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*J Immunol* published online 3 February 2014
http://www.jimmunol.org/content/early/2014/01/31/jimmunol.1301286

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/02/03/jimmunol.1301286.DCSupplemental

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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

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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 glycan-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.
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/6 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 (Coralville, IA) and were transfected into cells according to the manufacturer’s instructions. For heparinase treatment, cells were incubated with heparinase (heparin lyases 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 (eBioscience) at 37˚C, 5% CO2 for 1 h followed by filtration through a 40-μm cell strainer (BD Biosciences, San Jose, CA) 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 culture

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 FACS Calibur.

Traffic 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+/Prox1+/CreERT2 mutants and their Ndst1+/Prox1+/CreERT2 wild-type littermates (2 × 105 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-CD4 and allophycocyanin-labeled anti-CD8 Abs (eBioscience) and analyzed by FACS Calibur.

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

CD8 T cells were purified from spleens of OT-I (CD8+ T cells) 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 × 106/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 reactive peptides, OVA323-339 and OVA277-284 (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 0.2% type I collagenase at 37˚C for 1 h followed by filtration through a 40-μm cell strainer. Cells were then stained with allophycocyanin-labeled anti-CD4 or allophycocyanin-labeled anti-CD8 Abs (eBioscience) and analyzed by FACS Calibur.

BMDC interactions with hLECs under flow

The hLECs were seeded onto microfluidic channels of BioFlux plates (48-well, 0–20 dynes/cm2; Fluxion Biosciences, San Francisco, CA) precoated with Matrigel. Cells were allowed to adhere to the coverslips for 1 h at 37˚C and 5% CO2 and then stimulated with 50 ng/ml LPS. After 2 h, the supernatants were removed, and the hLECs were washed with serum-free EBM2 medium. BMDCs were labeled with calcein AM (Aldrich) and introduced into the microfluidic channels at a concentration of 106 cells/ml. After 40 h, images were acquired using the BioFlux plate reader and analyzed using the BioFlux software (Fluxion Biosciences, San Francisco, CA).
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 were prelabeled with calcein AM following the manufacturer’s instruction and were perfused into the channel at a concentration of 2 × 10⁶ cells/ml in EBM2 medium under an initial shear stress of 2 dynes/cm² for 2 min followed by a constant flow at 0.14 dynes/cm² for 15 min. After removing the residual BMDCs from the inlet well, the channel was washed with EBM2 medium at 1 dyn/cm² for 5 min to remove any unattached BMDCs. Adherent BMDCs across the whole 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 cells and presence of non-adherent BMDCs across the whole 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). The following capture of adhesion images at time 0 (t₀), transmigration of adherent BMDCs (defined by disappearance of cells and presence of non-adherent BMDCs across the whole 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). The following application of a solution containing skin allergen oxazolone together with the FITC, FITC+ DCs from the skin to the draining LNs were quantified by flow cytometric staining of FITC+CD11c+ cells. As a positive control, hLECs were either treated with heparinase, blocking Abs, or transfected with siRNA. The specific interactions between chemokines from CM and chemokine receptors on cytospin-collected BMDCs were determined with the Duolink proximity ligation assay (PLA; Olink Bioscience, Uppsala, Sweden) following the manufacturer’s instruction using the following primary Abs: anti-CCL21 (R&D Systems, MAB3661, 1:100), anti-CCR7 (Novus, St. Charles, MO, 1:250), anti-CXCL12 (R&D Systems, AF-310-NA, 1:100) and anti-CXCR4 (R&D Systems, MAB172, 1:200).

**Purification of HS from hLECs**

Purification of HS was performed as previously described (13). Briefly, hLECs in 10-cm tissue culture plates were transfected with different siRNA molecules, washed with PBS once, and digested in 1× Pronase buffer containing 167 µg/ml Pronase (Sigma-Aldrich), 40 mM sodium acetate, and 320 mM sodium chloride in PBS (10 ml buffer/10-cm plate) at 37°C for 24 h. The cellular digest was then filtered through 0.2-µm pore yethersulfone syringe filters (GE Healthcare, Piscataway, NJ) and run through a DEASephacel (GE Healthcare) column by gravity. After washing with DEASequilibration buffer (0.2 M sodium chloride, 20 mM sodium acetate [pH 6.0]), the glycan components were eluted from the column with DEAElution buffer containing 2 M sodium chloride and 20 mM sodium acetate (pH 6.0). The salt in the sample was then removed by running the eluent through the PD-10 desalting column (GE Healthcare) following the manufacturer’s instruction. The eluted glycans (containing both HS and chondroitin sulfate) were lyophilized, redissolved in chondroitinase buffer (50 mM Tris-HCl [pH 8.0], 50 mM sodium acetate), and digested with chondroitinase A, B, and C (a gift from Dr. Jeffrey Esko, University of California San Diego Department of Cellular and Molecular Medicine) at 37°C for 24 h. After another passage through DEASephacel and PD-10 desalting columns as described above, the purified and lyophilized HS was resuspended in PBS (500 µl/10-cm plate).

**Oligomerization analysis of CCL21 and CXCL12**

Purified PBS-resuspended HS (0.1 µM and 0.3 µM) was incubated separately with recombinant human CCL21 or CXCL12 (20 ng/reaction; PeproTech) in a total reaction volume of 30 µl at room temperature for 1 h followed by crosslinking using BS⁴ (Thermo Scientific, Rockford, IL.). After quenching the crosslinking reaction with 50 mM Tris, nonreducing protein loading buffer (5× concentration) was added to the samples, which were then boiled for 5 min and separated on 4–20% gradient gels (Bio-Rad). Samples were then electrotransferred from the gel onto a nitrocellulose membrane and probed with anti-CCL21 or anti-CXCL12 Ab (R&D Systems; 1:1000) followed by IRDye 800 secondary Ab (LI-COR, Lincoln, NE). The signal was detected using the Odyssey infrared imaging system (LI-COR).

**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 oxazolone painting and that from in situ–implanted BMDCs between mutant and littermate control mice was calculated using a two-tailed Student t test. Differences in LN colonization of skin DCs following FITC plus oxazolone painting and that from in situ–implanted BMDCs between mutant and littermate control mice was calculated using a two-tailed Student t test. Differences in LN colonization of skin DCs following FITC plus oxazolone painting and that from in situ–implanted BMDCs between mutant and littermate control mice was calculated using a two-tailed Student t test. Differences in LN colonization of skin DCs following FITC plus oxazolone painting and that from in situ–implanted BMDCs between mutant and littermate control mice was calculated using a two-tailed Student t test. Differences in LN colonization of skin DCs following FITC plus oxazolone painting and that from in situ–implanted BMDCs between mutant and littermate control mice was calculated using a two-tailed Student t test. Differences in LN colonization of skin DCs following FITC plus oxazolone painting and that from in situ–implanted BMDCs between mutant and littermate control mice was calculated using a two-tailed Student t test. Differences in LN colonization of skin DCs following FITC plus oxazolone painting and that from in situ–implanted BMDCs between mutant and littermate control mice was calculated using a two-tailed Student t test.

**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/loxP] mice) (13) with mice harboring a tamoxifen-inducible Cre allele driven by the lymph endothelial specific Prox1 promoter (Prox1Cre(loxP/Cre)) transgenic mice (5). Previous work has shown that in Ndst1[loxP/loxP] mutant mice, five consecutive 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.
HS in Cre\(^{e}\) mutant mice significantly reduced the number of FITC\(^{+}\) CD11c\(^{+}\) cells that colonized the draining LN, as compared to that in the Cre\(^{-}\) controls. To examine whether a reduction in lymphatic endothelial 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\(^{+}\) lymphatic vessels in the ear by whole-mount staining, and quantified CD11c\(^{+}\)MHC class II\(^{+}\) 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 DC trafficking 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\(^{+}\) DCs after 9-day culture in the presence of GM-CSF (Supplemental Fig. 1A). Further characterization showed that close to 99% of CD11c\(^{+}\) 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\(^{e}\) mutant mice as compared to that in Cre\(^{-}\) littermate controls.

**Genetic targeting of the N-sulfation of lymphatic endothelial-specific HS inhibits in vivo DC-dependent CD8\(^{+}\) 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 Ndst1, we found that CD8\(^{+}\) T cell populations were significantly lower in Ndst1\(^{Lox}^{+/}^{TeKcre}^{+}\) mutant mice than in Ndst1\(^{Lox}^{+/}^{TeKcre}^{-}\) control littermates (Fig. 2B). Upon restricting the mutation to solely the lymphatic endothelium in vivo (using an inducible Prox1\(^{Cre}\) transgenic model to drive lymphatic-specific disruption in Ndst1 expression), a similar reduction in CD8\(^{+}\) T cell proliferation in response to immunization was also observed in Ndst1\(^{Lox}^{+/}^{Prox1}^{Cre}^{ERT2}\) mutants as compared to that in Ndst1\(^{Lox}^{+/}^{Prox1}^{Cre}^{ERT2}\) littermate controls (Fig. 2C). In this model, when compared to vehicle (IFA)-injected mice, we observed a significant

![FIGURE 1](http://www.jimmunol.org/Downloadedfrom http://www.jimmunol.org/)
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 CD3+CD4+ and CD3+CD8+ T cells in draining LNs was compared between a sensitizing dose of OVA (500 μg/mouse) was administered i.p. Seven days later (day 19), 10 μg 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 panvasculature mutation in Ndst1. (B) Number of CD3+, CD3+CD4+, and CD3+CD8+ T cells in the draining LN was compared between Ndst1TekCre+ (n = 5) and Ndst1TekCre− control littermates (n = 5). *p < 0.05, as compared to Ndst1TekCre− control group. (C) Number of CD3+, CD3+CD4+, and CD3+CD8+ T cells in draining LNs was compared between Ndst1Prox1+/CreERT2 mutants (n = 8) and Ndst1Prox1−/CreERT2 littermates (n = 9) after either vehicle control (IFA only) or OVA challenge (OVA plus IFA). *p < 0.05, *p < 0.01.

increase in the total T cell (CD3+) population upon OVA challenge in both Ndst1Prox1+/CreERT2 mutants and Ndst1Prox1−/CreERT2 littermates (with only a slight but insignificant reduction in the response of mutants), indicating that T cell proliferation occurred in both groups of animals. Importantly, however, although a significant CD8+ T cell proliferation response occurred in Cre− wild-type littermates, the CD8+ T cell response was strikingly absent in Ndst1TekCre+ mutants (Fig. 2C, right panel). Alternatively, a relatively lesser degree of CD4+ T cell proliferation occurred in both groups (Fig. 2C, middle panel), although significance relative to the response in IFA-only controls was only achieved in the mutant group. Thus, the most dramatic effect in this model involving a highly lymphatic-specific deficiency in the N-sulfation of HS was the contrast between the CD8+ T cell proliferative response in wild-type mice and the complete deficiency in the mutants, indicating that T cell proliferation occurred in both genotypes (Fig. 2C, right panel).

The OVA model employed in Fig. 2 involves two-step stimulation with Ag. To more directly examine how a single immunization on the mutant background might, as a result of altered DC trafficking, affect lymph node T cell proliferation, we adoptively transferred CFSE-labeled OT-I CD8 and OT-II CD4 T cells (expressing transgenic T cell receptors for peptides OVA257–264 and OVA323–339, respectively) into Ndst1Prox1+/CreERT2 versus Ndst1Prox1−/CreERT2 mice. At 72 h following footpad injection of a mixture of two specific OVA-derived Ags, namely OVA257–264 and OVA323–339 peptides, we examined CD8 and CD4 proliferation within the draining popliteal lymph nodes by flow cytometry. As shown in Supplemental Fig. 2, we observed a significant reduction in the ratio of CFSEintCD8+ to CFSEhiCD8+ in Ndst1Prox1+/CreERT2 mutant mice as compared to that in Ndst1Prox1−/CreERT2 controls. Considering that CFSEintCD8+ cells represented adoptively transferred and actively proliferating CD8 T cells (as compared with nonproliferating CFSEhiCD8+ cells), the ratio of these two populations reflects the proliferative activity of the adoptively transferred CD8 T cells in vivo. Alternatively, no dramatic difference was noted in proliferating CFSEintCD4 T cells between the two genotypes.

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 Ndst1Prox1+/CreERT2 versus Ndst1Prox1−/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 Ndst1Prox1+/CreERT2 versus Ndst1Prox1−/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 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

**FIGURE 3.** Lymphatic endothelial HS critically regulates chemokine-dependent adhesion of BMDCs under low-level physiologic shear flow. hLECs were seeded into a BioFlux flow chamber (edges marked by dashed lines) and pretreated with neutralizing Abs (against CCL21, CCR7, or CCL5) or siRNA targeting the indicated HS biosynthetic enzymes (siDS). Day 9 BMDCs were labeled with calcein AM and introduced into the chamber at a constant shear stress of 0.14 dyne/cm² at 37˚C. After 15 min, nonadherent cells were washed off and the adherent BMDCs were imaged using fluorescence microscopy [(A) upper panels, fluorescence in white/gray over black flow channel background], quantified with Metamorph software, and normalized to hLEC control group [(B) upper graph in (B)] and to the siDS control group for siRNA-targeting experiments [(lower graph in (B))]. The transmigration of adherent BMDCs was recorded following an additional 30-min cell-free flow period (from t₀ to t₃₀), imaged under a fluorescence microscope [(lower panels in (A)) and quantified using Metamorph software, with the percentage transmigration calculated as [(no. adherent BMDCs at t₀ 2 no. adherent BMDCs at t₃₀)/no. adherent BMDCs at t₀] × 100%. All transmigration data were normalized to hLEC control group [(upper graph in (C)) and to the siDS control group for siRNA experiments [(lower graph in (C)]. *p < 0.05, **p < 0.01, as compared to hLEC control group in (B) and (C) upper graphs, and to siDS control group in (B) and (C) lower graphs.
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 hLEC HS (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 3-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 hLEC monolayer treated as indicated was quantified and normalized to NC. Anti-CCR7 [also aR7 in (F)], 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 a co-receptor).

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 Extl 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 Ndst1/+/TekCre+ mutant 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 (sHs3st1) are consistent with data regarding the importance of N-sulfation and 2-O-sulfation (both of which result from Ndst1 silencing) in terms of the CD8+ T cell phenotype in the LN was observed, highlighting the importance of lymphatic-specific HS in the immune phenotype.

FIGURE 6. Lymphatic endothelial HS is essential for optimal oligomerization of CCL21 and CXCL12. HS was purified from cultured hLEC monolayers (grown in 10-cm plates) that were transfected with either siDS or siRNA targeting the indicated HS biosynthetic enzymes, and resuspended in 500 μl PBS. Recombinant human CCL21 [hCCL21, 20 ng/reaction, molecular mass, 12.2 kDa (A)] or hCXCL12 [20 ng/reaction, molecular mass, 8 kDa (B)] was incubated with 0.1 μl and 0.3 μl of each HS prep, followed by BS3-mediated crosslinking, and separation on SDS-PAGE gels with detection by Western immunoblotting. Green signal, hCCL21 (A) or hCXCL12 (B) as detected by Western immunoblot; red signal, protein size marker. – and +, pure hCCL21 or hCXCL12 in the absence or presence of BS3 crosslinker, respectively.
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 extra-cellular 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 chemokine 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 vivo lymphatic chemokine–receptor interactions. 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 optimally interact with their receptors on the surface of trafficking DCs. This is supported by the fact that genetic alterations in LEC HS biosynthesis resulted in marked alterations in the ability of CCL21 to oligomerize on purified soluble HS from mutant LECs as opposed to HS from wild-type/control LECs (Fig. 6).

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.

Acknowledgments
We acknowledge assistance from Dr. Guillerano Oliver (St. Jude Children’s Research Hospital) for provision of inducible Prox1Cre transgenic mutant mice as well as Dr. Jeffrey Esko (University of California San Diego) for provision of heparin lyses. We acknowledge Steven Kendall and Fluxion Biosciences for assistance with BioFlux plates and microfluidics system as well as Dr. Jennifer Meerloo of the University of California San Diego Microscopy Shared Facility. We thank Dr. Klaus Ley of the La Jolla Institute for Allergy and Immunology for assistance with analysis of DC subsets in the lymph node and Dr. Stephen Hedrick for provision of OT-I and OT-II transgenic mice. We also thank Dr. Phil Gords for initial assistance with adoptive T cell transfers and Faye Nourollahi for additional assistance with mouse studies. We also acknowledge assistance and support from the Veterans Medical Research Foundation.

Disclosures
The authors have no financial conflicts of interest.

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