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Cytokines are systemic effectors of lymphatic function in acute inflammation ☆

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ABSTRACT

The response of the lymphatic system to inflammatory insult and infection is not completely understood. Using a near-infrared fluorescence (NIRF) imaging system to noninvasively document propulsive function, we noted the short-term cessation of murine lymphatic propulsion as early as 4 h following LPS injection. Notably, the effects were systemic, displaying bilateral lymphatic pumping cessation after a unilateral insult. Furthermore, IL-1β, TNF-α, and IL-6, cytokines that were found to be elevated in serum during lymphatic pumping cessation, were shown separately to acutely and systemically decrease lymphatic pulsing frequency and velocity following intradermal administration. Surprisingly, marked lymphatic vessel dilation and leakiness were noted in limbs contralateral to IL-1β intradermal administration, but not in ipsilateral limbs. The effects of IL-1β on lymphatic pumping were abated by pre-treatment with an inhibitor of inducible nitric oxide synthase, L-NIL (N-iminoethyl-L-lysine). The results suggest that lymphatic propulsion is systemically impaired within 4 h of acute inflammatory insult, and that some cytokines are major effectors of lymphatic pumping cessation through nitric oxide-mediated mechanisms. These findings may help in understanding the actions of cytokines as mediators of lymphatic function in inflammatory and infectious states.

1. Introduction

Inflammation is a response to pathogens, damaged cells, or irritation, resulting from infection, injury, autoimmune pathologies, or stress [1]. Cardiovascular and immune system responses to inflammation and infection are well documented, but the role of the lymphatic system is ill-defined, largely due to a lack of tools for studying the system. Responsible for fat absorption in the gut, and for transport of excess fluid, macromolecules, and cellular debris from the interstitial space to the hemovascular system, the lymphatics also play a critical role in mediating the immune response [2,3]. An imaging technique for noninvasively visualizing lymphatic vessel structure and function [4–6] now allows for investigation of the systemic response of the lymphatic circulatory system in infection and inflammation.

One model used for studying inflammation caused by exogenous pyrogens includes the use of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria. LPS binds to the CD14/TLR4/MD2 complex on many cells, especially macrophages, and results in signaling cascades directing the synthesis and release of cytokines, such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) [7]. The role that these cytokines and other molecular signals play in affecting lymphatic fluid and immune cell transport as part of the immune response is incompletely understood.

Although lymphangiogenesis is a well documented response to inflammation [8–13], not enough is known about the role of lymphatic transport in mediating and resolving inflammatory responses. The timely orchestration of lymph flow and cell movement within lymphatic vessels during different types of inflammatory events has yet to be thoroughly described. For example, dendritic cell (DC) and other immune cell transport to lymph nodes increases in response to chemokines expressed during inflammatory insults, yet some studies report a slowdown in lymph movement [14–17]. Most studies of lymphatic transport have reported effects using different inflammation models at limited time points or only in the local physiological environment sur-

Abbreviations: NIRF, near-infrared fluorescence imaging; L-NIL, N-iminoethyl-L-lysine; LPS, lipopolysaccharide; iNOS, inducible nitric oxide; Th1-type, T helper 1-type; CD14/TLR4/MD2, CD14/Toll-like receptor 4/MD2; ICG, indocyanine green; LECs, lymphatic endothelial cells; MCP-1/CCL2, monocyte chemoattractant protein-1/chemokine (C–C motif) ligand 2; IFN-γ, interferon-gamma; IL, interleukin.

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rounding the inflammatory insult [18–22]. A clear, macroscopic picture of systemic, temporal responses of lymph movement in acute, chronic, Th1-type (T helper 1-type), Th2-type, and Th17-type inflammation, together with information on the machinations of immune cell migration, is presently lacking.

In this study, using noninvasive, near-infrared fluorescence imaging (NIRF), we show transient changes in lymphatic transport in response to acute inflammation caused by unilateral LPS injection in the dorsal aspect of the hind paw in mice. We observed early, severe retardation of lymphatic pumping in response to a unilateral inflammatory insult, and, surprisingly, noted that the effect was systemic (bilateral)–not exclusively local. Additionally, we noted decreased systemic lymphatic propulsion after separate injection of IL-6, TNF-α, and IL-1β, cytokines that are elevated in serum after LPS injection, indicating that these cytokines may play an important role in control of lymphatic function. Curiously, distal contralateral lymphatic vessels were dilated and leaky after IL-1β treatment, while ipsilateral vessels were not. The action of IL-1β on lymphatic pumping was mitigated by inhibition of inducible nitric oxide synthase (iNOS), suggesting that cytokine effects on systemic lymphatic function in acute inflammation are driven by nitric oxide production.

2. Materials and methods

2.1. Mice

10–12 weeks-old female mice were obtained from Charles River (Wilmington, MA) and housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, according to institutional guidelines. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center-Houston. Female mice were used, as males tended to induce fight wounds on skin that diminished imaging quality. Mice were not tattooed, since tattooing can induce inflammation that lasts 14 days or more [23]. Mice were, however, eartagged several weeks before first imaging.

2.2. Reagents

Indocyanine green (ICG) was obtained from Akorn, Inc. (Lake Forest, IL) and diluted with sterile saline (Hospira, Lake Forest, IL) to 625 μM for use. Lipopolysaccharide (LPS) was purchased from Sigma–Aldrich (#L 3024, *E. coli* serotype 0111:B4, purified by ion exchange). Recombinant murine TNF-α, IL-1α, IL-1β, interferon-gamma (IFN-γ), monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2), and IL-6 were purchased from Peprotech (Rocky Hill, NJ). Recombinant human vascular endothelial growth factor-C (VEGF-C) was purchased from R&D Systems (Minneapolis, MN). L-NIL (N-iminoethyl-L-lysine) was purchased from Sigma–Aldrich.

2.3. LPS and cytokine injection

Mice were anesthetized with isoflurane, shaved, and covered with depilatory cream (Nair Sensitive) for 3 min. The cream was then rinsed off with warm water. Several days later, mice were anesthetized with isoflurane, and 10 μL of 625 μM ICG were injected intradermally with a 31-gauge needle/syringe (BD #328438, Fisher Scientific) at the base of the tail and/or on the dorsal side of the paw on both right and left sides of the mice to perform baseline NIRF imaging of the lymphatics. Two–three days after this initial imaging, mice were injected intradermally with 20 μL of 5 mg/mL (100 μg/mouse) of bacterial wall lipopolysaccharide, saline, or TNF-α (2 μg/mouse), IL-6 (50 ng/mouse), and/or IL-1β (1 μg/mouse) in saline in the dorsal aspect of the right hind paw. In separate experiments, interferon-gamma (IFN-γ, 400 ng/mouse), interleukin-1 alpha (IL-1α, 0.5 μg/mouse), monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2, 0.8 μg/mouse), and vascular endothelial growth factor-C (VEGF-C, 400 ng/mouse) were injected intradermally in the dorsal aspect of the right hind paw. The cytokine amounts were chosen based on previously published inflammatory or other effects [24–42]. In some experiments, TNF-α and IL-1β were heat-inactivated (85 °C, 15 min), in order to affirm that residual LPS in the cytokine materials did not account for effects observed [43–46].

2.4. Near-infrared fluorescent lymphatic imaging

NIRF images were collected with a custom-built system that employed illumination of tissue surfaces with 785-nm light from a laser diode (85 mA and 80 mW, DL7140-201, Sanyo) that was diffused to cover a circular area of approximately 8 cm in diameter [4,6]. Fluorescent light generated from the ICG within the lymphatic vasculature was collected with an EMCCD camera (electron-multiplying charge-coupled device, model 7827-0001, Princeton Instruments). Filter sets were used to reject backscattered and reflected excitation light. Images were acquired with V++ software (Total Turnkey Solutions, Sydney, Australia). The integration time for fluorescence images was 200 ms. 300 images were collected per side per mouse for lymph velocity and propulsive frequency measurements. Images were collected at zero and 4 h, and also at 1, 2, 4, and 7 days after LPS injection. Images were collected at 4 h after cytokine treatment with or without previous injection of iNOS inhibitor. In separate experiments, images were collected at 30, 60, 90, and 120 min after LPS or IL-1β injection, in order to compare kinetics of effects on lymphatic pumping.

2.5. Data analysis/statistics

Images were loaded into ImageJ software (NIH), and fluorescence intensity values were quantified and imported into Microsoft Excel for computation of propulsive frequencies and velocities as previously described [6]. Statistical significance for comparisons of propulsive frequencies was determined using ANOVA. Statistical significance for comparisons of propulsive velocities was determined using a linear mixed effects model.

2.6. Multiplex analysis of serum cytokines

Serum samples were obtained by retro-orbital bleeds of mice before LPS or saline injection, as well as at 1 day, 2 days, and 7 days post-injection (only one sample was collected per mouse). Approximately 200 μL blood per mouse was left at room temperature for 30 min to allow coagulation, and then spun at 350*g* for 10 min at room temperature. Serum was collected and stored at −80 °C until analysis. For multiplex analysis, serum samples were diluted and processed according to manufacturer's instructions (Millipore catalog #MPXMCYP2-73K-02 [MCP-5, IL-25], #MPXMCYTO-70K-15 [GM-CSF, IFN-γ, IL-1-α, IL-1-β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12p40, IL-17, MCP-1, RANTES, TNF-α, VEGF], #MPXMCYP3-74K-02 [IL-25, IL-27], and #MSCR-42K-03 [mVEGFR1, mVEGFR2, mVEGFR3]). Cytokine/chemokine levels were measured on a Luminex 200 instrument (Millipore, Billerica, MA).

2.7. Vessel dilation measurement

Four hours after dorsal i.d. cytokine (1 μg of IL-1β) injection in the right or left hind paw, mice were anesthetized with isoflurane, 1% Evan's blue dye was injected i.d. at the base of the tail and in the