SHORT COMMUNICATION

Inflammation and Lymphedema Are Exacerbated and Prolonged by Neuropilin 2 Deficiency

Patrick Mucka,^{*} Nicholas Levonyak,^{*} Elena Geretti,^{*†} Bernadette M.M. Zwaans,^{*} Xiaoran Li,^{*} Irit Adini,^{*†} Michael Klagsbrun,^{*†} Rosalyn M. Adam,^{†‡} and Diane R. Bielenberg^{*†}

From the Vascular Biology Program^{*} and the Urological Diseases Research Center,[†] Boston Children's Hospital, Boston; and the Department of Surgery,[‡] Harvard Medical School, Boston, Massachusetts

Accepted for publication
July 14, 2016.

Address correspondence to
Diane R. Bielenberg, Ph.D.,
300 Longwood Ave., Boston,
MA 02115. E-mail: diane.bielenberg@childrens.harvard.edu.

The vasculature influences the progression and resolution of tissue inflammation. Capillaries express vascular endothelial growth factor (VEGF) receptors, including neuropilins (NRPs), which regulate interstitial fluid flow. NRP2, a receptor of VEGFA and semaphorin (SEMA) 3F ligands, is expressed in the vascular and lymphatic endothelia. Previous studies have demonstrated that blocking VEGF receptor 2 attenuates VEGFA-induced vascular permeability. The inhibition of NRP2 was hypothesized to decrease vascular permeability as well. Unexpectedly, massive tissue swelling and edema were observed in *Nrp2*^{-/-} mice compared with wild-type littermates after delayed-type hypersensitivity reactions. Vascular permeability was twofold greater in inflamed blood vessels in *Nrp2*-deficient mice compared to those in *Nrp2*-intact littermates. The addition of exogenous SEMA3F protein inhibited vascular permeability in Balb/cJ mice, suggesting that the loss of endogenous Sema3F activity in the *Nrp2*-deficient mice was responsible for the enhanced vessel leakage. Functional lymphatic capillaries are necessary for draining excess fluid after inflammation; however, *Nrp2*-mutant mice lacked superficial lymphatic capillaries, leading to 2.5-fold greater fluid retention and severe lymphedema after inflammation. In conclusion, *Nrp2* deficiency increased blood vessel permeability and decreased lymphatic vessel drainage during inflammation, highlighting the importance of the NRP2/SEMA3F pathway in the modulation of tissue swelling and resolution of postinflammatory edema. (*Am J Pathol* 2016, 186: 2803–2812; <http://dx.doi.org/10.1016/j.ajpath.2016.07.022>)

The endothelium plays a crucial role in inflammatory reactions within tissues, and chronic inflammatory diseases (eg, psoriasis, eczema) are associated with neo-vascularization. The vasculature mediates two phases of acute inflammation: the leakage phase, modulated by endothelial cells (ECs), which results in edema and swelling, and the drainage phase, controlled by lymphatic ECs (LECs), which clears interstitial fluid and results in resolution and return to homeostasis.¹ The cutaneous delayed-type hypersensitivity (DTH) assay (also called the contact hypersensitivity assay) has been routinely used for studying the inflammatory endothelium. Inflamed vessels undergo remodeling characterized by increased permeability, increased flow, and an influx of immune cells.² The vascular endothelial growth factors (VEGFs) A and C pathways are known to be pivotal in regulating vascular permeability and lymphatic drainage, respectively, during

inflammation.^{3–6} For example, inflammation increases VEGFA, also called vascular permeability factor, which binds VEGF receptor (VEGFR)-2 in ECs to cause fluid leakage, resulting in edema.^{3,7} Keratin 14–VEGFA transgenic mice display excessive edema and psoriasis-like symptoms,^{3,8} and the inhibition of VEGFR2 with blocking antibodies during inflammation in hemizygous keratin 14–VEGFA mice inhibits the extent of edema.⁵ During resolution, fluid that leaks from blood vessels into the

This study was supported by the Vascular Biology Program at Boston Children's Hospital and NIH awards CA118732 (D.R.B.), CA155728 (D.R.B.), CA037392 (M.K.), and DK65298 (R.M.A.). The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

P.M. and N.L. contributed equally to this work.

Data in this manuscript were presented at the 2016 Experimental Biology National Meeting, San Diego, California, April 2–6, 2016.

interstitial space is drained by lymphatic capillaries through lymphatic ducts to lymph nodes. Lymphatic capillary dysfunction can result in lymphedema.⁹ Keratin 14–VEGFC transgenic mice stimulate lymphangiogenesis, providing a conduit for drainage and exhibit reduced lymphedema after inflammation,⁶ while antibodies used for blocking VEGFR3 during inflammation promote lymphedema.⁵

Neuropilins (NRPs) are transmembrane coreceptors that mediate both stimulatory signals from VEGF family proteins and inhibitory signals from class 3 semaphorin (SEMA3) ligands.^{10–12} ECs express two NRP receptors, NRP1 and NRP2, during development.^{11,13} NRP1 is essential for angiogenesis and cardiovascular development, and *Nrp1*-knockout (KO) mice die *in utero* from vascular defects.^{13,14} NRP2 is expressed in capillary (and venous) ECs of the blood and lymphatic system and is not required for developmental angiogenesis.^{13,15} *Nrp2*-deficient mice survive to adulthood and are fertile but smaller in size than are wild-type (WT) littermates.^{16,17} *Nrp2*^{−/−} embryos have fewer lymphatic vessels than do *Nrp2*^{+/+} embryos but do not show overt signs of edema.^{15,18}

Few studies have analyzed the physiologic function of the NRP2 receptor in ECs or LECs beyond developmental stages; however, mutations in the human *NRP2* gene have been found in families with primary lymphedema.¹⁹ Normally, NRP2 is down-regulated or absent in adult quiescent capillaries, but NRP2 can be up-regulated during ischemia.^{20,21} Since NRP2 is a mediator of the VEGFA and VEGFC pathways^{12,22,23} and is expressed in both ECs and LECs, we hypothesized that NRP2 may play a role in the regulation of fluid dynamics after inflammation during both the leakage and drainage phases. We predicted that inhibiting NRP2 would have effects similar to inhibiting VEGFR2 since both are coreceptors for VEGFA. However, our results show the opposite effect—*Nrp2*-deficient mice are hyperpermeable. Additionally, we demonstrate that adult *Nrp2*-mutant mice show extensive lymphedema under inflammatory conditions. These phenotypes are due to the promiscuous nature of the *Nrp2* receptor, which also binds to Sema3F, an endogenous angiogenesis and lymphangiogenesis inhibitor.^{24,25}

Materials and Methods

Mice

All mice were maintained under specific pathogen-free conditions in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The care and experimental procedures were conducted in compliance with the NIH's *Guide for the Care and Use of Laboratory Animals*²⁶ and were approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital (Boston, MA).

Nrp2^{+/*LacZ*} mice were a gift from Dr. Seiji Takashima (Osaka University Graduate School of Medicine, Osaka, Japan).²⁷ These mice were generated by replacing the first coding exon of *Nrp2* with a promoterless *Escherichia coli* β -galactosidase gene. These mice were backcrossed to the C57BL/6J strain for >10 generations. *Nrp2*^{*LacZ*/*LacZ*} pups die soon after birth. *Nrp2*^{+/*LacZ*} mice are of normal size and weight and are indistinguishable from *Nrp2*^{+/+} littermates.

Nrp2^{+/*gfp*} mice (also known as *Nrp2*^{*tm1.2Mom*}/*MomJ*; stock number 006700) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the C57BL/6J background. *Nrp2*^{*gfp/gfp*} mice (functionally referred to as *Nrp2*^{−/−} mice) are viable, fertile, and nonedematous at baseline. C57BL/6J and Balb/cJ mice were purchased from The Jackson Laboratory.

DTH Assay

DTH reactions were induced in the ears of female mice ($n = 5$ mice per group) at 8 to 12 weeks of age, as previously described.²⁸ DTH was performed on several strains, including Balb/cJ; C57BL/6J; *Nrp2*^{+/*LacZ*} and the resultant offspring from *Nrp2*^{+/*gfp*} $\times$ *Nrp2*^{+/*gfp*} matings, including *Nrp2*^{+/+} (WT), *Nrp2*^{+/*gfp*} (heterozygous, called *Nrp2*^{+/*−*}), and *Nrp2*^{*gfp/gfp*} (KO, called *Nrp2*^{−/−}). Mice were sensitized by the topical application of a 2% oxazolone (4-ethoxymethylene-2 phenyl-2-oxazoline-5-one; Sigma-Aldrich, St Louis, MO) solution in acetone/olive oil (4:1 vol/vol) to the shaved abdomen (50 μ L) and to each paw (5 μ L). Mice were challenged on the right ear 5 days after sensitization (day 0) by topical application of a 1% oxazolone solution (20 μ L total; 10 μ L on each side of the right ear), and left ears were treated with vehicle alone. The thicknesses of vehicle- and oxazolone-treated ears were measured using a Mitutoyo gauge, daily for up to 10 days, as described for the mouse ear-swelling test.²⁹ The increase in ear thickness over the baseline thickness (measured in microns) was used as a measurement of the extent of inflammation and plotted versus time. The experiment comparing WT, heterozygous, and KO mice was repeated three times.

Miles Assay

Miles assays were performed on female Balb/cJ mice ($n = 4$ mice per group) at 8 weeks of age. Mice were shaved 1 day before the experiment. Mice were anesthetized with tribromoethanol delivered i.p. Evans Blue dye (100 μ L of a 1% solution in normal saline) was injected via the tail vein. After 10 minutes, proteins (50 μ L) or vehicle were injected s.c. to induce permeability, including phosphate-buffered saline (PBS), recombinant human VEGFA₁₆₅ (200 ng/mL = 4.76 nmol/L) (R&D Systems, Minneapolis, MN), recombinant human VEGFA₁₆₅ (4.76 nmol/L) plus SEMA3F (95 nmol/L; 20-fold excess), recombinant human VEGFA₁₆₅

(4.76 nmol/L) plus SEMA3F (950 nmol/L; 200-fold excess), and recombinant human VEGFA₁₆₅ (4.76 nmol/L) plus bevacizumab (950 nmol/L; 200-fold excess) (Genentech, South San Francisco, CA). SEMA3F protein was purified as previously described.³⁰ After 20 minutes, the mice were euthanized in a CO₂ chamber, and the area of skin that included the extravasated dye was excised and imaged. Evans Blue dye was extracted from the skin pieces by incubation in formamide at room temperature for 5 days. The absorbance at 620 nm was measured using a spectrophotometer. This experiment was repeated three independent times with 4 mice per group in the first two experiments and 3 mice per group in the third experiment, for a total of 11 mice per group. The means $\pm$ SEM of each experiment were plotted and compared.

Modified Miles assays were performed on the female, adult (8- to 12-week-old) resultant offspring from *Nrp2*^{+/*gfp*} $\times$ *Nrp2*^{+/*gfp*} matings, including *Nrp2*^{+/+} (WT), *Nrp2*^{+/-} (heterozygous), and *Nrp2*^{-/-} (KO), on day 2 after DTH (*n* = 3 mice per group). Evans Blue dye (100 μ L of a 1% solution in normal saline) was injected via the tail vein. Dye leaked from blood vessels into the interstitial space in the challenged ears; vehicle-treated ears were used as controls. WT mice not undergoing DTH reactions were injected in the ear with recombinant human VEGFA₁₆₅ as a positive control. Extravasated dye was quantified in a spectrophotometer (620 nm).

Lymphangiography

Evans Blue dye (10 μ L of a 1% solution in normal saline) was injected into the tip of vehicle-treated or oxazolone-challenged ears on the 3rd day after DTH in isoflurane-anesthetized Balb/cJ mice (*n* = 5 mice). Mice were imaged within 5 minutes for the visualization of lymphatic vessels.

Dye-retention experiments were performed as described.⁶ Evans Blue dye (5 μ L of a 1% solution in normal saline) was injected into the tip of oxazolone-challenged ears on the 4th day after DTH in isoflurane-anesthetized *Nrp2*^{+/-} or *Nrp2*^{-/-} mice (*n* = 5 mice per group). After 16 hours, the mice were euthanized, and ear pieces of equal area were incubated in formamide for the extraction of the dye. After 48 hours, the absorbance was measured in a spectrophotometer at 620 nm.

Tissue Staining

Tissues were embedded in OCT compound and frozen in liquid nitrogen. Cryosections (8 μ m) were fixed in cold acetone for 5 minutes and washed with PBS. Endogenous peroxidases were blocked in a 3% H₂O₂ solution in methanol for 12 minutes and washed with PBS. Endogenous proteins were blocked with Tris-HCl, NaCl blocking buffer (PerkinElmer, Waltham, MA) for 20 minutes at room temperature. Sections were incubated overnight at 4°C in rabbit anti-mouse lymphatic vessel endothelial hyaluronic acid

receptor 1 antibody (catalog number 102-PA50; ReliaTech GmbH, Wolfenbuttel, Germany) in Tris-HCl, NaCl blocking buffer. The next day, tissues were washed in PBS and incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 hour. Sections were washed in PBS and incubated with alkaline phosphatase-conjugated avidin (Vectastain ABC-AP kit; Vector Laboratories) for 30 minutes. Sections were washed in PBS, visualized using Vector Red Substrate (Vector Laboratories), and counterstained with hematoxylin (Sigma-Aldrich).

Nrp2^{+/*LacZ*} mice cryosections were fixed in cold methanol for 5 minutes. β -Galactosidase activity was detected by incubation in X-gal reagent [1 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Gold Biotechnology, Olivette, MO) in dimethyl sulfoxide, 5 mmol/L K₃Fe(CN)₆, 5 mmol/L K₄Fe(CN)₆, 2 mmol/L MgCl₂ in PBS (pH 6.5)] overnight at 37°C. Sections were washed in PBS and counterstained in eosin (Sigma-Aldrich).

Paraffin sections (4 μ m) were dewaxed in xylene and rehydrated through a graded series of ethanol (100%, 95%, 70%, 50%) to water. Endogenous peroxidases and proteins were blocked as described in the previous paragraph. Sections were incubated overnight at 4°C in rabbit anti-SEMA3F antibody,³¹ rabbit monoclonal anti-mouse *Nrp2* antibody (catalog number 3366; Cell Signaling, Beverly, MA), or Syrian hamster anti-mouse podoplanin (ReliaTech) in Tris-HCl, NaCl blocking buffer. The next day, tissues were washed in PBS and incubated in biotinylated goat anti-rabbit IgG or anti-hamster IgG (Vector Laboratories) for 1 hour. Sections were washed in PBS and incubated with either horseradish peroxidase-conjugated avidin (Vectastain Elite ABC kit; Vector Laboratories) or alkaline phosphatase-conjugated avidin for 30 minutes. Sections were washed in PBS, visualized using the DAB (3,3'-diaminobenzidine) Substrate Kit (Vector Laboratories) or Ferangi Blue Chromogen Kit (Biocare Medical, Concord, CA), and counterstained with hematoxylin (Sigma-Aldrich).

Immunoblot Analysis

Protein lysates were separated with 7.5% SDS-PAGE, transferred to nitrocellulose membranes, blocked in skim milk for 30 minutes at room temperature, and incubated overnight at 4°C in rabbit monoclonal anti-mouse *Nrp2* (catalog number 3366; Cell Signaling) or in rabbit anti-SEMA3F antibody.³¹ Membranes were washed in Tris-buffered saline with Tween 20 and incubated in horseradish peroxidase-linked donkey anti-rabbit (catalog number NA934V; GE Healthcare Bio-Sciences, Pittsburgh, PA) for 1 hour at room temperature. Blots were washed in Tris-buffered saline with Tween 20 and exposed to Western Lightning Plus ECL (enhanced chemiluminescence) (PerkinElmer). Blots were stripped (Reblot Plus; EMD Millipore, Billerica, MA) and reprobed with anti-integrin α 5 (catalog number 4705; Cell Signaling) to normalize for protein loading.

Statistical Analysis

Significance was measured with an unpaired *t*-test.

Study Approval

Procedures were performed in compliance with NIH, Association for Assessment and Accreditation of Laboratory Animal Care, and Institutional Animal Care and Use Committee regulations at Boston Children's Hospital.

Results

Inflammation Induces Tissue Swelling, Edema, and Nrp2 Up-Regulation

DTH reactions in C57BL/6J mice resulted in tissue swelling that reached maximal levels by 1 day after challenge and caused hyperplasia and remodeling by day 3 (Figure 1, A and B). Inflammation-induced vascular permeability was evident by increased redness and swelling in the challenged ears (Supplemental Figure S1A). Lymphangiography showed pooled dye and retrograde lymph flow in inflamed ears compared to unchallenged ears (Supplemental Figure S1B).³² Immunoblot analysis of C57BL/6J mice ear proteins after DTH showed nearly twofold increased expression of Nrp2 by day 1 and nearly threefold by days 5 to 7 (Figure 1C). Moreover, Nrp2 expression (β -galactosidase activity) was up-regulated in inflamed endothelium in *Nrp2*^{+LacZ} mice (Figure 1D). Nrp2 is expressed in both vascular ECs and LECs (Supplemental Figure S1C). Nrp2 was found in inflamed blood capillaries (Figure 1E) and colocalized with podoplanin staining on lymphatic vessels after inflammation (Figure 1F). Noninflamed adult mouse ears expressed Nrp2 only in melanocytes of hair follicles (Figure 1D), not in capillaries, consistent with findings from previous reports.^{20,21,33} Nrp2 expression was absent in quiescent adult vasculature (Figure 1D) and in *Nrp2*^{-/-} mouse ear sections (Figure 1G).

Nrp2 Regulates Vascular Permeability and Edema

Adult female *Nrp2* KO and WT mice have ears of equal thickness (data not shown), suggesting that Nrp2-deficient mice are not normally edematous. In unchallenged ears, vascular permeability was slightly (but not significantly) greater in *Nrp2*^{-/-} vessels than in *Nrp2*^{+/+} vessels (Figure 2, A and B). However, on day 2 after inflammation, vascular leakage in inflamed ears, as measured by a modified Miles assay, was double in *Nrp2* KO mice compared to WT mice (Figure 2, A and B). Thus, Nrp2 deficiency increased edema due to increased vascular permeability.

NRP2 binds inhibitory SEMA3F proteins as well as VEGF proteins.^{34,35} We hypothesized that the edema seen in inflamed *Nrp2*^{-/-} mice may have been due to the lack of

endogenous SEMA3F signaling. Immunostaining and immunoblot analysis results demonstrated endogenous SEMA3F expression in normal mouse skin epithelium but not in endothelium or fibroblasts (Supplemental Figure S1, D and E). SEMA3F-transfected tumors served as positive controls for immunostaining (Supplemental Figure S1F).³² Additionally, exogenous SEMA3F protein inhibited vascular permeability in the skin in VEGFA-induced Miles assays in WT mice (Figure 2, C and D). At equal doses, SEMA3F protein was as effective as bevacizumab, a VEGFA-neutralizing antibody, in blocking vascular permeability.

Nrp2 Deficiency Prolongs Swelling and Lymphedema

DTH reactions in *Nrp2*^{+/+}, *Nrp2*^{+/-}, and *Nrp2*^{-/-} mice displayed maximal swelling by day 1 (Figure 3A). In WT and heterozygous mice, swelling was resolved within 4 to 5 days, while *Nrp2* KO mice exhibited significantly increased swelling and delayed resolution between 2 and 10 days (Figure 3A). Tissue thickness and hyperplasia were increased in *Nrp2* KO compared to WT mice (Figure 3B). Increased interstitial fluid pressure causes anchoring filaments to open junctions in LEC capillaries to allow for fluid uptake.³⁶ In normal mouse ears, lymphatic capillaries (identified by lymphatic vessel endothelial hyaluronan receptor 1-positive staining) were present below the epidermis and in the subcutis near cartilage (Figure 3C and Supplemental Figure S1G). Nrp2-deficient mice, on the other hand, lacked a superficial lymphatic capillary plexus (Figure 3C and Supplemental Figure S1H). Lack of lymphatic capillaries was likely due to guidance and sprouting defects during development, as evidenced by embryonic skin sections (Supplemental Figure S1, I and J) and previous reports.^{15,18} In fact, the mean depth below the epidermis to the nearest lymphatic capillary in embryonic day 18 skin was 2.5-fold greater in Nrp2-deficient mice than in WT mice ($P < 0.001$) (Figure 3E). This distance increased to 5-fold greater in inflamed adult Nrp2-deficient ears compared to WT ears ($P < 0.001$) (Figure 3D). The lymphatic capillary number was nearly half in adult *Nrp2* KO mice compared to WT mice after DTH (region of interest defined as the dorsal side of the ear in each 100 $\times$ field; mean vessels per area, 4 ± 1 in KO versus 7.5 ± 1 in WT). Additionally, both groups had dilated, open-lumened lymphatic vessels with similar total lymphatic capillary area per region of interest (WT, 5831 ± 1711 pixels²; KO, 6655 ± 3079 pixels²). Although the mean luminal areas of each lymphatic capillary were quite heterogeneous, in both groups there was a trend toward larger individual capillaries in the *Nrp2* KO mice compared to WT mice; however, the difference did not reach statistical significance (WT, 897 ± 1375 pixels²; KO, 1663 ± 1377 pixels²; $P = 0.2$). Therefore, the location of the capillary within the dermis dictated the overall drainage of the tissue bed.

Figure 1 Inflammation up-regulates neuropilin (Nrp)-2 in the endothelium. **A:** Ear swelling versus time after delayed-type hypersensitivity (DTH) reaction in C57BL/6 mice. **B:** Hematoxylin and eosin (H&E) staining of ear sections before (day 0; **top panel**) and after (day 3; **bottom panel**) DTH shows epidermal and dermal hyperplasia; **asterisks** denote dilated vessels. **C:** Immunoblot of ear lysates from C57BL/6 mice after DTH. Nrp2 receptor and soluble Nrp2 (**top panel**) increases by day 1 and is greatest on day 5 after DTH. Integrin $\alpha 5$, a loading control, is shown in the **bottom panel**. **D:** X-gal (blue) and eosin (pink) staining in *Nrp2*^{+/LacZ} mice ears before (day 0; **top panel**) and after (day 2; **bottom panel**) DTH. Nrp2-expressing vessels (**arrows**) are only present in inflamed ears. **E–G:** Nrp2 is up-regulated in endothelial cells (ECs) and lymphatic ECs. Immunostaining for Nrp2 (**E** and **G**; brown) or podoplanin (Pdpn) (**F**; blue). Tissue in **E** and **G** was counterstained with hematoxylin (blue nuclei). In serial sections of wild-type *Nrp2*^{+/+} ears on day 3 after DTH, **arrows** point to lymphatic vessels stained with Nrp2 and Pdpn; **asterisks** denote Nrp2-positive blood vessels negative for Pdpn staining. As a control, *Nrp2*^{-/-} ears on day 3 after DTH were negative for Nrp2 immunostaining (**G**, lack of brown); melanocytes in the skin are brown due to endogenous melanin. Data are expressed as means $\pm$ SD. $n = 5$ (**A**) Scale bars = 100 μ m [**B**, **D**, and **E–G** (scale bar in **E** applies to **F** and **G**)].

This difference in lymphatic structure and organization within the ear resulted in a 2.5-fold retention of fluid or functional lack of drainage, as measured by lymphangiography, in *Nrp2* KO mice compared to WT mice ($P < 0.05$) (**Figure 3F**).

Discussion

VEGFA stimulates EC leakage, and VEGFC stimulates LEC drainage.^{3,5} NRP2 is a receptor of both VEGFA and VEGFC^{12,23}; therefore, the deletion of NRP2 *in vivo* is

Figure 2 A neuropilin (Nrp)-2 ligand, semaphorin (Sema)-3F, inhibits vascular permeability. **A** and **B**: Vascular permeability was compared between *Nrp2*^{+/+} [wild type (WT)], *Nrp2*^{+/-} [heterozygous (HET)], and *Nrp2*^{-/-} [knockout (KO)] mice in modified Miles assays after delayed-type hypersensitivity (DTH). **A**: The mean leakage values of Evans Blue dye [optical density, 620 nm] extracted from unchallenged (-) and challenged (+) ears in the same mice from each group on day 2 after inflammation were plotted and compared to that of WT ears injected with exogenous vascular endothelial growth factor (VEGF) A (positive control) (representative data). **B**: Relative vascular permeability in each group compared with that in WT (combined data from 3 experiments). Baseline (unchallenged) vascular leakage was not significantly different among the three genotypes. Vascular permeability was doubled in *Nrp2*^{-/-} KO mice compared to that in *Nrp2*^{+/+} WT mice. **C** and **D**: Vascular permeability in Miles assays in Balb/cJ mice. Permeability was induced with phosphate-buffered saline (PBS; control), VEGF, or a mixture of VEGF and SEMA3F (S3F; at 20× or 200× of VEGF) or VEGF and anti-VEGF (at 200× of VEGF). **C**: Representative mouse skin image. **D**: Mean relative permeability values in the VEGF, VEGF + SEMA3F, VEGF + anti-VEGF groups compared to that in the PBS control group (from three independent experiments). VEGF SEMA3F and VEGF αVEGF were compared and found to be statistically similar. Data are expressed as means ± SEM. *n* = 3 mice per group (**A** and **B**); *n* = 11 mice total (**D**). **P* < 0.05.

predicted to inhibit leakage and drainage. However, our results demonstrate that the system is more complicated.

Inflammation increases VEGFA,⁷ which causes EC to leak, resulting in edema.³ VEGFR1 and VEGFR2 have been reported to be up-regulated in vessels after inflammation.³⁷ We report that the Nrp2 receptor is also up-regulated after inflammation. Previous studies have shown that blocking VEGFR1/VEGFR2 or Nrp1 in a DTH model resulted in reduced edema.^{3,5,28} Therefore, we hypothesized that blocking Nrp2 would lower edema since it is a coreceptor for VEGFA.¹² Nrp2-deficient mice showed no signs of edema before inflammation, yet once challenged, they responded with massive and prolonged edema. Our data suggest that NRP2 is a unique VEGFR in inflammatory endothelium.

NRP2 mediates both the VEGF and SEMA3F pathways. The inhibitory effect of Sema3F/Nrp2 in EC permeability

(Figure 2, C and D) is in sharp contrast to the stimulatory effect of SEMA3A/Nrp1 in EC permeability reported previously.³⁸ Our Sema3F immunostaining data (Supplemental Figure S1E) are in agreement with those from previous mRNA staining³⁹ and suggest that Sema3F is constitutively produced in the epidermis and acts, in a paracrine fashion, as an endogenous damper of inflammation and edema. SEMA3F protein has been shown to compete with VEGFA on NRP2³⁵ and to inhibit EC functions.^{33,40,41} Loss of the functional Sema3F receptor, Nrp2, increased edema and lymphedema after inflammation. This conclusion is based on findings from previous structural studies that have suggested that VEGFA binds between NRP receptor and VEGFR2 in a bridge formation.^{11,42,43} Additionally, an *in vitro* study showed that the addition of NRP2 to cells expressing VEGFR2 increases phosphorylation in response

Figure 3 Prolonged inflammation and lymphedema in mice lacking neuropilin (Nrp)-2. **A:** Ear swelling versus time after delayed-type hypersensitivity (DTH) reactions in *Nrp2*^{+/+} [wild type (WT); circle], *Nrp2*^{+/-} [heterozygous (HET); triangle], and *Nrp2*^{-/-} [knockout (KO); square] mice (representative of three independent experiments). **B:** Hematoxylin and eosin (H&E) staining of ear sections from WT (bottom panel) or *Nrp2* KO (top panel) mice after DTH (day 4). *Nrp2*^{-/-} ears are thicker than WT littermate ears; swelling is more pronounced in the outer ear. **C:** Cryosections stained for lymphatic vessel endothelial hyaluronin acid receptor (LYVE)-1 (red) 1 day after challenge reveal lymphatic capillaries. Ear sections from WT mice (left panel) show dilated superficial lymphatic capillaries near the epidermis (arrows) and in the deeper dermis near the cartilage. *Nrp2* KO mice (right panel) show only deeper lymphatic vessels and lack superficial capillaries. **D** and **E:** The distance from the epidermis to the nearest lymphatic capillary was measured in inflamed ears in adult mice (**D**) and in normal embryonic day 18 (E18) skin (**E**). Lymphatic capillaries were 5-fold deeper in the inflamed ears and 2.5-fold deeper in normal embryonic skin. **F:** The retention of Evans Blue dye was quantified after 16 hours in WT and *Nrp2* KO ears on day 4 after DTH. Lymphatic drainage was 2.5-fold less in the *Nrp2* KO mice compared to that in the WT mice. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group (**A** and **F**); $n = 4$ mice per group (**D** and **E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars = 100 μm .

to VEGFA stimulation compared to cells expressing VEGFR2 alone.¹²

An alternative explanation is that NRP2 acts as a decoy receptor for VEGFA and sequesters VEGFA away from VEGFR2. When NRP2 is deficient, as in the case of the *Nrp2* KO mice, there could be an increase in the total bioavailable VEGFA in the microenvironment, which could then signal via VEGFR2 more readily to increase permeability. In WT mice, increased Sema3F expression could compete with VegfA for Nrp2 binding and thereby free up VegfA to bind to VegfR2, again resulting in increased

permeability. This alternative explanation negates the inhibitory role of SEMA3F signaling via NRP2 and plexin, and is therefore less likely in our opinion.

Little is known about the regulation of NRP2 and/or SEMA3F *in vivo*. NRP2 expression is under the regulation of the transcription factors GATA2 and LMO2 (LIM domain only 2) during development.²² Hypoxia has been reported to inhibit the expression of NRP2,⁴⁴ whereas hyperoxia in a retinal ischemia model resulted in augmented NRP2 expression.⁴⁵ Other factors speculated to regulate NRP2 may include superoxides,^{46–48} nitric oxide,⁴⁹ growth factors such as

platelet-derived growth factor and heparin-binding epidermal growth factor–like growth factor,⁵⁰ and epithelial–mesenchymal transition–related pathways.^{51,52} Zinc finger E-box–binding homeobox 1 and inhibitor of DNA-binding protein 2 have been shown to down-regulate SEMA3F in tumor cells,^{30,53} but SEMA3F regulation in normal epithelial cells and its effects on ECs and vascular permeability are unclear.

Interstitial fluid pressure causes junctions in LECs to open and allows for fluid uptake in capillaries, whereas ducts do not take up fluid. The prolonged lymphedema in *Nrp2* KO mice is likely due to architectural defects from improper LEC sprouting during development and the lack of a superficial lymphatic capillary plexus necessary for competent fluid drainage, in agreement with findings from previous studies.^{15,18} *Nrp2* deficiency phenocopied the effect of VEGFR3-blocking antibodies in the DTH model,⁵ suggesting that *Nrp2* and VEGFR3 are essential for proper VEGFC/D signaling in lymphatic vessels. Together, these data suggest that *Nrp2* is necessary for proper lymphatic architecture and adequate fluid drainage.

Our results demonstrate that NRP2 is the only VEGFR that, when inhibited, increases edema (initial swelling after acute inflammation resulting from blood vessel leakage), and increases lymphedema (longer-term swelling after acute inflammation resulting from the lack of proper drainage from lymphatic vessels). In comparison, the inhibition of VEGFR1/VEGFR2 or NRP1 improves edema, and the inhibition of VEGFR3 worsens lymphedema but not edema.^{3,5,27}

In conclusion, our data suggest that NRP2 is important for normal vascular function in adults. In this mouse model, *Nrp2* deficiency during acute inflammation enhanced edema and lymphedema due to a loss of endogenous *Sema3F* signaling. We speculate that down-regulation of SEMA3F or its receptor may be involved in skin disorders such as vascular or lymphatic malformations, dermatitis, and/or eczema. In fact, mutations in the *NRP2* gene have been found in patients with primary lymphedema.¹⁹ Future studies on the role of the SEMA3F/NRP2 axis in chronic inflammation or lymphedema are encouraged.

Acknowledgments

The authors acknowledge Ricasan Rowley Histology Consulting for histology sections and H&E staining, Kristin Johnson for imagery and graphics, and Melissa Anderson for editing and administrative assistance.

P.M., N.L., E.G., B.M.M.Z., and X.L. performed experiments and analyzed data; I.A. designed experiments and analyzed data; M.K. provided funding and edited the manuscript; R.M.A. analyzed data, provided funding, and wrote and edited the manuscript; D.R.B. designed experiments, performed experiments, analyzed data, provided

funding, and wrote the manuscript; and all authors read and approved the final manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.07.022>.

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