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Normal Dendritic Cell Mobilization to Lymph Nodes under Conditions of Severe Lymphatic Hypoplasia

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To address the requirement for lymphatic capillaries in dendritic cell (DC) mobilization from skin to lymph nodes (LNs), we used mice bearing one inactivated allele of vascular endothelial growth factor receptor 3 (VEGFR3) where skin lymphatic capillaries are reported absent. Unexpectedly, DC mobilization from the back skin to draining LNs was similar in magnitude, and kinetics to control mice and humoral immunity appeared intact. By contrast, DC migration from body extremities, including ear and forepaws, was ablated. An evaluation in different regions of skin revealed rare patches of lymphatic capillaries only in body trunk areas where migration was intact. That is, whereas the ear skin was totally devoid of lymphatic capillaries, residual capillaries in the back skin were present though retained only at ∼10% normal density. This reduction in density markedly reduced the clearance of soluble tracers, indicating that normal cell migration was spared under conditions when lymphatic transport function was poor. Residual lymphatic capillaries expressed slightly higher levels of CCL21 and migration of skin DCs to LNs remained dependent on CCR7 in Chy mice. DC migration from the ear could be rescued by the introduction of a limited number of lymphatic capillaries through skin transplantation. Thus, the development of lymphatic capillaries in the skin of body extremities was more severely impacted by a mutant copy of VEGFR3 than trunk skin, but lymphatic transport function was markedly reduced throughout the skin, demonstrating that even under conditions when a marked loss in lymphatic capillary density reduces lymph transport, DC migration from skin to LNs remains normal. The Journal of Immunology, 2013, 190: 4608–4620.

Lympathic vessels mediate clearance of macromolecules and immune cells, such as Ag-transporting dendritic cells (DCs), from peripheral tissues (1–3). Absorptive initial lymphatic capillaries, consisting of a single layer of endothelial cells that form blind-ended termini, are present in most organs (2). These capillaries transition into collecting lymphatic vessels (2) characterized by valves and specialized muscle cells (3). Collecting vessels contact the subcapsular sinus of the regional lymph node (LN) and subsequently drain into efferent vessels and eventually to the thoracic duct, where lymph is returned to venous blood. Many questions remain unaddressed or unanswered in the nascent field of lymphatic biology. Lymphedematous diseases typically target skin and stem from impaired lymphatic transport (4). However, relatively little analysis has investigated immunological alterations in lymphedema patients, including whether immune cell transport to LNs is severely decreased, as might be expected. Addressing this issue will promote a better understanding of the array of defects that occur in these diseases, including lymphedema associated with breast cancer therapy or filariasis where maintaining immune defense is critical.

DCs enter the lymphatic vasculature through lymphatic capillaries (5–9), gaining access through “button-like” junctions found in initial lymphatic capillaries (10). DCs preferentially seek out areas associated with breast cancer therapy or filariasis where maintaining immune defense is critical.

It is widely assumed that impaired lymphatic transport of macromolecules would be paralleled by impaired immune cell trafficking (13). In this report, we studied DC migration from skin to LNs in a mouse model (Chy mice) bearing an inactivating mutation in the tyrosine kinase domain of vascular endothelial growth factor receptor 3 (VEGFR3), which is mutated in the form of primary lymphedema called Milroy’s disease (14). Chy mice have a loss of lymphatic transport from skin, reportedly because of a devoid lymphatic capillary network in the skin (15). We illustrate in this study that body extremities in Chy mice are indeed devoid of lymphatic capillaries, but body trunk skin retains lymphatic capillaries at ∼10% normal density. This residual density was insufficient to sustain normal lymphatic transport of macromolecules, but it was remarkably sufficient to permit normal DC migration from skin to LNs.
Areas of skin without lymphatic capillaries supported no DC trafficking as expected. Thus, it appears that the lymphatic capillary density needed to sustain normal DC migration to LNs is much lower than the density needed to maintain normal molecular transport.

Materials and Methods

Mice

Male Chy (heterozygote) mice on a mixed C3H background, obtained from the Medical Research Council Mammalian Genetics Unit Embryo Bank (Harwell, U.K.), were crossed with wild-type (WT) littermates to obtain heterozygote Chy offspring. Chy mice were also crossed 10 times with C57BL/6J mice (The Jackson Laboratory). Experiments were performed in 6- to 12-wk-old Chy mice on both backgrounds, and no differences were observed other than a reduced frequency (~10%) compared with ~50% of mutant offspring in the C57BL/6J colony. Differences between sexes were also not observed. The Chy mutation was identified by PCR using 5'-GACCTTGTAGTCT-3'/5'-AGGCIAAGTGCGCA-3' primer sequences. CCR7+/− mice were obtained from Jackson Laboratories, mice expressing enhanced (e)GFP under the control of the β-actin promoter were provided by M. Merad (Mount Sinai School of Medicine, New York, NY), and K14-VEGFR3-Ig (16) were provided to M.A.S. by K. Alitalo (University of Helsinki, Helsinki, Finland). Mice were housed in a specific pathogen-free environment and were used in accordance with protocols approved by animal welfare oversight committees at Mount Sinai, Ecole Polytechnique Fédérale de Lausanne, or Washington University.

FITC painting assay

Epikutaneous application of FITC to study DC migration was performed on the ears and on two areas of each side of the mouse back skin as described previously (17). Briefly, FITC (5 mg/ml) was dissolved in acetone and dibutyl phthalate (Sigma-Aldrich) and applied in 25-μl aliquots. Recovered LNs were teased and digested in 2.68 mg/ml collagenase D (Roche) for 25 min at 37°C. Then, 100 μl 100 mM EDTA was added for 5 min, and cells were passed through a 100-μm cell strainer, washed, counted, and stained for flow cytometry.

Adoptive transfer of DCs

WT or CCR7+/− bone marrow–derived DCs, generated by culture in GM-CSF and IL-3 (18), were pulsed overnight with green or red fluorescent polystyrene beads described above and were injected i.d. into both ears or front footpads or the shaved back skin or cheek skin overlying the region of the brachial, inguinal, or auricular LN of WT or Chy mice. Three days later, left and right brachial, inguinal, or auricular LNs were respectively pooled from each injected mouse and analyzed by flow cytometry (19).

Lymphatic clearance of dextran

A total of 5 μl 1% 70-kDa TRITC-conjugated, lysine-fixable dextran (Molecular Probes) was injected i.d. into ear and back dermis. 5 min after injection, auricular and brachial LNs were excised and snap-frozen in OCT (Tissue-Tek). To quantify lymphatic transport of dextran, we developed a simple clearance assay and performed it on mice to remove hair from ears and back skin 1 d earlier. A total of 1 μl Cy5–dextran (5000 kDa; Naugatuck) at a concentration of 2 mg/ml in sterile PBS was injected via a Hamilton syringe i.d. into the ear, or 5 μl of the same tracer at 0.2 mg/ml was injected into back skin. Fluorescence was observed through skin using a fluorescence stereomicroscope (M205FA Leica), and images of the skin were acquired each minute for 15 min using constant exposure time. Fluorescence intensity and exposure times were adjusted to ensure that intensity values were linearly proportional to the actual fluorescence. Images were processed using ImageJ and Fiji software, and the rate of clearance was determined by first calculating the area under the curve of fluorescence intensity in the injection region at each time point, normalized to the initial value. The normalized rate of fluorescence decay was then calculated from the slope of area under the curve versus time. This was considered proportional to the actual rate of Cy5–dextran clearance. Left and right assessments were made at each site (ear or back) in each mouse, and the two normalized values were averaged to generate one mean value per mouse per site.

Flow cytometry

Anti-CD11b, anti-CD11c, anti-CD8a, anti-Ly6G, and anti–I-Ab/E–M Abs were from BD Biosciences. Conjugated isotype-matched control mAbs were obtained from eBioscience or BD Biosciences. Intracellular staining for CD207 was performed after cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4°C and then washed in Perm/Wash buffer (BD Biosciences) and incubated for 20 min at 4°C with control goat IgG (R&D Systems) or goat anti-lyangerin Ab (Santa Cruz Biotechnology) diluted 1:400 in the Perm/Wash buffer. Cells were washed twice in Perm/Wash buffer and incubated with 1:300 anti-goat FITC Ab (Invitrogen) diluted in Perm/Wash buffer for 20 min at 4°C.

Immunohistochemistry

Ten-micron cross-sections of WT/Chy back skin and LNs were fixed in 4% PFA for 10 min, stained with primary Abs for 30 min and secondary Ab for 30 min, or sections were stained with Hema-3 solution (Fisher). For whole-mount staining, ears were dissected, hair was chemically removed, and the ear was split into dorsal and ventral sheets. Excess fat and cartilage was removed under a dissection microscope, and the tissue was incubated in 0.5 mM ammonium thiocyanate in H2O for 20 min before the epidermis was peeled off. The ears were then fixed in 100% acetone for 5 min, followed by 80% methanol for 5 min before blocking. For the GFP transplant experiments, ears were fixed in 1% PFA/20% sucrose (Fisher) for 1 h to preserve GFP signal. Both ears and 60-μm-en face frozen sections of back skin were blocked overnight and incubated with primary Ab overnight, and then, secondary Ab was added for 2 h. Primary Abs were anti-mouse podoplanin (Angiobio), goat anti-CCL21 (R&D Systems), rabbit anti–lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (Abcam), anti-smooth muscle actin (Sigma-Aldrich), rabbit anti-collagen IV (Abcam), rat anti-V-E-cadherin (BD Biosciences), anti-lyangerin, anti-CD3, and anti–β230 (BD Biosciences). These were detected with aminomethylcoumarin acetate, Cy2-, Cy3-, or Cy5-conjugated secondary Abs (Jackson Immunoresearch). Tissue was imaged by confocal microscopy using a Leica SP5 DM, and three-dimensional reconstructions and iso-surfaces were rendered using Volocity software.

Quantification of lymphatic capillary density and CCL21 intensity

Thick sections of frozen back skin overlaid the brachial LN were stained as above with anti–LYVE-1 and anti-CCL21. Using Volocity software, CCL21+ tissue maps were generated based on signal intensity, and the total area of CCL21+ vessel coverage was quantified. Ten regions were imaged per animal, and the mean of these readings was taken as the value for one animal and eight animals per group. For local density, only the mean of regions of Chy mice that contained any lymphatic capillaries were included and compared with WT mice where all regions contained vessels. CCL21 intensity was measured using ImageJ (National Institutes of Health, Bethesda, MD) and reported as mean normalized fluorescence intensity of CCL21+ lymphatic endothelial cells. Measurements were taken from three mice per group.

Inflammation and immunization

To induce inflammation, 1 μl emulsified CFA (diluted 1:1 in PBS; Sigma-Aldrich) was injected into the right ear dermis of WT and Chy mice using a Hamilton syringe. In the left ear of the same animals, 1 μl PBS was injected as a control. Ear thickness was measured using Absolute Digital im Pacta calipers (Mitutoyo). For immunization, 4 μg OVA mixed with 25 μg Ultrapure 0111:B4 LPS (InvivoGen, Nunningen, Switzerland) or 50 μg OVA in emulsified CFA (diluted 1:1) was injected into the back skin dermis of Chy and littermate WT mice. Fourteen to 21 d later, serum was harvested and assessed for total anti-OVA IgG Ab by ELISA as described previously (20). Fold increase in reactivity (OD) was normalized by dividing the OD value from various dilutions of plasma from the immunized mouse by the OD reading in the nonimmunized state.

Skin digestion for flow cytometry

Four hours after injection of emulsified CFA into the ear or back skin dermis, the skin was excised and cut into small segments using scissors. This was then incubated in digestion solution (Liberase [1.75 mg/ml] and 2% FCS in RPMI 1640 medium) for 25 min at 37°C.
Skin transplantation model

Mice were anesthetized, and the ear skin was cleaned. In the donor animal, a 0.25-cm incision was made in the dorsal side of the ear, and a small region of skin was removed. After an identical area of ear skin was removed from the recipient mice, the donor skin was sutured, using 9-0 polyamide monofilament sutures, onto the recipient ear. This was conducted on both ears of the same animal. As a control for the procedure, a "sham surgery" group of mice had an incision made on both ears. After a period of 14 d, 1-μm microspheres were injected into the ear dermis adjacent to the transplant, and the mice were sacrificed 3 d later. To assess lymphatic capillary density, five regions (one within the transplant site) of the ear were imaged per animal and the mean of these readings was taken as the value for one animal.

Statistical analysis

Statistical comparisons were made by using the Student t test. One-way ANOVAs were used for multiple comparisons, with a Tukey posttest. The nonparametric Kruskal–Wallis test was used to analyze the aggregate data in the humoral immune response, and a two-way ANOVA was used for repeated measures in the footpad swelling experiments. All experiments contained three or more replicate mice per experimental parameter.

Results

Normal proportions of lymph-trafficng DCs in some LNs of Chy mice

Skin-draining LNs, particularly the inguinal LN, of Chy mice exhibited reduced cellularity compared with WT littermates (Fig. 1A), and the popliteal LN, draining the footpad, which had the most overt lymphedema (15), could not be found in most Chy mice. CD11c+LN DC subsets can be broadly divided into "resident" and "lymph-migratory" DCs based on the expression of higher levels of MHC class II (MHC-II) on the latter (21). LN-resident DC subsets arrive to LNs through high endothelial venules (HEVs) (22) rather than lymphatics (23). Such resident DCs were present in normal proportions in all Chy LNs examined, including the auricular (which drains the ear), brachial (which drains the upper back skin and front paws), and inguinal (which drains the lower back and belly skin) LNs of Chy mice (data not shown). Unexpectedly, the proportions and numbers of lymph-migratory MHC-II+ DCs were also similar between WT and Chy mice in the brachial and inguinal LNs draining the trunk skin (Fig. 1B; data not shown), but their frequency was reduced in the ear-draining auricular LN (Fig. 1B). As a distinct approach to identifying lymph migratory DCs, we also analyzed LNs for the frequency of CD207 (Langerin)+ DCs. CD207 is expressed only identifying lymph migratory DCs, we also analyzed LNs for the frequency of CD207 (Langerin)+ DCs. CD207 is expressed only frequencies of CD207+ DCs. CD207+ DCs were present in normal proportions in all T cell zones of skin-draining LNs (Fig. 1C). As for MHC-II+ DCs, proportions and numbers of CD207+ DCs were reduced in the auricular LN of Chy mice relative to WT littermates but were similar in the brachial and inguinal LNs (Fig. 1B, 1D, 1E; data not shown). However, CD207 can be expressed by CD8α+ DCs (27), which may be LN-resident DCs, and thus, we assessed CD8α expression by this population. In this study, we found that all CD207+ DCs expressed CD8α in both genotypes (Fig. 1F); however, the majority of CD8α+ DCs (60–80%) failed to express CD207 (data not shown). Furthermore, because DCs constitute a minor proportion of the LN cellularity, we examined whether the reduced cellularity of Chy LNs reflected a reduction in numbers of other cell types. Thus, we assessed the total number of B and T lymphocytes in skin-draining LNs in Chy mice, shown in Table I. In this study, we found that the numbers of all subsets of lymphocytes, including B cells and CD4+ and CD8+ T cells, were reduced in Chy mice. These results starkly contrasted with findings that MHC-I+ and CD207+ lymph-migratory DCs are completely absent from all skin-draining LNs in K14-VEGFR3-Ig mice (Fig. 1G) (20), which, like Chy mice, have been reported to lack lymphatic capillaries in skin and exhibit reduced LN cellularity (16). Thus, against our expectations, lymph-migratory DCs were not absent in any Chy LNs and were capable of normally populating several Chy LNs.

No kinetic delay in DC migration to LNs in Chy mice

Although lymph migratory DC frequency in Chy LNs was normal in resting LNs draining the back skin, it remained possible that migration of DCs to Chy LNs was slower than under normal conditions. Thus, we set out to quantify DC migration from skin to draining LNs and assess its kinetics in Chy mice. We used the established “FITC painting” assay that allows for robust assessment of DC migration with known kinetics. Peak accumulation of FITC+ DCs in LNs occurs at 18 h, with DC arrival just getting under way at 12 h (17, 28). At 18 h after painting the back skin and ear, accumulation of FITC+ DCs was nearly abrogated in the ear-draining auricular LNs of Chy mice, but surprisingly, similar proportions of DCs appeared in brachial and inguinal LNs of Chy and WT mice (Fig. 2A, 2B). The total number of migrated DCs in the inguinal, but not brachial, LN was reduced in Chy mice (data not shown), reflecting our findings that several LNs were reduced in overall cellularity (Fig. 1A) and that DC migration to LNs from lymphatics is typically maintained in a manner proportional to LN cellularity (29).

Even at 12 h after FITC application, the time of earliest appearance of FITC+ DCs in LNs, no significant differences existed between Chy and WT mice (Fig. 2C). In a second migration assay, we quantified the mobilization of monocyte-derived DCs from skin to LNs in Chy mice by assessing their transport of i.d. injected, 1-μm fluorescent beads (19, 30). Intradermal 1-μm beads do not freely flow to the LN but are carried there by CD11c+CD11b+MHC-II+ DCs (19). Three days after bead injection i.d., bead+ DCs were absent in the auricular LN of Chy mice, but similar proportions and numbers of bead+ DCs were found in brachial and inguinal LNs of Chy and WT mice (Fig. 2D, 2E; data not shown), as seen after FITC skin painting. Thus, DC mobilization to draining LNs appeared to be normal in Chy mice in a highly region-dependent manner.

Surprised to detect migration of DCs to trunk skin-draining LNs, we explored whether DC migration in Chy mice involved CCR7, the master regulator of DC migration to and within WT LNs (21, 31, 32). We thus competed CD45.1 WT and CD45.2+CCR7+– splenic CD11b+ cells, labeled with Cell Tracker Orange, after they were cotransferred i.d. into the back skin of CD45.2+ WT or CD45.2+ Chy mice. Gating on Cell Tracker Orange+ transferred cells in the brachial LN revealed that donor cells were exclusively derived from WT mice, demonstrating DC mobilization in Chy mice is CCR7 dependent (Fig. 2F, 2G). To confirm this result, we labeled WT and CCR7–/– DCs with respectively different colored beads, injected them s.c., and assessed their migration. Although migration from the s.c. injection site up toward the dermis occurred independently of CCR7 in both genotypes (Fig. 2H), only WT DCs progressed to the LN (Fig. 2I). Thus, using three different assays that examined endogenous and adoptively transferred DCs, we conclude that DC migration is kinetically normal and dependent on CCR7 from the back skin of Chy mice. From the ear, however, DC migration is abrogated.

Regionalized lymphatic defects dictate DC mobilization

Given that some lymph-migratory DCs still populated steady-state auricular LNs of Chy mice (Fig. 1C–E), but migration of DCs from the ear that drains to this LN was ablated (Fig. 2B, 2E), the origin of these DCs in the auricular LN was unclear. Thus, we addressed whether the auricular LN drained other sites that may have intact lymphatic transit of DCs. To this end, we compared endogenous DC migration to the auricular LN from the ear and
cheek skin sites simultaneously using green and red beads, respectively, deposited i.d. in the ear or cheek. We found both ear and cheek bead-transporting DCs in WT littermate auricular LNs but only cheek-derived bead + DCs in the auricular LNs of Chy mice (Fig. 3A, 3B). We then carried out a similar approach to examine DC migration from the forepaw extremities. In this study, we injected the two differently colored beads, respectively, in the back skin or front footpad and assessed the brachial LN for migratory bead + DCs. Bead + DCs derived from both peripheral sites were observed in WT LNs; however, only DCs derived from the back skin and not the front footpad were observed in Chy mouse brachial LNs (Fig. 3C, 3D). Thus, profound defects in DC migration in Chy mice are confined to regionalized areas, notably the body’s extremities, limbs, and ears, but migration remains intact when DCs originate from the body trunk and facial skin.

Rare lymphatic capillaries exist in the back skin dermis of Chy mice

We next analyzed distinct anatomical regions for lymphatic capillaries by microscopy. Because macrophages can also express LYVE-1,
tissues stained for LYVE-1 (Fig. 4A, 4B) were costained with podoplanin, CCL21, and/or Prox-1 to identify lymphatic capillaries (Fig. 4C, 4D; data not shown). Indeed, LYVE-1+ lymphatic capillaries were completely absent in the ear dermis (Fig. 4A), as reported previously (15). LYVE-1+ lymphatic capillaries were also not seen in the back skin dermis of Chy mice by cross-section (Fig. 4B), where the subadipose layer was markedly thinned (Supplemental Fig. 1A). However, all Chy skin-draining LNs examined contained LYVE-1+ terminal lymphatic capillaries (Supplemental Fig. 1B) (1).

In thick en face sheets of back skin or whole-mount preparations of ear dermis that allowed a larger region to be analyzed, uniform lymphatic capillary networks were observed in WT mice (Fig. 4C, left panel). Most regions of back skin in Chy mice were devoid of lymphatic capillaries (Fig. 4C, middle panel) and in the ear entirely, but sparse clusters were present in back skin (Fig. 4C, right panel). These vessels still expressed the CCR7 ligand CCL21 (Fig. 4D) as in WT mice (12). Quantification of lymphatic capillary density revealed a ~90% reduction in density in Chy dermis (Fig. 4E). Even in scattered clusters of lymphatic vessels, the local density was reduced by ~60% compared with WT (Fig. 4F). CCL21 intensity was modestly elevated in Chy mice (Fig. 4G). Thus, regions of skin in Chy mice that support migration of skin DCs contain very rare networks of lymphatic capillaries, whereas the regions of skin containing no lymphatic capillaries allow no transit of DCs from skin. We also examined lymphatic precollecting/collecting vessels, which are characterized by smooth muscle coverage and little or no LYVE-1 expression (33). By whole-mount imaging, we observed podoplanin−/−LYVE-1− smooth muscle actin (SMA)+ collecting vessels in the ear and back skin dermis of both WT and Chy mice (Fig. 4H, 4I). These collectors were less frequent in the ear and back skin dermis of Chy mice and most appeared blind-ended without connection to LYVE-1+ capillaries (data not shown).

Molecular transport from back skin lymphatic vessels of Chy mice is impaired

The observation that DC migration was normal under conditions of severe lymphatic hypoplasia led us to wonder whether the rare lymphatic vessels in Chy mice had developed compensatory mechanisms to restore transport function in general. However, button-like junctions and portals appeared normal (Fig. 5A–D), suggesting no major compensatory changes at this level. Qualitatively, soluble TRITC–dextran transport from the ear was ablated in Chy mice but still occurred from the back skin (Fig. 5E, 5F). To quantify transport of macromolecules from the skin, we injected Cy5–dextran into ear or back skin and monitored its clearance over time at the same sites from which we earlier injected DCs. The median normalized rate of clearance was more profoundly impaired from the ear than from the back skin (Fig. 5G), but transport from the back skin was nonetheless less than half the rate observed in WT littermates (Fig. 5G). Thus, Chy lymphatic vessels do not develop a fully sufficient compensatory mechanism at the level of macromolecular transport to overcome severe lymphatic hypoplasia.

Classical inflammation drives limited lymphangiogenesis but fails to rescue DC mobilization in Chy mice

Because excess levels of vascular endothelial growth factor (VEGF)-C can rescue the loss of lymphatic capillaries in the ear of Chy mice (15), and VEGF-C levels are increased during inflammation (34, 35), we assessed whether a classical inflammatory stimulus could induce lymphangiogenesis and DC migration in mutant mice by injection of a small volume of CFA into the ear dermis. CFA is upstream of lymphangiogenesis around LNs and enhances DC migration (29, 36). We first assessed whether inflammatory cell recruitment to inflamed ear or back skin occurred normally in Chy mice and found that, 4 h after CFA injection, neutrophils could be found in similar proportions and numbers to WT mice in both sites (Fig. 6A, 6B, Supplemental 2A, 2B). Neutrophils can also enter afferent lymph and arrive in LNs rapidly (37), although other neutrophils may arrive through HEVs. When we assessed the accumulation of neutrophils in the brachial and auricular LNs at 4 h, the frequency of neutrophils in both skin-draining LNs was similar to WT mice (Supplemental Fig. 2C–F), suggesting that neutrophil migration in response to an inflammatory stimulus was intact.

Following CFA administration, we observed tissue edema in the ear in both genotypes as expected. However, this inflammation was notably increased and resolved more slowly in Chy mice (Fig. 6C). Moreover, the expected lymphadenopathy that occurs in WT mice treated with CFA (29) failed to occur in draining auricular LNs of Chy mice (Fig. 6D), and this was similar in the brachial LN after CFA injection in the back skin (data not shown). Lymphatic density was increased in the ears of WT mice treated with CFA, but this failed to reach significance (Fig. 6E). Despite the strong inflammatory response, there was a significant but minor increase in lymphatic density in Chy mutant mice, with some isolated vessels present in the ears of some mice (Fig. 6E; data not shown). However, DC mobilization from the ear remained abrogated in Chy mice 15 d after CFA administration (Fig. 6F, 6G). Thus, induction of general inflammation by CFA injection is not sufficient to promote restorative lymphangiogenesis in regions of Chy mouse skin that are devoid of lymphatics.

Humoral immunity is diminished in K14-VEGF3-1g mice following dermal immunization at sites where DC migration is entirely abolished (20). When Chy and WT littermates were immunized with OVA mixed with LPS or CFA in the back skin dermis or the footpad and assessed for anti-OVA IgG Abs in the serum 14–21 d later, footpad immunizations where there are no lymphatic vessels led to abrogated induction of anti-OVA IgG
FIGURE 2. No kinetic delay in DC migration to LNs in Chy mice. (A) Representative dot plots of FITC+CD11c+ cells in skin-draining LNs of WT and Chy mice 18 h after FITC application to the dorsal ear and upper and lower back skin. (B) Percentage of CD11c+FITC+ cells in LNs of WT and Chy mice. \( n = 5-7 \). (C) Percentage of CD11c+FITC+ cells in brachial LNs of WT and Chy mice 12 h after FITC application to upper back skin. (D) Representative dot plots of MHC-II+bead+ cells in skin-draining LNs of WT and Chy mice 3 d after i.d. injection of 1 \( \mu m \) green fluorescent beads in the ear and back skin. (E) Percentage of green bead+ cells in LNs of WT and Chy mice. \( s, WT \) mice; \( d, Chy \) mice. \( n = 4-7 \). (F) Spleen CD11b+ cells from CD45.1 WT and CD45.2 WT (left panel) or CD45.2 CCR7\(^{+/-} \) (middle and right panels) mice were labeled with Cell Tracker Orange and mixed at 1:1 ratio and then injected into the upper back skin dermis of WT/Chy CD45.2+ mice. At 18 h, CD45.1 and CD45.2 expression was examined in brachial LNs on total Cell Tracker Orange+ cells (F). (G) Results are shown as the mean percentage ± SD of Cell Tracker Orange+ cells that were CD45.1+ versus CD45.2+ in WT and Chy brachial LNs. (H) WT (red) and CCR7\(^{-/-} \) (green) bone marrow-derived DCs were pulsed overnight with 1-\( \mu m \) beads, stimulated with (Figure legend continues)
DC mobilization from regions with no previous drainage can be rescued by skin transplantation

Finally, we assessed whether we could rescue DC mobilization from the most severely affected regions by directly introducing a limited number of lymphatic capillaries. Specifically, we attempted to introduce lymphatic capillaries by transplantation of a small region of WT (or Chy as a negative control) ear skin onto the ear of Chy mice (Fig. 7A). We waited 14 d and then assessed lymphatic density and DC mobilization. Lymphangiogenesis was induced in the ear even in regions beyond the transplant area (regions 2–5 in Figure 7A, 7B) and to a limited degree in a proportion of Chy recipients that received Chy mutant donor skin (Fig. 7C; data not shown). However, the density of lymphatic capillaries in transplant recipients of WT donor skin was still less than half the density observed in unmanipulated WT mice (Fig. 7C). The superior lymphangiogenesis in Chy mice transplanted with WT skin indicated that the donor vessels expand rather than the surgery driving endogenous lymphatic expansion. However, to assess this directly, we transplanted dorsal ear skin from β-actin–eGFP mice onto Chy recipients and assessed the lymphatic network for host versus donor origin 2 wk later. In this study, we found that the majority (61%) of the LYVE-1+ capillaries, both within and outside the transplanted region, were of donor origin (Fig. 7D, 7G; data not shown). However, we could observe a lower number (24%) of recipient eGFP+LYVE-1+ vessels (Fig. 7E, 7G) and some vessels that appeared to be derived from both donor and recipient endothelium (15%) (Fig. 7F, 7G). However, because the donor is not a lymphatic-specific reporter, it remains possible that the few GFP+ cells in Fig. 7F are not lymphatic endothelial cells.

By i.d. injecting beads adjacent to the transplanted region, we assessed DC migration following skin transplantation or a sham operation (Fig. 7H–J). WT skin transplanted onto Chy recipients increased migration significantly over mice transplanted with mutant skin and sham-operated mice (Fig. 7I, 7J), and indeed, when we compared transplant or sham transplant data to the migration observed in Fig. 2, we found that transplant of WT onto Chy mice restored migration significantly enough that the difference to unmanipulated WT mice was no longer observable. However, migration in the sham-operated mice or recipients of Chy donor skin remained significantly reduced relative to that in WT mice. Although even transplantation of Chy skin onto Chy mice appeared to restore migration and induce lymphangiogenesis in some mice, WT skin used in the transplant was more often effective. Indeed, if we defined restoration of migration as migration that was quantitatively within 1 SD of the mean DC migration in unmanipulated WT mice, WT skin transplanted onto Chy mice rescued migration robustly in 64.7% of the recipients, whereas Chy skin transplanted onto Chy mouse ears did so only 21.4% of the time, and sham was minimal at 9.1% (data not shown). To ensure that the VEGFR3 mutation had no effects other than on the formation of initial lymphatic capillaries, we transplanted Chy skin onto WT recipients. In this study, we found that both lymphatic density and DC mobilization were comparable to unmanipulated WT mice (data not shown). Taken together, these data indicate that transplantation effectively restores DC migration.

Discussion

As anticipated, our data suggest that the presence of lymphatic capillaries in skin is essential for skin DC mobilization to LNs. However, contrary to expectations, even rare, sparsely localized lymphatic capillaries can be sufficient to support DC mobilization without obvious kinetic delay or reduction in magnitude. Steady-state migration of lymph-trafficking MHC-II+ or CD207+ DCs from the back skin, but not the ear, in Chy mice was similar to WT mice. However, CD207+ can be expressed by CD8α+ DCs (27), which may be LN-resident DCs seeding the LN through HEVs. We found that all CD207+ DCs expressed CD8α+ in both genotypes (Fig. 1F); however, the majority of CD8α+ DCs (60–80%) failed to express CD207. These data suggest that CD8α+CD207− DCs are the LN-resident DCs that enter LNs as precursors through HEVs (22), whereas CD207+CD8α+ DCs likely correspond with lymph-migratory DCs. Indeed, CD8α expression by lymph-migratory
DCs recently has been demonstrated in the mesenteric afferent lymphatics (38). Furthermore, migration of DCs after contact sensitization and migration of monocyte-derived DCs was also identical to WT mice from the back skin.

The major difference between skin of the extremities versus that of the body trunk was the presence of rare lymphatic capillaries in the trunk skin compared with their complete absence in extremities such as the ear. There were no obvious differences in button-like
junctions (Fig. 5A, 5B) or portals (Fig. 5C, 5D) of residual lymphatic capillaries in Chy mice, but these vessels displayed slightly more CCL21. Although this increase may augment DC migration in the face of lymphatic hypoplasia, the increase may be too modest to be considered a compensatory change. Furthermore, it is unlikely that prelymphatic fluid channels (2) evolved to greater efficiency in Chy mice, because such a modification would be expected to translate into improved lymphatic transport of macromolecules. Instead, we found that clearance of soluble lymphatic tracers was markedly reduced in Chy mice, arguing against compensatory improvements in processes associated with both molecular and cellular transport. Thus, based on the present findings, we propose that lymphatic capillaries in WT mouse skin evolved to the density they did to allow for optimal molecular transport and that the threshold of lymphatic density needed to support the migration of actively motile DCs, and perhaps other immune cells, is far lower. This conclusion, although unexpected, now seems logical given that macromolecular transport through lymphatic vessels occurs on a very different time scale (minutes) to cellular transport (hours). That the rate of DC migration is

FIGURE 5. Molecular transport from back skin lymphatic vessels of Chy mice is impaired. (A and B) Images show staining with LYVE-1 (green) and VE-cadherin (red) on lymphatic capillaries in the back skin dermis of WT and Chy mice. Scale bar, 24 μm. (B) Enlargement of inset in (A) showing that the structural pattern is highlighted more clearly after isosurface rendering of the image. Scale bar, 5.2 μm (left panel) and 5.08 μm (right panel). (C) Images show staining of back skin lymphatic capillaries with collagen IV (white) and CCL21 (red) in WT and Chy mice. Scale bar, 24 μm. (D) Enlargement of inset in (C). Yellow arrowheads indicate lymphatic portals. Scale bar, 11.1 μm (left panel) and 12 μm (right panel). (E and F) Sections of auricular (E) and brachial (F) LNs 5 min after injection of 50 μg TRITC–dextran (red) into the ear and back i.d. Counterstained with DAPI (blue). Scale bar, 60 μm. (G) Lymphatic transport measured in the ear and back skin. Each symbol represents pooled left and right data from a single mouse, and the bar shows the median normalized rate of fluorescence decay. Measurements are from one to four mice per group in two independent experiments. **p < 0.01, assessed using a one-tailed Student t test.
regulated by other features of lymphatic vessels such as adhesion of DCs within the lumen of lymphatic capillaries (12) is consistent with the concept that DC migration is rate-limited by different processes than molecular transport.

Chy LNs draining the skin exhibited a reduced cellularity in the face of lymphatic hypoplasia. That the popliteal LN, which drains only the rear footpad and not the body trunk where lymphatics are at least partly intact (39), is actually absent in Chy mice suggests that functional lymphatics are needed for a LN to form properly or be sustained. Although LN anlagen may be Prox-1 and therefore lymphatic vessel independent (40), maintenance of LN integrity may require lymph flow. Indeed, this possibility is consistent with reduced LN size when afferent lymphatics are surgically severed (41), which also results in altered HEV maturation and T–B cell compartmentalization. Furthermore, DCs, including those from the lymph, coordinate LN T cell homeostasis at least in part through enhancing HEV formation (42–44). However, critical elements derived from lymph likely include more than migratory DCs because skin-draining LNs still form in K14-VEGFR3-Ig mice that lack lymph-migrating DCs (Fig. 1G) (20).

Indeed, we observed that the typical lymphadenopathy associated with immunization failed in Chy mice. Although DCs have been linked to lymphadenopathy, our data suggest that intact DC migration may not be sufficient to coordinate lymphadenopathy in Chy mice. Earlier work from our laboratory revealed that lymphangiogenesis was associated with and required for lymphadenopathy (29). Thus, the failure of Chy mice to exhibit lymphadenopathy may be due to attenuated signaling of VEGFR3 by lymphatic endothelial cells. In the face of smaller baseline LNs in the Chy mouse, and their inability to exhibit lymphadenopathy in response to immunization, we find normal humoral immune responses at sites where DC migration remains proportional to overall LN cellularity, a result that is distinct from the impaired humoral responses observed in mice devoid of lymphatic capillaries in skin and devoid of lymph-trafficking DCs. This finding, in our view, highlights the greater importance of LN cellular composition, such as balanced ratios between lymph-homing and resident DCs and lymphocytes, than overall cellularity in driving an effective immune response.

Chy mice carry a dominant-negative mutation in one allele. They serve as a useful tool to examine the VEGFR3 axis in lymphatic...
biology, because mutations in Milroy patients with primary lymphedema are found in the vegfr3 gene (15). The regionalized lymphatic defects in Chy mice are reminiscent of primary lymphedema patients, where body extremities, particularly lower extremities, are typically most affected (45, 46). The regional differences in lymphatic capillaries of the Chy mouse also adds a layer of complexity to the role of VEGFR3 in lymphatic development and highlights differences in anatomic regions even within the skin. Chy-3 mice, which are haploinsufficient for vegfc, also exhibit a regionalized form of lymphedema primarily affecting the lower limbs (47). VEGFR3 signaling is thought to be modulated through the coreceptor neuropilin (NP)-2 in the control of anatomic differences in lymphatic hypoplasia (15). NP-2, which binds VEGF-C and VEGF-D and is internalized with VEGFR3 upon ligand stimulation (48), is expressed by intestinal but not cutaneous lymphatic vessels (15). NP-1 was recently shown to regulate the recycling of VEGFR2 (49). Because haploinsufficient Chy mice are not completely devoid of VEGFR3 signaling, regional differences in receptor recycling and other pathways that affect signaling output could be crucial to the development of residual, functional lymphatic capillaries.

Through skin transplantation, we demonstrated that the introduction of a limited number of lymphatic capillaries restores DC mobilization, suggesting this approach may prove to have therapeutic value. Indeed, some microsurgery techniques have shown success in lymphedema patients (50, 51) as well as in experimental

**FIGURE 7.** DC mobilization from regions with no previous drainage can be rescued by skin transplantation. (A) Representative photomicrograph after WT (or Chy) dorsal ear skin was transplanted onto the dorsal ear dermis of Chy recipient mice. Five regions (as indicated) were examined for the presence of LYVE-1 lymphatic capillaries. (B) Representative LYVE-1 (green) and SMA (red) staining of ear dermis within (left panel, region 1) and outside (right panel, region 4) the transplanted region from Chy mice transplanted with WT donor skin. Scale bar, 170 μm. (C) Lymphangiogenesis was assessed 17 d after surgery using Velocity software, and the area of ear dermis covered by LYVE-1+ vessels was measured in unmanipulated WT and Chy mice, Chy recipients of WT and Chy donor skin, and mice undergoing sham surgery. n = 3–19. (D–F) Representative images of ear dermis of Chy recipients of β-actin–eGFP donor skin 2 wk posttransplant. Images show LYVE-1 (red, left panel), eGFP signal (green, middle panel), and the colors merged (right panel). Note the presence of LYVE-1+ vessels, which are GFP+ (D), GFP− (E) and a mix of the two (F). Scale bar, 28 μm (D), 80 μm (E), 37 μm (F). (G) LYVE-1+ vessels present in the recipient ear dermis were assessed for GFP signal and quantified, and the percentage of vessels in each group is shown. (H) Representative dot plot of MHC-II+ green bead+ cells in the auricular LN of Chy recipients of WT donor skin 3 d after i.d. injection of 1-μm green fluorescent beads into the ear. Beads were injected 14 d posttransplant. (I and J) Results shown are the proportion (I) and total number (J) of green bead+ cells present in the auricular LN of transplant recipients or mice undergoing sham surgery. Each circle represents one individual transplant and two transplants per mouse were performed. Results are representative of four independent experiments. n = 11–17. *p < 0.05, **p < 0.01.
models of skin transplantation to areas of secondary lymphedema (52). In addition to restoration of DC migration, we also observed lymphatic capillaries in and adjacent to the transplant in the WT–Chy group and, to a lesser extent, the Chy–Chy group. The lymphangiogenesis was higher in the WT–Chy group compared with the Chy–Chy sham surgery groups, suggesting that, at least to some extent, donor LECs may proliferate and migrate to form new vessels. Indeed, our GFP donor experiments lend credence to this possibility, and the presence of collecting vessels in the ear of Chy mice (Fig. 4H) suggest donor capillaries may anastomose with endogenous collectors. Consistent with the heightened lymphangiogenesis in WT–Chy transplants, most lymphangiogenesis in adults occurs through sprouting from existing vessels (53). The more limited restoration, which occurred in the groups lacking pre-existing lymphatic vessels (Chy transplanted tissue or Chy sham-operated mice), may be due to local increases in VEGF-C as part of the surgery-associated inflammation. It is possible that the appearance of a low number of LYVE-1+ lymphatic capillaries observed after Chy–Chy transplant (Fig. 7C) either results from sprouting, or of anastomosis, with the collecting vessels already present (Fig. 4H), thereby facilitating DC migration in a small number of these recipients.

In summary, we show that the presence of very rare lymphatic capillaries in Chy mouse skin retards molecular transport through lymphatics but allows DCs to mobilize from the skin to LNs through the lymphatic vasculature normally and without delay. Furthermore, these results reveal regional differences in lymphatic capillaries in the Chy mouse, with residual capillaries found on the body trunk while they are absent in the extremities. These data importantly add to our understanding of the requirements for DC transit through the lymphatic vasculature, aid in defining how the immune system is affected by lymphatic hypoplasia, and may have a translational aspect and lend further credence to surgical treatment of patients with peripheral lymphedema.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Material

**Supplementary Figure 1. Back skin histology and intranodal lymphatics in Chy mice.** (A) Representative histologic staining using Hema-3 of cross-sections of the back skin of WT and Chy mice. (B) LYVE-1 staining (green) of auricular LNs from WT and Chy mice. Bar=60μm. Results are representative of 2 independent experiments.

**Supplementary Figure 2. Neutrophil accumulation in the skin and draining lymph nodes during inflammation.** (A-B) Proportions of live CD45+ cells that were CD11bLy6G+ in the ear (A) and back skin (B) 4 hours after injection of PBS (white bars) or CFA (black bars). (C-E) Proportions (C+E) and total numbers (D+F) of CD11b+Ly6G+ cells in the auricular (C+D) and brachial (E+F) LNs 4 hours after injection of PBS (white bars) or CFA (black bars) into the ear or back skin dermis. ns=not significant. Results are representative of 2 independent experiments.