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Lymphatic Specific Disruption in the Fine Structure of Heparan Sulfate Inhibits Dendritic Cell Traffic and Functional T Cell Responses in the Lymph Node

Xin Yin,*,‡ Scott C. Johns,*,‡ Daniel Kim,*,§ Zbigniew Mikulski,*, Catherine L. Salanga,‖ Tracy M. Handel,‖ Mónica Macal,# Elina I. Zúñiga,*, and Mark M. Fuster*,‡

Dendritic cells (DCs) are potent APCs essential for initiating adaptive immunity. Following pathogen exposure, trafficking of DCs to lymph nodes (LNs) through afferent lymphatic vessels constitutes a crucial step in the execution of their functions. The mechanisms regulating this process are poorly understood, although the involvement of certain chemokines in this process has recently been reported. In this study, we demonstrate that genetically altering the fine structure (N-sulfation) of heparan sulfate (HS) specifically in mouse lymphatic endothelium significantly reduces DC trafficking to regional LNs in vivo. Moreover, this alteration had the unique functional consequence of reducing CD8+ T cell proliferative responses in draining LNs in an ovalbumin immunization model. Mechanistic studies suggested that lymphatic endothelial HS regulates multiple steps during DC trafficking, including optimal presentation of chemokines on the surface of DCs, thus acting as a co-receptor that may function “in trans” to mediate chemokine receptor binding. This study not only identifies novel glycans-mediated mechanisms that regulate lymphatic DC trafficking, but it also validates the fine structure of lymphatic vascular-specific HS as a novel molecular target for strategies aiming to modulate DC behavior and/or alter pathologic T cell responses in lymph nodes. The Journal of Immunology, 2014, 192: 000–000.

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; CM, conditioned medium; DC, dendritic cell; hLEC, human lymphatic endothelial cell; HS, heparan sulfate; LEC, lymphatic endothelial cell; LN, lymph node; NdSt, N-deacetylsulfotransferase-1; PL-A, proximity ligation assay; siDS, control small interfering RNA; siRNA, small interfering RNA.

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chain initiation or chain sulfation significantly reduces chemokine-dependent trafficking of tumor cells in the lymphatic system (5). So far, however, our understanding of how lymphatic traffic by DCs is modulated in the lymphatic microenvironment, including mechanisms that control multiple chemokines, remains very limited. For CCL21 in particular, work in our laboratory as well as a study examining the effects of a global vascular disruption of HS chain elongation on multiple forms of cell traffic to the LN, showed that altering HS chains on the lymphatic endothelial cell surface impaired adhesion between CCL21 and lymphatic endothelium (5, 6). Additionally, gradients of CCL21 in the perilymphatic space have been shown to be altered following chemical disruption of HS in the extracellular matrix (7, 8). These observations raise questions as to what a lymphatic vascular-specific genetic disruption in the fine structure of HS might do not only to DC traffic, but also to the ultimate T cell responses in the LN upstream from an antigenic stimulus.

In this study, we hypothesized that disruption of lymphatic HS will inhibit the trafficking of Ag-loaded DCs to regional LNs as well as Ag-dependent T cell activation. We also hypothesized that specific fine structural modifications of lymphatic HS (which can be genetically targeted) may uniquely affect DC trafficking. To test our hypothesis, we examined the significance of genetically altering the sulfation of lymphatic endothelial HS on the trafficking of DCs to regional LNs, and we explored underlying mechanisms. We also assessed the subsequent effects on T cell immunity. Our results reveal novel glycan-specific modifications and mechanisms that regulate lymphatic DC traffic, and they validate the fine structure of lymphatic HS as a potential molecular target for therapeutic approaches to modulate DC behavior and/or correct pathologic immune responses.

Materials and Methods

Cell culture and treatments

Primary human lung LECs (hLECs; Lonza, Basel, Switzerland) were cultured in EBM2 endothelial basal medium supplemented with an EGM2 bullet kit (Lonza). Bone marrow–derived dendritic cells (BMDCs) were isolated from the femurs and tibias of C57BL/6J mice (8–12 wk of age) and differentiated with GM-CSF (20 ng/ml; PeproTech, Rocky Hill, NJ) as previously described (9). Unless otherwise stated, BMDCs at day 9 of differentiation were used for experiments. All small interfering RNA (siRNA) duplexes were from Integrated DNA Technologies (Coraville, IA) and were transfected into cells according to the manufacturer’s instructions. For heparinase treatment, cells were incubated with heparinase (heparin isoyases I, II, and III; 2.5 mU/ml; provided by Dr. Jeffrey D. Esko, University of California San Diego) in serum-free EBM2 medium at 37°C, 5% CO2 for 1 h. To block specific chemokine or chemokine receptor signaling, cells were incubated with Abs against CCL21 (1:100), CXCL12 (1:50), CCCL5 (1:100), CXCR4 (1:100), or CCR7 (1:100; R&D Systems, Minneapolis, MN) at 37°C, 5% CO2 for 1 h (for adhesion and transmigration assays under shear flow), 6 h (for transwell migration assays), or overnight (for transwell invasion assays).

Animals

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California San Diego. Mice between 4 and 8 wk of age were used in this study. Lymphatic-specific Ndst1-prox1CreERT2 (Prox1CreERT2) and their Ndst1+/- conditional mutant mice (kindly provided by Dr. G. Oliver at St. Jude Children’s Research Hospital, Memphis, TN) extensively backcrossed onto the C57BL/6 background with Ndst1+/- conditional mutant mice. To induce the activity of Cre recombinase, tamoxifen (Sigma-Aldrich, St. Louis, MO) dissolved in corn oil was i.p. injected into the mice at 0.12 mg/g body weight daily for 5 consecutive days. Unstimulated tamoxifen-induced mutant versus wild-type littermates (2 littermate pairs) were sacrificed, and their ears were dissected and separated into dorsal and ventral sheets. The sheets were fixed in 1% paraformaldehyde, permeabilized with PBS containing 0.3% Triton X-100 (PT buffer), blocked with PT buffer supplemented with 3% goat serum, and then incubated with rabbit anti-Lyve1 Ab (1:800; Abcam, Cambridge, MA) overnight at 4°C. After three washes in 1% BSA/PT buffer, Cy3-conjugated anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated at room temperature for 2 h. At least five random images were acquired from each sample using the fluorescence microscope, and the Lyve1+ lymphatic vascular area per image was quantified using National Institutes of Health ImageJ software.

Skin painting

FITC (6.6 mg/ml; Sigma-Aldrich) and oxazolone (30 mg/ml; Sigma-Aldrich) were dissolved in 95% ethanol and painted onto the shaved abdomen of mice (200 μl/mouse). After 40 h, inguinal and axillary lymph nodes were isolated and digested with 0.2% type I collagenase at 37°C for 1 h. Digested samples were filtered through a 40-μm cell strainer and then stained with PE-labeled anti-mouse CD11c Ab (eBioscience, San Diego, CA), and PE/FITC double-positive cells were analyzed by FACSCalibur (BD Biosciences, San Jose, CA).

Whole-mount immunofluorescence staining of mouse ear

Skin tissues were mechanically chopped into small pieces and digested in DMEM medium containing 0.8% trypsin and 20 μg/ml DNase I (Sigma-Aldrich) at 37°C for 1 h on a rocking plate. Digested cells were filtered through a 40-μm cell strainer (BD Biosciences), stained with FITC-labeled anti-mouse CD11c Ab as well as PE-labeled anti-mouse MHC class II Ab (eBioscience), and FITC/PE double-positive cells were analyzed on a FACSCalibur.

Trafficing of in situ–implanted BMDCs

BMDCs at day 9 of differentiation in culture were labeled with calcine AM (eBioscience) and injected into the left foot dorsum of Ndst1+/-Prox1CreERT2 mutants and their Ndst1+/-Prox1CreERT2 wild-type littermates (2 × 10^6 cells/mouse). After 40 h, the left popliteal LN was isolated, imaged under the fluorescence microscope, and digested with 0.2% type I collagenase into a single-cell suspension. Cells from each LN digest were resuspended into 100 μl PBS with 1.5 μl spotted onto the well of a Terasaki microtiter plate (Robbins Scientific, Sunnyvale, CA) and imaged under the fluorescence microscope. The number of calcine+ cells was quantified using National Institutes of Health ImageJ software.

In vivo T cell proliferation in draining LNs

OVA (Sigma-Aldrich) was dissolved into PBS at 5 mg/ml and mixed thoroughly with IFA (Sigma-Aldrich; v/v = 1:1) until a uniform oil-in-water emulsion was formed. The emulsion was then injected i.p. into the mice (200 μl/mouse). Seven days later, 40 μl freshly prepared OVA/IFA emulsion was injected intradermally into the footpad. Five days later, the mice were sacrificed, with popliteal and inguinal LNs from the injected side isolated, digested into single-cell suspensions, stained with Alexa Fluor 488–labeled anti-CD3 together with allopurinol-labeled anti-CD4 or allopurinol-labeled anti-CD8 Abs (eBioscience) and analyzed by FACSCalibur.

Adaptive transfer of CFSE-labeled T cells and their proliferation in vivo

CD8+ T cells were purified from spleens of OT-I mice and CD4+ T cells from spleens of OT-II mice (both OT-I and OT-II mice were kindly provided by Dr. Stephen Hedrick, University of California San Diego Division of Biological Sciences) using corresponding Dynabeads Untouched cell isolation kits (Invitrogen, Carlsbad, CA). The purified T cells were labeled with CFSE following an established protocol (10), and a mixture of equal numbers of CFSE-labeled CD4 and CD8 cells (3 × 10^5/mouse) were adoptively transferred into the mice via retro-orbital injection as previously described (11). After 24 h, the mice were injected with a mixture of the OT-I and OT-II respective peptides, OVA323-339 and OVA257-264 (1 mg/ml in normal saline and mixed thoroughly with IFA at v/v = 1:1) into the footpad (40 μl/footpad). After 72 h, mice were sacrificed and the popliteal LN on the injected side was isolated, digested into a single-cell suspension using a sequentially following tamoxifen injections. All mice were maintained in a pathogen-free facility on a 12-h light/dark cycle with food and water provided ad libitum.

Skin painting

FITC (6.6 mg/ml; Sigma-Aldrich) and oxazolone (30 mg/ml; Sigma-Aldrich) were dissolved in 95% ethanol and painted onto the shaved ab...
with Matrigel (1:50 in PBS; BD Biosciences). At ~90% confluence, hLECs were either treated with heparinase, blocking Abs, or transfected with siRNA. For adhesion assays, BMDCs prelabelled with calcine AM following the manufacturer’s instruction were perfused through the insert at a concentration of 2 × 10^5 cells/ml in EBM2 medium under an initial shear stress of 2 dynes/cm^2 for 2 min followed by a constant flow at 0.14 dynes/cm^2 for 15 min. After removing the residual BMDCs from the inlet well, the channel was washed with EBM2 medium at 1 dyne/cm^2 for 5 min to remove any unattached BMDCs. Adherent BMDCs across the whole transwell channel were imaged using a PerkinElmer UltraVIEW VoX spinning disk confocal microscope (×100; University of California San Diego Light Microscopy Facility) and quantified with Metamorph software (Molecular Devices, Sunnyvale, CA). Following the capture of adhesion images at time 0 (t₀), transmigration of adherent BMDCs (defined by disappearance of and for cells visibly from the surface of the hLEC monolayer to the space beneath the monolayer, as opposed to detachment and carriage out of the viewing field under flow) was observed under a constant shear of 0.14 dynes/cm^2 of cell-free EBM2 medium for another 30 min using the same microscope, with the end point image taken at that time (tₚ). The percentage transmigration was calculated as [(no. adherent BMDCs at t₀) – no. adherent BMDCs at tₚ)]/no. adherent BMDCs at t₀ × 100%.

Transwell invasion and chemotaxis migration assays

To assess the mobility of BMDCs toward hLECs in vitro, a modified transwell invasion and chemotaxis assay was performed as previously described (5). For collagen matrix-based invasion assays, 1 × 10^6 hLECs were embedded into 100 μl type I collagen gel containing 3 mg/ml PureCol (Advanced Biomatrix, San Diego, CA) in DMEM (pH 7.3), applied to cover the lower side of a transwell insert (3.0 μm in pore size; Corning), and allowed to solidify in a 37°C, 5% CO₂ incubator for 6 h. The insert was then inverted and placed into a 24-well plate containing pre-warmed serum-free EBM2 (500 μl/well). For chemotaxis assays, 5 × 10^5 hLECs/well were seeded directly into the bottom of a 24-well plate and treated. For both assays, 2–5 × 10^5 BMDCs prelabelled with calcine AM were resuspended in 100 μl EBM2 and loaded on top of the insert. For the invasion assay, the plates were placed in a 37°C, 5% CO₂ incubator overnight and for 6 h in the case of the chemotaxis assays. At the end of the invasion/chemotaxis period, transwells were transferred to a new 24-well plate and treated with either 0.2% type I collagenase (Sigma-Aldrich) for 1 h for collagen matrix-based invasion assay or trypsin (Invitrogen, Grand Island, NY) for 5 min (for chemotaxis assay) with gentle rocking at 37°C. Bottom-well solutions were collected, transferred to clean Eppendorf tubes, and centrifuged at 500 × g for 5 min. Cell pellets were resuspended in 20 μl PBS with 1.5 μl aliquots loaded onto a 96-well microplate containing pre-warmed serum-free EBM2 (500 μl/well) for confocal microscopy (100×; University of California San Diego Light Microscopy Facility) and quantified with MetaMorph software (Molecular Devices, Sunnyvale, CA). The following capture of adhesion images at time 0 (t₀), transmigration of adherent BMDCs (defined by disappearance of and for cells visibly from the surface of the hLEC monolayer to the space beneath the monolayer, as opposed to detachment and carriage out of the viewing field under flow) was observed under a constant shear of 0.14 dynes/cm^2 of cell-free EBM2 medium for another 30 min using the same microscope, with the end point image taken at that time (tₚ). The percentage transmigration was calculated as [(no. adherent BMDCs at t₀) – no. adherent BMDCs at tₚ)]/no. adherent BMDCs at t₀ × 100%.

Reverse transcription followed by quantitative real-time PCR

Total RNA was extracted from cells using an RNAqueous-4PCR kit (Ambion) and reverse transcribed into cDNA with a SuperScript III kit (Invitrogen) according to the manufacturers’ instructions. Real-time PCR was performed with an iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA). The primer sequences (5’ to 3’) used for real-time PCR were as follows: human Ndst1, forward, GGACATCTGGTCTAAG, reverse, GATGCCTTTGTGATAG; human XylT2, forward, ACGTTCAACCGCAAACTACC, reverse, ATTGCTCCTTTGTGATAG; human Prox1, forward, GGACATCTGGTCTAAG, reverse, GATGCCTTTGTGATAG; and human Cre, forward, GGACATCTGGTCTAAG, reverse, GATGCCTTTGTGATAG. The PCR was performed in triplicate with each sample. Statistical analysis

Quantitative data are presented as means ± SD for three replicates or three independent experiments where indicated. Significance between groups was calculated using a two-tailed Student t test. Differences in LN colonization of skin DCs following FITC plus oxazalone painting and that from in situ–implanted BMDCs between mutant and littermate control mice were examined using the rank order test. A p value <0.05 was considered statistically significant.

Results

Tissue-specific inhibition in the sulfation of lymphatic HS impairs in vivo DC trafficking

To test the degree to which the sulfation of HS on lymphatic endothelium plays a role in regulating DC trafficking in vivo, we inducibly and selectively knocked down the gene Ndst1 in LECs. The Ndst family of enzymes is involved in N-sulfation of glucosamine residues during the biosynthesis of nascent HS chains on proteoglycan core proteins (14). The LEC-specific knockdown of Ndst1 was achieved by crossing mice bearing both loxP-flanked alleles of Ndst1 (Ndst1[loxP] mice) (13) with mice harboring a tamoxifen-inducible Cre allele driven by the lymphatic endothelial specific Prox1 promoter (Prox1[CreERT2] transgenic mice) (5). Previous work has shown that in Ndst1[Prox1[CreERT2]) mice, mutant mice, five consecutive and selectively reduced Ndst1 expression in lymphatic endothelial cells, as compared to that of Ndst1[Prox1[CreERT2] wild-type control mice (5). After topical application of a solution containing skin allergen oxazalone together with the FITC, FITC⁺ DCs from the skin to the draining LNs were quantified by flow cytometric staining of FITC⁺CD11c⁺ cells. As shown in Fig. 1A, targeting the sulfation of lymphatic endothelial...
HS in Cre<sup>e</sup> mutant mice significantly reduced the number of FITC<sup>+</sup> CD11c<sup>+</sup> cells that colonized the draining LN, as compared to that in the Cre<sup>e</sup> controls. To examine whether a reduction in lymphatic endothelial \textit{Ndst1} expression in pre-existing unstimulated lymphatic vasculature might lead to any alterations in skin lymphatic vascular density or basal numbers of DCs within skin, we determined the density of Lyve1<sup>+</sup> lymphatic vessels in the ear by whole-mount staining, and quantified CD11c<sup>+</sup>MHC class II<sup>+</sup> cells in skin by flow cytometry. As shown in Fig. 1B and 1C, at baseline, there were no significant differences in either lymphatic vascular density or total DC quantity within the skin, suggesting that the reduction in the biosynthesis of lymphatic endothelial HS in pre-existing unstimulated lymphatic vasculature does not alter the conduit by which DCs travel or the basal peripheral pool of DCs prior to Ag uptake, but rather the trafficking process itself.

Considering that painted FITC may not only be taken up by skin-resident DCs, but also might freely diffuse from the skin, reach the draining LN via afferent lymphatic vessels, and be taken up by LN-resident DCs, we also examined in vivo coordinates by implanting mutant versus wild-type mice with equal numbers of fluorescence-labeled BMDCs into the foot dorsum, and we quantified DCs that trafficked to the draining LNs by fluorescence microscopy. To achieve this, we followed a well-established protocol for DC differentiation (15) and obtained ~80% marrow-derived CD11c<sup>+</sup> DCs after 9-day culture in the presence of GM-CSF (Supplemental Fig. 1A). Further characterization showed that close to 99% of CD11c<sup>+</sup> BMDCs were also positive for CD11b but negative for B220 (Supplemental Fig. 1B), consistent with the myeloid immunophenotype of these BMDCs. Fig. 1D shows that by 40 h after in situ DC injection, a significantly lower number of BMDCs travel to the popliteal LN in Cre<sup>e</sup> mutant mice as compared to that in Cre<sup>e</sup> littermate controls.

**Genetic targeting of the N-sulfation of lymphatic endothelial-specific HS inhibits in vivo DC-dependent CD8<sup>+</sup> T cell proliferation upon OVA challenge**

To test the biological significance of targeting lymphatic endothelial HS on DC trafficking–dependent T cell responses, we sensitized the mice with an initial dose of the classical Ag OVA. Following a reapplication of OVA into the footpad, we examined the populations of distinct T cell subtypes within the draining LN (Fig. 2A). Using initially a model characterized by a pan-endothelial mutation in \textit{Ndst1}, we found that CD8<sup>+</sup> T cell populations were significantly lower in \textit{Ndst1<sup>f/f</sup>Prox1<sup>Cre</sup>} mutant mice than in \textit{Ndst1<sup>f/f</sup>Prox1<sup>Cre</sup>ERT2} control littermates (Fig. 2B). Upon restricting the mutation to solely the lymphatic endothelium in vivo (using an inducible \textit{Prox1Cre} transgenic model to drive lymphatic-specific disruption in \textit{Ndst1} expression), a similar reduction in CD8<sup>+</sup> T cell proliferation in response to immunization was also observed in \textit{Ndst1<sup>f/f</sup>Prox1<sup>1CreERT2</sup>} mutants as compared to that in \textit{Ndst1<sup>f/f</sup>Prox1<sup>1CreERT2</sup>} littermate controls (Fig. 2C). In this model, when compared to vehicle (IFA)-injected mice, we observed a significant

**FIGURE 1.** Lymphatic endothelial-specific mutation impairing the sulfation of HS inhibits in vivo trafficking of DCs. (A) \textit{Ndst1<sup>f/f</sup>Prox1<sup>CreERT2</sup>} mutant mice (n = 11) and their \textit{Ndst1<sup>f/f</sup>Prox1<sup>1CreERT2</sup>} littermates (n = 12) were painted with FITC plus oxazolone on the abdomen. After 40 h, the draining inguinal and axillary LNs were isolated and digested into a single-cell suspension. Percentages of FITC<sup>+</sup>CD11c<sup>+</sup> cells within LNs were analyzed by flow cytometry and averaged following normalization to the mean value of the controls. To examine whether a reduction in lymphatic endothelial HS in pre-existing unstimulated lymphatic vasculature does not alter the conduit by which DCs travel or the basal peripheral pool of DCs prior to Ag uptake, we determined the density of Lyve1<sup>+</sup> lymphatic vessels in the ear by whole-mount staining, and quantified CD11c<sup>+</sup>MHC class II<sup>+</sup> cells in skin by flow cytometry. (A) Percentage of CD11c<sup>+</sup>MHC class II<sup>+</sup> DCs in the skin of \textit{Ndst1<sup>f/f</sup>Prox1<sup>1CreERT2</sup>} mutants (n = 7) and \textit{Ndst1<sup>f/f</sup>Prox1<sup>1CreERT2</sup>} littermate controls was determined by flow cytometry. (B) BMDCs at day 9 of differentiation in the presence of GM-CSF were labeled with calcein AM and injected into the left foot dorsum of \textit{Ndst1<sup>f/f</sup>Prox1<sup>1CreERT2</sup>} mutants (n = 6) and \textit{Ndst1<sup>f/f</sup>Prox1<sup>1CreERT2</sup>} littermate controls (n = 9) (2 × 10<sup>6</sup> cells/mouse). After 40 h, the left popliteal LN was isolated, imaged under the fluorescence microscope. The number of calcein<sup>+</sup> cells was quantified using National Institutes of Health ImageJ software, plotted, and averaged for each genotype (horizontal bars in (D)). *p < 0.05, **p < 0.01, as compared to the \textit{Ndst1<sup>f/f</sup>Prox1<sup>1CreERT2</sup>} group.
FIGURE 2. Lymphatic endothelial-specific reduction in the $N$-sulfation of HS results in the inhibition of CD8$^+$ T cell proliferation upon OVA challenge. (A) Scheme of the experiment. All animals were pretreated with daily tamoxifen for 5 consecutive days to activate Cre recombinase. On day 12, a sensitizing dose of OVA (500 $\mu$g/mouse) was administered i.p. Seven days later (day 19), 10 $\mu$g OVA was delivered to the left footpad by intradermal injection. On day 24, the left popliteal LNs were isolated, with distinct populations of T cells examined by flow cytometry. The experiment was first carried out in animals bearing a panvascular mutation in Ndst1. (B) Number of CD3$^+$, CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$ T cells in draining LNs was compared between Ndst1$^{1f/f}$TekCre$^+$ (n = 5) and Ndst1$^{1f/f}$TekCre$^-$ control littermates (n = 5). *p < 0.05, as compared to Ndst1$^{1f/f}$TekCre$^-$ control group. (C) Number of CD3$^+$, CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$ T cells in draining LNs was compared between Ndst1$^{1f/f}$Prox1$^{1+/CreERT2}$ mutants (n = 8) and Ndst1$^{1f/f}$Prox1$^{-/CreERT2}$ littermates (n = 9) after either vehicle control (IFA only) or OVA challenge (OVA plus IFA). *p < 0.05, *p < 0.01.

In addition to a phenotype that appears to depend on the trafficking of DCs in distinct lymphatic beds in the two-step model in Fig. 2, secondary effects by cell types other than DCs in the model nevertheless have the potential to influence T cell responses. Accordingly, we characterized the baseline level of distinct cellular components within the peripheral blood (by complete blood count) and the skin-draining lymph nodes (by flow cytometry) from Ndst1$^{1f/f}$Prox1$^{1+/CreERT2}$ versus Ndst1$^{1f/f}$Prox1$^{-/CreERT2}$ mice following tamoxifen injection. The two genotypes at baseline showed no significant differences in the complete blood count profile (Supplemental Table 1A), and there were no significant differences in baseline levels of T/B cells, NK cells, and monocytes within the skin-draining LNs (Supplemental Table 1B). We also compared distinct DC subpopulations, including CD8$^+$ resident DCs, PDCA1$^+$ plasmacytoid DCs, and migratory DCs in the skin-draining LNs of Ndst1$^{1f/f}$Prox1$^{1+/CreERT2}$ versus Ndst1$^{1f/f}$Prox1$^{-/CreERT2}$ mice at baseline, and identified no dramatic differences (Supplemental Table 1C).
Altering lymphatic endothelial HS affects DC adhesion but not transmigration under low shear flow

To understand which biological processes during DC trafficking are affected by targeting lymphatic endothelial HS, we first set up an in vitro flow system whereby DCs derived from bone marrow progenitor cells were introduced under low shear flow into a chamber lined with a confluent layer of hLECs. To target hLEC HS in vitro, two general approaches were applied: 1) enzymatic pretreatment of hLECs with heparinase [destroys HS chains (16)], and 2) altering HS biosynthesis (including specific sulfate modifications) in the hLECs through efficient siRNA targeting of specific HS biosynthetic enzymes (Supplemental Fig. 3A). After 15 min under shear flow, a moderate number of DCs were adherent to hLECs, whereas this was dramatically reduced if the hLECs were pretreated with heparinase or blocking Ab to CCR7 (Fig. 3A, upper panels, 3B, upper graph). Significant reduction in DC adhesion was also observed for hLECs pretreated with CCL21-neutralizing Ab (Fig. 3B, upper graph). When hLECs were transfected with siRNA targeting Ndst1 or XylT2 (the latter is required for initiating the biosynthesis of glycosaminoglycan chains on core proteins), adhesion was also significantly reduced (Fig. 3B, lower graph). However, pretreatment with CCL5-neutralizing Ab or transfection with siRNA targeting Hs3st1 (enzyme responsible for glucoronyl 3-O-sulfation of HS) did not alter adhesion. Following DC adhesion under flow, we monitored DC transmigration across the hLEC monolayer. In addition to the effect on adhesion, Abs for CCR7 or CCL21 markedly inhibited DC transmigration (Fig. 3A, lower panels, 3C, upper graph). In contrast, neither heparinase nor siRNA targeting the different HS biosynthetic enzymes significantly affected DC transmigration (Fig. 3C). These findings imply that CCL21–CCR7 binding and signaling plays an essential role in mediating both DC adhesion to and transmigration across hLEC layers. Alternatively, it appears that HS-mediated adhesion, at least in this model, is not required for transmigration. (Note that the force of gravity in such studies may also potentially contribute to engagement interactions that may be necessary for diapedesis.)

Lymphatic endothelial HS promotes the directional invasion and chemotaxis of DCs

We also assessed the role of lymphatic endothelial HS in modulating DC invasion/chemotaxis transwell-based systems in vitro. To mimic the in vivo physiological step by which DCs invade into extracellular matrix surrounding LECs, we designed a modified in vitro collagen matrix-based transwell assay (5) where hLECs were embedded in type I collagen on the underside of transwell...
filters, and fluorescence-labeled BMDCs were loaded on top of the insert. Invading BMDCs into the collagen gel were quantified under various conditions that alter or inhibit HS produced by hLECs (Fig. 4A, 4B). The presence of normal hLECs in the collagen was able to drive invasion by DCs >200-fold over basal invasion into hLEC-free collagen. If invasion proceeded in the presence of blocking Ab to either CCR7 or its cognate ligands CCL19 and CCL21, invasion was significantly reduced, suggesting that CCR7-mediated signaling (in addition to the presence of the cognate CCR7 chemokine ligands) plays an important role in the invasion of DCs toward hLECs across collagen. The production of HS by hLECs was required for DC invasion, as initially evidenced by marked inhibition in the setting of heparinase-treated hLECs. Targeting the HS chain-initiating enzyme XylT2 or the sulfating enzyme Ndst1 also led to significant reductions in DC invasion. In contrast, Ab blockade of CCL5 or treatment with siRNA targeting Hs3st1 did not lead to significant reduction in DC invasion. These findings suggest distinct and specific requirements with respect to chemokines as well as the fine structure of lymphatic HS in the system.

Because LEC-derived HS may either be associated with core proteins (e.g., syndecans or glypicans) on the lymphatic cell surface or secreted into the extracellular space (e.g., perlecans), we further investigated whether targeting HS secreted by LECs might alter the ability of DCs to migrate, with the idea that LEC-secreted HS might play a trans-acting role in DC migration. Specifically, fluorescence-labeled BMDCs on transwell filters were separated from the HS-targeted hLEC monolayer in lower wells by liquid medium, and DC migration into lower wells was quantified (5).

The presence of hLECs in the bottom well was sufficient to drive DC migration (Fig. 4C, 4D), and destroying HS with heparinase, blocking CCR7/CCL21 (Fig. 4C) and CXCR4/CXCL12 (Fig. 4D), or interfering with the biosynthesis of HS by hLECs (Fig. 4E, using siRNA targeting Ndst1 and XylT2) significantly reduced DC migration. In contrast, CCL5-blocking Ab, CCL19-blocking Ab, or treatment of hLECs with HS3st1 siRNA was not sufficient to alter migration (Fig. 4C–E). This suggests that the presence as well as specific sulfation properties (i.e., N-sulfation but not O-sulfation) of HS produced into the hLEC-CM is critical for mediating CCL21- and CXCL12-dependent migration of DCs toward the hLECs. Furthermore, when we combined targeting approaches, that is, heparinase treatment of the hLECs together with blocking Abs to CCR7 or CCL21, we detected no further reduction in BMDC chemotaxis as compared to that noted for each individual treatment alone (Fig. 4F), suggesting that the CCL21/CCR7-mediated effects under these conditions appear to fully depend on lymphatic endothelial HS.

**FIGURE 4.** Invasion and migration of BMDCs depends on lymphatic HS. (A and B) Invasion of BMDCs at day 9 of differentiation into a collagen gel containing either no cells (NC) or hLECs treated as indicated was quantified and normalized to NC. (C–F) Transwell migration of BMDCs into wells containing NC or hLECs monolayer treated as indicated was quantified and normalized to NC. Anti-CCR7 [also aR7 in (E), anti-CCL19, anti-CCL21 [also a21 in (F)], anti-CXCR4, anti-CXCL12, or anti-CCL5 indicates blocking Abs to CCR7, CCL19, CCL21, CXCR4, CXCL12, or CCL5, respectively; H’ase, hLECs pretreated with heparinase; siDS, hLECs transfected with control siRNA; siNdst1, siXylT2, or siHs3st1, hLECs transfected with siRNA targeting corresponding HS biosynthetic enzymes. *p < 0.05, **p < 0.01, as compared to hLEC control group in (A), (C), (D), and (F), and to siDS control group in (B) and (E).
Vehicle medium, the basal medium used to collect CM from hLECs, produced minimal CCL21/CCR7 or CXCL12/CXCR4 complexes (Fig. 5A, 5C), implying that PLA signal depends on the presence of these chemokines in the CM. Given that transient transfection of hLECs with different siRNA did not dramatically change the expression of CCL21 (Supplemental Fig. 3B) or its secretion into CM (5), the PLA data suggest that the optimal binding of chemokine to receptor on the BMDC surface depends on the presence of intact lymphatic HS produced into the CM (where it essentially acts as trans as a coreceptor).

**Lymphatic endothelial HS facilitates oligomerization of lymphatic chemokines**

The diversity of chemokine oligomerization has been shown to initiate distinct signaling responses and is known to be stabilized by HS glycosaminoglycans (17). To understand whether targeting lymphatic endothelial HS leads to alterations in chemokine oligomerization, we purified HS from confluent monolayers of hLECs transfected with either siDS, siXylT2, siNdst1, or siHs3st1 and incubated two different doses of the various purified siRNA-targeted HS species with recombinant human CCL21 (molecular mass, 12.2 kDa). We then examined the pattern of CCL21 oligomerization following crosslinking and gel electrophoretic separation of multimeric products. As shown in Fig. 6A, at the lower dose tested, HS from siDS- or siHs3st1-transfected hLECs led to robust CCL21 oligomerization, with distinct bands separated by approximately the size of a CCL21 monomer and the highest detectable oligomer being what appears to be an octamer (and a smear-like extension toward possibly larger products). In contrast, the same dose of HS from siXylT2- or siNdst1-transfected hLECs could only facilitate the formation of CCL21 trimers and tetramers, respectively. In the latter case, even at the higher HS dose, the purified siNdst1-altered HS could not support the formation of CCL21 oligomers greater than tetramer size (Fig. 6A), suggesting that targeting lymphatic endothelial HS biosynthesis at the level of initial chain formation or N-sulfation, but not 3-O-sulfation, dramatically reduces its capability to support the complexing of larger chemokine oligomers in this assay. A similar HS effect on CXCL12 (molecular mass, 8 kDa) oligomerization was also observed (Fig. 6B), with HS isolated from mock-transfected (siDS) control cells, but not from siXylT2 or siNdst1-transfected cells, strongly supporting CXCL12 multimerization.

**Discussion**

In this study, we provide genetic evidence that targeting the N-sulfation of lymphatic endothelial HS significantly reduces in vivo DC trafficking from the periphery to the draining LN, with the functional consequence of inhibiting CD8+ T cell proliferation in the draining LN in response to OVA immunization. Mechanistically, lymphatic endothelial HS may mediate DC trafficking at multiple steps, including adhesion to LECs under flow as well as chemokine-dependent migration toward lymphatic endothelium. Moreover, appropriately sulfated lymphatic HS appears to be required for chemokine oligomerization and optimal presentation of certain chemokines such as CCL21 and CXCL12 on the DC surface.

The development of effective approaches to manipulate DC traffic has been limited by a lack of understanding of the molecular controls for this process. A few studies, including our own, have demonstrated that altering vascular HS biosynthesis, either through an alteration in N-sulfation of nascent HS chains (Ndst1 mutation) or chain polymerization (Ext1 mutation), results in altered chemokine-dependent interactions of endothelial cells with trafficking neutrophils, DCs, and tumor cells as they home to sites of
inflammation or colonize lymphoid organs via blood vascular or lymphatic vascular routes (5, 6, 18). For example, a panendothelial mutation in Ext1 inhibits CCL21/CCL19 binding to LECs and impairs DC migration to the draining LN (6). Although this suggests what may occur when HS chains are absent or enzymatically ablated in the entire circulation, the degree to which lymphatic endothelial-specific alteration in HS affects DC traffic and specific T cell responses to Ag has not been reported. We stringently interrogated the role of lymphatic vascular-specific HS, separate from any role that blood endothelial HS might play in affecting T cell traffic through the blood vasculature (such as LN high endothelial venules). Moreover, HS is endowed with functional specificity for distinct ligands such as chemokines that may variably bind as a result of specific sulfate modifications. Targeting of the latter has also not been explored in the lymphatic system.

We inducibly knocked down Ndst1 in lymphatic vasculature and demonstrated impairments in DC traffic in models that allow examination of movement of endogenous DCs (utilizing FITC plus oxazolone skin painting) as well as the traffic of exogenously loaded BMDCs to regional LNs. Because pathologically stimulated lymphatic endothelium may potentially undergo impaired growth/remodeling responses in the setting of HS mutations (19), a control experiment confirmed that induction of lymphatic endothelial Ndst1 knockdown in the skin under basal/nonstimulated conditions does not alter lymphatic vascular density (Fig. 1B). Moreover, no significant changes in the total DC number within the skin were noted upon Ndst1 knockdown at baseline, suggesting that reduced DC colonization within the draining LN (Fig. 1A, 1D) is caused by the effect of lymphatic-specific HS mutation on the DC trafficking process itself. To what extent the mutation might result in a defect in slowing/arresting of DCs as they enter the interfollicular sinuses from afferent lymphatics entering the LN is unknown, although a sophisticated analysis of this may be worthwhile in future studies.

To test downstream DC-dependent immunologic events, we asked how targeting the N-sulfation of lymphatic HS would affect T cell responses in the draining LN upon Ag stimulation. In a sensitization-elicitation allergic reaction model employing OVA Ag, we observed significant reduction in the total CD8+ T cell population within the draining LN (Fig. 2). Although this is a complex immune response that involves in vivo lymphatic DC trafficking at both sensitization and elicitation steps, the lack of an LN CD8+ T cell response in lymphatic-HS mutants highlights the functional importance of appropriately sulfated lymphatic HS in modulating the magnitude of the DC-dependent LN immune response. Moreover, OVA contains multiple epitopes capable of activating distinct subtypes of T cells, including CD8+ cytotoxic and CD4+ helper T cells (20), potentially mediated by DCs of different subsets or at distinct maturation or activation stages. With this in mind, the specific reduction of a CD8+ T cell proliferative response but not that of the CD4+ T cells reveals the unique effect of silencing lymphatic HS on the LN balance of T cell responses. As a final control, we “dissected” the importance of lymphatic-specific HS from that of blood-vascular HS in this immunization process by repeating the experiment in Ndst1f/fTcRmutant mice (with a panendothelial deficiency in HS sulfation). A nearly identical result in terms of the CD8+ T cell phenotype in the LN was observed, highlighting the importance of lymphatic-specific HS in the immune phenotype.

The molecular diversity of HS is highlighted by the ability of its unique sulfate-modified structural motifs to facilitate and/or mediate protein interactions that are essential for a variety of biological processes (14). However, knowledge is lacking with respect to how specific HS modifications contribute to mechanistic steps involved in functional immune responses. In cell-based assays, we found that by targeting the integrity of HS with heparinase or by blocking LEC biosynthesis of HS via siRNA, DC adhesion under flow and LEC-driven collagen invasion and matrix-independent migration were all significantly reduced. This was associated with chemokine specificity as well as HS fine-structure specificity in the system. Blockade was achieved using Abs to CCR7/CCL21 or CXCR4/CXCL12 but not CCL5, and the effects of silencing N-sulfation of LEC HS as opposed to 3-O-sulfation (siHs3st1) are consistent with data regarding the importance of N-sulfation and 2-O-sulfation (both of which result from Ndst1 silencing) (5, 18) as opposed to 3-O-sulfation for binding of CCL21 or CXCL12 to immobilized HS on glycan arrays (8). The trends in specificity suggest that chemokines that drive this system may work cooperatively with key sulfate motifs on HS produced by the LECs.

Other soluble regulators that might affect DC trafficking to the LN include selectin adhesion molecules, integrin family members, metalloproteinases, and possibly other chemokines (21–24). Importantly, we found that upon disruption of lymphatic HS biosynthesis in a low shear-rate flow model of DC over primary LECs, DC–LEC adhesion events were markedly inhibited. Alternatively, HS-mediated adhesion did not appear to be required for transmigration in this model, whereas Ab-mediated blocking
of CCR7/CCL21 had a significant impact on both DC–LEC adhesion as well as DC transmigration across hLECs under flow. The lack of HS effect on transmigration in this in vitro assay, as opposed to our in vivo findings supporting the role HS in orchestrating DC entry into lymphatic vessels by modulating CCL21 haptotaxis, may also be attributed to the fact that most adhesion events required for subsequent transmigration in some in vitro models may be less dependent on HS-mediated chemokine sequestration because the adherent DCs are in direct contact with the LEC monolayer under the force of gravity alone, which might be sufficient to engage the necessary “machinery” for diapedesis. In contrast, in vivo DCs may require greater assistance from sequestered chemokines to navigate efficiently through tissues and make such contacts.

We followed phenotypic characterizations with mechanistic studies suggesting that LEC HS may essentially regulate chemokine-dependent signaling by DCs in multiple ways depending on the context in which HS is presented. Recent work demonstrates that perilymphatic CCL21 gradients likely depend on HS in the extracellular matrix (5–7). However, the reduced in vitro DC migration observed upon disrupting HS prompted us to look specifically into the importance of HS secreted by LECs in the presentation of chemokines to the migrating DCs. Expression of CCL21 by LECs in these systems did not appear to be reduced by treating the LECs with heparinase or transient transfection of LECs with siNdst1 or siXylT2 (Supplemental Fig. 3B). Nevertheless, we noted that the ability of CM from siXylT2 or siNdst1 targeted LECs to support partnerships between CCL21 and CCR7 (or CXCL12 and CXCR4) on the DC surface was markedly reduced in comparison to that of CM from siRNA-control LECs (Fig. 5). These observations suggest that LEC HS plays a critical role in supporting in trans presentation of these lymphatic chemokines to their receptors. A mechanistic explanation and extension of these findings highlights the possibility that LEC HS (whether bound to secreted HS proteoglycans or released as free chains) may serve as a soluble co-receptor for lymphatic chemokines to promote efficient through tissues and make such contacts.

The findings of this study contribute to our understanding of basic mechanisms that regulate DC migration and immunity. More generally, because DC traffic may become dysregulated in disorders such as autoimmunity, transplant rejection, or cancer, these findings suggest strategies for possibly reprogramming immunity through rational therapeutic interventions that modulate HS fine structure.

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Disclosures

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