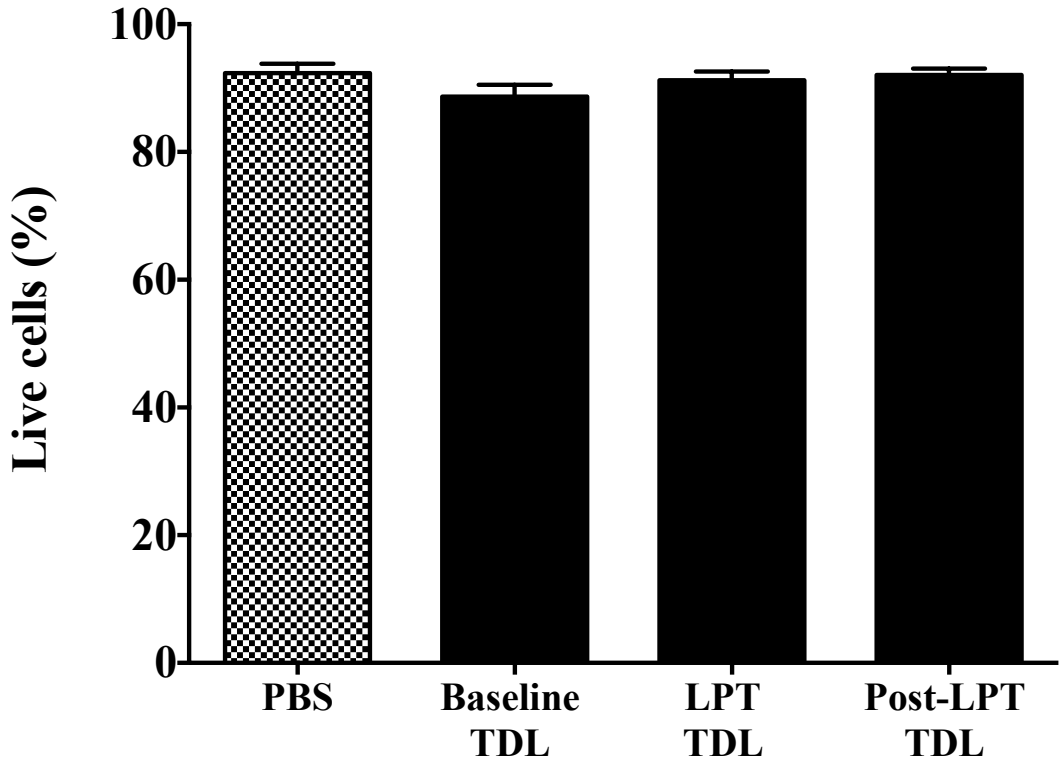

Live cells (%)
100
80
60
40
20
0
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.12. TDL suppressed the production of nitrite by RAW 264.7 macrophages activated with IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.05$) compared to PBS. N=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

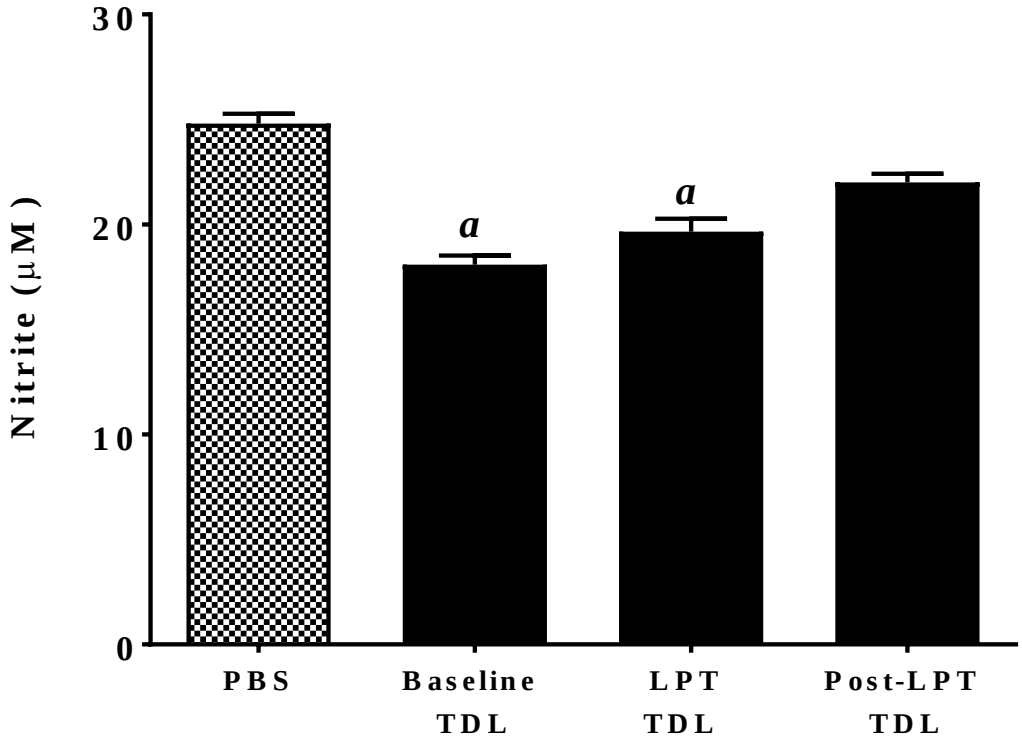

30
20
10
0
Nitrite (μM)
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.13. TDL suppressed the production of TNF-α by RAW 264.7 macrophages activated with IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.05$) compared to PBS. N=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

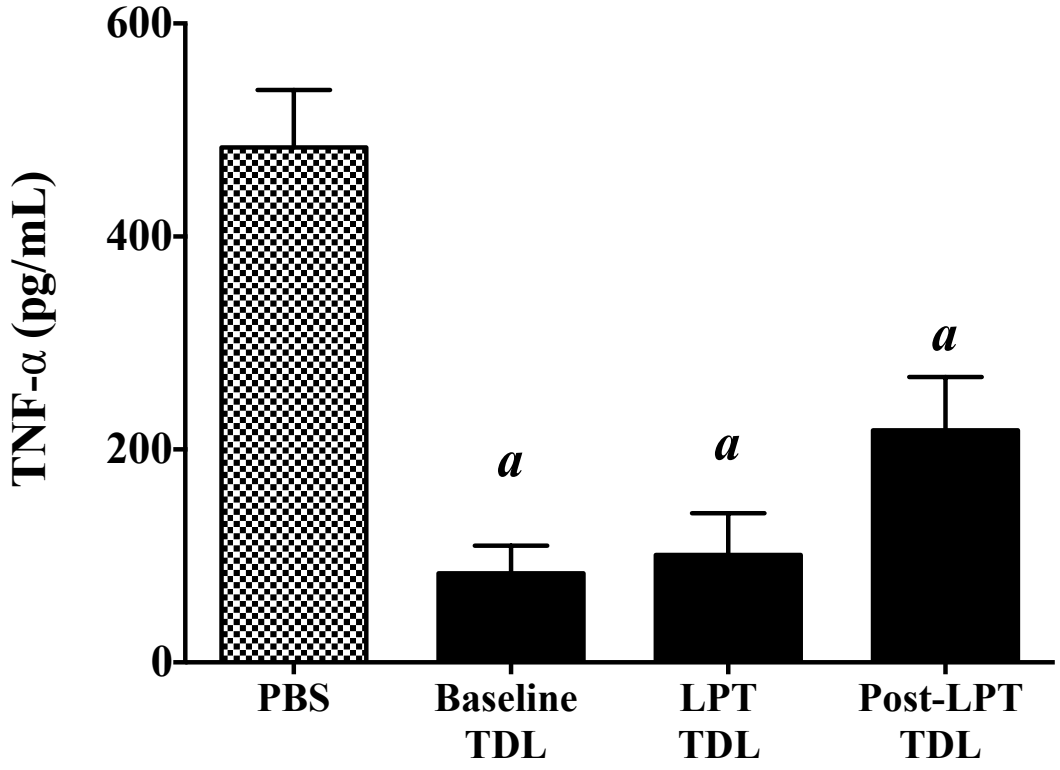

600
400
200
0
TNF-α (pg/mL)
a
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.14. TDL suppressed the production of IL-10 by RAW 264.7 macrophages activated with IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.0001$) compared to PBS. *N=3* dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

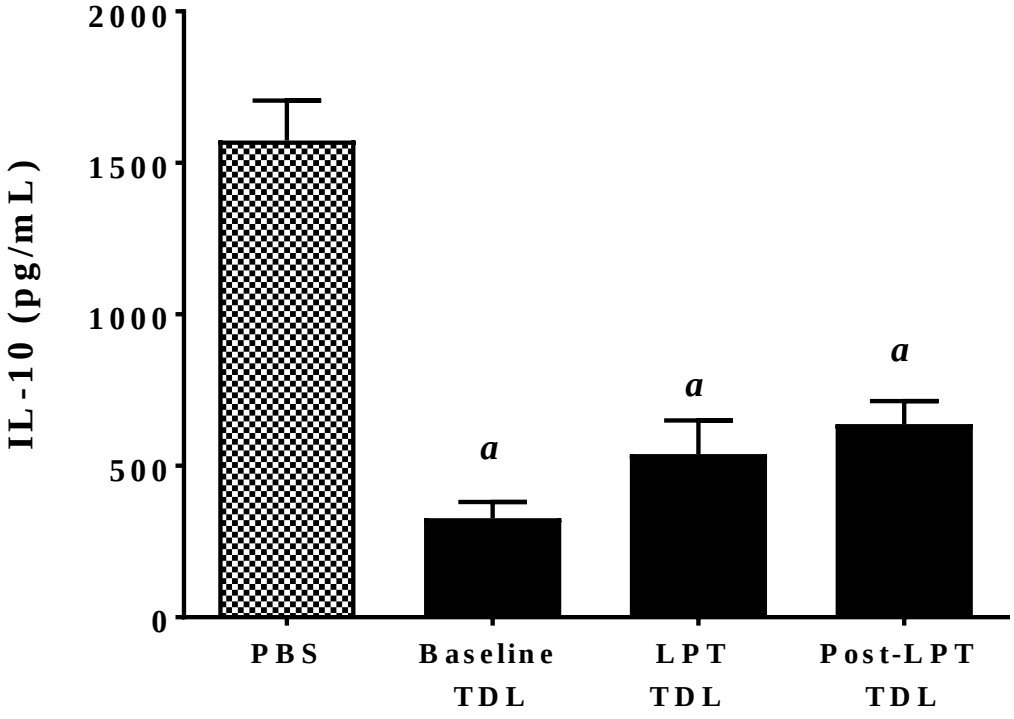

2000
1500
1000
500
0
IL-10 (pg/mL)
a
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.15. TDL did not alter MH-S macrophage viability in the presence of IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SD. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

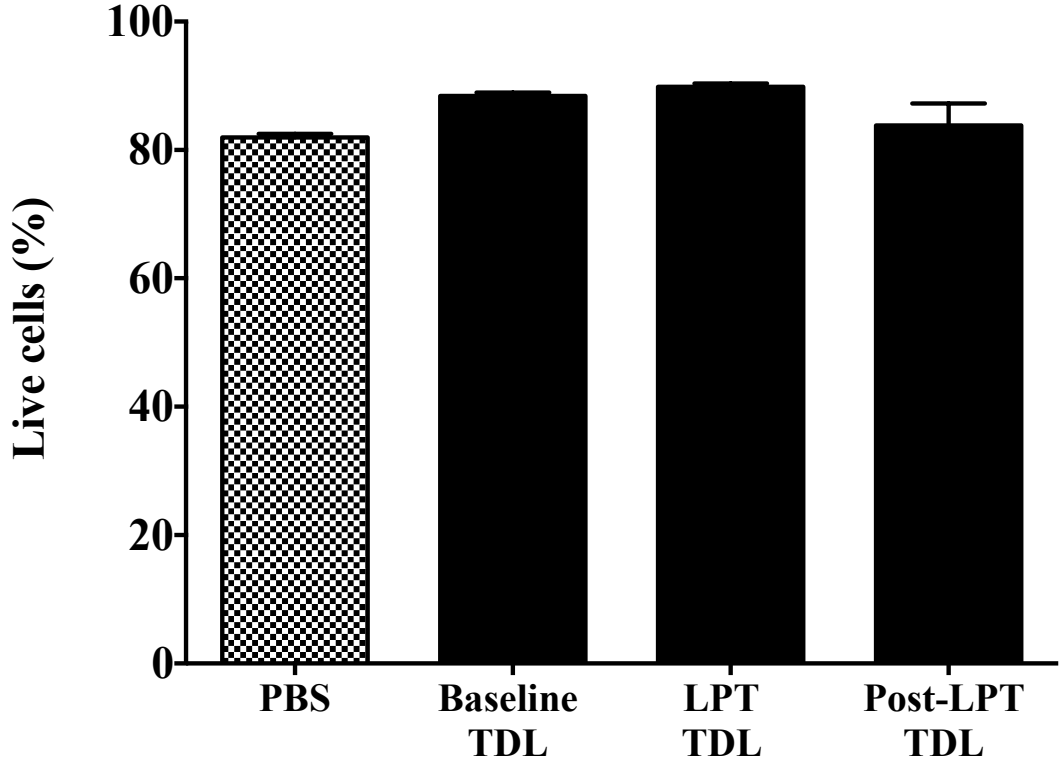

100
80
60
40
20
0
Live cells (%)
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.16. TDL suppressed the production of nitrite by MH-S macrophages activated with IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.0001$) compared to PBS. ^{b} denotes statistical increase ($P < 0.05$) compared to LPT TDL and Post-LPT. TDL N=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

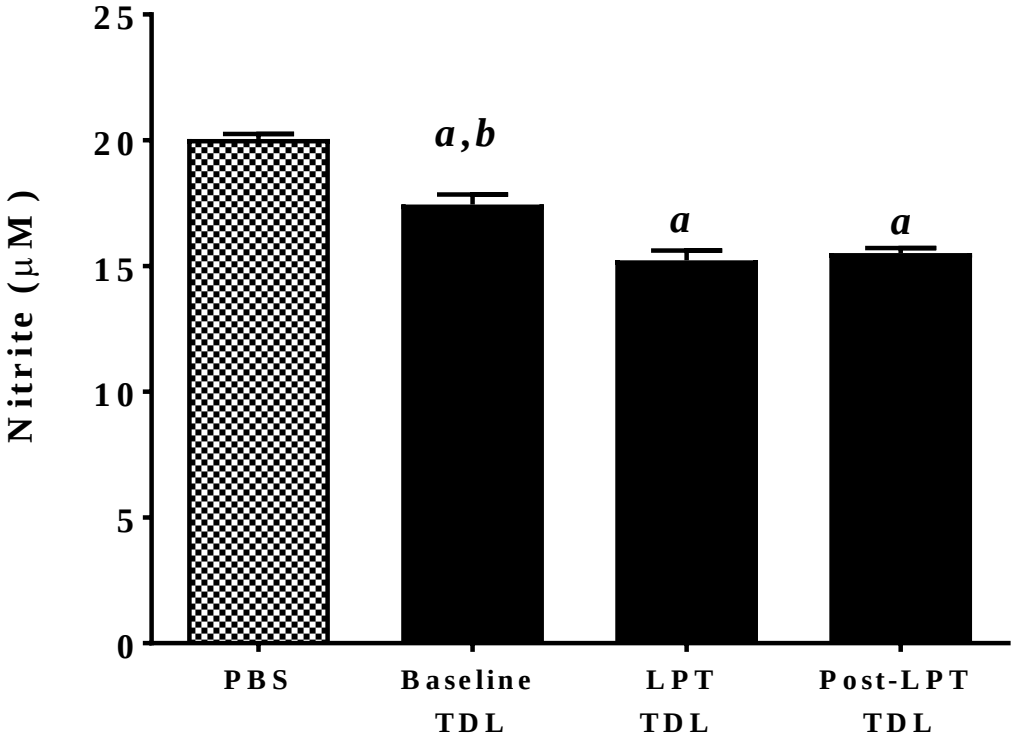

Nitrite (μM)
25
20
15
10
5
0
a,b
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.17. TDL suppressed the production of TNF-α by MH-S macrophages activated with IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. [a] denotes statistical decrease ($P < 0.0001$) compared to PBS. N=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.

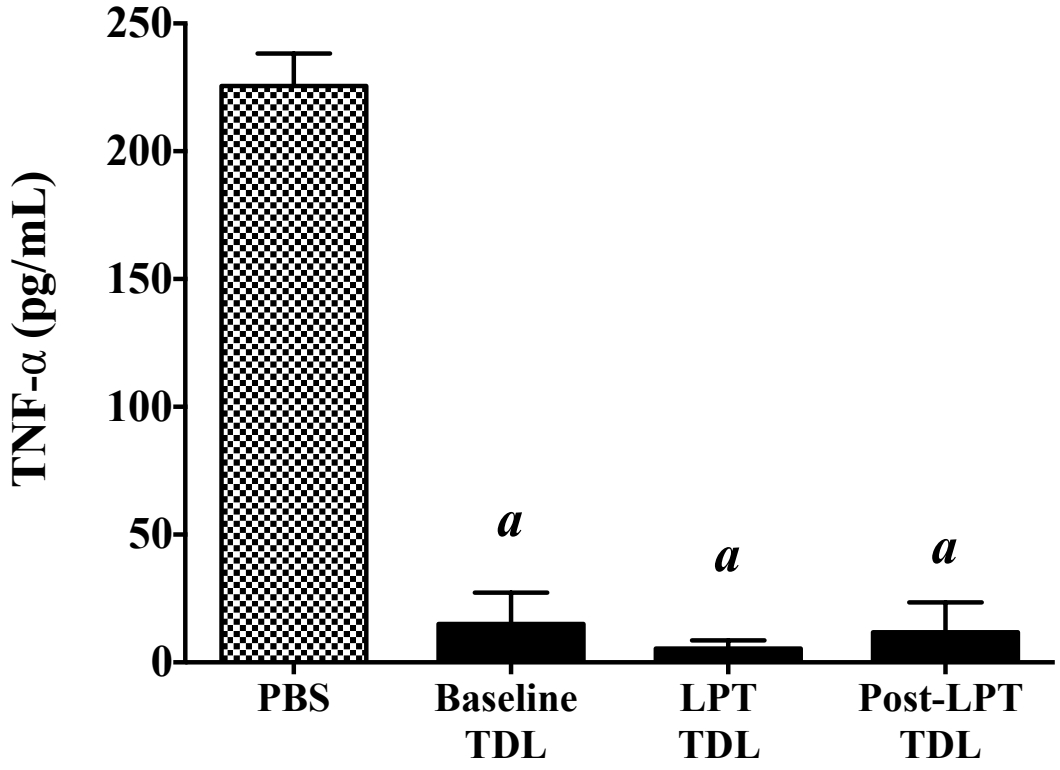
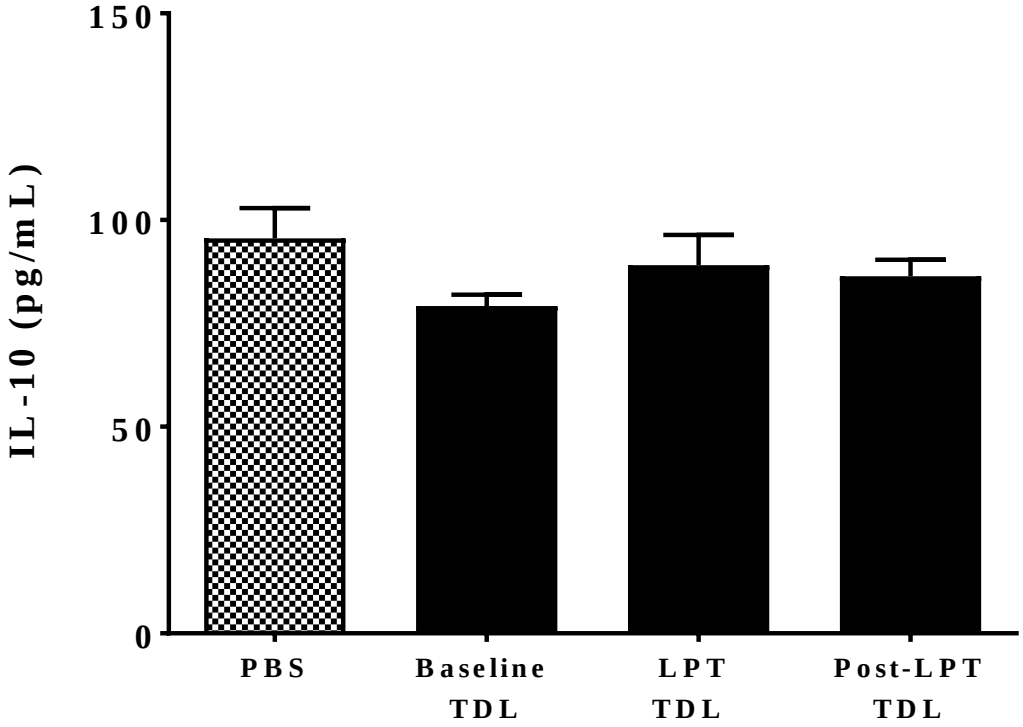

250
200
150
100
50
0
TNF-α (pg/mL)
a
a
a
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

Figure 5.18. TDL did not suppress the production of IL-10 by MH-S macrophages activated with IFN-γ. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. *N*=3 dogs for baseline, LPT, and post-LPT TDL from 1 experiment.


150
100
50
0
IL-10 (pg/mL)
PBS
Baseline TDL
LPT TDL
Post-LPT TDL

CHAPTER VI

DISCUSSION

The effect of LPT on antibiotics

Although OMT is used clinically and has been reported to be beneficial for respiratory disease [52, 53, 55], the mechanism of protection is unknown. Previous literature suggests that by enhancing lymph flow, LPT may promote the movement of lymph-borne factors through the thoracic duct into circulation and protect against pneumonia [53, 55, 58, 69]. One aim of these studies was to determine if abdominal LPT would facilitate the delivery of antibiotic the lung. We found that LPT did not alter the concentration of levofloxacin in the lung of naïve rats. Although these results do not support a synergistic effect between LPT and antibiotics, these findings may provide insight into an alternative mechanism of protection offered by LPT.

LPT may promote the entry of levofloxacin in the lung by increasing the concentration of levofloxacin in the lung tissue. Indeed, levofloxacin is a broad-spectrum fluoroquilone that passively diffuses through the blood-bronchus barrier to reach the pulmonary ELF [75]. However, it is important to note that LPT did not increase the concentration of levofloxacin significantly compared to sham treatment. Importantly, LPT may have increased the concentration of levofloxacin in the lung tissue. Therefore, future studies should be considered to measure levofloxacin in the lung tissue.

LPT increased levofloxacin concentrations in the ELF over time. Reaching adequate drug concentrations at the site of infection is an important consideration for predicting the efficacy of fluoroquinolones such as levofloxacin [94]. Therefore, delivering levofloxacin to the lung earlier may increase its efficacy. Furthermore, LPT may aid in sustaining drug concentrations at the site of infection if LPT is administered longer or repeated. These results suggest that LPT may be beneficial for drug delivery and support the use of LPT as an adjunctive therapy for pneumonia.

The multicenter osteopathic study in the elderly (MOPSE) supports the use of LPT as an adjunctive therapy for pneumonia. The combination of conventional care and OMT, which included LPT, reduced the length of hospital stay and duration of intravenous antibiotics compared to conventional care alone [53, 55]. The results of this study suggest that LPT may have protected independently of antibiotics. In support, LPT without antibiotics protected rats from *S. pneumoniae* infection when compared to sham treatment [5]. However, it is unknown if LPT altered levofloxacin concentration in the lungs of infected rats; therefore, future studies are need to determine the effect of LPT on antibiotic distribution during infection. Previous literature has proposed that antibiotic penetration into the lung is lower in patients with bronchitis compared to healthy subjects suggesting that the diffusion of antibiotic is impaired during pulmonary infection [76]. Future studies using a similar approach as this study in *S. pneumoniae* infected rats would be more translational and provide more insight into the mechanisms of protection offered by LPT during pneumonia.

Alternate mechanisms of protection offered by LPT

There is likely another mechanism of protection offered by LPT. Gut lymphatic vessels redistribute a large pool of lymph rich in immune cells, inflammatory mediators, and lipids.

Furthermore, LPT increases the lymphatic flux of inflammatory cytokines [65, 66]. Therefore, one possible mechanism of protection offered LPT is that it mobilizes lymph-borne factors to the lung that alter the inflammatory response of macrophages. Overall, baseline, LPT, or post-LPT TDL suppressed RAW 264.7 macrophage activity in response to LPS, LTA, or IFN-γ suggesting lymph suppresses the inflammatory response of interstitial macrophages or monocytes that migrate to the lung during infection. Suppressing the activity of infiltrating macrophages during *S. pneumoniae* infection may aid in reducing immunopathology. Indeed, persistent macrophage activity is correlated with mortality in murine models of pneumonia [46]. Overall, baseline, LPT, or post-LPT TDL suppressed MH-S macrophage activity in response to LPS, LTA, or IFN-γ suggesting lymph suppresses the inflammatory response of resident alveolar macrophages. Similarly, alveolar macrophages have been proposed to modulate the immune response to pneumonia and limit the production of inflammatory cytokines that lead to an increase in bacterial load, mortality, and inflammation [29]. Therefore, LPT may have mobilized protective factors in lymph to the lung to suppress macrophage activation during infection and protect the lung from immunopathology.

In a mouse model of pneumonia, alveolar injury and interstitial edema, attributed to pneumolysin, is seen in the lungs at 24 and 48 hours post infection. At 48 and 72 hours, monocyte and lymphocyte activity increase along with nitric oxide production that leads to bacterial proliferation and tissue damage [18]. It is possible that dampening the immune response of alveolar macrophages and recruited macrophages during *S. pneumoniae* infection may be beneficial to prevent immunopathology and decrease bacterial load. In support, inflammatory mediators, such as TNF-α can play a role in immunopathology. In mice, TNF-α is not necessary to clear pulmonary infection with *S. pneumoniae*, but TNF-α contributes to immunopathology

[49]. Furthermore, alveolar macrophages, interstitial macrophages, and monocytes contribute to pulmonary inflammation resulting in immunopathology and lung injury [20, 21, 22, 29]. The decrease of bacterial load in the lungs of rats infected with *S. pneumoniae* may be attributed to the mobilization of inhibitory mediators in lymph by LPT at 24, 48 and 72 hours post infection [58]. Pneumolysin binds to TLR4 therefore modulating the interaction of TLR4 and pneumolysin may protect the host from a strong and pathogenic immune response. Indeed, phospholipase A in pulmonary artery endothelium is activated by pneumolysin and mediates pulmonary inflammation and damage [43]. Furthermore, the lack of pneumolysin and interaction with TLR-4 reduces pulmonary inflammation and the bacterial load of *S. pneumoniae* in the nasopharynx, trachea, and lungs [110]. These findings suggest that limiting the interaction of pneumolysin and TLR-4 may be protective further supporting the notion that modulating the immune response by macrophages during pneumonia may lead to a reduction of bacteria.

Protective factors in lymph

The mobilization of anti-inflammatory mediators through TDL is not surprising. The tolerogenic environment in the gut contains immune regulators such as cytokines and lymphocytes. Immune suppression is driven by intestinal dendritic cells that sample bacteria but promote the induction of unresponsive T cells and active T regulatory cells that inhibit proinflammatory responses [99]. IL-10 is a central cytokine secreted by T cells, B cells, and most myeloid-derived cells for intestinal homeostasis [100]. The lack of functional IL-10 leads to severe intestinal inflammation in humans and animal models [101]. Therefore, immune regulators, such as IL-10, could be released in the interstitial fluid of the gastrointestinal tissue, enter the lymphatic system and be found in thoracic duct lymph [101]. Intestinal lymph reaches

systemic circulation via the thoracic duct and comprises a large portion of TDL [30, 34, 103,104]. Indeed, our lab has previously shown that leukocytes from mesenteric lymph nodes can be found in mesenteric and thoracic duct lymph [64] and that gut-associated lymphocytes can be found in the cisterna chyli [68]. It is also important to note that IL-10 is also released by epithelial cells and regulates alveolar macrophage activation [29] and was previously measured in canine TDL used in these studies.

The gastrointestinal lymphatics are responsible for the uptake of lipids and their transport back into circulation with the aid of lipoproteins [102]. Since mesenteric lymph compromises the majority of thoracic duct lymph [103, 104], lipids and lipid-like molecules may be responsible for the inhibitory effect of thoracic duct lymph. Previous studies have shown lipids can suppress endotoxin-mediated inflammation *in vitro* [82]. High-density lipoproteins can inhibit the M1-like or inflammatory phenotype in macrophages [105]. Furthermore, Apolipoprotein A1, the major component of high-density lipoprotein, protected the lung from fibrosis and promoted resolution *in vivo* [106]. Lastly, lipid mediators, such as lipoxins, are endogenous anti-inflammatory factors released during inflammation that reduce tissue injury and chronic inflammation [107]. Collectively, these studies suggest lymph contains lipids or lipid-like molecules that can suppress inflammation [108].

Previous studies suggest complement is also present in lymph, which could be mobilized by LPT [77]. Complement in the lung may aid in the opsonization of *S. pneumoniae*. Indeed, alveolar macrophages express complement receptors capable of binding to components, C1q and C3b, involved in opsonization [109]. Interestingly, complement components C3a, C5a, and C5b-9 polarize macrophages towards an inflammatory phenotype while apoptotic or target cells and complement components C1q and C3b polarize cells towards an anti-inflammatory phenotype

[109]. Therefore, complement in TDL may suppress inflammation while enhancing phagocytosis of bacteria.

The lymph-borne factors responsible for the suppressive effect reported in these studies are unknown which is a limitation. Therefore, in future studies, experimental methods described in Cheng *et al* (2006) and Gonzalez *et al* (2000) could be utilized to determine if the lipid component is responsible for the suppressive effect of lymph [81,82]. The lipid portion of lymph can be removed using a lipid removal reagent to obtain lipid-free lymph [82]. Therefore, baseline, LPT, or post-LPT lymph could be exposed to a lipid removal reagent to obtain lipid-free baseline, LPT or post-LPT lymph. Lipid-free lymph can be cultured with RAW 264.7 or MH-S macrophages as described in these studies to determine if lipid-free lymph has a similar suppressive effect during LPS, LTA, or IFN-γ activation. Lipids can also be extracted chemically from lymph to obtain the lipid component of lymph [81]. Therefore, the isolated lipids from baseline, LPT, or post-LPT TDL can be cultured with RAW 264.7 or MH-S macrophages to determine if the lipid component can inhibit macrophage activation *in vitro*. These results would give some insight into the component in TDL responsible for its suppressive effect.

The effect of LPT on lymphatic flux

We acknowledge there may differences between baseline, LPT, and post-LPT TDL; however, LPT did not change TDL qualitatively. The more profound effect of LPT on TDL is likely through the increase in lymphatic flow and flux. LPT increased lymphatic flow and flux of proteins in TDL approximately 10-fold. One possible mechanism of LPT is that it mobilizes pools of lymph into circulation that may provide protection during pneumonia by promoting resolution and reducing immunopathology associated with pneumonia. By increasing lymph

flow, LPT may increase the concentration of protective factors in the blood. Once in circulation, extra-lymph borne factors in blood may diffuse into in the lung where they protect the lung from immmunopathology and aid bacterial clearance. This *in vitro* approach does not account for other mechanisms of protection by LPT. By compressing the abdomen and thoracic cage, LPT may also enhance bacterial clearance by stimulating mucociliary clearance which was not investigated in these studies; therefore, LPT may have another mechanism of protection independent of enhancing lymph flow.

Limitations

We acknowledge there were limitations to these studies that may have influenced the results. Clinically, levofloxacin is not administered subcutaneously which is a limitation of this study [75]. Therefore, future studies should consider intravenous administration of levofloxacin to determine if LPT enhances the delivery of antibiotic to the lung. LPT did not enhance the penetration of antibiotic into the lung suggesting enhancing lymph flow would not enhance the delivery of intravenously delivered antibiotics. However, the intestinal lymphatics have been proposed as absorptive pathways for orally administered pharmaceuticals [95, 96]; therefore, LPT may also increase the concentration of orally-administered levofloxacin in the lung. Therefore, future studies should also consider oral administration of levofloxacin to over this limitation.

LPT increased the concentration of antibiotic in the lungs of male rats; therefore it is unknown if the effect of LPT is influenced by sex. In clinical studies evaluating the effects of the OMT [53, 55] did not find significant differences in the demographics of their subjects; therefore, it is unknown if one gender benefits more from OMT. However, age-related decrease

in lymphatic pumping pressure in healthy subjects was more profound in post-menopausal women suggesting elderly female patients with pneumonia may benefit more from lymph-enhancing techniques [110]. Therefore, in future studies, both age and sex should be considered to identify the influence of sex and age on the effect of LPT during pneumonia.

It is unknown if limiting the production of inflammatory mediators by macrophages contributes to phagocytosis and bacterial clearance during infection, which is a limitation of this study. During *S. pneumoniae* infection, neutrophils are quickly recruited to the lung and rapidly phagocytose bacteria and play a major role in bacterial clearance [18, 24]. In this study, the effect of LPT mobilized lymph on neutrophils is unknown. However, previous studies suggest mesenteric lymph may prime and inhibit neutrophil apoptosis [81] suggesting TDL may enhance bacterial clearance by neutrophils. Therefore, future studies are warranted to identify the effect of lymph on phagocytes isolated from infected animals. In future studies, mice or rats could be infected with *S. pneumoniae*, as previously described [58, 59], and macrophages and neutrophils isolated from lung tissues at 24, 48, 72, and 96 hours post injection. Phagocyte function can be measured by culturing macrophages and neutrophils with TDL isolated before, during, and after LPT. These results will demonstrate if TDL can suppress the release of inflammatory mediators and enhance phagocytosis by macrophages and neutrophils isolated from mice infected with *S. pneumoniae*.

We acknowledge the use of cell lines is a limitation to these studies. Inconsistencies between studies using primary macrophages and macrophage cell lines suggest that TDL may have a different effect on primary macrophages. Previous studies also demonstrate that primary macrophages have a greater inflammatory response to infection compared to a macrophage cell line [111] suggesting primary macrophages should be used for host-pathogen interactions.

Furthermore, the duration of culture may influence cytokine expression in macrophages [112] further suggesting that freshly isolated macrophages may be more translational to evaluate macrophage activation and inhibition. Overall, the differences between primary cells and cell lines suggest that future studies should consider using freshly isolated macrophages from mice to confirm the inhibitory effect of thoracic duct lymph.

The cross species approach used in these studies is a limitation. However, in previous studies, normal rat mesenteric lymph blunted the response of human pulmonary endothelial cells to endotoxin suggesting lymph contains soluble protective factors, including lipoproteins, *in vitro* [82]. Normal canine mesenteric lymph infused into rats with endotoxin-mediated lung injury reduced cell-adhesion molecules, myeloperoxidase, and lung injury *in vivo* [37]. Similarly, normal canine mesenteric lymph reduced endotoxin-mediated hepatic tissue damage and kidney injury *in vivo* suggesting lymph infused intravenously protected more than one organ [35, 87]. Lastly, normal rat mesenteric lymph reduced apoptosis in human neutrophils suggesting lymph may contain soluble protective factors [81]. Consistent with other previous studies [35, 37, 82, 87]; the results of these studies also suggest that the suppressive factors in lymph are not species specific. However, we acknowledge this cross-species approach is a limitation to this study because the effect of a species-specific factor(s) in lymph is unknown. Therefore, future studies should consider macrophages and TDL from the same species.

We acknowledge sex differences may also influence inflammatory responses to respiratory infection. Interestingly, in a murine model of influenza, female mice suffered from greater morbidity and mortality than male mice which was associated with the release of inflammatory mediators [113]. In these studies, both murine macrophage cell lines were generated from cells isolated from male mice; therefore it is unknown if TDL would have the

same inhibitory effect on macrophages isolated from female mice. Alveolar macrophages isolated from female mice have a higher expression of M2 genes compared to alveolar macrophages isolated from male mice [114] further suggesting the effect of TDL may be influenced by sex. Therefore, future studies should consider isolating macrophages and neutrophils from male and female mice.

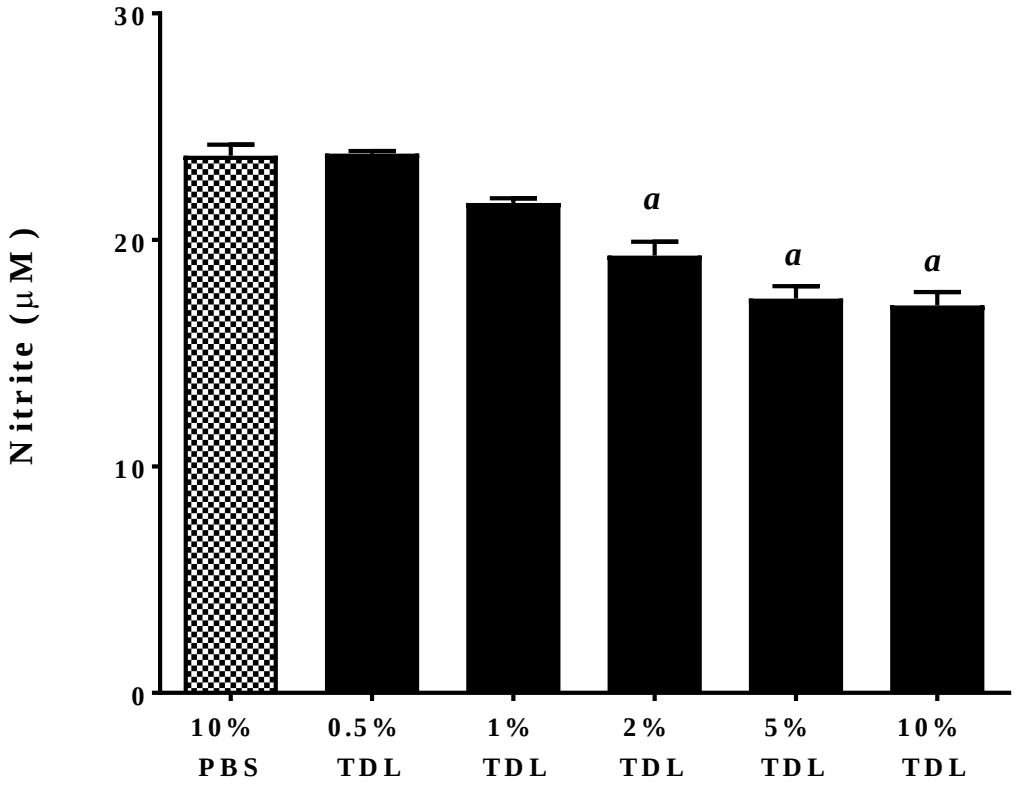
Conclusions

In conclusion, the results from previous studies and the novel findings presented in this dissertation, suggest LPT may provide protection during pneumonia by 1) reducing bacterial load, 2) redistributing pools of protective lymph that may protect the lung from immunopathology, and 3) facilitating the delivery of antibiotic to the lung. These studies further support the existence of a gut-lung axis and provide evidence that lymph-borne factors may influence pulmonary immunity. Lastly, these studies support the use of LPT as an evidence-based therapy for the treatment of pneumonia. By enhancing lymph flow, LPT may facilitate the delivery of lymph-borne factors and pharmaceuticals to the lung which may aid in reducing hospitalization time, the use of antibiotics, and respiratory failure in patients with pneumonia. These studies are essential to support the use of LPT by physicians for the treatment of respiratory infection and inflammation and guide physicians in clinical practice.

APPENDIX I

The murine macrophage cell line, RAW 264.7, was cultured with baseline TDL to determine the optimal dose of TDL to use in Chapters III-V. RAW 264.7 macrophages were grown in Dulbecco's modified Eagle high glucose medium with 10% fetal bovine serum (Hyclone) at 37°C under 5% CO_2. RAW 264.7 macrophages were passaged 5-15 times by scraping adherent cells. After 24 hours of culture, RAW 264.7 were counted using trypan blue (Corning) exclusion and $1x10^5$ cells per well were cultured for one hour to allow the cells to acclimate. After one hour, cell-free baseline TDL was added at 0.5%, 1%, 2%, 5%, or 10% total volume per well from six dog donors (N=6) and co-cultured with or without 500ng of LPS (Sigma) for 24 hours. As a control, RAW 264.7 macrophages were cultured for one hour to allow cells to acclimate then sterile 1X PBS (Hyclone) at 10% total volume per well added with or without 500 ng of LPS per well for 24 hours. After 24 hours of culture, cell-free supernatants were collected by centrifugation at 400G for 5 minutes at 4°C and measured for nitrite using a Promega Griess reagent system.

Figure AI.1. Increasing baseline TDL suppresses the production of nitrite by RAW 264.7 macrophages activated with LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE. ^{a} denotes statistical decrease ($P < 0.05$) compared to 10% PBS, 0.5% baseline TDL, and 1% baseline TDL. N=6 dogs for baseline TDL


Nitrite (μM)
30
20
10
0
a
a
a
10% PBS
0.5% TDL
1% TDL
2% TDL
5% TDL
10% TDL

APPENDIX II

DEXTROSE-BASED PROLOTHERAPY INDUCES APOPTOSIS AND REDUCES THE RELEASE OF INFLAMMATORY MEDIATORS FROM MACROPHAGES

Prolotherapy has been used clinically to treat musculoskeletal, arthritic, and tendon/joint laxity conditions. Although clinical studies support the use of dextrose prolotherapy, the mechanism remains unclear. Proliferant agents have been proposed to promote irritation and inflammation followed by tissue healing, improved ligament mechanics, and decreased pain. Previous studies suggest dextrose induces the infiltration of inflammatory leukocytes to healthy ligaments, increases collagen and growth factors in fibroblasts, and increase expression of inflammatory mediators in tenocytes *in vitro*. However, the effect of dextrose on macrophage activity is unknown. Proliferant agents and high glucose may lead to macrophage apoptosis. Therefore, the purpose of this study was to examine the effect of dextrose-based prolotherapy on the viability and the inflammatory response of macrophages *in vitro*. We hypothesized that dextrose would reduce macrophage viability and decrease the production of inflammatory mediators by macrophages. To test this hypothesis, RAW 264.7 macrophages were cultured with 2.5%, 5%, or 10% of a dextrose solution with or without lipopolysaccharide for 24 hours. After culture, macrophages were collected to measure viability and cell-free supernatants to measure the production of inflammatory mediators, nitrite and TNF-α. Five percent or 10% dextrose significantly decreased macrophage viability and the production of nitrite and TNF-α. This study

suggests that dextrose-prolotherapy induces apoptosis in RAW 264.7 macrophages thereby reducing the production of inflammatory mediators. Understanding the mechanism of action of prolotherapy solutions, such as dextrose, will provide scientific support for their clinical use.

Introduction

Prolotherapy is an injection-based complementary and alternative medicine therapy that has been used in clinical practice for over eighty years to treat various chronic musculoskeletal, arthritic, and tendon/joint laxity conditions. The therapy includes the injection of an irritant, such as dextrose, into painful joint insertions, or into adjacent joint spaces, over the course of multiple sessions. Clinically, prolotherapy has been shown to provide a protective effect when used to treat tendinopathies, low back pain, joint osteoarthritis and spinal pain due to ligament dysfunction [115-117]. Rabago *et al* (2013) conducted a randomized controlled trial that supported the use of prolotherapy for knee osteoarthritis. Ninety adults with knee osteoarthritis were randomized to a blind injection of dextrose prolotherapy or saline or at home exercise. Patients receiving dextrose prolotherapy had improved osteoarthritis index scores and decreased knee pain compared to groups receiving saline or exercise [118]. Additionally, other randomized controlled trials concluded that dextrose prolotherapy decreased pain in osteoarthritis patients and supported the use of prolotherapy for osteoarthritis [119]. Although, anecdotal and clinical evidence supports the use of prolotherapy for lower back pain, tendinopathies, and osteoarthiritis, the inflammatory mechanism of action of prolotherapy is unclear.

Prolotherapy is coined from the term proliferant therapy and has been proposed to promote irritation and inflammation followed by the growth of connective tissue leading to tissue healing, improved ligament mechanics, and decreased pain [120-123]. However, there have only

been a few mechanistic studies demonstrating the inflammatory effect of prolotherapy. The injection of dextrose into rat knee ligaments induced the infiltration of inflammatory leukocytes to the healthy ligaments and significantly increased the cross-sectional area of injured ligaments [120]. Furthermore, exposure to dextrose increased expression of collagen and growth factors in human fibroblasts *in vitro* [124]. Additionally, dextrose decreased metabolic activity, collagen production, and cell migration and increased expression of inflammatory mediators in human tenocytes *in vitro* [125]. However, the inflammatory mechanism of action of prolotherapy is unclear. Furthermore, the effects of prolotherapy have only been studied using fibroblasts and tenocytes; therefore the effect of prolotherapy on macrophages has not been investigated.

Macrophages initiate inflammation by releasing proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-12 (IL-12). In addition to cytokines, macrophages release chemokines, leukotrienes, prostaglandins and anti-bacterial factors such as nitric oxide. Locally, these inflammatory mediators increase vascular permeability and recruit neutrophils and monocytes to the site of inflammation. However, these inflammatory mediators have also been associated with immunopathology therefore modulating their production is crucial to resolution and a return to homeostasis. Macrophages also secrete anti-inflammatory cytokines such as interleukin-10 (IL-10) and tumor growth factor-beta (TGF-β) that target macrophages and various other target cells. The absence of anti-inflammatory cytokines have been implicated during systemic inflammation [126].

Apoptosis is a key feature during activation of the innate immune response and resolution. Macrophage apoptosis and phagocytosis of apoptotic cells by macrophages promotes resolution at sites of inflammation [127-129]. Proliferant agents have been shown to decrease

cell numbers and the production of collagen in fibroblasts [130]. Furthermore, high glucose induced apoptosis in macrophages [131, 132]. Therefore, proliferant agents leading to macrophage apoptosis may reduce inflammation and pain and promote healing.

Pre-clinical and clinical studies support the use of prolotherapy for the treatment of osteoarthritis. Furthermore, studies suggest that cell types other than resident cells may be involved in the protective effect of prolotherapy. Activation of macrophages and their production of proinflammatory cytokines have been implicated as risk factors for clinical symptoms and progression of osteoarthritis [133]. These studies suggest the infiltration and accumulation of macrophages in joints of osteoarthritis patients is pathogenic; therefore the effect of prolotherapy on macrophage activity should be elucidated. The purpose of this study was to examine the effect of dextrose-based prolotherapy on the inflammatory response of macrophages *in vitro.* Understanding the mechanism of action of prolotherapy solutions, such as dextrose, will provide scientific support for their clinical use.

Methodology

Cell culture and reagents

The murine macrophage cell line, RAW 264.7 (ATCC), were grown in Dulbecco's modified eagle high medium (ThermoFisher Scientific) supplemented with characterized fetal bovine serum (FBS) (Hyclone) at 37°C under 5% CO_2 and passaged 5-15 times before cell stimulations. One hundred thousand cells per well were cultured with 50% dextrose solution in water (Hospira Incorporated, Lake Forest, IL) at 2.5%, 5% and 10% total volume per well with and without 500 ng per well of lipopolysaccharide (LPS) (Sigma-Aldrich) for 24 hours. As a control, RAW 264.7 macrophages were cultured with sterile saline (Hospira Incorporated) or

sterile deionized water at 2.5%, 5% and 10% total volume per well with and without 500 ng per well of LPS. After 24 hours, cell-free supernatants were collected using centrifugation and stored at -80°C. Each condition was cultured in triplicate and the experiment repeated for a total of 6 wells per condition from 2 separate experiments.

The concentration of nitrite, an indirect measure of nitric-oxide, tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) in cell free supernatants were measured using commercially available kits. Cell-free supernatants were assayed for NO_2^- using Griess reagent (Promega Corporation) and TNF-α and IL-10using ELISA (BD Biosciences). The minimum detectable nitrite concentration for this assay is 2.5 μM. The minimum detectable concentrations for TNF-α and IL-10 were 15.5 pg/mL and 31.3 pg/mL, respectively.

Cell viability and apoptosis

After cell stimulation, RAW 264.7 cells were collected and isolated by scraping centrifugation. To determine cell viability and apoptosis, cells were immunofluorescently stained with annexin V and propidium iodide (BD Biosciences). Cells were subjected to a BD LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJO software. The percentages of live cells were determined by gating on intact cells not positive for annexin V or propidium iodide. The percentages of apoptotic cells were determined by gating on intact cells positive for annexin V only and double-positive for annexin V and propidium iodide. The percentages of cell debris or dead cells were not statistically different between cells cultured with dextrose, saline, water or LPS.

Statistical analysis

Data are presented as arithmetic means ± standard error (SE). Values from replicate wells from respective culture conditions were averaged, and the mean values are shown in figures. Data were subjected to analyses of variance followed by a Tukey's multiple comparisons test to evaluate statistical significance. Statistical analyses were performed with GraphPad Prism version 7 (GraphPad Software). Differences among mean values with $P \leq 0.05$ were considered statistically significant.

Results

Dextrose decreases macrophage viability

In the absence of LPS, RAW 264.7 macrophage viability was approximately 91.6 ± 0.4 %, 90.1 ± 0.4%, and 89.3 ± 0.8% when cultured with 2.5%, 5%, and 10% saline, respectively. The addition of 5% dextrose (64 ± 1.4%) and 10% dextrose (5 ± 2%) significantly decreased ($P<0.001$) the viability of RAW 264.7 macrophages compared to saline groups. In the presence of LPS, the addition of 5% dextrose (47.5 ± 3.6%) and 10% dextrose (0.4 ± 0.2%) significantly decreased ($P<0.001$) the viability of RAW 264.7 macrophages. The addition of 2.5% dextrose did not significantly decrease ($P>0.05$) viability when RAW 264.7 macrophages were cultured with (89.6 ± 0.7%) or without (90.3 ± 0.9%) LPS (Figure AII.1). Furthermore, the addition of 2.5%, 5% and 10% water did not significantly decrease ($P>0.05$) viability when RAW 264.7 macrophages were cultured with (87.9 ± 0.6%, 86.2 ± 1.6%, 88 ± 1.6%, respectively) or without LPS (91.8 ± 0.6%, 91.8 ± 0.3%, 91.7 ± 1%, respectively) (Data not shown).

Consistent with the viability data above, the percentage of apoptotic RAW 264.7 was approximately 7.6 ± 0.5 %, 8.8 ± 0.6%, and 8.9 ± 0.7% when cultured with 2.5%, 5%, and 10% saline, respectively, in the absence of LPS. The addition of 5% dextrose (34.8 ± 1.6%) and 10% dextrose (92.9 ± 2.9%) significantly increased (P<0.001) the percentage of apoptotic RAW 264.7 macrophages compared to saline groups (data not shown). Similarly, in the presence of LPS, the addition of 5% dextrose (50.3 ± 3.8%) and 10% dextrose (97.2 ± 0.2%) significantly increased (P<0.001) the percentage of apoptotic RAW 264.7 macrophages. As above, the addition of 2.5% dextrose did not significantly increase (P>0.05) the percentage of apoptotic RAW 264.7 macrophages cultured with (9.1 ± 1.1%) or without (8 ± 1.3%) LPS (Figure AII.2). The addition of 2.5%, 5% and 10% water did not significantly increase (P>0.05) the percentage of apoptotic RAW 264.7 macrophages cultured with (11.71 ± 0.6%, 13.6 ± 1.5%, 12 ± 1.6%, respectively) or without LPS (7.5 ± 0.5%, 7.9 ± 0.4%, 8 ± 1%, respectively).

Dextrose decreases macrophage activity

To examine the effect of dextrose on macrophage activity, the murine macrophage cell line, RAW 264.7, was cultured with 2.5%, 5%, and 10% dextrose for 24 hours and the inflammatory mediators nitrite and TNF-α, were measured in cell-free supernatants. The production of nitrite was below the assay limit of detection in all saline groups and dextrose groups in the absence of LPS (data not shown).

The production of nitrite significantly increased (P<0.0001) when RAW 264.7 macrophages were cultured with LPS and 2.5% saline (16.9 ± 0.4 uM), 5% saline (16.4 ± 0.6 uM), and 10% saline (17 ± 0.3 uM).

However, the addition of 5% dextrose (10.7 ± 0.4 uM) and 10% dextrose (0.3 ± 0.4 uM) significantly decreased (P<0.001) the production of nitrite by RAW 264.7 macrophages compared to saline and water controls (Figure AII.3). In the absence of LPS, The addition of 2.5% dextrose did not significantly decrease (P>0.05) the production of nitrite (17.1 ± 0.3 uM) by RAW 264.7 macrophages. The addition of 2.5% and 5% water did not significantly decrease (P>0.05) the production of nitrite (16.8 ± 0.4 uM, 14.8 ± 0.3 uM respectively).

Similarly, the production TNF-α was below the assay limit of detection in all saline groups and dextrose groups in the absence of LPS. The production of TNF-α significantly increased (P<0.0001) when RAW 264.7 macrophages were cultured with LPS and 2.5% saline (2534 ± 180 pg/mL), 5% saline (2293 ± 214 pg/mL), and 10% saline (2453 ± 306 pg/mL). However, the addition of 5% dextrose (1310 ± 67.9 pg/mL) and 10% dextrose (16.8 ± 14.6 pg/mL) significantly decreased (P<0.05) the production of TNF-α by RAW 264.7 macrophages compared to saline and water controls. As above, the addition of 2.5% dextrose did not significantly decrease (P>0.05) the production of TNF-α (2634 ± 65 pg/mL) by RAW 264.7 macrophages. The addition of 2.5%, 5%, and 10% water did not significantly (P>0.05) decrease the production of TNF-α (2164 ± 260 pg/mL, 1855 ± 56 pg/mL, 1972 ± 283 pg/mL, respectively) (Figure AII.4).

Discussion

To our knowledge, this is the first study to demonstrate the effect of dextrose on macrophage activity and viability. Dextrose decreased the production of nitrite and TNF-α in activated macrophages suggesting dextrose has an anti-inflammatory effect. We also found that dextrose reduced macrophage viability. In support, proliferant agents decreased cellular function

in tenocytes and decreased viability of fibroblasts *in vitro* [121, 126]. Furthermore, high glucose induced macrophages apoptosis [127, 128]. Collectively, these studies suggest dextrose induces a signaling cascade leading to apoptosis and the lack of production of pro-inflammatory mediators in response to endotoxin.

Macrophages cultured with saline, dextrose or water (data not shown) did not increase the production of nitrite and TNF-α. Culture with 10% water significantly decreased the production of nitrite but did not affect TNF-α, IL-10 or macrophage viability suggesting water in the dextrose solution was not responsible for the suppression seen in this study. However, activated macrophages cultured with 5% and 10% dextrose had a lower production of nitrite and TNF-α suggesting a higher concentration of dextrose was required to inhibit activation. Similarly, the addition of 5% and 10% dextrose decreased macrophage viability when activated with LPS. The decrease in macrophage viability was approximately 41% with 5% dextrose and 88% with 10% dextrose. Consistent with the decrease in viability, the decrease of nitrite with 5% dextrose and 10% dextrose was 34% and 98%, respectively. Similarly, the decrease of TNF-α with 5% dextrose and 10% dextrose was 46% and 99%, respectively. These results suggest, the decrease of nitrite and TNF-α are likely due to the decrease in cell viability induced by dextrose.

The decrease in viability corresponded with the increase in apoptosis of macrophages cultured with 5% and 10% dextrose with and without LPS. Apoptosis is a key feature during the resolution of inflammation. The phagocytosis of apoptotic cells does not activate the inflammatory response and has been suggested to suppress the production of pro-inflammatory cytokines [124]. Specifically, macrophages exposed to apoptotic cells increased their production of transforming growth factor beta-1 and decreased their production of pro-inflammatory cytokines. Furthermore, the induction of apoptosis in pro-inflammatory macrophages can limit

inflammation in other models [125]. Therefore, dextrose-based prolotherapy may induce apoptosis in macrophages, limiting their production of inflammatory mediators and promoting resolution in inflamed tissues. Phagocytes infiltrating the site of injection may also be suppressed upon the phagocytosis of apoptotic cells. The results from this study report an anti-inflammatory effect of dextrose on macrophages. This novel finding supports the use of dextrose-based prolotherapy to promote the resolution of inflammation.

Figure AII.1. Dextrose reduced RAW 264.7 macrophage viability in the presence of LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE in triplicate wells from 2 separate experiments. ^{a} denotes statistical decrease ($P < 0.05$) compared to all saline groups, ^{b} denotes statistical decrease ($P < 0.05$) compared to all groups.

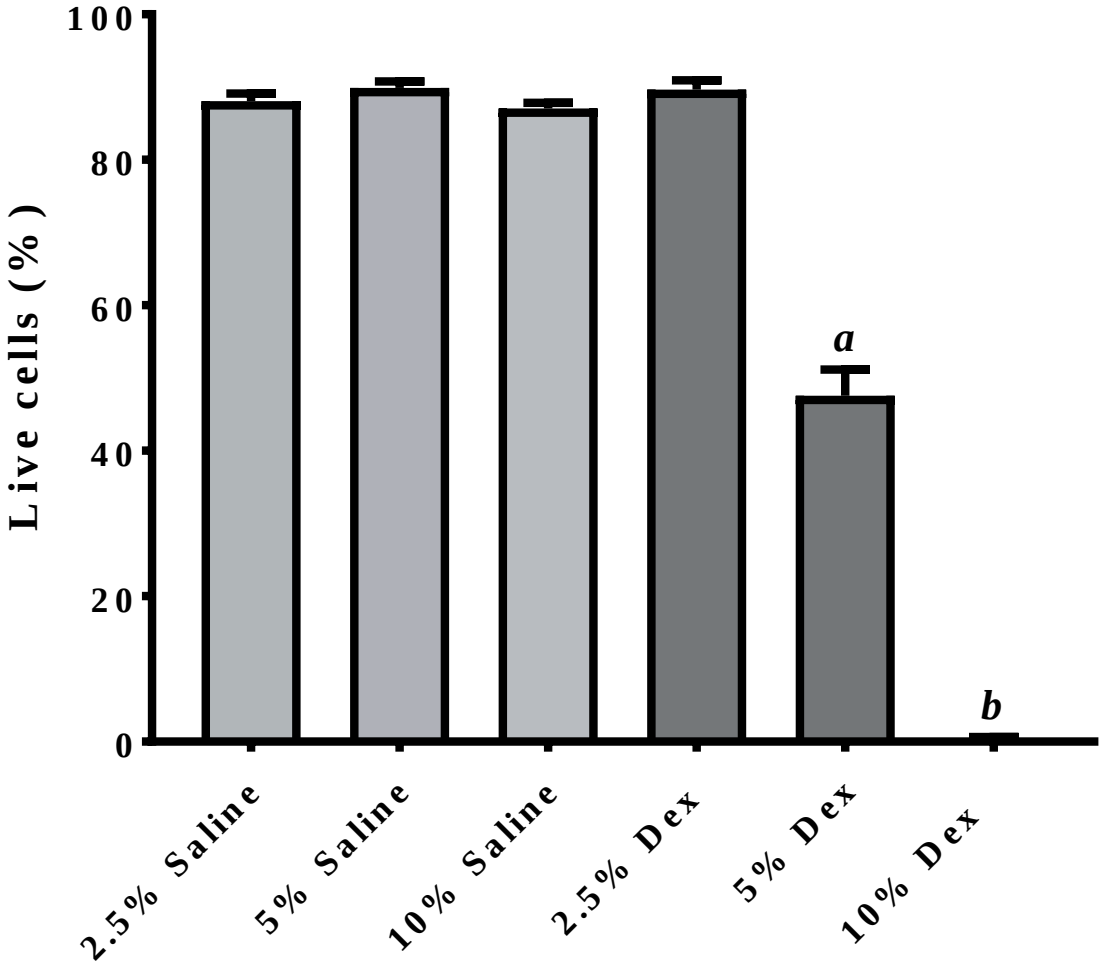

100
80
60
40
20
0
Live cells (%)
a
b
2.5% Saline
5% Saline
10% Saline
2.5% Dex
5% Dex
10% Dex

Figure AII.2. Dextrose induced RAW 264.7 macrophage apoptosis in the presence of LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE in triplicate wells from 2 separate experiments. ^{c} denotes statistical increase ($P < 0.05$) compared to all saline groups, ^{d} denotes statistical increase ($P < 0.05$) compared to all groups.

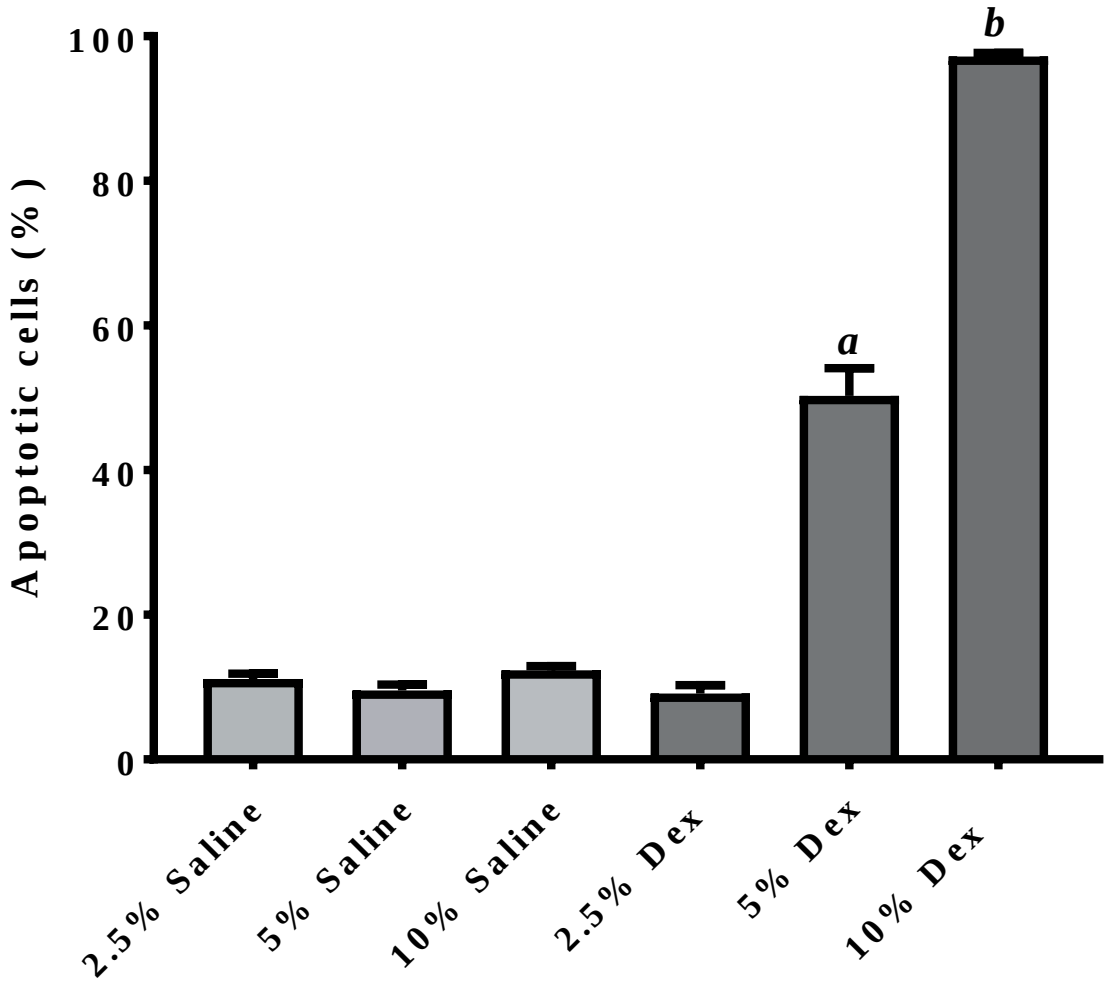

Apoptotic cells (%)
100
80
60
40
20
0
a
b
2.5% Saline
5% Saline
10% Saline
2.5% Dex
5% Dex
10% Dex

Figure AII.3. Dextrose reduced the production of nitrite by RAW 264.7 macrophages activated with LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE in triplicate wells from 2 separate experiments. ^{a} denotes statistical decrease ($P < 0.05$) compared to all saline groups, ^{b} denotes statistical decrease ($P < 0.05$) compared to all groups.

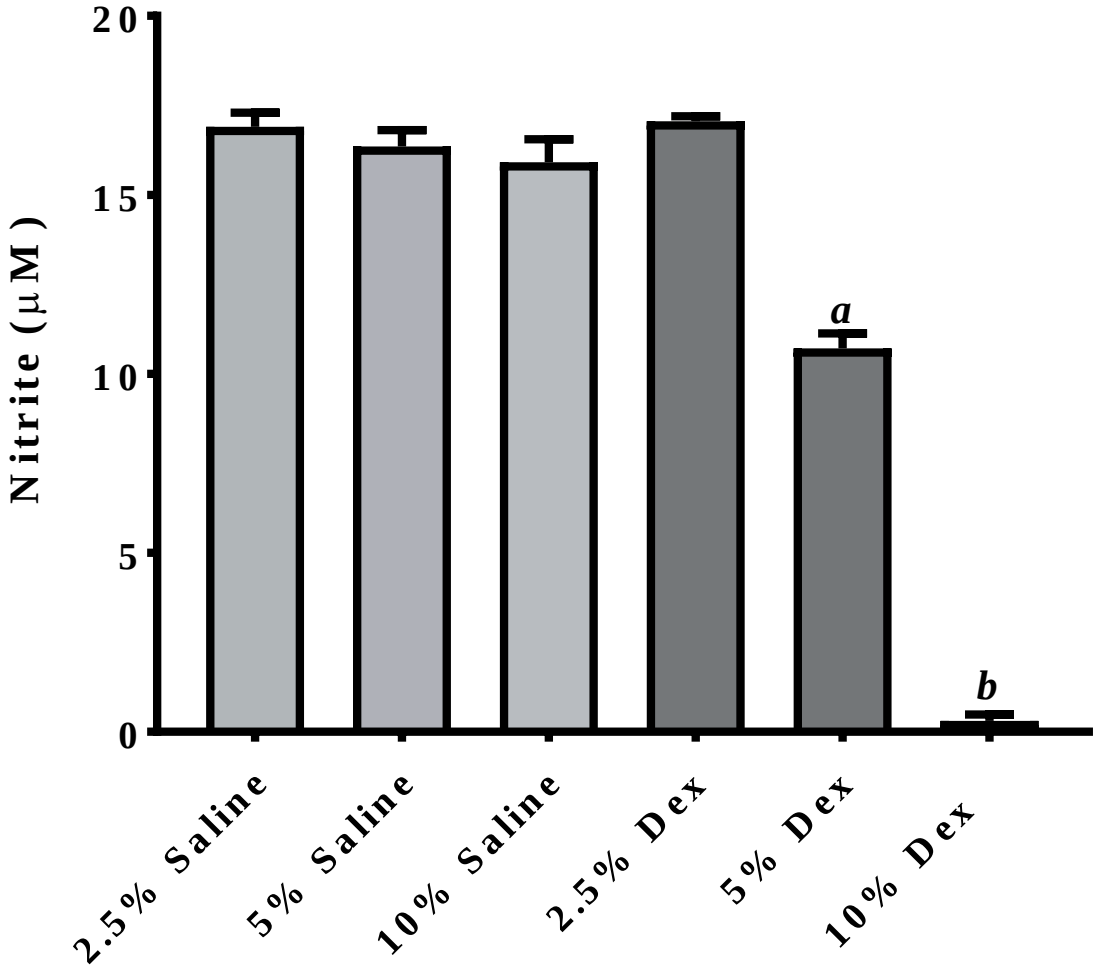
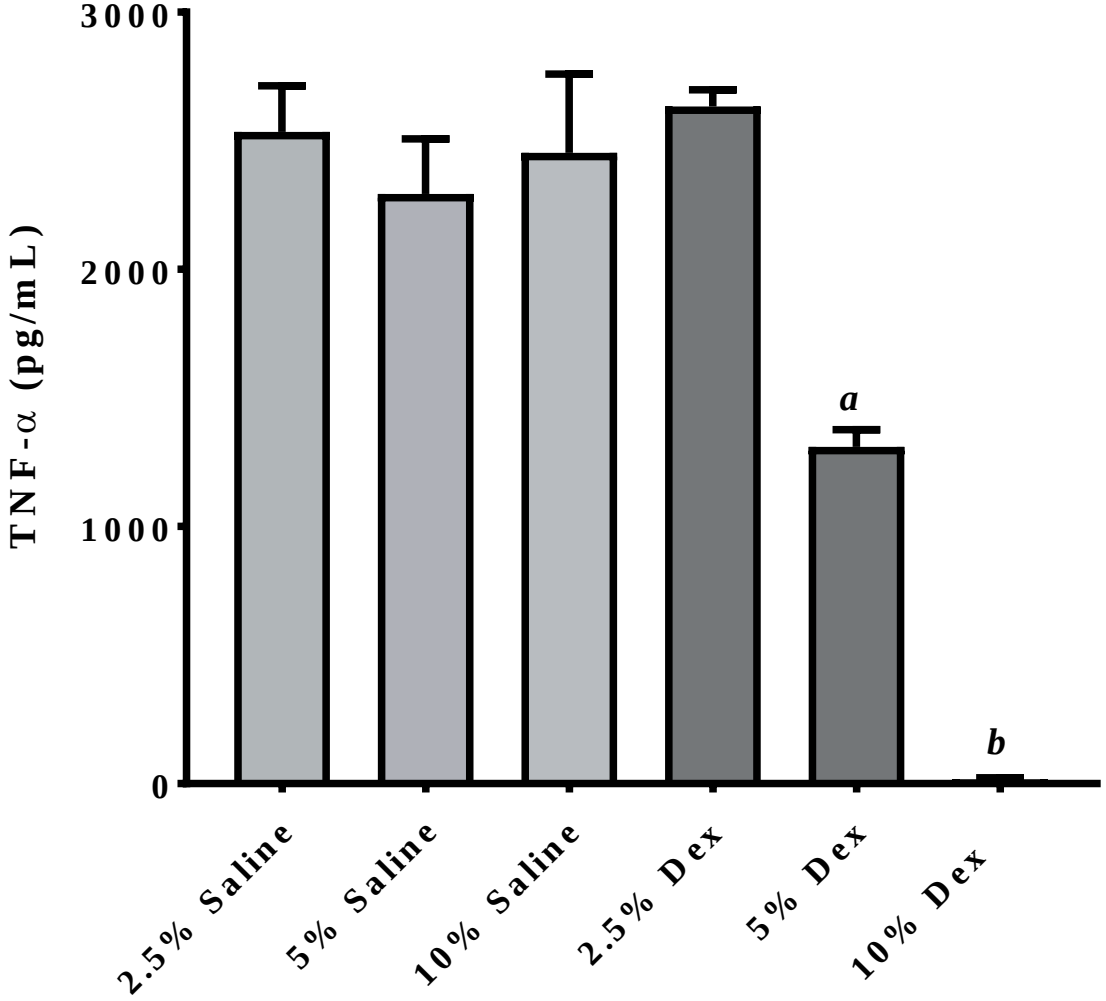

Nitrite (μM)
20
15
10
5
0
a
b
2.5% Saline
5% Saline
10% Saline
2.5% Dex
5% Dex
10% Dex

Figure AII.4. Dextrose reduced the production of TNF-α by RAW 264.7 macrophages activated with LPS. Data were analyzed by analysis of variance followed by a Tukey post-test. Data are presented as means ± SE in triplicate wells from 2 separate experiments. ^{a} denotes statistical decrease ($P < 0.05$) compared to all saline groups, ^{b} denotes statistical decrease ($P < 0.05$) compared to all groups.


3000
2000
1000
0
TNF-α (pg/mL)
a
b
2.5% Saline
5% Saline
10% Saline
2.5% Dex
5% Dex
10% Dex

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