Regulation of Inflammation and Fibrosis by Macrophages in Lymphedema

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Abstract:

Introduction: Lymphedema, a common complication of cancer treatment, is characterized by inflammation, fibrosis, and adipose deposition. We previously have shown that macrophage infiltration is increased in mouse models of lymphedema. Because macrophages are regulators of lymphangiogenesis and fibrosis, this study aimed to determine the role of these cells in lymphedema using depletion experiments.

Methods: Matched biopsy specimens of normal and lymphedema tissues were obtained from patients with unilateral upper extremity breast cancer-related lymphedema and macrophage accumulation was assessed using immunohistochemistry. In addition, we used a mouse tail model of lymphedema to quantify macrophage accumulation and analyze outcomes of conditional macrophage depletion.

Results: Histological analysis of clinical lymphedema biopsies revealed significantly increased macrophage infiltration. Similarly, in the mouse tail model, lymphatic injury increased the number of macrophages and favored M2 differentiation. Chronic macrophage depletion using lethally irradiated wild-type mice reconstituted with CD11b-DTR mouse bone marrow did not decrease swelling, adipose deposition, or overall inflammation. Macrophage depletion after lymphedema had become established significantly increased fibrosis, accumulation of CD4+ cells, and promoted Th2 differentiation while decreasing lymphatic transport capacity and VEGF-C expression.

Conclusion: Our findings suggest that macrophages home to lymphedematous tissues and differentiate into the M2 phenotype. In addition, our findings suggest that macrophages have an anti-fibrotic role in lymphedema and either directly or indirectly regulate CD4+ cell accumulation and Th2 differentiation. Finally our findings suggest that lymphedema associated macrophages are a major source of VEGF-C and that impaired macrophage responses after lymphatic injury results in decreased lymphatic function.
Introduction

Lymphedema is a disease characterized by lymphatic fluid stasis, inflammation, and fibroadipose deposition that occurs commonly after lymphatic injury during the course of cancer treatment. Although breast cancer survivors make up the largest cohort of patients with secondary (i.e. iatrogenic) lymphedema, this complication also occurs commonly in patients treated for a variety of other solid tumors including gynecological cancers, sarcomas, and melanomas (5, 22). In these individuals, progressive adipose deposition and tissue fibrosis results in increasing limb volume, heaviness, functional difficulties, increased susceptibility to infections, and rare but deadly secondary tumors.

A number of clinical findings suggest that the pathology of lymphedema is a multistep sequence with lymphatic injury serving as the initiator of these events. This hypothesis is supported by the fact that lymphedema develops only in a subset of patients who undergo lymphadenectomy rather than uniformly in all patients who suffer lymphatic injury(21). In addition, some patients develop lymphedema even after seemingly trivial injury to the lymphatic system, suggesting that even minor disturbances in lymphatic function can initiate the pathologic sequence (12). Finally, the development of lymphedema in the majority of patients occurs in a delayed fashion, usually months to years after the initial surgical injury, implying that intervening secondary events are necessary for the development of this pathologic process (19).

Recent studies in our lab have suggested that tissue fibrosis is a key step in the development of lymphedema. This hypothesis is supported by the fact that fibrosis is a hallmark of lymphedema clinically (27) and progression of disease is characterized by deposition of fibroadipose tissues and luminal obliteration of collecting lymphatic vessels with proliferative smooth muscle cells (13). In addition, it is well recognized that fibrosis is a common cause of end-organ failure in a number of organ systems including liver, lung, skin, kidney, and heart suggesting that lymphedema may simply represent “end-organ failure” of the lymphatic system due to fibrosis (32). Fibrosis also provides a rationale for the delayed development of lymphedema since the progressive abnormal collagen deposition necessary to cause organ dysfunction takes time to accumulate. Finally, recent work in our lab has shown that interventions designed to decrease tissue fibrosis in lymphedema can
potently increase lymphatic function and decrease the pathologic consequences of lymphatic injury in pre-clinical mouse models (1-4, 38). Thus, there is a strong rationale to understand the pathological mechanisms that regulate fibrosis in lymphedema as understanding these concepts may provide novel preventative or treatment options for a disease that currently has no cure.

Our previous studies have characterized the inflammatory cell infiltrate associated with lymphedema both clinically and in mouse models. Using a variety of techniques, we have shown that more than 70% of the cells that accumulate in chronically lymphedematous tissues are CD4+ cells and that neutralization of these cell types using depleting antibodies or surgical lymphedema models in CD4 knockout mice markedly decreases fibrosis and lymphatic dysfunction after lymphatic injury (3). However, we have also previously reported that the number of macrophages in the lymphedematous tissues is increased substantially as compared with controls (37). In addition, in a recent report using a mouse tail model of lymphedema, we found that animals lacking toll-like receptors (TLR) had decreased macrophage accumulation and increased fibrosis/adipose deposition (35). These findings are consistent with previous studies demonstrating that macrophages play active roles in the regulation of fibrosis in other organ systems (6, 33). In addition other studies, consistent with known roles of macrophages in the regulation of lymphangiogenesis (7, 8, 23), we have shown that macrophages in lymphedematous tissues strongly express VEGF-C (33-36). However, while it is clear that lymphedema promotes macrophage infiltration, the precise role of these cells in the regulation of fibrosis and lymphangiogenesis in response to sustained lymphatic fluid stasis has not been assessed through direct loss of function studies.

The purpose of the present study was therefore to determine the role of macrophages in the regulation of pathologic changes in response to sustained lymphatic fluid stasis after lymphatic injury. Using a conditional ablation model of macrophages expressing diphtheria toxin receptor (DTR), we show that decreasing macrophage accumulation after lymphatic injury results in increased fibrosis and impaired lymphatic function. In addition, we report that loss of macrophages in this setting is associated with increased CD4+ cell infiltration and expression of pro-fibrotic cytokines. Finally, we report that conditional ablation of macrophages results in decreased expression of VEGF-C in the mouse tail lymphedema model.
**MATERIAL AND METHODS**

*Clinical lymphedema specimens*

After informed consent and approval by the Institutional Review Boards of the MD Anderson and Memorial Sloan Kettering Cancer Centers, six women with stage 2 or 3 upper extremity breast cancer-related lymphedema were recruited and underwent full thickness skin biopsy of their normal and lymphedematous limbs. Based on the relative amount of lymphedema in the various regions of the arm, matched 5mm biopsies (i.e. harvested from the same region of the arm on both sides) were obtained from a point approximately 5-10cm above or below the elbow on the dorsal surface of the limb. Specimens were fixed overnight in 10% formalin, paraffin-embedded, sectioned and stained with the macrophage marker EMR-1 (Abcam, Cambridge, MA) using our previously published immunohistochemistry protocols (3).

*Animals and mouse tail model of lymphedema*

All experimental protocols were reviewed and approved by the IACUC committee at Memorial Sloan Kettering Cancer Center. Adult female C57BL/6J and CD11b-DTR (B6.FVB-Tg(ITGAM-DTR/EGFP)34Lan/J) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All animals were maintained in a temperature and light controlled environment with *ad libitum* access to food and water. Surgery was performed on animals ranging in age from 14-20 weeks.

Tail lymphedema was established in mice according to our previously published methods (3). Briefly, a 2-3mm portion of skin was circumferentially excised in a full thickness manner at a point located 10cm distal to the base of the tail. Deep lymphatic collecting vessels located along the lateral tail veins were identified and then microsurgically ligated taking care to avoid injury to the vascular structures. The wounds were then covered with a sterile dressing and allowed to heal by secondary intention. Control animals underwent a circumferential tail skin incision without deep lymphatic ligation or injury.
We used the CD11b-DTR mice as our model system to enable systemic, chronic macrophage depletion. These animals have been previously characterized and are transgenic animals that express the simian diphtheria toxin receptor (DTR) gene under the regulation of the CD11b promoter, a gene that is highly expressed on macrophages (6, 31). Because the primate DTR is 100,000 times more avid at binding diphtheria toxin (DT) than the murine equivalent, administration of minute doses of the toxin results in targeted cell death and systemic depletion of CD11b expressing cells. Although this system has been used previously in a number of settings for depletion of various cell types, the dosing and schedule of DT administration requires optimization in various transgenic lines. In addition, in some circumstances repeated dosing of DT can cause systemic toxicity requiring extensive optimization if chronic cellular depletion is necessary. Therefore, in extensive preliminary experiments we optimized the dosing schedule of DT (Sigma-Aldrich, St. Louis, MO) and used a treatment schedule of 2.5ng/g intraperitoneally (i.p.) three times per week. Control animals received intraperitoneal injections of vehicle only (phosphate buffered saline) over the same time period.

In order to avoid systemic toxicity from repeat injections and to avoid potential confounding effects of DT ablation on non-macrophage CD11b expressing cells, we created bone marrow chimeras in wild type animals reconstituted with bone marrow cells derived from CD11b-DTR mice using standard methods (17). Briefly, adult female wild-type mice were irradiated (950 rads) to ablate their bone marrow cells using a whole animal irradiator and 24 hours later were reconstituted with 5x10^6 donor bone marrow cells harvested from the femurs and humeri of female CD11b-DTR mice and delivered via lateral tail vein injection. Mice were allowed to recover for four weeks prior to use in experiments.

**Flow cytometry**

Flow cytometry was performed on tail tissues and lymph nodes as previously described (37). Briefly, single cell suspensions were created from whole lymph nodes by crushing the tissues between two glass slides in RPMI and filtering the suspension through a 70-micron filter. Single cell suspensions were prepared from tail skin and subcutaneous tissue specimens by digesting tissues in a mixture of Collagenase D, Dispase and DNaseI for 45 minutes (Roche, Nutley, NJ). The cell suspension was then passed through a 70-micron filter. All filtered single cell suspensions were re-
suspended in a 2% FCS solution. Cells were then incubated in CD16/CD32 (eBiosciences, San Diego, CA) to block endogenous Fc receptors and analyzed using a Fortessa multi-color flow cytometer (BD, San Jose, CA) with BD FACSDiva and FlowJo software (Tree Star, Ashland, OR). Cell populations were analyzed and defined using the cell surface markers B220, CD11b, CD45, CD206, F4/80, and Ly6G (all BioLegend, San Diego, CA) using 3-5 animals/group/experiment.

**Tail Volumes and Lymphoscintigraphy**

Tail volumes measurements were performed weekly using digital caliper measurements at 1 centimeter intervals beginning at the distal zone of lymphatic injury and calculated using the truncated cone formula (3). Lymphoscintigraphy using technetium -99m sulfur colloid (Tc\textsuperscript{99}) was used to analyze lymphatic transport function and performed using our previously reported methods (24). Briefly, clinical-grade unfiltered Tc\textsuperscript{99}-sulfur colloid was purchased from Nuclear Diagnostic Products (Rockaway, NJ) and used as supplied. The radiochemical purity of the Tc\textsuperscript{99}-sulfur colloid was confirmed by the manufacturer using thin-layer chromatography (Whatman 3MM paper developed in normal saline) and was uniformly 92% or better. The preparation was also checked visually under a microscope to verify the absence of any aggregates. The administered activity for each mouse imaged was assayed in a radioisotope calibrator. Using a high molecular weight conjugated tracer insured that activity was only seen in conjunction with the colloid (i.e. uptake limited to the lymphatics). Tc\textsuperscript{99} was injected in a small volume and under constant pressure in the distal portion of the tail and decay adjusted uptake was measured in the sacral lymph nodes using an X-SPECT camera (Gamma Medica, Northridge, CA). Analysis was completed using ASIPro software (CTI Molecular Imaging, Knoxville, TN) and region-of-interests (ROI) were quantified to measure peak and rate (i.e. slope of the best fit line) uptake of Tc\textsuperscript{99}.

**Histology, cell counts and fibrosis**

Tail tissues were harvested, briefly fixed in 4% paraformaldehyde (Affymetrix, Cleveland, OH), decalcified, and paraffin embedded using our previously published methods (3). Hematoxylin and eosin staining was completed using standard protocols. Adipose deposition area in the subcutaneous tissues was outlined and calculated using Pannoramic Viewer (3DHISTECH, Budapest, Hungary). Dermal thickness was measured in multiple quadrants in the lymphedematous tail and was quantified using Pannoramic Viewer.
Immunohistochemical and immunofluorescent staining was performed using our previous methods for CD45, DTR (both from R&D Systems, Minneapolis, MN), Gata3 (BD), CD4, Collagen I, LYVE-1, and F4/80 (all from Abcam) (3). Negative control sections were incubated with isotype control antibody or secondary antibody alone and specificity was confirmed using known positive and negative controls. Positively stained cells in the dermis and immediate subcutaneous areas were identified and cell counts were performed in 3 random high powered fields/animal by two reviewers who were blinded to the experimental groups. For analysis of lymphatic vessel counts, LYVE-1+ vessels were identified in the dermis of cross-sectional histological sections located 1.5 cm distal to the zone of lymphatic injury using scanned images and a minimum of 3 areas were analyzed in each animal. For analysis of lymphatic vessel area, LYVE-1+ vessels were identified in the dermis of cross-sectional histological sections and the luminal area was calculated in a random selection of 5-8 vessels in each quadrant of the distal tail.

Sirius Red scar index was calculated to analyze fibrosis and collagen deposition patterns as previously reported (1, 3). Briefly, tissues were stained with Sirius Red (Direct Red 80; Sigma-Aldrich), washed extensively, and imaged using an Axio Scope microscope (Carl Zeiss, Germany). Birefringence patterns and hue, reflecting patterns of collagen deposition and organization, were analyzed in the dermis using Metamorph Offline Software (Molecular Devices Corporation, Sunnyvale, CA) in a minimum of 3 sections per animal per group. Scar index was calculated by comparing the ratio of orange/red to yellow/green staining and is expressed as an arbitrary number with higher numbers representing more fibrosis.

Protein and RNA analysis

VEGF-C protein expression was quantified in total protein isolated from the tail tissues using ELISA (eBioscience) as previously described (3). Briefly, skin and subcutaneous tissues were harvested and lysed using the Qiagen DNA/RNA/Protein mini kit using the manufacturer’s methods (Qiagen, Valencia CA). Protein concentration was quantified using the Bradford method and VEGF-C expression was analyzed in triplicate samples of 20 \( \mu \)g of total protein per animal.

For analysis of tissue mRNA expression, a 5 mm section of distal tail skin was harvested, snap frozen in liquid nitrogen, and homogenized in Trizol (Life Technologies, Carlsbad, CA). Total cellular RNA was extracted and RNA quality and integrity were assessed using the Agilent
bioanalyzer (Santa Clara, CA). Equal amounts of RNA were reverse transcribed into cDNA and RT-
PCR was performed with validated primers for Prox-1, IL-13, LYVE-1, and GAPDH using the 
Taqman system as previously described (all reagents Life Technologies) (36). Results were obtained 
using the Viia7 software (Life Technologies) normalized to GAPDH and analyzed with the delta-
delta method to compare groups (25). Results are represented as fold-change as compared with 
controls.

**Statistical analysis**

The Student’s T-test was used to compare differences between 2 groups while comparison 
of multiple groups was performed using analysis of variance (ANOVA) with post-hoc tests (Tukey 
Kramer) to compare differences between individual groups. Data was analyzed and presented 
graphically using GraphPad software (La Jolla, CA). Graphs are presented as box-and whisker plots 
with the box representing the 25%-75% data range, the line representing the sample median, and the 
whiskers representing the data minimum and maximum. In all experiments, a p-value of less than 
0.05 was considered significant. All experiments were performed in triplicate and for each individual 
experiment 6-10 animals were used for each group unless otherwise noted.
RESULTS

Lymphedema results in increased macrophage infiltration

Analysis of matched skin biopsies from patients with upper extremity breast cancer-related lymphedema demonstrated a more than 3-fold increase in the number of macrophages (EMR-1+ cells) in the subcutaneous and dermal tissues of lymphedematous tissues as compared with controls (Figure 1A, B; p<0.01). Although macrophages were primarily present in the dermal areas, they could also be observed adjacent to adipocytes just below the dermis. Similarly, we found that lymphatic injury in the mouse tail model also results in significant accumulation (3.3-3.5 fold increase) of macrophages (F4/80+) in the dermis at an early time point after surgery and is sustained even 6 weeks later (Figure 1C, D; p<0.01). Using flow cytometry on single cell suspensions harvested from mouse tail tissues 6 weeks after surgery, we found that the lymphedema results in a relative decrease in the percentage of M1 cells (CD11b+, CD206lo) and an increase in the percentage of M2 cells (CD11b+, CD206hi) as compared with controls (Figure 1E, F).

Chronic ablation of macrophages in CD11b-DTR mice is effective but has systemic toxicity

As expected, acute treatment of the CD11b-DTR mouse with DT resulted in significant and dose dependent depletion of macrophages 24 hours after toxin administration. One dose of 25 ng/g of DT led to a marked decrease (nearly 80%) in splenic and lymph node macrophages (not shown) while smaller doses (2.5ng/g) led to an approximately 35% decrease in macrophage number. The highest doses of DT administered caused minor systemic toxicity when administered as a one-time dose; there was no discernable systemic effect from the lower doses used (<5ng/g; not shown). Maximum macrophage depletion occurred by 24 hours after DT administration and cell numbers rebounded by 72 hours (not shown). Therefore, we optimized a schedule of dosing to enable chronic and sustained depletion of macrophages. This was achieved with a dose of 2.5ng/g administered IP every 3 days and resulted in a nearly 75% depletion of macrophage numbers that was sustained for long periods (2-3 weeks; not shown). Animals tolerated this dosing schedule well without evidence of systemic toxicity or weight loss for a period of 2-3 weeks. However, when dosing was continued for longer periods as required by our study, we encountered increasing toxicity and decreased survival beginning at the 3 week time point (not shown). Because long-term and sustained macrophage depletion was a primary goal of our studies, we devised other approaches to decrease systemic toxicity and achieve our goal.
Chimeric CD11b-DTR have sustained macrophage depletion without systemic toxicity

Macrophages are hematopoietic cells, therefore we reasoned that creation of bone marrow chimeras transferring the bone marrow from CD11b-DTR mice into wild-type mice would decrease the toxicity and off target effects of chronic DT treatment while still enabling sustained macrophage depletion (Figure 2A). In the chimeric animals, bone marrow immunofluorescence revealed that over 75% of the macrophages co-expressed DTR (Figure 2B). Furthermore, more than 80% of macrophages present in lymphedematous tail tissue were bone marrow derived and co-expressed F4/80 and DTR (not shown). Consistent with this, we found that chimeric mice were highly sensitive to DT treatment such that even low doses of DT administered three times per week (2.5 ng/g) resulted in a more than 3-fold decrease in the percentage of macrophages in peripheral lymph nodes as assessed by flow cytometry (Figure 2C, D; p<0.01). This treatment also substantially decreased the number of macrophages in lymphedematous tail tissues as assessed by immunohistochemical localization of F4/80+ cells (>2 fold decrease; Figure 2E, F; p<0.01). More importantly, chimeric mice displayed no evidence of systemic toxicity or weight loss (not shown). Because CD11b is also expressed in variable levels in other cell types derived from the granulocyte lineage, we also found modest (30%) though significant reductions in the number of bone marrow neutrophils after DT administration (not shown; p<0.05).

Depletion of macrophages does not alter tail volumes or adipose deposition

In order to determine the temporal effects of macrophage depletion after lymphatic injury, chimeric WT/CD11b-DTR animals underwent tail skin and deep lymphatic excision and were treated with DT to deplete macrophages beginning either immediately after surgery (immediate DT) or starting 3 weeks postoperatively (3 week DT). Control animals were treated with vehicle only (PBS; i.e. not depleted). DT treatments were continued every 3 days for 3 weeks and animals were sacrificed 6 weeks after surgery.

We were somewhat surprised to find no significant differences in tail volume measurements at any time point postoperatively (Figure 3A). Similarly, although there was a modest increase in adipose area and dermal thickness in the 3 week DT group, these differences only achieved statistical significance for dermal thickness when compared with controls and the relative differences were modest (Figure 3B-D). In addition, analysis of inflammation 6 weeks after surgery using the pan-leukocyte marker CD45 in histological sections showed no differences in overall tissue inflammation as a consequence of immediate or 3 week DT macrophage depletion (Figure 3 E,F). Taken
together, these findings suggest that macrophage infiltration either immediately after lymphatic
injury or even after lymphatic stasis has become established does not significantly contribute to
adipose deposition or overall inflammatory reactions associated with interstitial fluid stasis.

Depletion of macrophages increases tissue fibrosis and impairs lymphatic function

Consistent with our observation of increased dermal thickness in the 3 week DT animals,
histological analysis of tissue sections from DT treated and control animals harvested 6 weeks after
surgery demonstrated that depletion of macrophages beginning at 3 weeks postoperatively resulted
in a significant increase in collagen type I deposition as compared with control animals (Figure 4A,
B). Thickened collagen bundles could easily be observed in the subdermal and dermal areas of the
skin surrounding capillary lymphatics in this area. This observation was confirmed with Sirius Red
staining demonstrating increased scar index indicative of increased organized collagen fiber
deposition in the 3 week DT group (Figure 4C, D). We also noted decreased lymphatic function in
the 3 week DT group as reflected by markedly decreased Tc<sup>99</sup> transport (Figure 4 E-H). This
analysis demonstrated a decrease both in peak nodal uptake by the sacral lymph nodes (p<0.01 for 3
week DT group) as well as a significant decrease in the rate of Tc<sup>99</sup> uptake in all macrophage
depleted animals suggesting that interstitial transport capacity is greatly diminished after macrophage
depletion.

Macrophage depletion increases CD4<sup>+</sup> cell infiltration and Th2 differentiation

We have previously shown that CD4<sup>+</sup> cells play a significant role in the regulation of fibrosis
in lymphedema (3, 37). Therefore, we next sought to determine how macrophages regulate CD4<sup>+</sup>
cell responses. Consistent with our observation of increased fibrosis in the 3 week DT group, we
found that depletion of macrophages 3 weeks after lymphatic injury resulted in modest though
significant increases in the number of CD4<sup>+</sup> cells infiltrating the distal lymphedematous tail tissues
(Figure 5A, B). In addition, we noted increased numbers of cells that stained positive for Gata-3, a
transcription factor necessary for Th2 differentiation of naïve CD4<sup>+</sup> cells, suggesting that Th2
differentiation was increased in animals depleted of macrophages either immediately after or
beginning 3 weeks after surgery (Figure 5C, D). This observation was supported by a more than
3-fold increase in the gene expression of IL-13, a Th2 cytokine, in macrophage depleted animals
(Figure 5E; p<0.05). Taken together, these findings suggest that macrophage accumulation after
lymphatic injury can regulate or modulate CD4<sup>+</sup> cell infiltration and Th2 differentiation.

Macrophage depletion decreases VEGF-C expression
Analysis of LYVE-1+ vessel counts in the tail tissues of experimental and control mice demonstrated that depletion of macrophages 3 weeks after surgery modestly (1.5-fold), though significantly, decreased the total number of capillary lymphatic vessels (Figure 6A, B; p<0.05). However, the lymphatic vessel luminal area was unchanged (Figure 6C). Consistent with the decreased number of LYVE-1+ vessels in the 3 week DT group, we noted a decrease in the expression of VEGF-C protein in tissue samples harvested from animals in this group (Figure 6D). These changes were reflected in our qPCR findings analyzing Prox-1 and LYVE-1 expression among the control, immediate DT and 3 week DT groups. Using the control group as the standard for the delta-delta method, we found a nearly 4-fold decrease in the expression of these lymphatic markers in the 3 week DT group as compared with control animals (Figure 6E, F). In contrast, we found no significant differences in VEGF-C or Prox-1 expression in animals treated with DT immediately after surgery.

**Depletion of macrophages after lymphedema is established decreases adipose deposition**

In our initial set of experiments, we sought to determine the role of macrophages on the development of pathological changes associated with lymphedema beginning shortly after lymphatic injury. We next sought to determine how changes in macrophage infiltration modulate the pathologic effects of sustained lymphatic stasis after lymphedema had become established. To accomplish this, we performed tail skin and lymphatic excision surgery on WT/CD11b-DTR bone marrow chimera mice and allowed them to recover for a period of 6 weeks. We have previously shown that at 6 weeks mice have significant lymphedema changes in the tail including adipose deposition, fibrosis, and persistent CD4+ cell accumulation(3). Beginning at the 6 week time point, animals were depleted of macrophages using three times weekly DT injections for a period of 3 weeks and pathologic changes were assessed.

Similar to our findings with immediate or early macrophage depletion, we found no significant differences in tail volumes after DT administration in animals treated after lymphedema had become established (Figure 7A). However, in contrast to our earlier findings we did note a modest (1.5 fold), though significant, decrease in adipose deposition in these animals after macrophage depletion while dermal thickness was increased (Figure 7B, C). Although we found no overall differences in tissue inflammation as assessed by CD45+ cell counts (Figure 7D, E) we again noted that there was a significant increase in the number of CD4+ cells in tissues obtained from macrophage depleted animals (Figure 7F, G). There were no significant differences between groups when comparing LYVE-1+ vessel counts or LYVE-1 vessel area, however, similar to our
earlier observation we noted a decrease in the concentration of VEGF-C protein by ELISA (Figure 7H-K).

**Late macrophage depletion increases fibrosis and impairs lymphatic function**

Consistent with our observations in animals depleted of macrophages shortly after lymphatic injury, we found that late depletion of these cells also results in increased fibrosis as assessed by collagen I immunostaining and Sirius Red birefringence (Figure 8A-D). Similar to our earlier observations, we noted dense collagen fiber deposition in the dermal areas with prominent staining surrounding capillary lymphatic structures. This increase in fibrosis was reflected in a significant impairment in lymphatic transport as assessed by Tc$^{99}$ lymphoscintigraphy. Macrophage depleted animals displayed a more than 4-fold decrease in peak nodal uptake and a greater than 12-fold decrease in the rate of Tc$^{99}$ uptake as compared with controls (Figure 8E-H). In addition, although we found no differences in LYVE-1$^+$ vessel number or diameter, we did note significant decreases in VEGF-C protein expression after macrophage depletion (Figure 8I-L). Taken together, these findings suggest that macrophage inflammatory reactions after lymphedema has become established contribute either directly or indirectly to adipose deposition, exert an anti-fibrotic effect, and regulate VEGF-C expression.
DISCUSSION

In the current study, consistent with our previous publications and the work of others, we have shown that lymphedema, both clinically and in the mouse tail model, results in a significant accumulation of macrophages. Although the mechanisms that regulate macrophage homing, proliferation, and differentiation in lymphedema remain unknown, several lines of evidence suggest that this process is regulated by T cell inflammatory reactions. For example, our group has previously shown that depletion or loss of T cells in general, or CD4+ cells in particular, results in significant attenuation of macrophage accumulation in the mouse tail model of lymphedema (3). We have also shown that lymphedema-induced CD4+ cell inflammatory response is necessary for increased expression of inflammatory cytokines (e.g. interferon gamma, IL-4, IL-6) that regulate macrophage migration and proliferation (3, 37). In addition, we have previously shown that lymphedema increases Th2 inflammatory responses, thus providing a rationale for the M2 biased macrophage inflammatory responses we observed in lymphedematous mouse tail tissues in the current study (3). The hypothesis that T cell inflammatory responses are necessary for macrophage migration and proliferation in lymphedema is also supported by studies on adipose tissue inflammation in obese individuals and mouse models demonstrating that T cell inflammatory reactions precede, and are necessary for, macrophage infiltration (16). Because a major component of the pathology of lymphedema involves abnormal adipose deposition, it is possible that similar inflammatory reactions to adipose inflammation drive macrophage migration to lymphedematous tissues. It is likely, however, that macrophage infiltration in lymphedema is regulated by multiple mechanisms since it is known that inflamed adipose tissues, as is characteristic of lymphedema and obesity, can directly promote macrophage migration and proliferation through the release of free-fatty acids by necrotic adipocytes and elaboration of macrophage chemotactic factors such as monocyte chemoattractant protein-1 (10, 28). Taken together, these studies suggest that T cells and adipose tissue deposition play critical roles in the regulation of macrophage migration and proliferation in lymphedema. Future studies should address these pathways in more detail.

An interesting observation of our study was that lymphedema in the mouse tail model favored M2 differentiation of tissue macrophages. This finding is important because M2, or alternatively activated macrophages, are known to play key roles in the regulation of lymphangiogenesis by producing VEGF-C and because these cell types contribute to tissue remodeling by regulating production of matrix metalloproteinases and collagen by other cell types.
Our observation is supported by previous studies demonstrating that tissue macrophages that accumulate in response to lymphedema express high levels of VEGF-C, consistent with M2 differentiation status (29, 35, 36). In addition, the known roles of M2 macrophages provide a rationale for our finding that depletion of macrophages after lymphatic injury significantly decreases the expression of VEGF-C and expression of lymphatic markers while increasing tissue fibrosis as compared with controls. The combined effect of these changes may therefore be responsible for diminished lymphatic function that we observed after macrophage depletion. Taken together, these findings suggest that macrophage infiltration in lymphedema is reactive to ongoing inflammatory responses, and macrophages in this setting act to augment lymphangiogenesis and diminish fibrosis. This role is in contrast to CD4+ cells, which our lab has previously shown to contribute, either directly or indirectly, to the pathology of lymphedema.

Although a variety of techniques have been described for macrophage depletion, we chose the CD11b-DTR transgenic model because this protocol enabled us to effectively and chronically deplete tissue macrophages for long periods of time in a cost effective manner. This approach required a significant amount of work for optimization, however, when optimized we were able to successfully deplete macrophages systemically and in lymphedematous tissues. In contrast, although the use of clodronate liposomes is well described and effective for systemic macrophage ablation (11, 30), previous studies have shown that this approach is less effective for depletion of tissue macrophages as compared with circulating cells because liposomes do not cross endothelial cell membranes (9). Similarly, other studies have depleted macrophages systemically by using small molecule inhibitors or neutralizing antibodies directed against colony-stimulating factor 1 or its receptor as this signaling pathway is essential for macrophage proliferation and differentiation (20). However, while this approach is effective and decreases macrophage cell populations significantly in short term studies, the use of these treatments for more chronic conditions such as lymphedema is hampered by the relative high cost of these reagents when administered over long periods of time.

We were somewhat surprised to find that macrophage depletion in the mouse tail model did not significantly decrease overall inflammatory responses but in fact was associated with increased CD4+ cell infiltration. Although this finding is somewhat counterintuitive, a possible explanation may be that macrophages that accumulate in response to lymphedema play an anti-inflammatory or immunosuppressive role. This concept is supported by our observation of M2 biased macrophage responses as these cells are known to have immunosuppressive effects and limit inflammatory responses in other physiologic settings (14). Thus, while macrophage depletion in our study...
decreased the number of tissue macrophages, the generalized inflammatory responses (and more specifically T infiltration) to lymphedema may have been augmented due to loss of M2 macrophages resulting in a net lack of difference in overall number of inflammatory cells. Alternatively, it is possible that depletion of macrophages had little effect on overall inflammatory response to lymphedema because macrophages make up only a small proportion of cells in the inflammatory milieu of chronic lymphedema. This hypothesis is supported by our previous studies demonstrating that the vast majority (>70%) of leukocytes present in lymphedematous tissues are CD4+ cells (3).

An interesting finding in the current study was that depletion of macrophages was associated with a significant increase in collagen deposition, fibrosis, and impaired lymphatic function. Previous studies have shown that macrophages can promote both pro- and anti-fibrotic effects and that these responses are not only organ specific but also differ temporally even within the same organ system (6). For example, macrophages have been shown to promote liver and renal fibrosis by producing pro-fibrotic cytokines such as transforming growth factor beta-1 (TGF-β), activating fibroblasts, promoting migration of myofibroblasts, increasing collagen synthesis, and decreasing extracellular matrix turnover (26, 33). In other circumstances, macrophages can aid in reversal of fibrosis by phagocytosing apoptotic or injured cells, directly removing collagen from the extracellular matrix, promoting breakdown of extracellular matrix products by increasing production of matrix metalloproteinases, and decreasing the expression of pro-fibrotic cytokines. Although the precise mechanisms by which macrophages regulate fibrosis in lymphedema remains unknown and requires additional study, our current study suggests that this response involves interactions with CD4+ cells (more specifically with Th2 differentiated cells) since macrophage depletion significantly increased these responses. This concept is supported by previous studies in pathogenic responses and fibrosis in response to schistosomiasis infections (18).

In conclusion, using a mouse model as well as clinical specimens of lymphedema, we have shown that macrophage infiltration is significantly augmented in response to lymphatic injury and lymphedema. In addition, we have demonstrated that the majority of macrophages present in lymphedema are M2 differentiated and that depletion of macrophages either before or after lymphedema is established results in decreased VEGF-C expression and increased fibrosis, collagen deposition, and CD4+ cell infiltration. Our findings, therefore, suggest that macrophage infiltration in response to lymphedema is reactive aiming to decrease overall inflammation and inhibit fibrosis. However, further studies are needed to test this hypothesis and define the mechanisms that regulate macrophage migration and function after lymphatic injury.
**FIGURE LEGENDS**

**Figure 1. Lymphedema results in increased macrophage infiltration.**

A. Representative high power (100x) histological sections of matched control (normal limb) and lymphedematous tissues obtained from a patient with unilateral upper extremity breast cancer-related lymphedema. Red arrows show EMR positively stained macrophages.

B. Cell counts of EMR⁺ macrophages in control and lymphedematous limbs of patients with breast cancer-related upper extremity lymphedema (n=8; *p<0.01).

C. Representative high power (80x) histological images of tissues obtained from mice that underwent tail incision without lymphatic ligation (control) and lymphedematous tails 2 and 6 weeks after surgery stained for F4/80⁺ macrophages.

D. Cell counts of F4/80⁺ cells in control and lymphedematous tail tissues 2 and 6 weeks after tail surgery (n=10; *p<0.01).

E. Representative flow diagrams of tail tissues obtained from control and lymphedematous tail tissues 6 weeks after surgery identifying cells that express CD206 and CD11b.

F. Quantification of M1 (CD11b⁺CD206⁻) and M2 (CD11b⁺CD206⁺) macrophages in control and lymphedematous tail tissues 6 weeks after surgery (n=8; *p<0.01).

**Figure 2. Chimeric CD11b/DTR have sustained macrophage depletion without systemic toxicity.**

A. Diagrammatic representation of bone marrow chimeras created from wild type mice reconstituted with bone marrow from CD11b-DTR mice.

B. Immunofluorescent staining of chimeric bone marrow showing co-staining of CD11b and diphtheria toxin receptor (DTR).

C. Representative flow diagram of macrophages (B220⁺/CD11b⁺) harvested from the peripheral lymph nodes of animals treated with vehicle control or diphtheria toxin (3 weeks after three times a week treatment).

D. Quantification of macrophages in peripheral lymph nodes of mice treated with or without DT for three weeks (*p<0.01).
E. Representative immunohistochemical staining of lymphedematous tail tissues harvested 3 weeks after 3 times a week treatment with or without DT. Arrows show positively stained macrophages.

F. Quantification of F4/80+ cells per high powered field (80x magnification) in lymphedematous tissues of mice treated with or without DT for 3 weeks (n=8; *p<0.01).

Figure 3. Depletion of macrophages does not alter tail volumes or adipose deposition.

A. Tail volumes of mice treated with vehicle control, DT beginning immediately after surgery (immediate DT), or DT beginning 3 weeks after surgery (3 week DT).

B. Representative cross-sectional histological sections of control, immediate DT, and 3 week DT treated mice (2.5x magnification) harvested 6 weeks after surgery. Sections were harvested 1.5 cm distal to the zone of lymphatic injury.

C. Quantification of adipose area in control, immediate DT, and 3 week DT groups from cross-sectional histologic sections harvested 6 weeks after surgery.

D. Dermal thickness of various groups analyzed in cross-sectional histologic sections (n=8; *p<0.05).

E. Representative high powered photomicrograph (80x) of tail tissues stained for CD45.

F. Quantification of CD45+ cells in tail sections of mice treated with vehicle control, immediate DT, or DT 3 weeks after surgery.

Figure 4. Depletion of macrophages increases tissue fibrosis and impairs lymphatic function.

A. Representative high-power photomicrographs (40X) of tail sections stained for type I collagen.

B. Quantification of type I collagen staining in tail sections of mice treated with vehicle control, immediate DT, or DT beginning 3 weeks after surgery (n=8; *p<0.01).

C. Representative photomicrographs of tail sections stained with Sirius red and imaged using polarized light microscopy (40X magnification).

D. Scar index of various groups quantified from Sirius red stained tissues (n=8; *p<0.01).

E. Representative Tc99 heat maps of tails from control, immediate DT, and 3 week post-surgery DT treated animals. Hot spot at the bottom of the photograph is the injection site. Small areas of uptake at the top of the picture are the sacral lymph nodes (white arrow).

F, G, H. Graphs depicting decay adjusted uptake (F), peak nodal uptake (G) (n=8; *p<0.01), and rate of uptake (H) (n=8; *p<0.05) in the sacral lymph nodes of animals in various groups.
**Figure 5.** Macrophage depletion increases CD4$^+$ cell infiltration and Th2 differentiation.

A. Representative high-powered (80X) photomicrographs of tail sections stained for CD4$^+$ cells.

B. Quantification of CD4$^+$ cells per high powered field in tissue sections harvested from animals in various groups (n=8; *p<0.01).

C. Representative high-powered (80X) photomicrographs of tail sections stained for Gata-3$^+$ cells.

D. Quantification of Gata-3$^+$ cells per high powered field in various experimental groups (n=8; *p<0.01).

E. IL-13 gene expression relative to control animals in experimental animals treated with DT either immediately after surgery or beginning 3 weeks postop. Data is presented as fold change versus control corrected for GAPDH expression (n=8; *p<0.05).

**Figure 6.** Macrophage depletion decreases VEGF-C expression.

A. Representative photomicrographs (40X) of tissue sections stained with LYVE-1 in various experimental groups.

B. Quantification of the number of LYVE-1$^+$ vessels in tail tissue sections (n=8; *p<0.01).

C. Quantification of LYVE-1$^+$ vessel area in tail tissue sections.

D. VEGF-C protein expression in total cellular protein harvested from tail tissue sections of various experimental groups (n=8; *p<0.01).

E, F. Expression of Prox-1 (E) and LYVE-1 (F) in tail tissues in various experimental groups. Values are expressed as mRNA levels fold change relative to control animals that were not treated with DT (n=8; *p<0.05).

**Figure 7.** Depletion of macrophages after lymphedema is established decreases adipose deposition.

A. Tail volumes of mice treated with or without DT beginning 6 weeks after surgery when lymphedema had become established.

B. Representative cross-sectional histology of tail sections stained with hematoxylin and eosin (2.5x magnification).

C. Quantification of adipose area in control and depleted animals (n=8; *p<0.01).
D. Quantification of dermal thickness in control and depleted animals (n=8; *p<0.05).
E. Representative high-powered photomicrographs (80x) of tail tissues harvested from control and DT depleted animals stained for CD45.
F. Quantification of CD45+ cells in tail tissues of control and macrophage depleted animals.
G. Representative high-powered photomicrographs (80X) of tail tissues harvested from control and DT depleted animals stained for CD4.
H. Quantification of CD4+ cells per high powered field in tail tissues of control and macrophage depleted animals (n=8; *p<0.01).
I. Representative photomicrograph of tail tissues from control and macrophage depleted animals stained with LYVE-1
J. Quantification of LYVE-1+ vessels/HPF in control and experimental animals.
K. Quantification of LYVE-1+ vessel areas in control and experimental animals.
L. VEGF-C protein expression in total cellular protein harvested from tail tissue sections of experimental and control animals (n=8; *p<0.01).

Figure 8. Late macrophage depletion increases fibrosis and impairs lymphatic function.
A. Representative photomicrographs (40X) of tail tissues from control and macrophage depleted animals stained for type I collagen.
B. Quantification of type I collagen staining area in experimental and control animals (n=8; *p<0.01).
C. Representative photomicrographs (40X) of tail sections stained with Sirius red and imaged using polarized light microscopy.
D. Scar index of control and experimental animals quantified from Sirius red stained tissues (n=8; *p<0.05).
E. Representative Tc99 heat maps from control and macrophage depleted animals.
F, G, H. Graphs depicting decay adjusted uptake (F), peak nodal uptake (G), and rate of uptake (H) in the sacral lymph nodes of animals in control and macrophage depleted animals (n=8; *p<0.05).
I. Representative photomicrographs (40X) of tissue sections stained with LYVE-1 in various experimental groups.
J. Quantification of the number of LYVE-1+ vessels in tail tissue sections.
K. Quantification of LYVE-1+ vessel area in tail tissue sections.
VEGF-C protein expression in total cellular protein harvested from tail tissue sections of control and macrophage depleted animals (n=8; *p<0.01).
REFERENCES


Figure A: Change in Tail Volume (%)

- Control
- Depleted

Figure B: Histological Sections

Control vs. Depleted

Figure C: Adipose Area (mm²)
P = 0.01

Figure D: Dermal Thickness (μm)
P = 0.001

Figure E: G0/G1 cells (%)

Figure F: Cidea cells (μm²)

Figure G: Control vs. Depleted

Figure H: CD8⁺ cells (μm²)

Figure I: Histological Sections

Control vs. Depleted

Figure J: Vessel number (μm²)

Figure K: Vessel area (μm²)

Figure L: VEGFC (pg/μl)

*(statistically significant difference)