Lymphatic Pump Treatment Mobilizes Bioactive Lymph That Suppresses Macrophage Activity In Vitro

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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 disease.[3] Many osteopathic manipulative treatment techniques were designed to promote lymph circulation.[4-6] Lymphatic pump treatment (LPT) is used to manage congestive heart failure, upper and lower gastrointestinal tract dysfunction, respiratory tract disease, infection, and edema.[4] 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.[6]

Previous studies[7-10] 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.[9,10] Collectively, results of these studies[7-10] 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,[11-13] blunt the pulmonary inflammatory response to endotoxins,[12] inhibit neutrophil apoptosis in vitro,[11,12] increase endothelial cell permeability,[14,15] and redistribute leukocytes, cytokines, and chemokines to distant organs.[15] 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.[16] 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.[16]

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*.[17] 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.[18]

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 500 ng 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 the Table. 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 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 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

Table.
TDL Flow, Protein Concentration, and Protein Flux Measurements Before, During, and After LPT in Dogs (N=6)[a]

TDL Measurements	Baseline	During LPT	After LPT
Flow, mL/min	0.62 (0.11)	6.80 (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: LPT, lymphatic pump treatment; TDL, thoracic duct lymph.

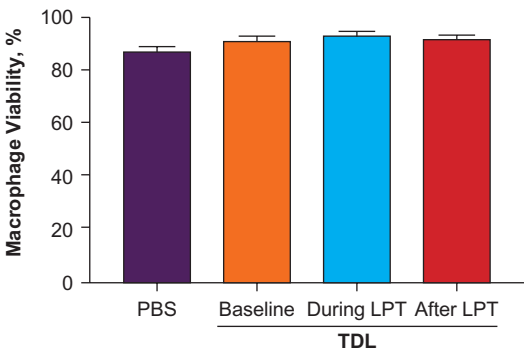

Figure 1.
The viability percentage of RAW 264.7 macrophage cultured with 5% phosphate-buffered saline (PBS) and cell-free thoracic duct lymph (TDL) taken before, during, and after lymphatic pump treatment (LPT) and cocultured with 500 ng per well of lipopolysaccharide.

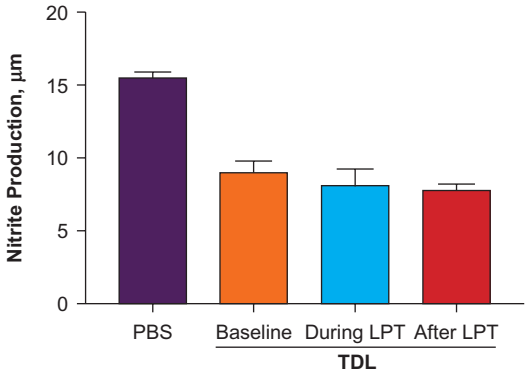

Figure 2.
Production of nitrite by RAW 264.7 macrophages cultured with 5% phosphate-buffered saline (PBS) and cell-free thoracic duct lymph (TDL) taken before, during, and after lymphatic pump treatment (LPT) and cocultured with 500 ng per well of lipopolysaccharide.

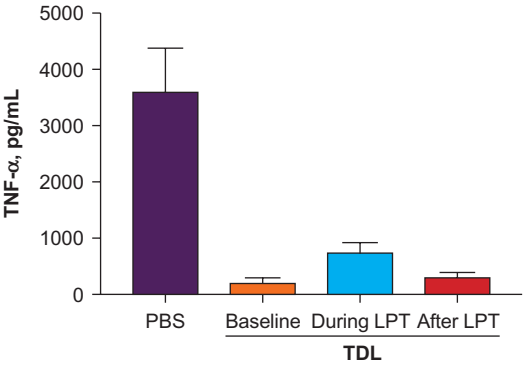

Figure 3.
Production of tumor necrosis factor α (TNF-α) by RAW 264.7 macrophages cultured with 5% phosphate-buffered saline (PBS) and cell-free thoracic duct lymph (TDL) taken before, during, and after lymphatic pump treatment (LPT) and cocultured with 500 ng per well of lipopolysaccharide.

[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**).

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 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.[14] Furthermore, the transfusion of normal mesenteric lymph into rats alleviated LPS-induced lung injury in vivo.[13] 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[12,13] 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,[9,10] LPT did not increase the concentration of specific proteins, such as cytokines and chemokines. However, consistent with previous studies,[7-10] 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,[19] who asserted that the normal circulation of body fluids is essential to normal

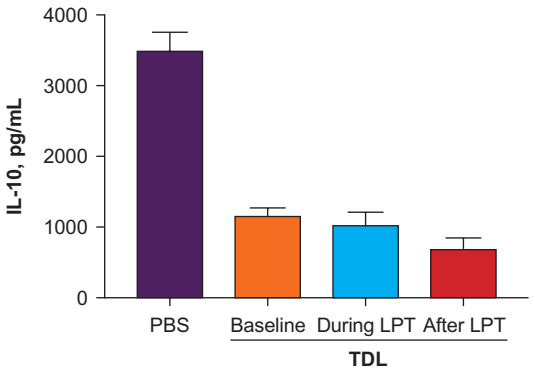

Figure 4.
The production of interleukin 10 (IL-10) by RAW 264.7 macrophages cultured with 5% phosphate-buffered saline (PBS) and cell-free thoracic duct lymph (TDL) taken before, during, and after lymphatic pump treatment (LPT) and cocultured with 500 ng per well of lipopolysaccharide.

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.[15] However, Davidson et al[20] found that the bioactive mediator in lymph from rats subjected to hemorrhagic shock was not a cytokine. Other reports[11,12,21] 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.

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Author Contributions

All authors provided substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; all authors drafted the article or revised it critically for important intellectual content; all authors gave final approval of the version of the article to be published; and all authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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