Original Research

Lymphatic pump manipulation mobilizes inflammatory mediators into lymphatic circulation

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Abstract

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

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

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Introduction

Osteopathic physicians have developed osteopathic manipulations collectively known as lymphatic pump techniques (LPTs), which are designed to enhance lymph flow.[1,2] By increasing lymph flow, LPT is thought to aid in the removal of metabolic wastes, toxins, exudates and cellular debris that accumulate in the tissue interstitial fluid during infection or edema.[3] Clinically, LPT has been shown to enhance vaccine specific antibodies,[4,5] and reduce the length of hospital stay and the duration of antibiotic use in elderly patients with pneumonia.[6]

During infection and edema, inflammatory cytokines, chemokines, reactive oxygen species (ROS), such as superoxide dismutase (SOD), and reactive nitrogen species (RNS), such as nitrotyrosine (NT) are generated. The proinflammatory cytokines and chemokines, interleukin-2 (IL-2), IL-4, IL-6, IL-8, tissue necrosis factor-α (TNF-α), interferon-γ (IFN-γ), monocyte chemotactic protein-1 (MCP-1) and keratinocyte chemoattractant (KC) induce leukocyte activation, migration and cell-mediated immune responses to pathogens,[7,8] whereas anti-inflammatory cytokines such as IL-10 limit inflammation by suppressing cell-mediated immune responses.[7,8]

Recent use of animal models has provided insight into the mechanisms by which LPT affects the lymphatic and immune systems.[2,9–12] Previously, we reported that LPT enhances thoracic duct lymph (TDL) and mesenteric duct lymph (MDL) flow and leukocyte concentrations in dogs and rats.[2,10,11,13] The purpose of this study was to determine if LPT would mobilize inflammatory mediators into the lymphatic circulation.[7,8] In addition, SOD and NT were measured. The results of this study provide support for the clinical application of LPT to enhance function of the immune system, and may explain, in part, a mechanism by which LPT protects against infection and edema.

Materials and methods

Animals

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

Surgical techniques

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

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

Lymphatic pump technique

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

Measurements of TDL and MDL

A commercially available multiplex assay (Millipore, Billerica, MA, USA) was used to determine the concentrations of cytokines and chemokines in TDL and MDL. Specifically, the cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α, and the chemokines MCP-1 and KC were measured. A range of standards, provided with the multiplex assay, was used, and the assay was analyzed using the Luminex® 200 System with the xPONENT Software Interface (Millipore). The minimum detectable concentrations for IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α, MCP-1 and KC were 6.4, 28.8, 12.1, 20.3, 1.6, 4.4, 0.4, 8.6 and 1.6 pg/mL, respectively. To compute the cytokine/chemokine flux in TDL and MDL, the respective concentration was multiplied by lymph flow during each minute for each condition, and these values were averaged.

Thoracic lymph concentrations of SOD (Cayman Chemicals, Ann Arbor, MI, USA) and NT (Molecular Probes, Inc, Eugene, OR, USA) were measured using commercially available kits. The SOD assay measures all three forms of SOD by utilizing a tetrazolium salt for the detection of xanthine oxidase and hypoxanthine-derived superoxide radicals. One unit of SOD is defined as the amount of enzyme necessary to cause 50% dismutation of the superoxide radical. The SOD minimum detectable concentration for this assay is 0.025 U/mL. NO reacts with superoxide to form peroxynitrite.[14] Subsequently, peroxynitrate reacts with proteins, resulting in measurable NT. The minimum detectable concentration for NT of this assay is 2 nmol/L SOD and NT was measured only in TDL, since the samples of MDL were not sufficient for both these measurements and the Luminex assays. To compute SOD or NT flux in TDL, the respective concentration was multiplied by lymph flow during each minute for each condition, and these values were averaged.

Statistical analysis

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

Results

LPT increased intestinal and TDL flow

Similar to our previous reports,[10,11] LPT enhanced TDL and MDL flow. LPT increased TDL flow from 0.90 ± 0.19 mL/min during pre-LPT to 5.65 ± 0.93 mL/min ($P < 0.001$) and the flow subsequently decreased to 2.07 ± 0.28 mL/min during post-LPT ($P < 0.01$). LPT also increased MDL flow from 0.30 ± 0.03 mL/min during pre-LPT to 2.71 ± 1.01 mL/min ($P < 0.05$) and the flow subsequently decreased to 0.32 ± 0.25 mL/min during post-LPT ($P < 0.05$).

LPT increased the concentrations of MCP-1 in TDL

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

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

LPT increased lymphatic cytokine and chemokine flux

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

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

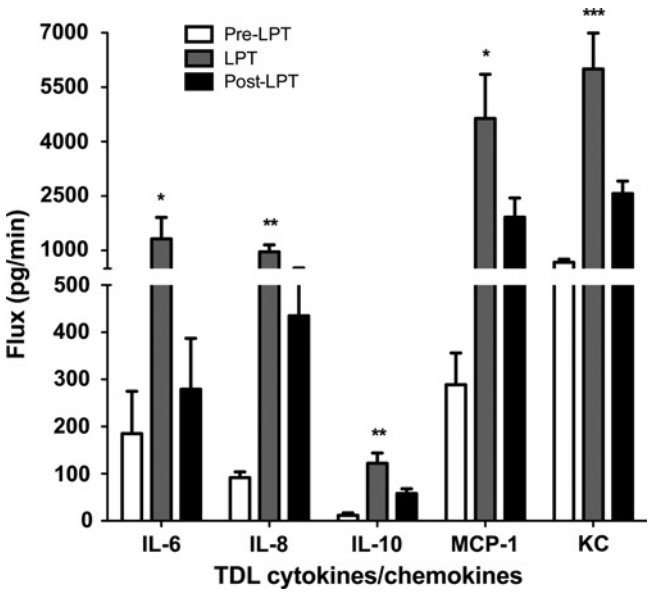

Figure 1 LPT increased cytokine and chemokine flux in thoracic duct lymph (TDL). Data are means ± SE ($n = 6$). *Greater than respective pre-LPT and post-LPT ($P < 0.05$). **Greater than respective pre-LPT and post-LPT values ($P < 0.01$). ***Greater than respective pre-LPT and post-LPT values ($P < 0.001$). Repeated measures ANOVA with Student–Newman–Keuls post-test. LPT, lymphatic pump technique; ANOVA, analysis of variance

Table 1 LPT significantly altered the concentration of MCP-1, but did not significantly alter other cytokine, chemokine and reactive oxygen species concentrations in lymph

	Pre-LPT	LPT	Post-LPT
Thoracic duct lymph (TDL)			
IL-6 (pg/mL)	193 ± 65	217 ± 74	158 ± 59
IL-8 (pg/mL)	183 ± 25	240 ± 48	217 ± 42
IL-10 (pg/mL)	24 ± 9	31 ± 5	29 ± 5
MCP-1 (pg/mL)	578 ± 135	1160 ± 304*	958 ± 266
KC (pg/mL)	1351 ± 165	1501 ± 247	1284 ± 172
SOD (U/mL)	0.135 ± 0.038	0.176 ± 0.026	0.173 ± 0.019
NT (mmol/L/mL)	3.93 ± 2.54	6.79 ± 1.93	2.86 ± 0.76
Mesenteric duct lymph (MDL)			
IL-6 (pg/mL)	217 ± 43	241 ± 116	333 ± 115
IL-8 (pg/mL)	396 ± 85	543 ± 114†	392 ± 54
IL-10 (pg/mL)	51 ± 20	72 ± 24	47 ± 17
MCP-1 (pg/mL)	3094 ± 749‡	3482 ± 685‡	3007 ± 473‡
KC (pg/mL)	2389 ± 554	2522 ± 506	2270 ± 526

LPT, lymphatic pump technique; MCP-1, monocyte chemotactic protein-1; IL, interleukin; KC, keratinocyte chemoattractant; SOD, superoxide dismutase; NT, nitrotyrosine
Data are means ± SE ($n = 6$)
*Greater than respective pre-LPT ($P < 0.05$)
†Different from respective TDL value ($P < 0.05$)
‡Different from respective TDL value ($P < 0.01$)
Repeated measures analysis of variance with Student–Newman–Keuls post-test

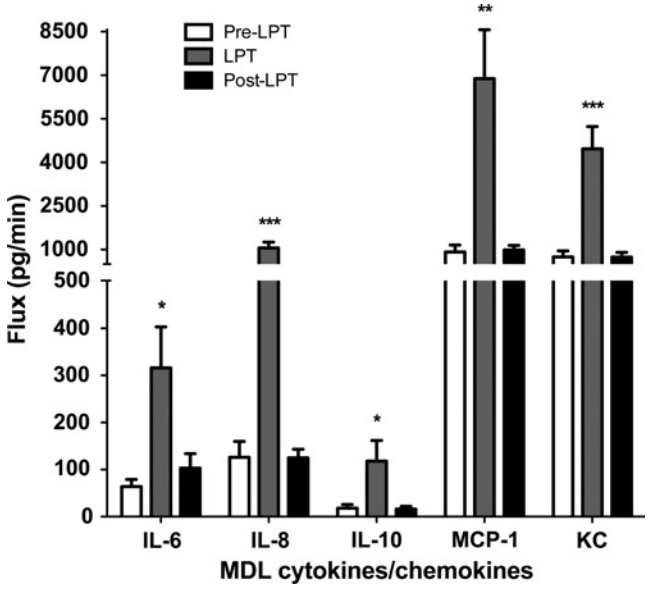

Figure 2 LPT increased cytokine and chemokine flux in mesenteric duct lymph (MDL). Data are means ± SE ($n = 6$). *Greater than respective pre-LPT and post-LPT ($P < 0.05$). **Greater than respective pre-LPT and post-LPT values ($P < 0.01$). ***Greater than respective pre-LPT and post-LPT values ($P < 0.001$). Repeated measures ANOVA with Student–Newman–Keuls post-test. LPT, lymphatic pump technique; ANOVA, analysis of variance

LPT increased the flux of ROS and RNS in TDL

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

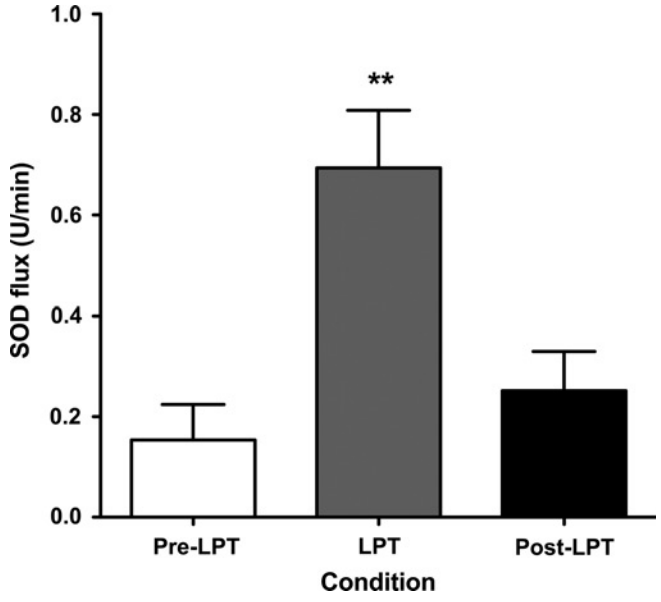

Figure 3 LPT increased SOD flux in thoracic duct lymph. Data are means ± SE ($n = 6$). **Greater than respective pre-LPT and post-LPT values ($P < 0.01$). Repeated measures ANOVA with Student–Newman–Keuls post-test. LPT, lymphatic pump technique; ANOVA, analysis of variance; SOD, superoxide dismutase

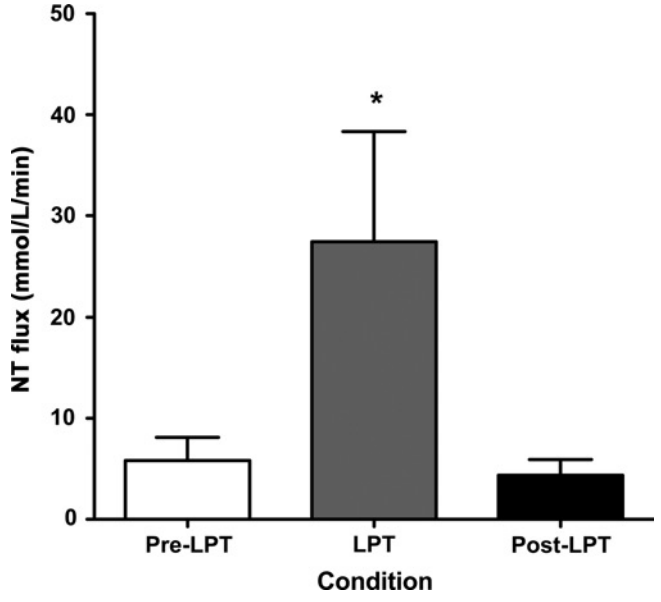

Figure 4 LPT increased NT flux in thoracic duct lymph. Data are means ± SE ($n = 6$). *Greater than respective pre-LPT and post-LPT values ($P < 0.05$). Repeated measures ANOVA with Student–Newman–Keuls post-test. LPT, lymphatic pump technique; ANOVA, analysis of variance; NT, nitrotyrosine

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

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

Discussion

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

Cytokines, chemokines, ROS and RNS are generated during the innate immune response to pathogens. During infection, the cytokines IL-6, IL-8, MCP-1 and KC induce inflammation by recruiting and activating leukocytes, while IL-10 regulates the inflammatory response.[7,8,15–17] During acute inflammation, inflammatory cytokines

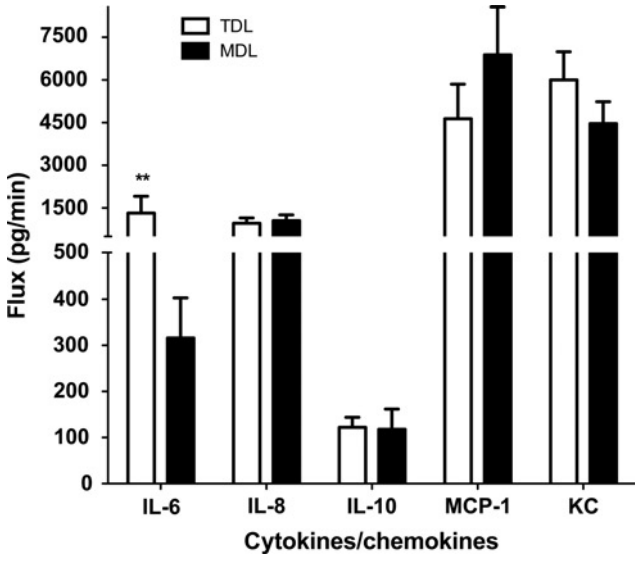

Figure 5 LPT created a difference in measurable IL-6 in TDL versus MDL. Data are means ± SE ($n = 6$). **Greater than respective MDL value ($P < 0.01$). ANOVA with Student–Newman–Keuls post-test. LPT, lymphatic pump technique; ANOVA, analysis of variance; IL-6, interleukin-6; MDL, mesenteric duct lymph; TDL, thoracic duct lymph

stimulate the formation of edema by accumulating in the interstitial fluid, which initially lowers the interstitial fluid pressure, setting the stage for the influx of proteins and plasma fluid.[18,19] Therefore, LPT may suppress edema by mobilizing inflammatory mediators out of interstitial fluid into the lymphatic circulation, as well as directly increasing lymph flow and removing excessive interstitial fluid.[2,12]

LPT is used to treat infection,[4–6,20,21] but the mechanisms by which LPT protects against infectious diseases are unclear. LPT may enhance protection against infection by increasing mesenteric-derived inflammatory mediators in circulation, enabling the re-distribution of these mediators to other tissues. In support of this notion, lymph has been shown to re-distribute mesenteric-derived cytokines and chemokines to distant organs.[22–25] Furthermore, it has been shown *in vitro* that mesenteric lymph can activate neutrophils and increase endothelial cell permeability.[26] It is not surprising, that LPT would enhance this re-distribution and potentially enhance immune function.

Previously, we reported that LPT releases leukocytes from mesenteric lymph nodes into TDL and enhances leukocyte flux in MDL and TDL.[10] Following exposure to microorganisms, phagocytes, such as macrophages and neutrophils, release ROS and RNS which are bactericidal.[7] Thus, by enhancing the lymphatic flux of leukocytes, cytokines, chemokines, ROS and RNS, LPT may facilitate cell-mediated clearance of infection.

It has been hypothesized that following tissue injury, lymph flow quickly increases and provides the earliest signal in the lymphatic system to induce the inflammatory response.[27] It has been documented that lymphedema impairs immune cell trafficking and increases susceptibility to infection.[28] Recently, transmural flow across lymphatic endothelia was shown to regulate cell and fluid transport functions of lymphatic endothelium.[29] Specifically, transmural flow increased chemokine ligand secretion, influenced dendritic cell migration into lymphatic vessels, increased vessel permeability and upregulated cell adhesion molecules on lymphatic vessels.[29] The resulting increase in shear stress induces endothelial nitric oxide expression in human lymphatic endothelial cells;[30] so elevated lymph flow causes release of endogenous nitric oxide from lymphatic endothelial cells.[31,32] Therefore, in addition to releasing leukocytes into lymphatic circulation, by enhancing lymph flow and NT release into lymph, LPT may signal the lymphatic system to increase immune cell trafficking.

We also compared the lymphatic cytokine and chemokine composition between thoracic and mesenteric lymph. The thoracic duct is a large vessel and transports lymph drained from abdominal visceral organs (mainly the liver and intestines), skin and skeletal muscle.[7,8,33] We found that the concentrations of cytokines and chemokines were higher in MDL (Table 1), which is consistent with the prior report that most of the lymph and protein in the thoracic duct is derived from the mesenteric lymph.[34] This result suggests that compared with mesenteric lymph, lymph derived from the liver and other tissues contains low concentrations of inflammatory mediators, and thus dilutes mesenteric-derived cytokines in TDL. It is important to note that these were healthy animals; therefore, during infection or inflammation, the concentrations of inflammatory mediators in TDL and MDL may vary.

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

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

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