Supplementary materials and methods

Isolation of lymphatic endothelial cells (LECs) from mouse ears, footpad and lymph nodes

Dorsal and ventral sides of ears were separated with forceps. Both sides of the ear was made to float on 2 ml of 4U/ml dispase (Gibco) and incubated at 37°C for 90 min. Following incubation, the dermal layers were minced and digested in 0.1 mg/ml of Liberase in RPMI + 1% FCS for 45 min at 37°C.

Footpads were minced into small pieces with scissors. The footpads were digested with a mix that contains collagenase IV 2mg/ml and dispase 2U/ml (both from Gibco) in RPMI-1600 at 37°C for 60 minutes with gentle agitation.

Isolation of LECs from LNs was carried out as previously described. Briefly, LNs were digested in HBSS medium containing 4mg/ml collagenase IV (Gibco) at 37°C for 45 minutes with gentle agitation.

Reagents and antibodies used for flow cytometry analysis

More details of antibodies used for flow cytometry analysis can be found in supplementary table 1. BrdU uptake was detected using the BrdU flow kit (BD Biosciences) as per manufacturer’s instructions.

Cell counts were determined using Count Bright® Absolute Counting Beads (Molecular Probes, Invitrogen). FACS analysis was performed using a CyAn ADP Analyzer (Beckman Coulter) and analyzed with Flowjo software (Treestar).

Reagents and antibodies used for immunofluorescence

Endogenous avidin and biotin were quenched using the Avidin/ Biotin blocking kit (Vector Laboratories). Blocking with Mouse on Mouse™ (M.O.M.™) Immunodetection Kit (Vector Laboratories) was performed to minimize non-specific
signals during immunostaining with the mouse anti-mouse VEGF-A-VEGFR2 complex antibody. More details of antibodies used for immunofluorescence can be found in supplementary table 2.

**Antibodies used for immunoblotting**

The following purified primary antibodies were used for immunoblotting: anti-MMP-9 (Abcam), anti-TIMP-1 (R&D Systems), anti-heparanase (Abcam) and anti-VEGF-C antibody (Millipore). HRP-conjugated anti-rabbit IgG and anti-goat IgG were used as secondary antibodies. More details of antibodies used for immunoblotting can be found in supplementary table 3.

**Depletion of heparanase activity from conditioned media**

Briefly, 10μg of anti-heparanase antibody (Abcam) was added to 500μg total protein of CM and left overnight at 4°C. Following this, protein G agarose beads was added at a concentration of 20mg of Rabbit IgG/ml agarose and left for 4 hours at 4°C. Agarose beads and the supernatant comprising of heparanase-depleted CM were separately collected. The beads were washed with PBS before boiling to release the anti-heparanase-heparanase immunocomplex.

**Gelatin Zymography**

After electrophoresis, the gel was re-natured for 1 hour with a 50mM Tris, 4.5mM CaCl₂, ZnCl₂ and 2.5% Triton-X buffer and subsequently left overnight at 37°C in a 50mM Tris, 4.5mM CaCl₂ and ZnCl₂ buffer. The gel was stained with 0.1% Coomassie Blue and subsequently destained with 30% Ethanol/ 10% Acetic Acid.
Microscopy and image analysis

Area densities (percentage of total tissue area) of LYVE-1⁺ lymphatic vessels in footpad whole mounts were quantified for 5 regions per footpad, each 1 mm² in area, using ImageJ software (http://rsb.info.nih.gov/ij). The average area density of lymphatics within these 5 regions was taken as representative of the footpad sample. Images of 3-4 individual fields of view under a 100x magnification were acquired per ear section to determine the average density of lymphatics. Mean fluorescence intensity of VEGF-A: VEGFR2 complexes were measured using images from 3-4 individual fields of view per sample. Images were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA) for adjustment of image brightness.
### Supplementary tables

#### Table 1. List of antibodies used for flow cytometry

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#### Table 2. List of antibodies used for immunofluorescence

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**Supplementary figure legends**

**Supplementary figure 1. Neutrophils drive lymph node lymphangiogenesis in the absence of B cells.** (A) Increases in LN cellularity in WT and μMT mice at various time-points post-immunization. Increase is expressed as fold change over time-matched non-immunized mice. (B) Increases in T and dendritic cell populations at various time-points post-immunization. Increase is expressed as fold change over time-matched non-immunized mice (C-D). Typical dot plots representing myeloid cells (C) and neutrophils (D) in WT and μMT WT LNs on day 14 post-immunization. (E) Typical dot plots representing circulating Ly6G⁺ CD11b⁺ neutrophils in μMT mice treated with NIMP-R14 MAb or control rat IgG after immunization. (F) Typical dot plots representing BrdU⁺ LEC populations in LNs from μMT mice treated with NIMP-R14 MAb or control rat IgG. Data from (B) are pooled from 3 independent experiments with 4-5 mice per group in each experiment (n=12-15). Bars represent mean ± SD. **: p<0.01. In (C-E), numbers next to gates indicate percentage of cells relative to the total live single cells population. In (F), numbers next to gates indicate percentage of BrdU⁺ LECs.

**Supplementary figure 2. Attenuating neutrophil accumulation in footpads from WT mice following CFA/ KLH immunization.** (A) Typical dot plots representing circulating neutrophils in immunized WT mice treated with NIMP-R14 MAb or
control rat IgG. (B) Typical dot plots representing neutrophils and non-granulocytic myeloid cells in footpads. Cells in dot plots are gated on the CD45+ CD3− B220− fraction. CD11b+ Ly6G+ cells are neutrophils while CD11b+ Ly6G− cells are non-granulocytic myeloid cells. (C) Neutrophil populations in immunized footpads from NIMP-R14 or control rat IgG-treated mice. (D) Typical dot plots representing BrdU+ LEC populations in footpads of mice. In (A-B), numbers next to gates indicate percentage of cells relative to the total live single cells population. Data from (C) consists of 4-5 mice per group. Bars represent mean ± SD. *: p<0.05, **: p<0.01. In (D), numbers next to gates indicate percentage of BrdU+ LECs.

**Supplementary figure 3. Attenuating neutrophil accumulation in ears from WT mice following CHS.** (A) Typical dot plots representing neutrophils in DNCB-challenged ears of WT mice treated with NIMP-R14 or control rat IgG. Cells in dot plots are gated on the CD45+ CD3− B220− fraction. CD11b+ Ly6G+ cells are neutrophils while CD11b+ Ly6G− cells are non-granulocytic myeloid cells. (B) Typical dot plots representing BrdU+ LEC populations in DNCB-challenged ears of mice treated with NIMP-R14 or control rat IgG. In (A), numbers next to gates indicate percentage of cells relative to the total live single cells population. In (B), numbers next to gates indicate percentage of BrdU+ LECs.

**Supplementary figure 4. Blocking VEGFR2 and VEGFR3 signaling exacerbated footpad swelling after CFA/KLH immunization.** Footpad swelling in immunized mice treated with anti-VEGFR2, anti-VEGFR3 or control rat IgG. Data is representative of 2 independent experiments with 3 mice per group in each
experiment (n=6). Scale bar in represent 2mm.

Supplementary figure 5. Amount of VEGF-C in CFA/KLH immunized footpads is unaffected by attenuating neutrophil accumulation. (A) Western blot detecting for VEGF-C (native and/ or processed) in supernatant harvested from non-stimulated or fMLP-stimulated neutrophils. Immunized footpad serves as positive control. Immunoblot is representative of 4 independent experiments (n=4). NS, non-stimulated. (B) ELISA of VEGF-C in footpad homogenates from NIMP-R14 and control rat IgG-treated mice at days 7, 10 and 14 post-immunization. Data are pooled from 2 independent experiments with 3 mice per group in each experiment (n=6) and represent mean ± SD. NS, not significant. (C) Western blots detecting VEGF-C proteins (native and/ or processed) in footpad homogenates from NIMP-R14 and control rat IgG-treated mice at days 10 and 14 post-immunization. Each lane represents 1 individual mouse. GAPDH is used as a loading control.

Supplementary figure 6. Association of TIMP-1 with neutrophils and MMP-9. TIMP-1 did not associate with Ly6G+ neutrophils nor neutrophil-derived MMP-9 in inflamed footpad sections. Images are representative of 5 independent experiments (n=5). Scale bar represent 50μm.

Supplementary figure 7. Inhibiting MMP-9 and heparanase activity by using a MMP-9 inhibitor and heparanase depletion. (A) Gel zymogram showing that inhibition of MMP-9 activity in the CM was optimal using 5mM of the MMP-9 inhibitor. (B) Western blot showing efficient depletion of heparanase in the CM by immunoprecipitation with an anti-heparanase antibody. ND refers to non-depleted
CM. (C) Gelatin zymography showing that heparanase depletion from the CM did not affect MMP-9 activity. (D) Gel zymogram showing that footpad homogenates obtained 1 day after treating neutrophil-depleted mice with CM lacking MMP-9 activity, were devoid of MMP-9 activity. Lanes 1 and 2: footpad homogenates obtained one day after treating 2 neutrophil-depleted mice with CM lacking MMP-9 activity; Lane 3: CM that have not been treated with MMP-9 inhibitor; Lane 4: footpad homogenate from CFA/ KLH immunized WT mice.

Supplementary figure 8. Presence of VEGF-A: VEGFR2 complexes, phosphorylated VEGFR2 and co-localization with lymphatic vessels. VEGF-A: VEGFR2 complexes resulted in VEGFR2 phosphorylation (activation). Co-localization of VEGF-A: VEGFR2 complexes and phosphorylated VEGFR2 with lymphatic vessels. Images are representative of 5 mice per treatment group (n=5). Scale bars represent 50µm.

Supplementary figure 9. Proposed model for how neutrophils may contribute to lymphangiogenesis. (A, upper panel) During inflammation, epithelial cells, dendritic cells and macrophages are the major cellular sources of VEGF-A and/ or C. Most of the secreted VEGF-A is bound to the ECM. Although activated neutrophils may secrete VEGF-A and/ or C, it is likely that they are more important as the producer of VEGF-D. (A, lower panel) Neutrophils secrete MMP-9 and heparanase which liberate VEGF-A from the ECM. The bioavailable VEGF-A binds to VEGFR2 on lymphatic vessels to induce lymphangiogenesis. Neutrophil-derived VEGF-D also binds to VEGFR3 to induce lymphangiogenesis. (B, upper panel) During inflammation when neutrophil accumulation is reduced, cells such as epithelial cells,
dendritic cells and macrophages will continue to secrete VEGF-A and/or C. The amount of VEGF-D is however greatly reduced. (B, lower panel) MMP-9 and heparanase in the inflamed tissues are reduced when neutrophil accumulation is attenuated. VEGF-A remains bound to the ECM and is not biologically active. Although some VEGF-C may be present, lymphangiogenesis is greatly inhibited.
Supplementary figure 3

A

Control rat IgG  

NIMP-R14

Ly6G  

CD11b

Neutrophils (%)  
Non-granulocytic myeloid cells (%)

B

Non-immunized  

Immunized

Control rat IgG  

NIMP-R14

BrdU  

FSC-A

BrdU+ LECs per ear (%)  
BrdU+ LECs per ear (%)

Non-granulocytic myeloid cells (%)
Supplementary figure 4

Control rat IgG

Anti-VEGFR2 IgG

Anti-VEGFR3 IgG
Supplementary figure 5

A

Precursor VEGF-C (67 kDa)
Various cleaved forms of VEGF-C
Mature VEGF-C

B

NS  NS  NS

Control Day 7 Day 7 Day 10 Day 14 Day 14

VEGF-C (pg per mg of tissue)

Control
Immunized
Control rat IgG
Immunized
NIMP-R14

C

Day 10

Control rat IgG  NIMP-R14

Precursor VEGF-C (61 kDa)
Processed VEGF-C (55 kDa)
Processed VEGF-C (43 kDa)
Mature VEGF-C (12 kDa)

Day 14

Control rat IgG  NIMP-R14

Precursor VEGF-C (61 kDa)
Processed VEGF-C (55 kDa)
Processed VEGF-C (43 kDa)
Mature VEGF-C (12 kDa)
GAPDH
Supplementary figure 6
Supplementary figure 8

Control rat IgG + media

NIMP-R14 + CM
Supplementary figure 9

A. Inflammation

B. Inflammation, neutrophils accumulation attenuated

Figure legends:
- Dendritic cell
- Lymphatic endothelial cell
- Neutrophil
- Epithelial cell
- Macrophage
- Extra-cellular matrix
- VEGF-A
- VEGFR-2
- VEGF-C
- VEGFR-3
- VEGF-D
- Cleavage
- Release
- Possible release