THE EFFECT OF LYMPHATIC PUMP TREATMENT ON ANTI-TUMOR IMMUNE RESPONSES

THESIS

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CHAPTER I

INTRODUCTION AND BACKGROUND

The lymphatic system's significance in maintaining health has been focused on by the osteopathic medical profession for years. Osteopathic manipulative treatments (OMT), specifically lymphatic pump techniques (LPT), aim at increasing lymphatic circulation and improving the clearance of interstitial fluid, inflammatory agents, and protein from the interstitial space.[1,2] However, certain osteopathic manipulative techniques, such as LPT, are contraindicated in the presence of cancer with metastatic potential because it is thought that by accelerating the flow of lymph through the lymphatic vessels, tumor cells may metastasize throughout the body via the lymphatics.[2] However, this theory lacks scientific proof.

Studies previously conducted by our lab show that LPT increases thoracic duct lymph flow and leukocyte numbers[3] in the rat and significantly decreases solid tumor formation and increases leukocyte concentrations in the lungs of rats with tumors (unpublished data). It is possible that increased lymph flow along with an increased number of circulating leukocytes can improve immune surveillance, providing recognition of and protection against pathogens and disease. Therefore, we hypothesized that administration of LPT would enhance anti-tumor immune responses in the lungs of rats with pulmonary tumors. In order to test this hypothesis,

this thesis focused on one specific aim: to determine if LPT enhances leukocyte activation and/or function, thereby enhancing anti-tumor activities, such as tumor lysis and cytokine secretion.

Cancer and the Immune System

The immune system has been shown to be a crucial element in the surveillance of oncogenesis and in the elimination and control of cancer.[4] The immune system can be divided into innate and adaptive components, with extensive crosstalk between the two. The innate response serves as the first line of defense against infection and includes complement proteins, granulocytes, mast cells, macrophages, dendritic cells, and natural killer (NK) cells.[5,6] Components of the innate immune system use pattern-recognition receptors and other germline-encoded cell-surface molecules to detect tumor cells.[6,7] NK cells can lyse tumor cells through the perforin-granzyme pathway or through death ligands such as tumor necrosis factor related apoptosis related ligand (TRAIL).[7,8] Macrophages can lyse tumors through the production of nitric oxide and reactive oxygen species.[7,8] Alveolar macrophages, the most abundant antigen presenting cells in the airways and alveolar spaces[9], constitute one of the first lines of defense against pulmonary neoplasia. Compared to macrophages from other organs, alveolar macrophages are characterized by a higher capacity to phagocytose foreign material, increased production of reactive nitrogen and oxygen species, and increased production of tumor-necrosis-factor-alpha (TNF-α).[10] Dendritic cells are important in cross-priming tumor specific T cells and serve as a bridge between the innate and adaptive immune responses. Dendritic cells accomplish this by taking up tumor cell antigens or debris and re-routing them into the MHC class I presentation pathway, where they can present the tumor antigens via MHC class I to CD8+ T cells, producing tumor-specific lymphocytes.[6,7]

The adaptive immune response is mediated by antigen-specific B cells and CD4+ and CD8+ T cells. The adaptive immune response is antigen-specific and can produce memory cells

to more efficiently and quickly kill tumor cells or pathogens. CD8+ T cells recognize peptides presented by MHC class I molecules and can kill tumor cells through the perforin-granzyme pathway or the TRAIL pathway.[7,11] CD4+ T cells, also known as T helper cells, specifically T helper 1 (Th1) cells, produce cytokines which can enhance CD8+ T cell and macrophage cytotoxic functions.[6-8] CD4+ T helper 2 (Th2) cells can enhance antibody production by B cells, which can produce antibodies against tumor antigen.[8] Antibody coated tumor cells can be killed by NK cells and macrophages via antibody-dependent cellular cytotoxicity (ADCC).[7]

Cytokines are secreted or membrane-bound proteins that play in important role in leukocyte activation and trafficking into tissues. They serve as a method of communication between cells of the immune system. Several cytokines involved in the immune response against cancer include interleukin-2 (IL-2), interleukin-10 (IL-10), TNF-α, and interferon-gamma (IFN-γ). IL-2, produced by T cells, plays an important role in T cell activation, proliferation, and can enhance NK cell and CD8+ T cell function.[7,12] Thus, the antitumor effects of IL-2 are derived from its ability to expand and activate lymphocytes with antitumor activity.[12] IL-10 is important in the control of inflammation and maintaining immune homeostasis.[13] IL-10 can suppress immune responses by inhibiting antigen presentation, inhibiting MHC class II expression, inhibiting dendritic cell maturation, and inhibiting proinflammatory cytokine production.[14] However, not all IL-10 bioactivity results in a suppression of immune responses. IL-10 can costimulate B cell activation, prolong B cell survival, contribute to B cell class switching, costimulate NK cell proliferation and cytokine production, and can stimulate proliferation of certain subsets of CD8+ T cells.[14] TNF-α, secreted by macrophages and CD4+ Th1 cells activates macrophages, aids in lymphocyte recruitment into tissues, and can induce tumor cell apoptosis.[7,15] IFN-γ, secreted by NK cells, CD8+ T cells, and CD4+ Th1 cells facilitates anti-

tumor immune responses such as inhibiting tumor cell proliferation, enhancing tumor cell apoptosis, improving antigen presentation, and inhibiting angiogenesis.[7] Given the importance of the immune system in the control of cancer, therapies which enhance immune surveillance may also promote the recognition and elimination of cancer.

The Lymphatic System

The lymphatic system is an organized network of cells, vessels, and fluids which function in draining excess interstitial fluid and returning it to the blood.[16] After fluid passes from interstitial spaces into lymphatic vessels, it is called lymph.[17] Lymph flow is important in clearing excess interstitial fluid, inflammatory agents, and protein from the interstitial space.[1]

The lymphatic network begins with the lymphatic capillaries, or terminal lymphatics, that branch through peripheral tissues.[18] Lymphatic capillaries are lined by overlapping endothelial cells.[17] This unique structure functions as a one-way valve. It permits the entry of fluids and solutes, while preventing their return to the intercellular spaces.[18] From the lymphatic capillaries, lymph flows into successively larger and thicker-walled channels— first collecting vessels, then trunks, and finally, the ducts.[19] Two sets of lymphatic vessels, the superficial lymphatics and the deep lymphatics, collect blood from the lymphatic capillaries.[18] These vessels eventually converge to form larger vessels called lymphatic trunks that in turn empty into two main channels, the thoracic duct and the right lymphatic duct.[17] The right lymphatic duct drains lymph from the upper right side of the body into venous blood via the right subclavian vein.[17] The much larger thoracic duct, which begins as a small dilation called the cisterna chili, receives lymph from the rest of the body and returns it to venous blood via the left subclavian vein.[17]

Under normal conditions, lymphatic vessels are low-pressure conduits, and the same mechanisms which promote the return of venous blood to the heart, skeletal muscle contractions and respiratory pumps, also promote lymph flow.[19] Lymphatics are usually bundled together in

connective tissue sheaths along with blood vessels, and pulsations of nearby arteries also promote lymph flow.[19] Moreover, smooth muscle in the walls of the lymphatic trunks and thoracic duct contracts rhythmically, helping promote the flow of lymph.[17,19] Additionally, physical activity, passive limb movements, and body-based manipulative medicine techniques can increase lymph flow.[1,19-22] When lymphatic flow is impeded, edema can occur and metabolic waste products accumulate which can adversely affect cellular activity, predisposing cells to dysfunction and disease.[1]

In addition to functioning to maintain the extravascular homeostasis of the body and regulating tissue fluid volume and chemical composition, the lymphatic system also plays a vital role in facilitating immune responses.[16] Lymphocytes are generated in the bone marrow and migrate to the central lymphoid organs for maturation and differentiation.[15] Once mature, lymphocytes are carried in the bloodstream to the peripheral lymphoid organs, such as the lymph nodes, spleen, and mucosal lymphoid tissues.[15,18] Lymphocytes recirculate between the blood and these organs until they encounter their specific antigen in a process called immune surveillance.[15]

Lymph nodes are highly organized lymphoid organs located at the points of convergence of vessels of the lymphatic system and contain resident lymphocytes, macrophages, and dendritic cells.[15,19] As lymph is transported back to the bloodstream through lymphatic vessels, it is filtered through the lymph nodes.[19] In this way, antigen and antigen-bearing cells from infected tissues are carried in the lymph to the peripheral lymphoid organs, such as the lymph nodes, where they activate antigen specific lymphocytes.[15,18] The activated lymphocytes then undergo a period of proliferation and differentiation, after which most leave the lymph nodes as effector cells via the efferent lymphatic vessel.[15] This eventually returns them to the blood where they

can travel to sites of infection where they will act.[15] Impaired lymph drainage can inhibit leukocyte trafficking and delay antigen specific immune responses.[23] Therefore, therapies designed to improve lymph flow should enhance immune cell interaction with and response to infection and inflammation.

Cancer and the Lymphatic System

Lymphangiogenesis, or the growth of the lymphatic vessels, is regulated by lymphangiogenic growth factors such as those in the vascular endothelial growth factor (VEGF) family. In particular, VEGF-C, a secreted glycoprotein that is a ligand for vascular endothelial growth factor receptor-3 (VEGFR-3), promotes vascular permeability, angiogenesis, and lymphangiogenesis.[24] Lymphangiogenesis induced by tumor associated lymphangiogenic factors, such as VEGF-C, could promote the growth of new vessels into tumors, providing an escape route for tumor cells which could invade the lymphatics and subsequently enter the bloodstream via the thoracic duct.[24] The overexpression of VEGF-C by several tumor cell lines has been correlated with intratumoral lymphangiogenesis and increased incidence of tumor metastases.[24] Several studies on various human cancers suggest a correlation between high serum VEGF-C levels and the presence of lymph node metastasis.[25-28] Therefore, serum VEGF-C levels may be useful as a prognostic indicator of tumor metastasis. While lymphangiogenesis is associated with tumor metastasis, the effects of increased lymph flow on tumor development and metastasis are unknown.

Lymphedema is one of the most distressing and debilitating complications of breast cancer therapy, characterized by an abnormal accumulation of protein-rich, lymph fluid in the interstitial spaces of the affected tissue.[29,30] Treatments for lymphedema are aimed at increasing lymphatic transport, promoting lymphatic drainage, and encouraging removal of excess interstitial fluid.[31] Many treatments for lymphedema aim at increasing lymphatic transport and

may promote the lymphogenous spread of cancer cells.[32] While there is no scientific evidence supporting this theory, it is believed that the movement of lymph through the lymphatic vessels can include cancerous cells, and therefore promote the spread of cancer via the lymphatics.[32] Therefore, treatments that enhance lymph flow are believed to be a contraindication in the presence of cancer with metastatic potential.[2]

While there are no studies that demonstrate increased lymph flow can promote lymphatic metastasis, exercise has been shown to increase thoracic duct lymph flow[21,33] and has been associated with a positive prognosis in patients with cancer.[34,35] Specifically, studies examining exercise and cancer have shown that exercise can increase NK killing of tumor cells, decrease lung metastasis in mice in an NK-dependent manner, increase lymphocyte activation, enhance survival, and reduce tumor size in rats with cancer.[35-37] Therefore, therapies which enhance lymph flow may provide enhanced immune protection against tumors.

Osteopathic Manipulative Medicine

Homeostasis and the interrelationship of body systems are two of the basic tenets of osteopathic medical theory.[1] The osteopathic medical profession has long recognized the importance of the lymphatic system in upholding these tenets.[1] Osteopathic manipulative techniques aimed at enhancing the lymphatic circulation, such as LPT, help to mobilize fluids, enhance removal of metabolic waste, quicken recovery from infections, prevent potential disease-producing processes, and possibly boost immunity.[1,2] LPT is thought to enhance lymphatic return by enhancing the gradient for lymph flow and assisting the return of lymph from the lung, abdomen, and other tissues.[1,2] Several clinical studies provide evidence for the association of LPT with a shorter duration of hospital stay in elderly patients with pneumonia, enhanced vaccine-specific antibody responses, and increased levels of peripheral blood

leukocytes.[2,38-40] However, many of these were pilot studies involving different types of LPT, varied time-points of sample collection and experimental designs, and subjects with a wide range of demographic characteristics, making it difficult to ascertain the role of LPT at enhancing the immune response and protecting against infectious disease.

The first study to show direct, real time increases in thoracic duct lymph flow during LPT was published in 2005 by Knott et al. in which they demonstrated that both exercise and LPT significantly enhanced thoracic duct lymph flow to a similar extent in a dog model.[20] Following that study, our lab demonstrated that LPT significantly increased both thoracic duct lymph flow and leukocyte count, thus enhancing lymphatic leukocyte flux in a dog model.[22] Furthermore, studies from our lab have demonstrated that LPT increases the release of leukocytes from the gut-associated lymphoid tissues (GALT) into lymphatic circulation of rats and dogs.[3,41] This is important because antigen-specific lymphocytes primed in the gastrointestinal tissue can migrate to other mucosal tissues, such as the respiratory tract, and provide protection during infection or inflammation.[15] Together, these studies suggest that measures which increase lymph flow, such as LPT, enhance the mobilization of leukocytes, which could improve immune surveillance and provide enhanced immunity against disease.

Model to study the effects of LPT on cancer

Our lab has developed a rat model that allows us to determine the effects of LPT on the lymphatic and immune systems.[3] This was achieved by anesthetizing rats and placing a catheter in the thoracic duct while LPT was applied. Consistent with our previous studies in dogs[20,22,41], in this model we have shown that LPT significantly increases thoracic duct lymph flow and leukocyte count in rats and that a significant number of these mobilized leukocytes originate from the GALT.[3] Collectively, these studies show that the application of LPT to both the dog and rat has a similar effect and results in a significant increase in thoracic duct lymph flow and

leukocyte numbers, and thus an increase in thoracic duct leukocyte flux. Therefore, using this established rat model of LPT, the current study aimed to determine the immunological effects of LPT on cancer.

To study the immunological effects of LPT on cancer, we utilize the MADB106 tumor cell model in the Fischer 344 (F344) rat. MADB106 is an NK-sensitive, chemically induced mammary adenocarcinoma originally obtained from a pulmonary metastasis of a F344 rat.[42,43] Furthermore, MADB106 metastasizes to the lungs after intravenous (IV) injection and is commonly used to study tumor metastasis in rats.[44,45] When injected IV, lung tumor metastases can be seen in the lungs as early as seven days post-injection.[45] NK cells have been shown to play a significant role in the host defense against MADB106 tumor cells.[43] NK cell depletion results in a significantly increased number of tumor cells retained in the lungs and a five-fold increase in lung metastases.[43] In addition to NK cells, B cells have also been shown to play an important role in immune defense against MADB106 tumors. When antibody to B lymphocytes is administered to neutralize these cells, the number of MADB106 lung metastases is significantly increased.[45] Furthermore, when B cells are removed from lung lymphocyte cultures, there is diminished tumor lysis and IFN-γ secretion.[46]

Pilot studies from our lab using this tumor model have shown that application of LPT reduces solid tumors in the lungs and increases the number of leukocytes in the lungs of rats with pulmonary tumors (unpublished data). Specifically, the application of LPT for seven days after tumor cell injection resulted in a 47% reduction in the number of solid tumors in the lungs. Furthermore, the application of LPT resulted in a significant increase in the total number of pulmonary leukocytes. LPT produced approximately a three-fold increase in macrophages, NK cells, B cells, CD4+, and CD8+ T cells, with no preferential increase in any one cell type.

Therefore, given the results from our pilot studies, in the current studies, we wished to identify the immune mechanisms responsible for the clearance of tumors in rats given LPT.

CHAPTER II

THE EFFECTS OF LPT ON LEUKOCYTE FUNCTION IN RATS WITH PRIMARY MADB106 LUNG TUMORS

Introduction

Previous data collected by our lab indicates that LPT increases thoracic duct lymph flow and leukocyte numbers in a rat and dog model.[3,22] In addition, our lab has found that LPT mobilizes leukocytes from the GALT into lymphatic circulation.[41] Studies have demonstrated that both exercise and LPT enhance thoracic duct lymph flow to a similar extent in dogs.[20,33,47] Furthermore, exercise has been shown to enhance anti-tumor activities and survival in rats with cancer.[35-37] Collectively, these reports suggest that enhancing the lymphatic and immune systems may increase protection against cancer.

Studies from our lab have shown that LPT significantly decreases solid tumor formation and increases leukocyte numbers approximately two-fold in the lungs of rats with tumors (unpublished data). It is likely that by enhancing the lymphatic release of leukocytes, LPT is enhancing the trafficking of leukocytes with anti-tumor activities into the lungs of rats with pulmonary tumors. Therefore, the focus of these studies was to determine the immune mechanisms, both locally and systemically, responsible for this protection. Specifically, we

aimed to determine if LPT enhanced leukocyte activation and/or function, thereby enhancing anti-tumor activities, such as tumor lysis and cytokine secretion.

Given the lack of scientific evidence supporting or refuting the use of LPT in patients with cancer, it is important to investigate and provide scientific evidence documenting the effect of LPT in patients with cancer, as it could prove to be a valuable adjunctive therapy to be used to treat cancer patients.

Materials and Methods

Animals. Immune competent male inbred Fischer 344 (F344) rats (Charles Rivers Laboratories, Wilmington, MA) with jugular vein catheters weighing between 250-350 grams were used in these studies. Catheterized rats were used in order to avoid repeated administration of intraperitoneal anesthesia, minimizing pain and distress. Rats were housed and fed according to the Institutional Animal Care and Utilization Committee (IACUC) of the University of North Texas Health Science Center in the barrier facility.

Tumor Cell Culture and Inoculation. MADB106 is an NK-sensitive, chemically induced mammary adenocarcinoma originally obtained from a pulmonary metastasis of a F344 rat.[42,43] The MADB106 cell line was maintained in monolayer cell culture at 37°C with 5% CO_2 in RPMI complete medium (HyClone, Logan, UT) supplemented with 10% heat inactivated fetal bovine serum (HyClone), 1X antibiotics/antimycotics (HyClone), 1X L-glutamine (CellGro™ Mediatech, Inc, Herndon, VA), and 1X hepes (Fisher Scientific, Fair Lawn, NJ). Cells were harvested from culture flasks by using sterile PBS (Hyclone) and a cell scraper to remove the adherent cells from the surface of the flask. Tumor cells were washed twice and suspended in sterile PBS for hemacytometer cell count prior to being injected into the animals. Tumor cell

viability was determined by Trypan blue (Sigma-Aldrich, St. Louis, MO) staining. On day 0 rats were injected utilizing the indwelling jugular vein catheter with $1x10^6$ MADB106 tumor cells in log phase per rat in sterile PBS. Previous studies in our lab have shown that using this method, solid tumors form in the lungs by the eighth day.

Treatment Groups. Rats were divided into one of three treatment groups 24 hours following tumor inoculation. Treatment groups consist of control, sham, and LPT. On days 1-7 the control group received no treatment or anesthesia, the sham group received a daily sham treatment consisting of intravenous administration of propofol anesthesia (Hospira Inc., Lake Forest, IL) followed by four minutes of light touch, and the LPT group received four minutes of LPT daily under propofol anesthesia. For the LPT and sham treatment, the propofol anesthesia was administered at a dose of 10 mg/kg body weight through the jugular vein catheter. To perform LPT, the operator contacted the rat with the thumb and fingers placed bilaterally at the costal-diaphragmatic junction. Sufficient pressure was exerted medially and cranially to compress the lower ribs until significant resistance was met against the diaphragm, then the pressure was released. Compressions were administered at approximately one per second for the duration of the four minute treatment.

***Lymphocyte Isolation*.** Eight days following tumor injection, lungs and spleens were removed. To prepare lung single-cell suspensions, lungs were placed in RPMI wash media (5% FBS, 1X antibiotics/antimycotics, 10 mM Hepes) and finely minced for subsequent tissue dissociation using a gentleMACS dissociator (MACS Miltenyi Biotec, Auburn, CA). Lung cell suspensions were then passed through a nylon mesh filter to remove any non-dissociated tissue. After lung

cell suspensions were washed twice with RPMI wash media, they were layered over a Lympholyte Rat gradient (Cedarlane Laboratories Limited, Burlington, NC) to purify lymphocytes by density gradient centrifugation. Samples were centrifuged at 1200g for 20 minutes, 25°C, slow break. The lymphocytes separated on the gradient were collected for enumeration using a Hemavet 950 Cell Analyzer (Drew Scientific, Waterbury, CT). The lung lymphocytes were used for *in vitro* stimulations and *in vitro* tumor lysis assays.

A single cell suspension of spleen cells was prepared by passing spleens through a nylon mesh filter. Cells were then washed twice in RPMI wash media (5% FBS, 1X antibiotics/antimycotics, 10mM Hepes) and red blood cells were removed using ACK lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA). Cells were collected and washed 2 more times in RPMI wash media, then counted and resuspended in culture media. The spleen lymphocytes were used for Hemavet total cell count, *in vitro* stimulations, and for *in vitro* tumor lysis assays.

Flow cytometry. Immunofluorescent staining of lung and spleen lymphocytes was performed using monoclonal antibodies against CD3+ T cells, CD4+ T cells, CD8+ T cells, CD49d, CD61, macrophages, granulocytes, B cells, and NK cells. For each stain, 1 x 10^6/100µL of lung or spleen cells were incubated with the optimal concentration of monoclonal antibody for 30 minutes in the dark at room temperature. Individual lung and spleen cell aliquots were stained with either 20µL of anti-rat CD3-FITC isotype control, or 5 µL of anti-rat CD4-RPE isotype control. Additionally, separate 100µL aliquots of each sample were stained with 2µL of CD3, 2.5µL of CD4, 2.5µL of CD8, and 10µL of CD49d or CD61. Separate aliquots were stained with 2µL CD3 and 2.5µL of CD4, 2µL of CD3 and 2.5µL of CD8, 10µL of CD49d and CD61,

20µL of MO, 2µL of B cell, and 2µL of NK cell. All flow cytometry antibodies were obtained from BioLegend (San Diego, CA), except CD49d and CD61 from SeroTec (Raleigh, NC). One 100µL lung and spleen aliquot was left unstained to serve as a negative control. Individual 100µL aliquots of each of the samples were washed two times with 2mL of staining buffer (PBS with 2% fetal bovine serum) at 400g, 5 minutes, 4°C. Following removal of supernatant, lung and spleen cells were fixed with 0.5% paraformaldehyde (Sigma) in PBS and stored at 4°C until flow cytometry analysis. Cells were analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA). Lymphocyte gates and detector voltages were set using unstained lung and spleen cell controls. Cell populations were detected from histogram peaks or dot plot cell clusters. Each cell population was expressed as the percentage of the number of stained cells. To calculate the total number of a specific lymphocyte population, their percentage was multiplied by the total number of cells in lung or spleen samples as determined by hemacytometer or hemavet count.

Leukocyte Counts. Blood, lung, and spleen samples were analyzed for leukocyte numbers. To prepare lung and spleen tissue samples for leukocyte enumeration, tissue samples were treated as described in the previous section. Cardiac blood samples were collected eight days after tumor injection. Total leukocytes and differential leukocytes counts for each blood and spleen sample and total leukocytes counts of each lung sample were determined by a Hemavet 950 Cell Analyzer (Drew Scientific).

Cell Stimulations. An *in vitro* cell stimulation was used to determine the *in vitro* cytokine production of lung and spleen leukocytes in response to stimulation with Concanavalin A (Con

A) or MADB106 tumor cells. To prepare lung and spleen tissue samples for stimulation, samples were treated as described in the previous two sections. $1x10^6$ lung or spleen cells were added per well to 96 well culture plates. Cells were cocultured for 48 hours at 37°C and 5% CO_2 with either 10µg/ml Con A (Sigma), 10^4 MADB106 tumor cells, or RPMI complete culture media supplemented with 10% heat inactivated fetal bovine serum, 1X antibiotics/antimycotics, 1X L-glutamine, and 1X hepes. After 48 hours of coculture, the plates were centrifuged at 400g for 10 minutes at 4°C and cell stimulation supernatants were analyzed for IL-2, IL-6, IL-10, IFN-γ, and TNF-α by multiplex enzyme-linked immunosorbent assays (ELISA).

Cytokine Multiplex ELISA Assay. The amount of cytokine production in blood serum and in *in vitro* spleen and lung stimulation supernatants was determined by multiplex ELISA. Rat IL-2, IL-6, IL-10, IFN-γ, and TNF-α cytokine levels were measured using rat cytokine Milliplex MAP kits (Millipore, Billerica, MA). Briefly, 96-well filter plates were prewet with 200µl assay buffer per well and mixed on a plate shaker for 10 minutes at room temperature. Following removal of assay buffer by vacuum, 25µl standard or control was added to appropriate wells, 25µl assay buffer was added to background and sample wells, and 25µl sample was added to sample wells. Next, 25µl matrix was added to background, standard, and control wells. After the addition of matrix, 25µl of antibody-immobilized beads was added to each well and plates were sealed and incubated overnight on a plate shaker at 4°C. Plates were then washed 2 times with 200µl per well of wash buffer. After washing, 25µl detection antibodies were added to each well and plates were incubated on a plate shaker for 2 hours at room temperature. Following incubation, 25µl streptavidin-phycoerythrin was added to each well and plates were incubated for 30 minutes at room temperature on a plate shaker. Plates were then washed 2 times with 200µl per well of

wash buffer. After washing, 150µl of sheath fluid was added to each well and beads were resuspended on a plate shaker for 5 minutes. Plates were then read on a Bio-Plex 100 system (Bio-Rad, Hercules, CA). Cytokine levels were determined by analysis of the median fluorescent intensities (MFI) using a weighted 5-parameter logistic curve-fitting method and analyzed using Bio-Plex Manager 4.0 software (Bio-Rad).

VEGF-C ELISA Assay. The VEGF-C levels in the blood serum were determined using a rat VEGF-C ELISA kit (Bender MedSystems GmbH, Vienna, Austria). Briefly, pre-coated 96 well microtiter plates were washed twice with 400µl wash buffer per well. Next, 100µl of the standards and blood serum samples diluted in sample diluent were then added into the appropriate wells. Next, 50µl biotin-conjugate was added to each well. Plates were then covered and incubated at room temperature for 2 hours. Following incubation, plates were washed 6 times with 400µl wash buffer per well. 100µl of diluted streptavidin-HRP was then added to all wells and plates were incubated for 1 hour at room temperature. Following incubation, plates were washed 6 times as before and 100µl TMB substrate solution was added to all wells. Plates were then covered and incubated at room temperature for 30 minutes. Subsequently, 100µl of stop solution was added to each well and plates were immediately read at an absorbance of 450nm using a MRX Microplate Reader (Dynex Technologies, Inc, Chantilly, VA). VEGF-C levels were determined by comparison with standard curves determined by 5-parameter curve fits.

Chromium Release Assay. A chromium release assay was used to quantify cell-mediated tumor lysis by lung and splenic leukocytes. The standard xenogeneic YAC-1 mouse lymphoma cell

line (ATCC, Manassas, VA) was used to measure *in vitro* NK cytotoxicity. This cell line is the standard target cell line for assessing NK cytotoxicity *in vitro* in rodents.[48] The YAC-1 cells were maintained in 5% CO_2 at 37°C suspension cultures in RPMI complete medium (HyClone) supplemented with 10% heat inactivated fetal bovine serum (HyClone), 1X antibiotics/antimycotics (HyClone), 1X L-glutamine (CellGro™ Mediatech, Inc), and 1X hepes (Fisher Scientific).

Single cell suspensions of lung and spleen cells were prepared as described previously. Target YAC-1 tumor cells were labeled by incubating $1x10^6$ cells with 2 MBq of $Na_2{}^{51}CrO_4$ (PerkinElmer, Waltham, MA) for 90 minutes at 37°C and 5% CO_2. Separately, effector lung and spleen cells were incubated with ^{51}Cr-labeled target YAC-1 tumor cells at selected effector:target cell ratios (1:1, 10:1, 50:1 for lung cells and 1:1, 10:1, 50:1, 100:1 for spleen cells) in duplicate in round bottom 96 well plates. Cells were incubated for 4 hours at 37°C and 5% CO_2. After incubation, the plates were centrifuged at 280g for 5 minutes and supernatants were collected and their radioactivity was measured. The percentage of specific lysis was calculated by the following equation: $(a-b/c-b) \times 100$, where *a* is the radioactivity of the supernatant of target cells mixed with effector cells, *b* is that in the supernatant of target cells incubated alone, and *c* is that in the supernatant after lysis of target cells with 1% Nonidet P-40.

Preparation of Tumor Cell Lysate. A solution of MADB106 tumor cell lysate was prepared to be used to coat tumor antibody ELISA plates to detect tumor specific IgM and IgG. Briefly, aliquots of 1×10^6 MADB106 tumor cells/ml in 1 ml sterile PBS were placed in microcentrifuge tubes. Next, cells were frozen at -80°C for 15 minutes. Cells were then thawed in a warm water bath at 37°C for 4 minutes. These freeze-thaw cycles were repeated for a total for 4 times.

Following the last freeze-thaw cycle, the cells were centrifuged at 300g for 10 minutes and supernatant was collected and pooled together. Protein content in tumor cell lysate was quantified by Bradford protein assay (Bio-Rad).

Tumor Antibody ELISA Assay. The amount of tumor specific IgM and IgG were determined by an indirect ELISA assay. Briefly, 96-well microtiter plates were coated with 100 µl/well of 10 µg/ml tumor lysate diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) and incubated overnight at 4°C. Plates were then washed 3 times with 200 µl/well wash buffer (PBS/0.05% Tween 20). Plates were then blocked with 200 µl/well 3% powdered milk and incubated at room temperature for 2 hours. Plates were then washed 3 times with wash buffer as before and 100 µl rat serum samples serially diluted in 3% powered milk were added to wells. Plates were then incubated overnight at 4°C. The next day, plates were washed 3 times and 100 µl/well of goat anti-rat IgM: HRP or goat anti-rat IgG: HRP (AbD Serotec, Raleigh, NC) diluted 1:1000 in 3% powdered milk was added to the wells and plates were incubated for 2 hours at room temperature. Plates were then washed 3 times with wash buffer and 100 µl/well of TMB substrate solution was added to the plates which were then incubated for 7 min (IgM) and 1 hour (IgG) at room temperature. 50 µl stop solution was then added to each well and plates were read at 450 nm using a MRX microplate reader (Dynex Technologies, Inc.). End-point antibody titers were expressed as the reciprocal dilution of the last dilution giving an OD at 450 nm > 0.1.

Statistical analysis. Results obtained from control, sham and LPT groups were compared. To perform statistical analysis, results were analyzed by ANOVA to compare sham and control treatment values with LPT values. GraphPad Prism version 5.00 for Windows, GraphPad

Software was used (GraphPad Software, San Diego, CA). Differences among mean values with $P \leq 0.05$ were considered statistically significant. Data was expressed as mean ± SEM.

Results

LPT increases leukocyte numbers in the lungs

We previously found that LPT decreases solid tumor formation by 47% in the lungs of rats with pulmonary tumors. To find out if the decrease in solid tumors in the lungs of LPT animals was associated with an increase in the number of leukocytes in the lungs, rats were intravenously injected with tumor cells and given control, sham, or LPT treatment for seven consecutive days as previously described. On day eight post tumor injection, lung leukocyte populations were quantified by flow cytometry. LPT significantly increased the number of leukocytes in the lungs compared to control and sham treatment (Table 1). Specifically, the number of macrophages, natural killer (NK) cells, B cells, CD4+ T cells, and CD8+ T cells were all significantly increased in the lungs of LPT-treated rats. While LPT increased the absolute numbers of the different leukocyte populations in the lungs, it did not change the relative proportion of any of the leukocyte subsets (Table 1), suggesting that LPT does not preferentially promote the migration of any specific leukocyte population. In contrast to the lungs, spleen leukocyte numbers did not increase in response to LPT (Table 1), demonstrating that the effects of LPT are localized to the lung.

LPT enhances in vitro IL-2, IL-10, and IFN-γ production from lung-derived leukocytes

To determine if the leukocytes in the lungs of the LPT animals were more sensitive to cytokine production *in vitro*, rats were injected with tumors and received control, sham, or LPT as described earlier. Eight days after tumor injection, lung and spleen leukocytes were isolated and cocultured for 48 hours at 37°C in the presence of either culture media (negative control),

concanavalin A (ConA), or MADB106 tumor cells. Following coculture, cell supernatants were analyzed for IL-2, IL-6, IL-10, IFN-γ, and TNF-α by Multiplex ELISA assay. The lung-derived leukocytes from LPT treated rats produced significantly more IL-2 (Figure 1) and IL-10 (Figure 2) in response to stimulation with ConA compared to those from control and sham groups. Furthermore, increased IFN-γ production was also observed in lung-derived leukocytes from LPT treated rats in response to ConA and tumor stimulation when compared to control and sham groups, although this was not statistically significant (Figure 3). While tumor stimulated the most IL-6 production *in vitro*, there were no significant differences in IL-6 production by lung-derived leukocytes (Figure 4). There was little TNF-α produced by splenic and lung leukocytes, nor were there differences between groups (data not shown).

In contrast to the patterns observed in lung leukocytes, there was no statistical difference in *in vitro* production of any measured cytokines by splenocytes in response to any of the stimuli between control, sham, and LPT treatment groups (Figures 1-4). Although lung-derived leukocytes from LPT treated rats produced more IL-2, IL-10, and IFN-γ in response to ConA, splenocytes still produced a higher overall concentration of each of these cytokines. Overall, these results suggest that leukocytes isolated from the lung, but not the spleen, of LPT treated rats are more sensitive to cytokine secretion is response to ConA stimulation.

LPT mobilizes gastrointestinal-derived lymphocytes into the lungs

Previously, we have demonstrated that LPT increases the release of leukocytes from the GALT into lymphatic circulation of rats and dogs.[3,41] Therefore, to determine if LPT facilitates the trafficking of gastrointestinal-derived lymphocytes into the lungs of tumor-bearing rats, rats were injected with tumors and received control, sham, or LPT treatment for seven consecutive

days as described earlier. On day eight post tumor injection, lung leukocytes were stained for the gastrointestinal homing receptor integrin α4β7. LPT significantly increased the number of α4+β7+ lymphocytes in the lungs compared to control and sham treatment (Figure 5). This suggests that LPT increases the number of GALT-derived lymphocytes in the lungs of rats with pulmonary tumors.

LPT does not enhance in vitro tumor lysis

To determine if lymphocytes from LPT treated rats have enhanced killing activity against tumors, rats were injected with tumors and received control, sham, or LPT treatment as described previously. Eight days after tumor injection, *in vitro* tumoricidal activity of lung and splenic leukocytes was measured. Briefly, chromium-51-labeled YAC-1 tumor cells were incubated for four hours at 37°C with lung or splenic leukocytes at selected effector:target cell ratios (1:1. 10:1, 50:1, and 100:1), after which cell supernatants were analyzed for radioactivity to quantify the amount of tumor lysis. There were no differences in tumor lysis between treatment groups (Figure 6), suggesting that LPT does not enhance the capacity of leukocytes to kill tumor cells.

LPT does not increase serum VEGF-C

Vascular endothelial growth factor-C (VEGF-C) is a secreted glycoprotein that promotes vascular permeability, angiogenesis, and lymphangiogenesis.[24] Several studies on various human cancers suggest a correlation between high serum VEGF-C levels and the presence of lymph node metastasis.[25-28] Therefore, we measured serum VEGF-C levels as a potential indicator of tumor metastasis. There was no difference in the serum VEGF-C levels between treatment groups (Figure 7). Furthermore, no solid tumors were detected in extrapulmonary tissues. These

data suggest that LPT does not promote lymphangiogenesis and metastasis during solid tumor development during this time frame.

LPT does not enhance peripheral blood leukocyte numbers

To determine if LPT also increases the numbers of leukocytes in the blood, rats were injected with tumors and received control, sham, or LPT treatment for seven consecutive days as described previously. Eight days after tumor injection rats were sacrificed and cardiac blood was taken and total leukocytes and differential leukocyte counts were quantified using a Hemavet 950 cell analyzer. There were no significant differences in leukocyte counts in cardiac blood between treatment groups (Table 2). In addition, LPT did not increase the proportion of any of the analyzed cell populations in the cardiac blood.

LPT does not increase tumor specific antibody concentrations in the serum

To determine if LPT would increase tumor specific IgM or IgG in the serum, rats were injected with tumors and received control, sham, or LPT treatment for seven days. Eight days following tumor injection cardiac blood serum was isolated and analyzed for tumor specific IgM and IgG by ELISA. While there was a trend for LPT to increase tumor-specific serum IgM, this is not statistically significant (Figure 8). In addition, no tumor specific IgG could be detected in any of the serum samples, suggesting isotype switching had not yet occurred. Together, these data suggest that LPT does not increase tumor-specific antibody concentrations in the blood.

Discussion

Previously we demonstrated that LPT decreases solid tumors and increases leukocyte numbers in the lungs of rats with pulmonary tumors. The objective of this study was to determine if LPT enhanced anti-tumor leukocyte responses. Therefore we measured *in vitro*

cytokine production and tumor lysis by lung and splenic leukocytes and tumor specific antibody responses in the blood serum. We also determined leukocyte numbers both locally, in the lung, and peripherally, in the blood and spleen. Furthermore, we measured circulating VEGF-C levels to help determine if LPT promotes lymphangiogenesis during primary tumor development.

The most significant finding of this study was that LPT exerts a tissue specific response in the lung. This is demonstrated by two main findings, the first being that LPT significantly increased leukocyte concentrations in the lung but not the spleen. Secondly, LPT enhanced *in vitro* cytokine production from pulmonary, but not splenic leukocytes. Specifically, LPT enhanced IL-2, IL-10, and IFN-γ production by pulmonary leukocytes in response to ConA stimulation.

IL-2 is an important cytokine for T cell proliferation and can enhance NK cell and CD8+ T cell function.[7,12] IFN-γ facilitates anti-tumor immune responses such as inhibiting tumor cell proliferation, enhancing tumor cell apoptosis, improving antigen presentation, and inhibiting angiogenesis.[7] IL-10 is important for controlling inflammation and maintaining immune homeostasis.[13] While LPT increased the number of immune cells in the lungs, it did not alter the leukocyte populations (Table 1). Therefore, it is unlikely that the *in vitro* stimulations had varied pulmonary leukocyte populations between the groups. However, LPT did enhance IL-2 production *in vitro*, which may have enhanced T cell proliferation, which could subsequently increase T cell numbers.

While lung-derived leukocytes from LPT treated rats produced more IL-2, IL-10, and IFN-γ in response to ConA stimulation, splenocytes produced a higher overall concentration of each of these cytokines. In contrast, lung-derived leukocytes produced more IL-6 than splenocytes. Studies have found differences in cytokine secretion, cell activation, and cell

proliferation between the lungs and spleens of infected mice.[49,50] Importantly, a previous study using the MADB106 tumor model found that B cells from the lung and spleen differentially influenced *in vitro* cytokine production and tumor lysis, thus differentially affecting antitumor activity in each compartment.[46] Collectively, these studies demonstrate differences between the lung and splenic immune response, including cell activation, proliferation, and cytokine production, which is likely occurring in our model as well.

We have previously shown that LPT facilitates the release of leukocytes from the GALT.[22,41] In this study, LPT significantly increased the number of GALT-derived lymphocytes in the lungs of tumor-bearing rats. Antigen-specific lymphocytes primed in the gastrointestinal tissue can migrate to other mucosal tissues, such as the respiratory tract, and provide protection during infection or inflammation.[15] GALT-derived lymphocytes differ from peripheral blood lymphocytes in that they are in a higher state of activation due to constant stimulation by intestinal contents and gut flora.[51] Furthermore, GALT-derived lymphocytes have increased expression of genes associated with T cell activation and produce high levels of cytokines when stimulated via mitogen or CD2, when compared to peripheral blood lymphocytes.[52,53] Therefore, LPT may enhance the redistribution of leukocytes from the GALT into the lung.

Our results indicate that LPT does not enhance the ability of lymphocytes to kill tumors, since there were no differences in *in vitro* tumor lysis between treatment groups. However, we found that LPT significantly increases the numbers of both NK cells and CD8+ T cells in the lungs of tumor-bearing rats. Both of these cell types are important in the anti-tumor immune response. Specifically, NK cells and CD8+ T cells can lyse tumors through the release of enzymes such as perforin and granzyme as well as through apoptosis-inducing ligands such as tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL).[7] Therefore, by increasing the

number of CD8+ T cells and NK cells in the lungs, LPT may enhance cell-mediated killing of pulmonary tumors.

VEGF-C is a factor which promotes vascular permeability, angiogenesis, and lymphangiogenesis.[24] Overexpression of VEGF-C by various tumor cell lines and cancers has been correlated with intratumoral lymphangiogenesis and increased incidence of tumor metastases.[54] Several studies on various human cancers suggest a correlation between high serum VEGF-C levels and the presence of lymph node metastasis.[25-28] Therefore, we measured serum VEGF-C levels as a potential indicator of tumor metastasis. There was no difference in the serum VEGF-C levels between treatment groups. Furthermore, no extrapulmonary tumors were detected in any rats. These data suggest that LPT does not promote lymphangiogenesis and metastasis during solid tumor development.

LPT did not increase leukocyte numbers in the blood. A previous study using MADB106 found no change in leukocyte numbers in the blood and spleen, while those in the lung increased in response to tumor cell injection.[45] In addition, LPT moderately increased tumor-specific serum IgM but this was not statistically significant, suggesting that LPT does not enhance tumor-specific IgM in the serum. Furthermore, it appears that isotype switching has not yet taken place on day eight post tumor injection, due to the lack of detectable IgG. It is possible that LPT may enhance anti-tumor antibody concentrations at a later time point, after isotype switching has taken place. Collectively, these data suggest that LPT does not enhance peripheral blood immune responses, but rather increases pulmonary immunity.

Conclusion

Our data suggests that treatments that aim to enhance lymphatic flow and GALT redistribution of lymphocytes, such as LPT, may enhance immune surveillance and inhibit solid tumor formation in the lung. Mechanistically, we propose that by increasing leukocyte

trafficking into the lungs, LPT may facilitate the interaction between leukocytes and tumor antigens as well as promoting the entry of immune cells with anti-tumor activity into the lungs, hence enhancing the development of an effective anti-tumor immune response. Additionally, LPT enhances the mucosal redistribution of GALT-derived lymphocytes into the lungs of rats with pulmonary tumors. These redistributed GALT-derived lymphocytes may secrete cytokines that facilitate T cell proliferation, anti-tumor responses, and regulation of the inflammatory response. Furthermore, our results suggest that LPT does not facilitate the metastasis of cancer during primary tumor development; however, it is still unknown as to whether LPT will promote the metastasis of established tumors. Our results provide early experimental support for the use of LPT as an adjunctive therapy in patients with cancer.

CHAPTER III

ILLUSTRATIONS

Table 1. LPT increases leukocytes in the lungs of rats with pulmonary tumors.

	Spleen			Lung		
Cells x 10^5	**Control**	**Sham**	**LPT**	**Control**	**Sham**	**LPT**
Macrophages	192 ± 19	209 ± 39	252 ± 33	14 ± .75	15 ± 1.0	28 ± 1.4 ***
NK cells	450 ± 33	401 ± 63	479 ± 20	2.9 ± .38	3.3 ± .45	7.5 ± 1.1 **
B cells	608 ± 86	698 ± 90	879 ±77	2.9 ± .44	3.3 ± .59	7.3 ± 1.5 *
CD4+ T cells	773 ± 52	802 ± 73	998 ± 65	2.4 ± .21	2.5 ± .46	4.3 ± .72 *
CD8+ T cells	858 ± 60	995 ± 237	1155 ± 114	3.5 ± .41	3.5 ± .73	6.8 ± 1.2 *

	Spleen			Lung		
Percentage	**Control**	**Sham**	**LPT**	**Control**	**Sham**	**LPT**
Macrophages	4.2 ± .41	4.1 ± .50	4.4 ± .41	38 ± 2.1	36 ± 2.6	40 ± 2.0
NK cells	10 ± .37	8.2 ± .49	8.5 ± .20	8.2 ± 1.0	8.2 ± 1.1	11 ± 1.6
B cells	13 ± 1.7	15 ± .46	15 ± .53	8.0 ± 1.2	8.2 ± 1.5	10 ± 2.2
CD4+ T cells	17 ± .44	17 ± 1.3	18 ± .73	6.2 ± .67	6.2 ± 1.2	6.2 ± 1.0
CD8+ T cells	19 ± .42	20 ± 2.6	20 ± 1.2	9.8 ± 1.1	8.7 ± 1.8	9.7 ± 1.7

On day 0, rats were intravenously injected with 1 x 10^6 tumor cells in log phase. For days 1-7, the control group received no treatment or anesthesia, the sham group received 4 min of light touch under anesthesia, and the LPT group received 4 min of LPT under anesthesia. On day 8, rats were euthanized and the lungs removed to enumerate leukocyte populations. Data are means ± SEM total lung leukocytes x 1 x 10^5 (top table) and % of total lung leukocytes (bottom table). N=5 rats per group for spleen and N=13-17 rats per group for lung. *** denotes $p < 0.001$, ** denotes $p < 0.01$, and * denotes $p < 0.05$ compared to control and sham.

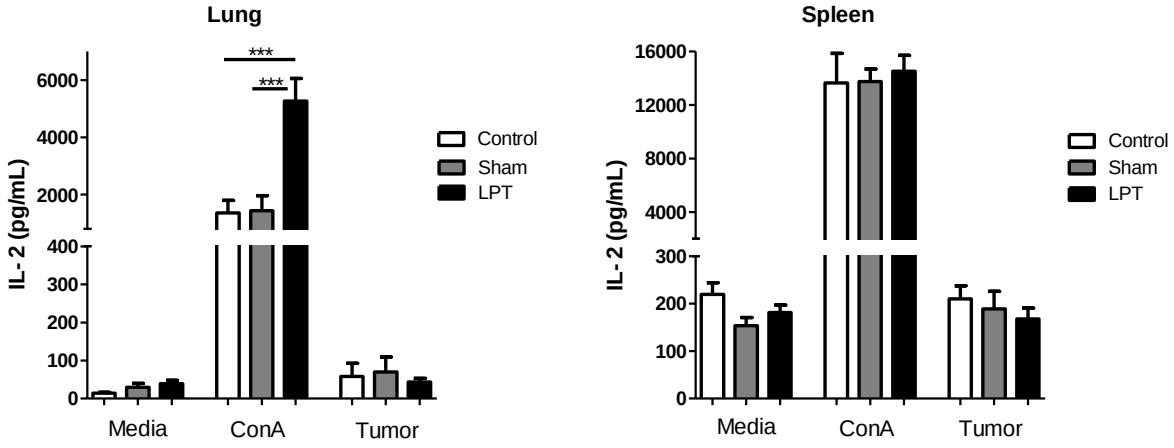

Figure 1. LPT enhances IL-2 secretion by pulmonary leukocytes *in vitro*. On day 0, rats were intravenously injected with 1 x 10^6 MADB106 tumor cells in log phase and received treatment for days 1-7. On day 8, rats were euthanized and splenic and lung leukocytes were isolated. The leukocytes were adjusted to a concentration of 10^6 leukocytes/well and co-cultured with either media, 10µg/ml ConA, or 10^4 MADB106 tumor cells/well. Cell culture supernatants were analyzed by multiplex assay. Data are means ± SEM IL-2 concentration (pg/ml) for 3 experiments. *** denotes $p < 0.001$. N=5-11 rats per group.

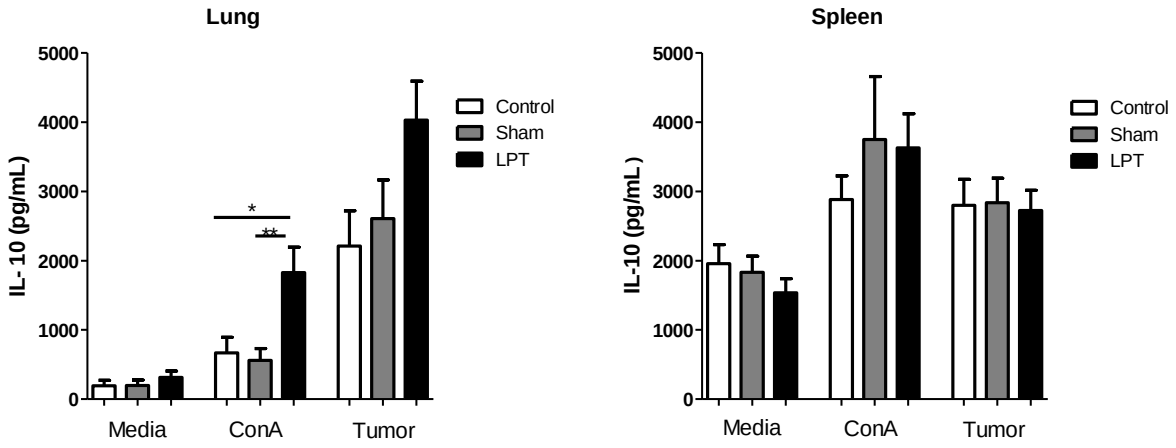

Figure 2. LPT enhances IL-10 secretion by pulmonary leukocytes *in vitro*. On day 0, rats were intravenously injected with 1 x 10^6 MADB106 tumor cells in log phase and received treatment for days 1-7. On day 8, rats were euthanized and splenic and lung leukocytes were isolated. The leukocytes were adjusted to a concentration of 10^6 leukocytes/well and co-cultured with either media, 10µg/ml ConA, or 10^4 MADB106 tumor cells/well. Cell culture supernatants were analyzed by multiplex assay. Data are means ± SEM IL-10 concentration (pg/ml) for 3 experiments. * denotes $p < 0.05$. ** denotes $p < 0.01$. N=5-11 rats per group.

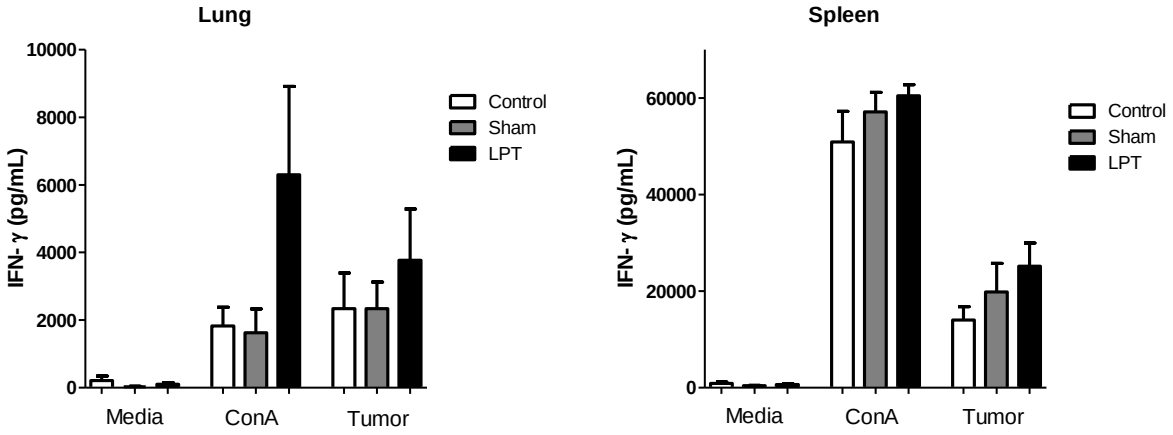

Figure 3. LPT enhances IFN-γ secretion by pulmonary leukocytes *in vitro*. On day 0, rats were intravenously injected with 1 x 10^6 MADB106 tumor cells in log phase and received treatment for days 1-7. On day 8, rats were euthanized and splenic and lung leukocytes were isolated. The leukocytes were adjusted to a concentration of 10^6 leukocytes/well and co-cultured with either media, 10µg/ml ConA, or 10^4 MADB106 tumor cells/well. Cell culture supernatants were analyzed by multiplex assay. Data are means ± SEM IFN-γ concentration (pg/ml) for 3 experiments. N=5-11 rats per group.

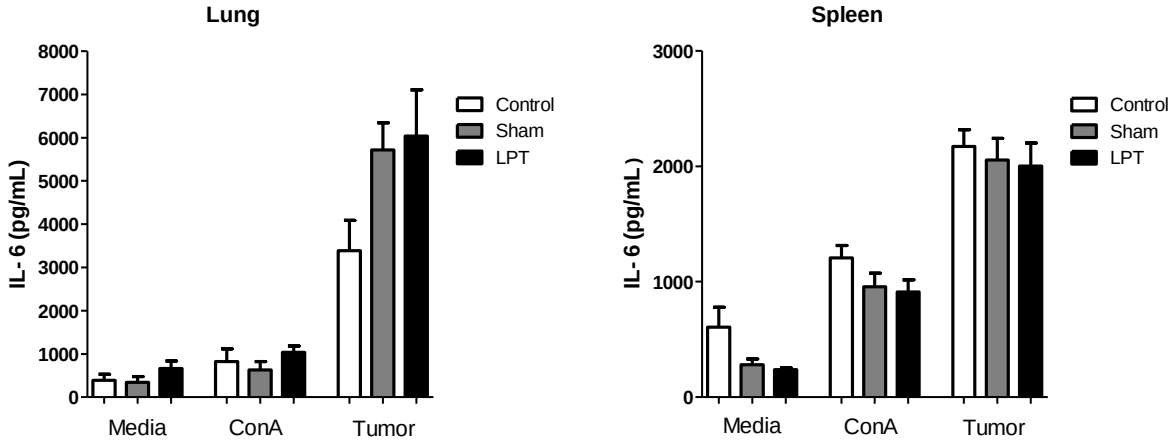

Figure 4. *In vitro* IL-6 production by pulmonary and splenic leukocytes. On day 0, rats were intravenously injected with 1 x 10^6 MADB106 tumor cells in log phase and received treatment for days 1-7. On day 8, rats were euthanized and splenic and lung leukocytes were isolated. The leukocytes were adjusted to a concentration of 10^6 leukocytes/well and co-cultured with either media, 10µg/ml ConA, or 10^4 MADB106 tumor cells/well. Cell culture supernatants were analyzed by multiplex assay. Data are means ± SEM IL-6 concentration (pg/ml) for 3 experiments. N=5-11 rats per group.

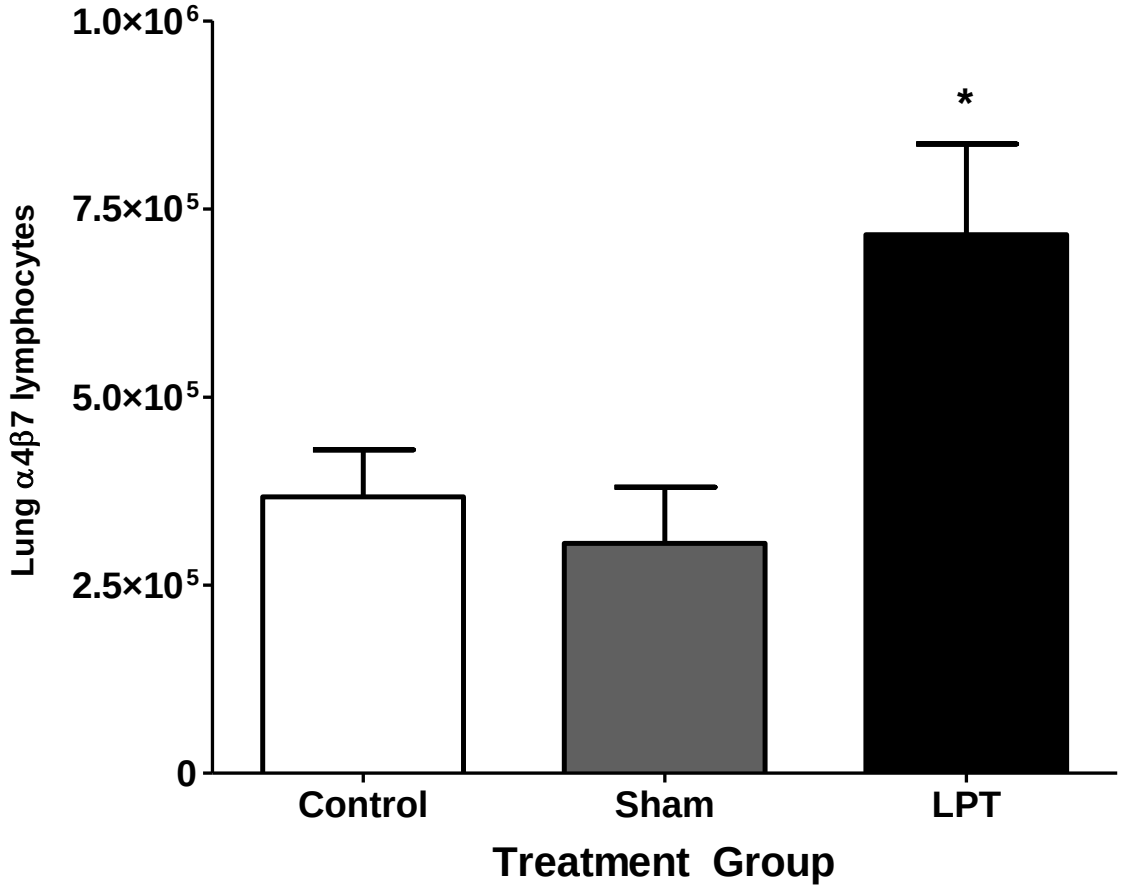

Figure 5. LPT enhances pulmonary trafficking of gastrointestinal-derived leukocytes. Rats were intravenously injected with 1 x 10^6 MADB106 tumor cells in log phase. For days 1-7, rats received control, sham or LPT. On day 8, rats were euthanized and the lungs were stained for the gastrointestinal lymphocyte homing receptor, integrin α4β7. Data are means ± SEM the number of total lung leukocytes (Control N=10, Sham N=8, LPT N=11). * denotes $p < 0.05$ compared to control and sham groups.

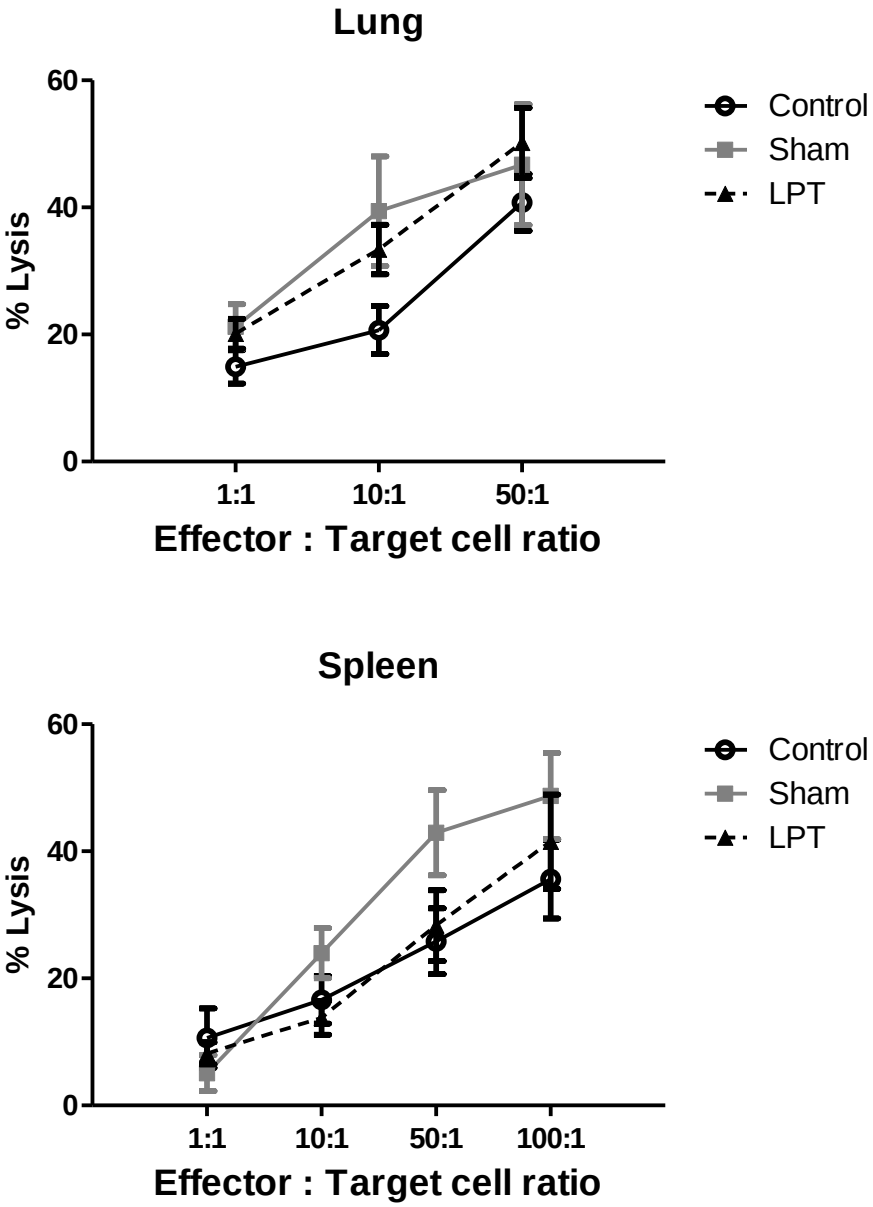

Figure 6. Lymphatic pump treatment does not enhance tumor lysis by pulmonary or splenic leukocytes *in vitro*. On day 0, rats were intravenously injected with 1x 10^6 MADB106 tumor cells in log phase and received treatment for days 1-7. On day 8, rats were euthanized and tumoricidal activity of pulmonary and splenic leukocytes against YAC-1 target cells was measured. Data are means ± SE the percent lysis of tumors. N=10.

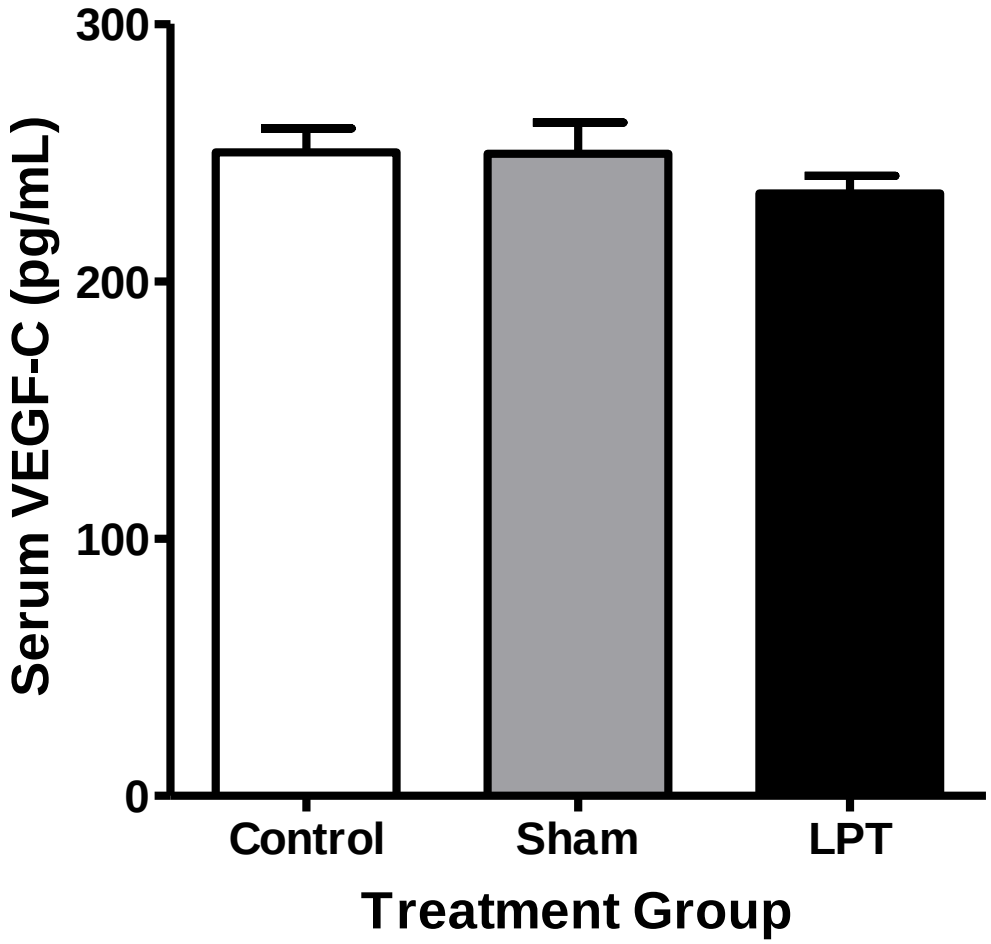

Figure 7. LPT does not increase serum VEGF-C. On day 0, rats were intravenously injected with 1x 10^6 MADB106 tumor cells in log phase and received treatment for days 1-7. On day 8, rats were euthanized and serum was assayed for the concentration of VEGF-C. Data are means ± SEM VEGF-C (pg/ml) from 2 experiments. N=8-9 rats per group**.**

Table 2. LPT does not enhance peripheral blood leukocyte numbers.

Cardiac Blood			
	Control	**Sham**	**LPT**
Leukocytes/ml	4.8 ± 0.37	5.1 ± 0.39	4.3 ± 0.47
Lymphocytes/ml	2.5 ± 0.18	2.64± 0.18	2.0 ± 0.17
Neutrophils/ml	2.2 ± 0.34	2.4 ± 0.36	1.9 ± 0.18
Monocytes/ml	0.23 ± 0.035	0.22 ± 0.030	0.21 ± 0.033

Cardiac Blood			
	Control	**Sham**	**LPT**
% Lymphocytes	49 ± 2.1	50 ± 3.1	47 ± 3.4
% Neutrophils	45 ± 2.2	46 ± 3.2	47 ± 3.5
% Monocytes	4.8 ± 0.48	4.2 ± 0.40	4.9 ± 0.58

On day 0 rats were intravenously injected with 1 x 10^6 MADB106 tumor cells in log phase and received treatment for days 1-7. On day 8, rats were euthanized and cardiac blood taken and analyzed for leukocyte counts by Hemavet. Data are means ± SE number of cells (x10^6)(top table) and % total leukocytes (bottom table) from 3 experiments. N=7-14 rats per group.

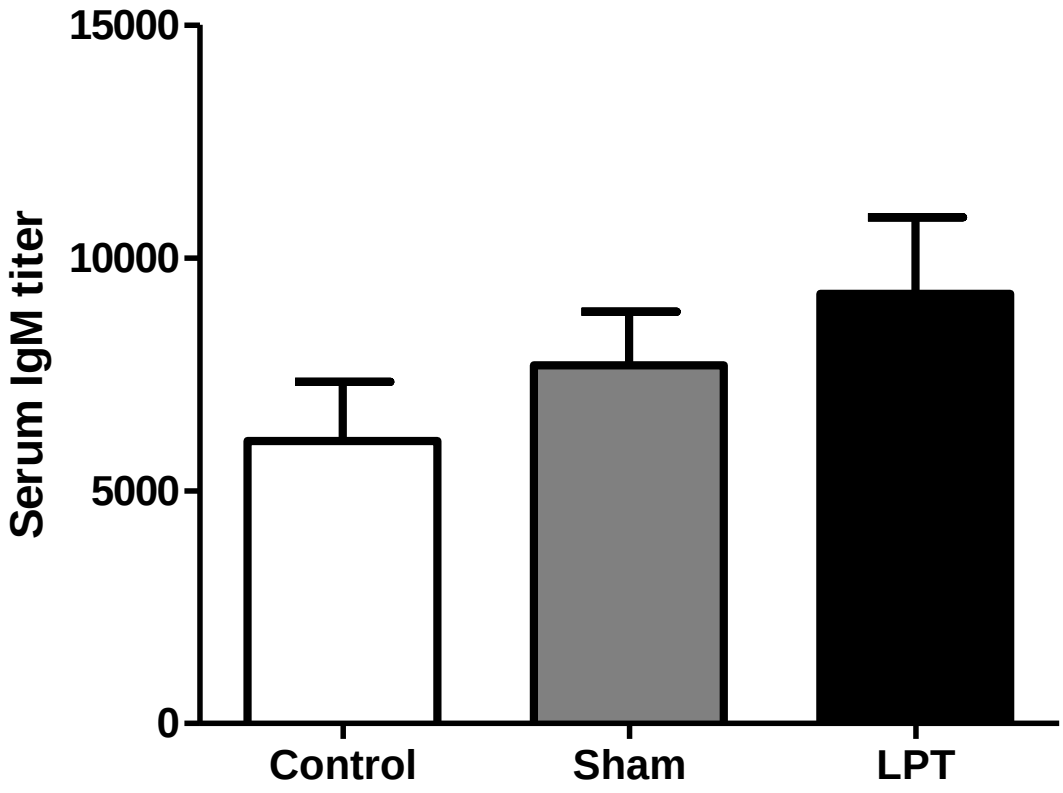

Figure 8. LPT does not increase tumor-specific IgM in the serum. On day 0 rats were intravenously injected with 1 x 10^6 MADB106 tumor cells in log phase and received treatment for days 1-7. On day 8, rats were euthanized and serum was analyzed for tumor specific IgM by ELISA. Data are means ± SEM of 3 experiments. N=13-14 rats per group.

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