Toll-like receptor deficiency worsens inflammation and lymphedaema after lymphatic injury

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Submitted 4 August 2011; accepted in final form 31 October 2011

Zampell JC, Elhadad S, Avraham T, Weitman E, Aschen S, Yan A, Mehrara BJ. Toll-like receptor deficiency worsens inflammation and lymphedaema after lymphatic injury. Am J Physiol Cell Physiol 302: C709–C719, 2012. First published November 2, 2011; doi:10.1152/ajpcell.00284.2011.—Mechanisms regulating lymphaedaema pathogenesis remain unknown. Recently, we have shown that lymphatic fluid stasis increases endogenous danger signal expression, and these molecules influence lymphatic repair (Zampell JC, et al. Am J Physiol Cell Physiol 300: C1107–C1121, 2011). Endogenous danger signals activate Toll-like receptors (TLR) 2, 4, and 9 and induce homeostatic or harmful responses, depending on physiological context. The purpose of this study was to determine the role of TLRs in regulating tissue responses to lymphatic fluid stasis. A surgical model of lymphedaema was used in which wild-type or TLR2, 4, or 9 knockout (KO) mice underwent tail lymphatic excision. Six weeks postoperatively, TLR KOs demonstrated markedly increased tail edema compared with wild-type animals (50–200% increase; P < 0.01), and this effect was most pronounced in TLR4 KOs (P < 0.01). TLR deficiency resulted in decreased interstitial and lymphatic transport, abnormal lymphatic architecture, and fewer capillary lymphatics (40–50% decrease; P < 0.001). Lymphedaematous tissues of TLR KOs demonstrated increased leukocyte infiltration (P < 0.001 for TLR4 KOs), including higher numbers of infiltrating CD3+ cells (P < 0.05, TLR4 and TLR9 KO), yet decreased infiltrating F4/80+ macrophages (P < 0.05, all groups). Furthermore, analysis of isolated macrophages revealed twofold reductions in VEGF-C (P < 0.01) and LYVE-1 (P < 0.05) mRNA from TLR2-deficient animals. Finally, TLR deficiency was associated with increased collagen type I deposition and increased transforming growth factor-β1 expression (P < 0.01, TLR4 and TLR9 KO), contributing to dermal fibrosis. In conclusion, TLR deficiency worsens tissue responses to lymphatic fluid stasis and is associated with decreased lymphangiogenesis, increased fibrosis, and reduced macrophage infiltration. These findings suggest a role for innate immune responses, including TLR signaling, in lymphatic repair and lymphedaema pathogenesis.

Endogenous danger signals

Lymphedaema is the pathological accumulation of interstitial fluid in tissues and, in the United States, occurs most commonly after lymph node dissection for cancer treatment. It is estimated that 30–40% of patients who undergo lymph node dissection will go on to develop lymphedaema, and that more than 5 million Americans currently suffer from this disabling disorder (38, 44).

Patients with lymphedaema suffer from functional issues, such as heaviness (6, 7), stiffness, and pain, as well as emotional problems, resulting in decreased quality of life (1). In fact, many cancer survivors consider dealing with lymphedaema more difficult than their original cancer treatment, since lymphedaema is a chronic and progressive disorder. Much of this frustration is likely due to the fact that treatment for lymphedaema remains palliative and based primarily on manual lymphatic massage and tight-fitting garments designed to physically prevent fluid accumulation. These treatments are time consuming and lifelong, serving as a constant reminder of cancer diagnosis. Similarly, although advances in microsurgical techniques and liposuction hold promise in the treatment of this disorder, the reported results from these procedures have been mixed, and most surgeons consider these options as palliative rather than curative (7, 9).

Development of novel, targeted treatments for lymphedaema has been hampered by the fact that the etiology of lymphedaema remains unknown. For example, while it is clear that lymphatic injury is the initiating event, it remains unknown why some patients develop lymphedaema while others do not. Similarly, it is unclear why, in many cases, lymphedaema develops in a delayed manner, usually 12–36 mo after surgery. In fact, although it is well known that the histological characteristics of lymphedaema are chronic inflammation, fibrosis, and lipodystrophy, the molecular mechanisms that regulate these changes remain unknown.

Toll-like receptors (TLRs) are a family of conserved pattern recognition molecules that recognize normal (i.e., endogenous) cellular components or pathogens to initiate inflammation. Endogenous molecules that activate TLRs are called endogenous danger signals and include a diverse group of molecules, including extracellular matrix breakdown products (hyaluronan fragments), heat shock proteins (HSPs), members of the S100 family of proteins, and nuclear proteins, such as high-mobility group box-1 (HMG1) (20, 34). Activation of TLRs and other receptor mechanisms by endogenous danger signals initiates sterile inflammatory reactions in a number of physiological circumstances, including ischemia-reperfusion injury, trauma, hemorrhagic shock, and autoimmunity (13, 19, 23, 24, 39, 48, 51). More recently, our laboratory has shown that endogenous danger signals, including HMG1 and HSP70, are activated by lymphatic fluid stasis and contribute to sterile inflammation and lymphangiogenesis (55). Furthermore, our laboratory has shown that gradients of lymphatic fluid stasis can induce nuclear translocation of nuclear factor-κ-light-chain enhancer of activated B cells (NF-κB), a downstream mediator of TLR activation (21).

Endogenous danger signal activation of TLRs can be either beneficial/homeostatic or harmful, depending on physiological milieu and context. For example, neutralization of TLR or endogenous danger signal function is protective in ischemia-reperfusion, trauma, hemorrhagic shock, and sepsis (24, 32,
METHODS

Mouse model of lymphatic stasis. Adult female, 10- to 12-wk-old wild-type C57BL/6, TLR2, TLR4, or TLR9 mice based on the same genetic background were obtained from Jackson Laboratories (Bar Harbor, ME). To study the effects of lymphatic fluid stasis in the various groups (n = 5–7/group), we excised a 2-mm-wide, circumferential full-thickness area of skin in the midportion of the tail using our laboratory’s previously published methods (4). In addition, we visualized and ligated the deep lymphatic system using a surgical microscope (Leica, StereoZoom SZ-4, Wetzlar, Germany). Care was taken to avoid injury to the lateral tail veins, and wounds were covered with a sterile dressing for the first 5 days and then left open. Tails were carefully monitored postoperatively, and any animals with evidence of ischemia were killed, excluded from analysis, and replaced with additional tail operations (~5%). Ischemic changes, if present, were obvious by the second postoperative week. Our laboratory has previously shown that this technique reliably causes sustained tail edema for at least 6 wk after surgery, resulting in histological changes consistent with lymphedema, including chronic inflammation, fibrosis, and fat deposition (4). All surgical procedures were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

To measure the extent of postsurgical edema, tail circumference measurements were performed at a distance of 1.5 cm from the distal end of the wound using a digital calliper. All measurements were performed in duplicate at weekly intervals for 6 wk. In addition, the tails were photographed with standard settings (i.e., distance to the tail from the camera lens) at the conclusion of the experiment.

Isolation of peritoneal macrophages. Elicited peritoneal macrophages were isolated from wild-type or TLR-deficient animals 5 days following peritoneal administration of 3% Brewer’s thiglycollate, according to standard protocols (15, 25, 56). Briefly, 5 days following 1-ml peritoneal injection of sterile Brewer’s thiglycollate medium, animals were injected with 5-ml phosphate-buffered saline twice directly into the peritoneum. Peritoneal fluid and cells were aspirated, washed, and placed in culture for 16 h. Floating cells were discarded, while monolayers of peritoneal macrophages were harvested for further analysis. Isolated macrophages were lyzed in lysis buffer (RLT buffer, Qiagen, Valencia, CA), and lysates were homogenized by drawing through a 25G/1.2-gauge needle; RNA was isolated using Allprep DNA/RNA/protein isolation kits, according to Qiagen protocols (Qiagen).

Microlymphangiography. We used our laboratory’s previously published methods to evaluate interstitial fluid flow in vivo (53). Briefly, 6 wk after surgery, animals were anesthetized, and 15 μl of a 2,000-kDa dextran solution conjugated to a fluorescein isothiocyanate molecule (10 mg/ml) was injected ~10 mm proximal to the tip of the mouse tail under constant pressure. Due to its large size, this molecule is excluded from the blood microcirculation, but is picked up and transported by the lymphatic system or by interstitial fluid flow. Capillary lymphatics were visualized using the Leica MZFL3 Stereo scope (Wetzlar, Germany). Fluorescent images were obtained at consistent magnification using Volocity software (Perkin-Elmer, Waltham, MA).

Tissue processing, histology, immunohistochemistry. Six weeks after surgery, animals were killed, and cross-sectional tissue was harvested 1.5 cm distal to the wound margin (i.e., same area at which tail circumference measurements were performed). Tissues were fixed overnight at 4°C in 4% paraformaldehyde, decalcified in Immunon (Decal Chemical, Tallman, NY), embedded in paraffin, and sectioned at 5-μm intervals. Histochemical stains (hematoxylin/eosin and trichrome) were performed using standard techniques. Immunohistochemical and immunofluorescent staining was performed using our laboratory’s previously published methods (11). We identified lymphatic vessels using polyclonal rabbit anti-mouse antibodies against podoplanin (a lymphatic specific marker; Abcam, Cambridge, MA). Proliferating cells were identified using polyclonal rabbit antibodies against proliferating cell nuclear antigen (PCNA; Abcam). Inflammatory cells were identified using an antibody against the pan-leucocyte marker CD45, while macrophages were stained using antibodies against F4/80 (both from Abcam). Collagen I and transforming growth factor (TGF)-β1 staining were performed using rabbit polyclonal antibodies to collagen I (Abcam) or TGF-β1 (Santa Cruz Biotech, Santa Cruz, CA). For immunofluorescent staining, Alexa Fluor secondary antibodies (Invitrogen Molecular Probes, Carlsbad, CA) were used. For immunohistochemistry (IHC) staining, secondary antibodies from the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) were used and developed using diaminobenzidine. Negative control sections were incubated with secondary antibody only. Coimmunohistochemistry for PCNA and podoplanin were performed using peroxidase and diaminobenzidine substrate (brown chromagen) development for PCNA and alkaline phosphatase and substrate (blue chromagen) for podoplanin. The percentage of PCNA+ lymphatic endothelial cells was determined per high-power field (HPF) (n = 3–5 animals/group; 5–6 HPF counted per animal), and mean percentages are represented graphically. Images were obtained using bright-field microscopy (Leica TCS) for IHC and a fluorescent microscope for immunofluorescence (Leica). Cell counts (for F4/80, CD45, CD3, or TGF-β1) and lymphatic vessel (podoplanin+) counts were performed in 3–5 HPF per section (n = 3–5/time point) by two blinded reviewers.

Subcutaneous tissue thickness analysis was performed using a modification of our laboratory’s previously published methods (4). Briefly, thickness of the dermis, including the subcutaneous fat layer, was analyzed using ImageJ Software (software available at http://rsweb.nih.gov/ij/) in three to five animals per group 6 wk after surgery by two blinded reviewers at ×10 magnification. A minimum of three measurements were performed for each animal. Lymphatic vessel diameter was determined as previously reported (4). Briefly, lymphatic vessels in the dermis and subcutaneous layer were identified, and the maximal diameter was measured using Mirax Imaging Software (Carl Zeiss). A minimum of three animals were evaluated per group with five to seven vessel measurements per animal. Collagen deposition was determined as a function of the number of brown pixels (defined as positive above a lower threshold) per unit area (μm²); 10–15 HPF were analyzed per group using Metamorph Imaging software (Metamorph Offline, Molecular Devices, Sunnyvale, CA).

PCR. Reverse transcription was performed using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA), followed by quantitative reverse transcriptase polymerase chain reaction using TaqMan Universal Mastermix (Applied Biosystems) and LightCycler thermocycler (Roche Diagnostics, Indianapolis, IN). Expression of VEGF-A (primer Mm00437308_m1; Applied Biosystems), lymphatic vessel endothelial hyaluronan
receptor-1 (LYVE-1) (primer Mm00475056_m1; Applied Biosystems), and VEGF-C (Mm01202432_m1; Applied Biosystems) was compared with 18S measured concurrently using the delta-delta technique ($2^{-\Delta\Delta Ct}$) for relative quantification (28). All experiments were performed in triplicate.

Statistical analysis. Statistical analysis was performed using one-way ANOVA to compare differences between groups. We used the Tukey-Kramer multiple-comparison post hoc test to compare differences between individual groups and considered $P < 0.05$ significant. All data are presented as means ± SD.

RESULTS

Loss of TLR function increases tail swelling. In line with our laboratory’s previous publications, we noted that, 6 wk following tail skin and lymphatic excision, wild-type animals had moderate gross swelling of the tail distal to the wound site (11). Similarly, TLR-deficient mice also displayed swelling of the tail distal to the wound site; however, this effect was more pronounced, particularly in TLR4 knockouts (KOs) (Fig. 1A). To confirm and quantify these observations, we performed tail circumference measurements 1.5 cm distal to the wound (Fig. 1B). We chose this site because our laboratory has previously shown that it is located away from the wound and, therefore, less reflective of swelling and tissue changes related to the wound (55). This analysis demonstrated that, on average, TLR-deficient mice had a 50–200% increase in tail circumference compared with wild-type animals. The differences in tail circumference between TLR2 KO and wild-type mice were significant 2, 3, 4, and 5 wk after surgery ($P < 0.01$, Fig. 1B). Similarly, TLR4 and TLR9 KO animals had significantly increased tail circumference at the 3-, 4-, 5-, and 6-wk time points ($P < 0.01$).

To further analyze tail edema in the various groups, we studied histological cross sections of the tail 1.5 cm distal to the wound (Fig. 2). Low-power photomicrograph analysis confirmed our gross observations, demonstrating markedly increased tail circumference in TLR-deficient mice (Fig. 2A). Compared with wild-type animals, TLR-deficient sections had more interstitial edema, dilation of capillary lymphatics, and subcutaneous fat accumulation (either from fat hypertrophy or hyperplasia) (Fig. 2B). In fact, subcutaneous tissue thickness, inclusive of adipose tissue, in the TLR KO mice was significantly increased in experimental mice compared with wild-type controls (Fig. 2C; $P < 0.001$). In addition, similar to gross observations, we noted that TLR4 KO mice had the highest mean subcutaneous tissue thickness, demonstrating a nearly twofold increase compared with wild-type animals ($P < 0.001$). Subcutaneous fat deposition and increased interstitial edema are histologically consistent with the characteristic histological changes of clinical lymphedema, suggesting that sustained lymphatic fluid stasis in this model, particularly in TLR-deficient mice, results in pathological changes that are seen clinically.

Loss of TLR function decreases interstitial fluid flow and results in dilatation of capillary lymphatics. To determine how loss of TLR function increases tail swelling after skin and lymphatic excision, we performed microlymphangiography. This technique entails injection of a large molecular weight colloid that is transported only by interstitial flow or by intact lymphatic vessels. Dermal lymphatic vessels take on a “honeycomb” appearance and can be seen under fluorescent light. Analysis of wild-type animals at the 6-wk time point demonstrated flow of fluorescent colloid across the wound from distal to proximal (Fig. 3A). Although we could not see distinct, newly formed lymphatics spanning the wounds of these animals (i.e., honeycomb appearance), interstitial fluid appeared to flow across the wound in most cases (4/5 animals). In addition, consistent with our gross and histological observations, we noted some pooling of the colloid distal to the wound site, indicating that the lymphatic channels had not fully regenerated. Analysis of TLR-deficient mice demonstrated markedly different patterns; in these animals we did not observe proximal flow of fluorescent contrast material across the wound in any of the animals of the various groups. Severe pooling of the contrast material was noted in all animals studied.

Lymphatic stasis is characterized histologically by dilation of capillary lymphatic vessels. Therefore, to analyze this feature in our model, we measured the diameter of capillary lymphatics in the various experimental groups by staining lymphatics with podoplanin (a lymphatic specific marker) and measuring the diameter of capillary lymphatics (Fig. 3, B and C). The average diameter of capillary lymphatics in wild-type animals was 69 ± 32 μm. In contrast, although there was some variability in lymphatic vessel diameter in the TLR-deficient mice, measurement of these vessels demonstrated a marked increase (nearly 300 μm or 4- to 5-fold increase) compared with wild-type animals ($P < 0.001$ in all comparisons). We noted similar changes in LYVE-1-stained lymphatics, another lymphatic marker (not shown).

A

Wild-Type TLR2 KO TLR4 KO TLR9 KO

B

Circumference (cm)

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Post-operative week

Fig. 1. Loss of Toll-like receptor (TLR) function increases tail swelling. A: representative gross photomicrograph of wild-type and TLR2, 4, or 9 knockout (KO) mice 6 wk after excision of capillary and collecting lymphatics. Note increased swelling in TLR KO mice. B: tail circumference measurements of wild-type and TLR KO mice. Weekly measurements were performed at a point located 1.5 cm distal to the wound edge, as shown in gross photograph and dotted line by blinded reviewers (5–6 animals/group). Values are means ± SD. *$P < 0.01$. 

AJP-Cell Physiol • doi:10.1152/ajpcell.00284.2011 • www.ajpcell.org
**Loss of TLR signaling decreases the number of capillary lymphatics.** When we examined podoplanin staining of capillary lymphatic vessels, we noted that wild-type animals tended to have less dilated lymphatic vessels and greater numbers of them (Fig. 4A). To quantify this observation, we counted podoplanin-stained capillary lymphatics in the various groups. This analysis demonstrated that wild-type animals had 9.3 ± 0.1 lymphatic capillary vessels/HPF (Fig. 4B). In contrast, TLR-deficient mice had a nearly twofold decrease in the number of capillary lymphatics (TLR2 KO = 5.7 ± 0.7, P < 0.01; TLR4 KO = 4.9 ± 0.7, P < 0.01; TLR9 = 5.6 ± 0.9; P < 0.05). To determine whether these differences were a result of proliferating lymphatic endothelial cells, we analyzed numbers of PCNA-podoplanin double-positive cells by IHC using our laboratory’s previously published methods (3, 11). This analysis revealed no significant differences among groups at 6 wk postoperatively (Fig. 4C), suggesting that loss of TLR signaling does not alter lymphatic endothelial cell proliferation at the 6-wk time point.

**Loss of TLR signaling decreases macrophase infiltration.** Clinical lymphedema is characterized histologically by chronic inflammation. We have previously shown that sustained lymphatic stasis in the mouse tail model similarly results in chronic mononuclear cell inflammation, and that the severity of this response depends on the degree of lymphatic stasis. Not surprisingly, we found evidence of chronic inflammation in all groups, including TLR KOs (Fig. 5, A–C). When quantified, mean numbers of CD45-positive cells were higher for all TLR KO groups compared with wild-type animals, but reached statistical significance only in the TLR4 KO animals (40% increase; P < 0.001; Fig. 5, A–C). To further evaluate differences in inflammatory responses in TLR KO mice, we analyzed T-cell infiltration using CD3 staining and found modest, although significant, increases in T cells/HPF in tissues isolated from TLR4 and TLR9 KOs (Fig. 5, D–F, *P < 0.05).

To further analyze differences in inflammation between wild-type and TLR KO animals, we performed Western blot analysis of tail tissues isolated from TLR2 KO mice. Consistent with our CD45 and CD3 cell counts by IHC, we found only subtle changes in Th1 (interferon-γ) and Th2 cytokines [ interleukin-4 (IL-4) and interleukin-13 (IL-13)] when comparing wild-type and TLR KO mice, suggesting that isolated TLR deficiency does not alter Th1/Th2 responses to lymphatic fluid stasis (Fig. 5G).
Macrophages are critical regulators of lymphangiogenesis, not only by producing lymphangiogenic cytokines, but also by directly trans-differentiating into lymphatic endothelial cells (30, 31). To analyze changes in macrophage infiltration we evaluate expression of F4/80, a macrophage marker (Fig. 5, H–J). This analysis demonstrated that TLR deficiency significantly decreased the number of infiltrating macrophages by three to fourfold in TLR2 (**P < 0.01), TLR4 (*P < 0.05), and TLR9 KO mice (**P < 0.01). Taken together, these findings suggest that TLR deficiency worsens lymphatic stasis and decreases lymphangiogenesis, and that this response is associated with diminished infiltration of macrophages.

Loss of TLR signaling decreases macrophage VEGF-C and LYVE-1 expression. We used a model of sterile peritoneal inflammation to further assess the effects of loss of TLR signaling in macrophages. We selected TLR2 KO mice for this analysis, given that they demonstrated the most significant reduction in macrophage infiltration in tail sections after lymphatic ablation. PCR analysis of isolated and enriched macrophages revealed a 45% decrease in VEGF-C mRNA (P < 0.01) and twofold reduction in LYVE-1 mRNA (P < 0.05) in TLR2 KO mice compared with wild-type controls (Fig. 6A). Interestingly, we noted a slight (5%), although statistically significant, increase in VEGF-A mRNA expression in TLR2 KO macrophages compared with wild-type controls (P < 0.01; Fig. 6A).

We performed Western blots of tail tissue sections to determine whether similar changes in VEGF-A/C expression occurred in response to lymph stasis. This analysis demonstrated only minor differences in VEGF-C and LYVE-1 expression 6 wk postoperatively (Fig. 6B); however, similar to our observations with peritoneal macrophages, we did note a 3.3-fold increase in VEGF-A expression in TLR2 KO mice compared with wild types. Taken together, these findings suggest that TLR signaling does play a role in the regulation of VEGF-A, VEGF-C, and LYVE-1 in macrophages; however, this regulation is complex, dependent on the local microenvironment, and possibly temporally regulated.

TLR KO animals have increased dermal fibrosis. Our laboratory has previously shown that lymphatic fluid stasis results in dermal fibrosis in the mouse tail model (3, 4). Furthermore, we have shown that the degree of fibrosis is associated with the severity of lymphatic fluid stasis. Previous studies have also shown that TLRs play a significant role in liver fibrosis (14, 41). Therefore, to determine whether loss of TLR function can alter the fibrotic response associated with lymphatic fluid stasis, we performed IHC for type I collagen, since this molecule is one of the
main components of fibrotic tissues. Interestingly, we found that TLR-deficient animals demonstrated markedly increased collagen I staining compared with wild-type controls (Fig. 7, A and B). Dense collagen bundles could easily be seen in the subdermal area in TLR-deficient animals. Consistent with the finding that loss of TLR4 function had the most significant effect on lymphatic stasis, we found that these animals also appeared to have the most intense staining for type I collagen (Fig. 7, B and C). We used ImageJ analysis to quantify relative collagen staining and found significant increases in dermal collagen deposition in TLR4 (2.2-fold; \( P < 0.001 \)) and TLR9 (1.7-fold; \( P < 0.01 \)) KO animals compared with wild-type controls (Fig. 7C). Although the mean staining intensity in TLR2 KO mice was also higher than that in wild-type animals, these differences were not statistically significant \( (P = 0.082) \).

We have previously shown that TGF-\( \beta_1 \) plays an important role in the regulation of tissue fibrosis in response to lymphatic fluid stasis (3, 4, 11). We, therefore, analyzed the expression of TGF-\( \beta_1 \) using IHC to determine whether increases in tissue fibrosis in TLR-deficient mice were associated with increased TGF-\( \beta_1 \) expression. Determination of numbers of TGF-\( \beta_1 \)-positive cells revealed a twofold increase in mean TGF-\( \beta_1 \) expression in TLR4 KOs \( (P < 0.01) \) and a fourfold increase in TGF-\( \beta_1 \) in TLR9 KOs \( (P < 0.001) \). (Fig. 7, D and E).

**DISCUSSION**

TLRs are a family of evolutionarily conserved pattern recognition receptors that enable the immune system to rapidly recognize foreign organisms, such as bacteria or viruses. To
date, 12 mammalian TLRs have been described, each recognizing a variety of signals. For example, TLR2 recognizes bacterial lipoproteins and cell membrane components. In contrast, TLR4 is activated by lipopolysaccharides, and TLR9 by unmethylated CpG DNA of bacteria. In addition, TLR2, 4, and 9 can bind endogenously produced molecules, such as extracellular matrix molecules, HSPs, reactive oxygen species, or DNA breakdown products released as a result of cellular stress or injury (14, 19). This is important, since our laboratory has previously shown that lymphatic fluid stasis can induce expression of endogenous danger signals, including HMGB1 and HSP70 (55). Transduction of TLR2 or 4 signaling requires coactivators such as MyD88 (myeloid differentiation primary-response gene), leading to activation of a variety of downstream pathways, including inhibitor of NF-κB kinase β-subunit, mitogen activated protein kinase, and extracellular signal-regulated kinase-1/2 (21). These kinases subsequently activate a variety of pathways and transcription factors, such as NF-κB and activator protein-1, to mediate downstream effects. Importantly, our laboratory has previously shown that lymphatic fluid stasis in the mouse tail model markedly increases NF-κB expression, suggesting that these pathways are activated in this process (55).

TLRs are expressed in a wide variety of cells, including most cells found in the skin, such as fibroblasts, keratinocytes, Langerhans cells, dendritic cells, mast cells, endothelial cells, and lymphatic endothelial cells (33, 55). It is, therefore, not surprising that TLR function has been shown to influence wound healing in some settings (21, 29, 45). However, in the present study, we did not find significant changes in overall wound-healing responses in the surgically created tail wounds of individual KOs of TLR2, 4, or 9. In fact, although excisional skin wound healing was not a primary endpoint of our study, we found that animals in all groups, including TLR-deficient mice, had reepithelialized their excised tail skin wounds by the 6-wk time point. This finding may be due to a number of causes. For instance, functional overlap exists between various TLR subtypes (2, 21), and it is possible that this overcomes the effects of loss of function in one receptor. This hypothesis is supported by the fact that deficiency of My88D (a downstream coactivator of TLR2 and TLR4), but not individual TLR subtypes, resulted in delayed wound healing in mice (29). Similarly, although stimulation of TLR9 with methylated oligodeoxynucleotides in wild-type animals can significantly improve wound healing in mice (40) and monkeys (45, 52), only modest delays in wound healing occur in TLR9-deficient mice compared with wild-type animals. In addition, differences in wound healing in mice deficient for multiple TLRs or TLR9 KO mice were only reported in the early time periods following skin wounding (1–3 wk) (45, 52). In contrast, in the present study, our analysis of lymphatic function was performed 6 wk after surgery, when differences in wound healing are less pronounced.

In the present study, we found that TLR-deficient mice, particularly TLR4 KO mice, had decreased numbers of podoplanin-positive capillary lymphatic vessels. In addition, we found that loss of TLR function delayed lymphatic repair and flow of interstitial fluid as assessed by microlymphangiography, and that TLR-deficient mice had dilated, ectatic lymphatic vessels displaying a more pathological phenotype compared with wild-type controls. Collectively, these findings suggest that loss of TLR function reduces lymphangiogenesis and lymphatic repair during wound healing and in response to lymphatic fluid stasis. A number of previous studies support this hypothesis. For example, previous studies have shown that activation of TLRs and adenosine agonists has a synergistic effect on VEGF-A expression as a result of HIF-1α stabilization (42). This finding is important since VEGF-A plays a critical role in both angiogenesis and lymphangiogenesis during wound repair and in response to inflammation (12). Finally, our laboratory has previously shown that HMGB1, an endogenous activator of TLR2, 4, and 9 (22, 36, 37, 47), is expressed by lymphatic endothelial cells in response to lymphatic stasis in our mouse tail model, and that inhibition of HMGB1 significantly decreases inflammatory lymphangiogenesis (55).
One potential mechanism responsible for decreased lymphangiogenesis in TLR-deficient animals may be reduced macrophage recruitment, as observed in our study. Macrophages are known to play a central role in lymphatic regeneration (12, 50) and thereby provide a potential mechanism for the decreased lymphangiogenesis that we observed. The seemingly contradictory response of increased leukocyte infiltration but reduced macrophage infiltration in TLR KOs may represent tissue responses to sustained lymphatic fluid stasis. This hypothesis is supported by our laboratory’s previous studies demonstrating that sustained lymphatic fluid stasis in the mouse tail model is associated with chronic T-cell inflammation, Th2 cell differentiation, and decreased macrophage infiltration compared with temporary lymphatic fluid stasis (4, 11). In light of the fact that TLR signaling (particularly TLR2 and 4) can regulate Th2 differentiation, it is also possible that loss of these signals alters tissue immune responses to lymphatic fluid stasis, including macrophage recruitment. This hypothesis is supported by the finding that patients with filariasis have immune dysfunction and lymphedema, and that these responses are associated with decreased expression and function of TLR1, 2, 4, and 9 on their inflammatory cells (5). It is additionally possible that TLR signaling is required for macrophage accumulation, as Lin et al. (26) demonstrate, for example, that TLR4-induced chemokine production was necessary for macrophage recruitment to corneal wounds. Taken together, this information suggests that macrophage recruitment to regions of lymphatic fluid stasis is attenuated in TLR-deficient animals, and this may be secondary to either greater lymphatic stasis, impaired TLR signaling, or a combination of these factors.

In support of our hypothesis that decreased lymphangiogenesis in TLR-deficient animals is related to altered macrophage recruitment and function, we found that isolated macrophages in TLR2 KO animals (animals with the most significant decrease in macrophage numbers in the lymphedematous tail) had decreased expression of VEGF-C and LYVE-1. Thus it is possible not only that macrophage recruitment is decreased in TLR-deficient mice, but also that these cells are less functional in terms of lymphangiogenic cytokine expression and possibly lymphatic differentiation. Interestingly, we found only subtle differences in VEGF-C expression in the tail tissues when comparing TLR2 KO and wild-type animals. In addition, TLR2-deficient animals paradoxically expressed higher quan-

Fig. 7. TLR KO animals have increased dermal fibrosis. A and B: representative low-power (×10; A) and high-power (×40; B) photomicrographs of collagen I immunohistochemistry in wild-type and TLR KO animals. Sections were harvested 1.5 cm distal to the wound (as shown in gross photograph) 6 wk following lymphatic ablation. Note marked increase in collagen staining in the dermis of TLR KO animals compared with wild types. C: ImageJ quantification of collagen deposition with subcutaneous tissues ***P < 0.001, *P < 0.01. Collagen deposition is expressed as the number of brown pixels per unit area (μm²). Scale bar represents 50 μm. D: representative low- (top) and high-power (bottom) photomicrographs of transforming growth factor (TGF)-β1 staining in tissues located 1.5 cm distal to the wound. Scale bar represents 50 μm. E: cell counts of TGF-β1+ cells (n = 3–5 animals per group; 5–6 HPF/animal). Values are means ± SD. ***P < 0.001, *P < 0.01.
ities of VEGF-A in the tail sections compared with wild-type animals. This particular finding is interesting, since it is known that VEGF-A expression is required for macrophage recruitment (and subsequent VEGF-C production). Thus it is possible that abnormal macrophage recruitment in TLR-deficient mice leads to increased VEGF-A expression by local tissues in a homeostatic manner. In fact, recent studies in our laboratory have shown that delayed lymphatic repair is also paradoxically associated with increased expression of lymphangiogenic cytokines (VEGF-A, VEGF-C, hepatocyte growth factor), and that decreased lymphatic function in this setting may be related to increased expression of anti-lymphangiogenic cytokines. Therefore, future studies will be needed to delineate the time course of lymphangiogenic and anti-lymphangiogenic cytokines to determine whether changes in these pathways contribute to altered lymphatic function in TLR-deficient animals.

Key clinical features of lymphedema include increased interstitial tissue edema, fat deposition, and tissue fibrosis. Indeed, in our present study, TLR-deficient animals had markedly increased tail circumference as a result of increased tissue edema, dilated lymphatics, subcutaneous fat deposition, and fibrosis with resultant dermal thickening. Interestingly, it appears that TLR signaling can either increase or decrease fibrosis, depending on local tissue characteristics and the physiological insult. For example, TLR blockade is protective of cardiac fibrosis, resulting from myocardial infarction or arterial occlusion (27, 43). Decreased cardiac fibrosis as a result of TLR activation is associated with a shift toward a Th1-differentiated state and downregulation of Th2 responses. In contrast, TLR activation worsens kidney fibrosis secondary to ureteral obstruction (8), augments bleomycin-induced lung fibrosis (49), and increases liver fibrosis, both clinically and in animal models (16, 17, 46). Similarly, TLR activation is required for bleomycin-induced skin fibrosis (54), and TLR expression is increased in fibroblasts obtained from hypertrophic scars (49). Changes in tissue fibrosis may result from interactions with other fibroitic molecules. For example, TLR activation in stellate cells of the liver increase fibrosis by sensitizing them to the profibrotic effects of TGF-β1 (17). Our laboratory has previously shown that TGF-β1 plays a critical role in the regulation of fibrosis in the mouse tail model of lymphatic fluid stasis and is associated with a shift toward Th2 differentiation (4). These mechanisms may also contribute to increased fibrosis in TLR-deficient animals, as we found increased expression of TGF-β1 in TLR KO mice compared with wild-type animals. Additional study is needed to dissect these fibrotic pathways and the interactions between T cells, macrophages, and fibrotic cytokine expression.

In conclusion, we have shown that TLR function plays an important role in homeostatic responses to lymphatic fluid stasis. These molecules regulate a variety of effects, ultimately regulating lymphangiogenesis, fat deposition, inflammation, and tissue fibrosis. These findings, together with our previous report demonstrating increased expression of endogenous danger signals that are known to activate TLR2, 4, and 9, suggest that these pathways play important roles in the regulation of tissue responses to lymphatic fluid stasis.

GRANTS
Sources of funding for this work are gratefully acknowledged and include an National Institutes of Health T32 grant for J. C. Zampell and E. Weitman, Society of Memorial-Sloan Kettering Grant for B. J. Mehrara, Plastic Surgery Education Foundation Research Fellowship Grants for J. C. Zampell and T. Avraham, and the Sloan-Kettering Institute Department of Surgery.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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