Shape and Function of Interstitial Chemokine CCL21 Gradients Are Independent of Heparan Sulfates Produced by Lymphatic Endothelium

Kari Vahtomeri\(^1,2\)*, Christine Moussion\(^1\), Robert Hauschild\(^1\) and Michael Sixt\(^1\)*

\(^1\) Institute of Science and Technology Austria (IST Austria), Klosterneuburg, Austria, \(^2\) Wihuri Research Institute and Translational Cancer Medicine Research Program, University of Helsinki, Biomedicum Helsinki, Helsinki, Finland

Gradients of chemokines and growth factors guide migrating cells and morphogenetic processes. Migration of antigen-presenting dendritic cells from the interstitium into the lymphatic system is dependent on chemokine CCL21, which is secreted by endothelial cells of the lymphatic capillary, binds heparan sulfates and forms gradients decaying into the interstitium. Despite the importance of CCL21 gradients, and chemokine gradients in general, the mechanisms of gradient formation are unclear. Studies on fibroblast growth factors have shown that limited diffusion is crucial for gradient formation. Here, we used the mouse dermis as a model tissue to address the necessity of CCL21 anchoring to lymphatic capillary heparan sulfates in the formation of interstitial CCL21 gradients. Surprisingly, the absence of lymphatic endothelial heparan sulfates resulted only in a modest decrease of CCL21 levels at the lymphatic capillaries and did neither affect interstitial CCL21 gradient shape nor dendritic cell migration toward lymphatic capillaries. Thus, heparan sulfates at the level of the lymphatic endothelium are dispensable for the formation of a functional CCL21 gradient.

Keywords: chemokine gradient, chemokine CCL21, chemotaxis, lymphatic system, lymphatic endothelium, dendritic cell, heparan sulfate

INTRODUCTION

Chemokines and growth factors form extracellular gradients, which guide migrating cells and morphogenetic processes. Endothelial chemokines regulate immune cell approach and transmigration across the blood and lymphatic endothelium. The evidence for mechanisms regulating the extracellular chemokine cues are emerging (1–3). However, the sparsity of endogenous gradients that can be detected at tissue level has hampered research on gradient formation. CCL21 is the only chemokine, which has been directly shown to form functional gradients in tissues at endogenous levels (2, 4), and thus presents a unique opportunity to study the mechanistic basis of formation and maintenance of chemokine gradients.

CCL21 is secreted by lymphatic endothelial cells (LEC), shows the highest concentration at the plasma- and basement membrane of the lymphatic capillary and forms a gradient decaying into the surrounding interstitium (4–7). CCL21 is essential for the guidance of antigen-presenting CCR7
positive dendritic cells (DC) from peripheral tissues to the lymphatic capillaries and further into the parenchyma of lymph nodes (2, 4, 5, 8, 9).

Most gradient forming proteins exist in a soluble and a glycan anchored pool. The binding to proteoglycans, especially heparan sulfates (HS), has been shown to shape fibroblast growth factor gradients by limited diffusion (10, 11). Accordingly, genetic deletion of the essential enzymes of HS synthesis resulted in defective morphogen gradient formation: deletion of Ext2 and Ext3, and thus absence of HS chain polymerization, lead to enhanced FGF diffusion in zebrafish (12), whereas hypomorphomic allele of Ext1 resulted in elevated range of HS binding Indian hedgehog in mouse embryos (13).

CCL21 interacts in vitro with glycans like HSs, chondroitin sulfate B and E and also collagen IV capillary, CCL21 sequestration at the CCL21 source, i.e. at the lymphatic carboxy-terminus (14, 15). Accordingly, intact lymphatic gradients by limited diffusion (10, 11). Accordingly, genetic laboratories.

Ears of the 4-OHT treated Prox1CreERT2;Ext1−/− mice followed by culture in R10 medium (RPMI1640 containing penicillin-streptomycin, glutamine, 10% fetal calf serum; all from Gibco) supplemented with GM-CSF hybridoma supernatant. Day 8 DCs were activated with LPS (200 ng/ml, Sigma-Aldrich) for 20 h. Activated DCs were labeled with 6.7 µM 5- and 6-carboxytetramethyl rhodamine, succimidyl ester (TAMRA, Molecular Probes, Life Technologies) in PBS at room temperature for 15 min. The staining reaction was quenched by the addition of R10, cells were collected by centrifugation and resuspended to the R10 + GM-CSF hybridoma supernatant.

**Dendritic Cell Preparation**

Mature dendritic cells (DCs) were generated by extracting bone marrow from femur and tibia of 8–12 weeks old wild type or Ccr7−/− mice by Taija Mäkinen (2012).

**Ear Sheet Preparation, Interstitial Dendritic Cell Migration Assay and Heparinase II Treatment**

Ear sheets were prepared as reported earlier (4). In short, ears were split and the ventral ear sheets were either treated with 1 mg/ml Collagenase A (Sigma-Aldrich) in DMEM supplemented with 1.3 mM CaCl2 at +37°C for 1 h. The collagenase A treatment was quenched with 10 mM EDTA at room temperature for 10 min, tissue lysates were stripped through 70 µm cell strainer, cells were collected by centrifugation and subsequently resuspended to FACS buffer (5 mM EDTA in PBS). FACS Aria (Becton Dickinson) was used to sort the cells to EGFP+ and tdTomato+ populations. Sorted cells were collected by centrifugation and cell pellets were directly lysed with genotyping sample buffer and used for genotyping of Ext1fl allele, Ext1 deleted allele and Oaz1 (loading control).

**Materials and Methods**

**Mice**

All the mice were on a C57BL/6J background. Wild type mice were purchased from Charles River and mTmG mice from Jackson laboratories. Ext1fl/fl mice were kindly provided by Yu Yamaguchi and Holger Gerhardt (18), Prox1CreERT2 mice by Taija Mäkinen (19) and Ccr7−/− mice by Reinhold Förster (20). Male and female mice were bred and maintained according to the local rules (Institutional Review Board approval BMWF-66.018/0005-II/3b/2012). Prox1CreERT2;Ext1fl/fl, Ext1floxflo, Prox1CreERT2;Ext1floxflo;mtMg and Ext1floxflo;mtMg mice were topically treated with acetone dissolved 4-OHT (10mg/ml) (Sigma-Aldrich) once a day at P2-5. 4-OHT treated Prox1CreERT2;Ext1floxflo and Prox1CreERT2;Ext1floxflo;mtMg mice are referred to as “Ext1ALEC” whereas 4-OHT treated Ext1floxflo and Ext1floxflo;mtMg are referred to as “control”. Ears were collected for further analyses or treated with FITC at the age of 4 to 6 weeks. In each experiment control and Ext1ALEC mice were littersmates.

**Sorting and Genotyping of Prox1CreERT2; Ext1floxflo;mtMg Ear Dermal Cells**

Ears of the 4-OHT treated Prox1CreERT2;Ext1floxflo;mtMg mice were collected at 4 weeks age. Ears were split into ventral and dorsal halves, fat layer was removed, ears were minced and treated with 1 mg/ml Collagenase A (Sigma-Aldrich) in DMEM supplemented with 1.3 mM CaCl2 at +37°C for 1 h. The collagenase A treatment was quenched with 10 mM EDTA at room temperature for 10 min, tissue lysates were stripped through 70 µm cell strainer, cells were collected by centrifugation and subsequently resuspended to FACS buffer (5 mM EDTA in PBS). FACS Aria (Becton Dickinson) was used to sort the cells to EGFP+ and tdTomato+ populations. Sorted cells were collected by centrifugation and cell pellets were directly lysed with genotyping sample buffer and used for genotyping of Ext1fl allele, Ext1 deleted allele and Oaz1 (loading control).
with PBS. The Ext1<sup>ALEC</sup> ears (Prox1CreERT2;Ext1<sup>fl<sup>ox</sup>fl<sup>ox</sup>;mTmG) were treated similarly, but the α-LYVE1 and α-rat Alexa 488 were omitted due to lymphatic endothelial EGFP expression (see Figure 1B, for the specificity of EGFP expression). Ears were imaged immediately.

For staining of LYVE1 and heparan sulfates (Figure 1D) the fixed ears were blocked with 1% BSA in PBS for 1 h. Before antibody staining, 1:50 diluted α-HS (mouse monoclonal IgM, k) (10E4; US Biological) was pre-incubated with biotinylated α-mouse antibody (vector labs) for 60 min at room temperature in blocking buffer. The α-HS (10E4)-anti-mouse antibody complex with or without α-LYVE1 (1:200 dilution; R&D MAB2125) were incubated on blocked ears in blocking buffer for 2 h. The ears were washed three times, incubated with α-rat Alexa-488 secondary antibody and streptavidin-647 and washed three times. All the images were captured with upright Zeiss LSM700 confocal microscopy by using Zen black imaging software.

**Image Analyses**

The margins of LYVE1 positive lymphatic vessels (LV) were manually drawn to allow the segmentation of overview images (Figure 2B). For the quantification of LV bound CCL21 (Figure 2C), the average CCL21 intensity overlapping with the LV mask in control and Ext1<sup>ALEC</sup> mice was quantified. For CCL21 gradients (Figure 2D), CCL21 intensity outside of the mask was measured as a function of distance from the nearest LV margin.

For the quantification of interstitial DC migration (Figure 2E), DCs were identified by thresholding TAMRA channel images. The distance of each identified DC was measured to the nearest LV margin. As a control of random DC distribution,
FIGURE 2 | Lymphatic endothelial heparan sulfates are not required for chemokine CCL21 gradient formation. (A) High magnification and (B) overview images of non-permeabilized CCL21 (white) and LYVE1 (green) stained control dermis or CCL21 (white) stained and EGFP (green) expressing Ext1^{ALEC} dermis. Yellow arrow indicates extracellular CCL21 deposits, which possibly represent the sites of DC triggered CCL21 secretion (5). Scale bars 20 and 200 µm, respectively. (C) Bar graph shows mean (+/− SD, p-value = 0.055) CCL21 intensity at the lymphatic vessel i.e. CCL21 staining overlapping with LYVE1 staining or EGFP signal (green). N= 8 independent control and Ext1^{ALEC} mouse ears. (D) Line graph shows a quantification of the mean (+/− SD) interstitial CCL21 intensity in control (black line) and Ext1^{ALEC} (red line) mouse ear dermis as a function of distance from the nearest lymphatic vessel margin. N= 4 independent control and five Ext1^{ALEC} mouse ears. (E) Images show LYVE1 stained (green) lymphatic vessels and TAMRA labeled DCs (white) after 60’ of migration. The associated bar graph shows mean (+/− SD) migration efficiency of DCs toward lymphatic capillaries in control and Ext1^{ALEC} ears (p-value = 0.93). The Ccr7^{−/−} DCs are unable to sense CCL21 and thus show random distribution (p-value<0.001). N=14 independent ears for “wt on control” 12 for “wt on Ext1^{ALEC}” and 6 for “Ccr7^{−/−} on control”. Scale bar 200 µm.
the LV mask was rotated 90° in relation to the DC image and distances were quantified. The efficiency of migration was evaluated by dividing the real mean distance of DCs to the LV by the mean distance of the control measurement. Finally, results were presented as normalized to the average of wild type DCs on control ears.

**FITC Painting and Analyses of Lymph Node Cellularity**

Ten percent FITC stock solution was dissolved 1:5 in a 1:1 mix of Acetone and DBP (Dibutyl-Phthalate). The 5 weeks old 4-OHT treated control (Ext1
<sup>ox/fl</sup>) or Prox1CreERT2;Ext1
<sup>fl/fl</sup> (Ext1
<sup>LEC</sup>) mice were anesthetized with isoflurane and both the ventral and dorsal sides of the ears were painted with 25 µl of the FITC suspension. Seventy-two hours later, cervical lymph nodes were harvested and placed on ice in RPMI1640 supplemented with 10% FCS and 5 mM EDTA. smashed, centrifuged and resuspended in RPMI1640 supplemented with 10% FCS and 5 mM EDTA. The 3*10<sup>6</sup> cells/96well were blocked with FITC suspension. Seventy-two hours later, cervical lymph nodes and total lymph node cellularity were quantified by dividing the real mean distance of DCs to the LV mask was rotated 90° in relation to the DC image and distances were quantified. The efficiency of migration was evaluated by dividing the real mean distance of DCs to the LV by the mean distance of the control measurement. Finally, results were presented as normalized to the average of wild type DCs on control ears.

**Statistics**

Normality of the data was tested with Shapiro-Wilk and Kolmogorov-Smirnov tests and the statistical significance was tested with the Student’s t-test with two-tailed distribution and Welch’s correction (Prism software, GraphPad software).

**RESULTS**

To study the contribution of lymphatic endothelial heparan sulfates to CCL21 gradient formation, we specifically prevented HS production in LECs by deletion of Ext1, a rate-limiting gene in the HS synthesis pathway. Lack of Ext1 expression leads to a loss of HSs in vivo and in vitro (18, 21, 22). To induce the deletion of Ext1 in the mouse ear dermis lymphatic endothelium, we generated Prox1CreERT2;Ext1<sup>fl/fl</sup>;mTmG mice, in which tamoxifen-induced recombination is reported by a switch on of EGFP and switch off of tdTomato (Figures 1A–C). Indeed, upon tamoxifen treatment, EGFP was observed exclusively in the lymphatic endothelium (Figure 1B), indicating that Prox1CreERT2 has been expressed specifically, and at sufficient levels, in all the LECs of lymphatic capillaries. Genotyping of the extracted mouse ear dermal LECs, marked by EGFP, detected only the deleted Ext1 allele whereas the non-recombined cell types contained only the non-deleted Ext1<sup>fl</sup> allele (Figure 1C). Wholemount immunofluorescence staining of mouse ears demonstrated that HSs specifically decorate lymphatic and (more intensely) blood endothelium of control mice. In line with the lymphatic endothelial-specific Ext1 deletion, HSs were absent (Figures 1C, D). These results suggest that LECs are the major source of perilymphatic endothelial HS-carrying proteins.
To study the consequence of loss of lymphatic endothelial produced HSs on CCL21 presentation by LECs in vivo, we wholemount stained the exposed mouse ear dermis. Extracellular CCL21 levels at the lymphatic capillary of Ext1\textsuperscript{ALEC} mice were only modestly decreased in comparison to control mice (Figures 2A–C). Thus, in contrast to earlier in vitro results (14, 16, 17), lymphatic endothelium produced HSs are largely dispensable for CCL21 binding to lymphatic capillaries in vivo.

Analyses of the interstitial CCL21 intensity showed similar CCL21 gradient shape in control and Ext1\textsuperscript{ALEC} mice (Figures 2B, D). Next, we functionally validated these data by ex vivo DC migration assays. Activated DCs were loaded onto exposed dermis of split ears, which triggers directed migration of DCs toward the lymphatic capillaries (4). Wild type DCs were as effective in approaching the lymphatic capillaries in control and Ext1\textsuperscript{ALEC} ears whereas Cer7 deficient DCs, which are unable to sense CCL21, were randomly distributed in control ears (Figure 2E).

To complement our ex vivo findings, we compared DC homing to lymph nodes of control and Ext1\textsuperscript{ALEC} mice 72 h after FITC-painting. The proportion of FITC+ migratory DCs of total lymph node cellularity and total lymph node migratory DCs was comparable in control and Ext1\textsuperscript{ALEC} mice (Figures 3A, B). Importantly, also the proportion of the FITC+ epidermal subpopulation of DCs, the Langerhans cells, was similar in control and Ext1\textsuperscript{ALEC} mice when compared to the lymph node total cellularity or lymph node migratory DCs (Figures 3C, D). Since the peak of the Langerhans cell homing to the draining lymph nodes is at day 4 after immunization (23), this result shows that the first Langerhans cells arrive in lymph nodes efficiently in time also in Ext1\textsuperscript{ALEC} mice, further supporting the existence of normal CCL21 guidance cues in the absence of lymphatic endothelial HSs. Also, the absolute number of lymph node total migratory DCs (FITC+ and FITC–) was unaltered upon deletion of Ext1 (Figure 3E).

DISCUSSION

Our studies show that, in our model system, LEC produced HSs are dispensable for the formation of the mesenchymal CCL21 gradient (Figures 2B, D). We deleted Ext1 before the establishment of a mature dermal lymphatic vessel network, which resulted in a drop of HSs, both at the LEC surface and basement membrane, below the detection limit (Figure 1D). Earlier, it has been shown that binding of CCL21 on the LEC surface in vitro is dramatically reduced upon Ext1 deletion (17). We show that in vivo Ext1 deletion results only in a modest reduction of the lymphatic capillary associated CCL21 (Figures 2A–C). Thus it is conceivable that also in vivo HSs are needed for CCL21 binding to the LEC surface, but that the peri-lymphatic capillary matrix, in an HS-independent manner, is the major site of CCL21 anchoring. In support of CCL21 binding on the extracellular matrix at the lymphatic capillary, an earlier study showed that lymphatic capillary associated CCL21 deposits are removed by collagenase treatment (24). The candidate molecules mediating CCL21 anchoring to the cell-matrix in the absence of HS include basement membrane component collagen IV and proteins bearing chondroitin sulfate B and E moieties (14, 15). Interestingly, gradient formation of Indian hedgehog is regulated by both HSs and chondroitin sulfates in mice in vivo (13, 25).

We show that in our model system LEC-produced HSs are dispensable for DC migration toward lymphatic capillaries (Figure 2E). In contrast, in an earlier study by Bao et al., TEEK-Cre-deleter driven Ext1 deletion in blood and lymphatic endothelium and leukocytes was shown to cause attenuated homing of intradermally injected wild type bone marrow-derived DCs to lymph nodes (17). It is conceivable that blood endothelium or leukocyte HSs are needed for dispersion or sequestration of systemic signals, which affect DC activation and/or migration to lymphatic vessels. In studies by Yin et al., lymphatic endothelial HS production was prevented by deletion of Ndst1, an enzyme downstream of EXT1 in the HS synthesis pathway, whose deletion results in altered HS fine structure by preventing N-sulfation. Deletion of lymphatic endothelial Ndst1 resulted in a decreased xenograft tumor cell metastases to lymph nodes and oxazolone induced lymph node homing of DCs possibly via defective CCL21 oligomerization and/or promotion of CCR7-CCL21 interaction rather than anchoring to the matrix (16, 26). It is noteworthy that DC CCL21-CCR7 signaling is dependent on the polysialylation of the receptor (27). Thus, it is conceivable that non-sulfated HSs glycan backbones, created upon Ndst1 deletion, could interfere with sialic acid-induced conformational changes in CCL21. Our studies also differ in terms of the onset of the prevention of the HS synthesis. Whereas we deleted Ext1 at early postnatal development to prevent LEC produced HS deposition to the lymphatic capillary basement membrane, Yin et al. deleted Ndst1 only after maturation of the dermal lymphatic capillary network and deposition of the basement membrane (26, 28).

Recently, Arokiasamy et al. showed a dramatic reduction in HS coverage of lymphatic capillaries upon inflammatory stimulus (29). Interestingly, a decrease in the HS coverage of lymphatic capillaries was essential for efficient tissue fluid drainage, but, importantly, not CCL21 dependent migration and entrance of neutrophils to lymphatic capillaries (29, 30). Together with our study, these results show that CCL21 dependent leukocyte migration cues are not affected by the changes in lymphatic endothelial HSs. However, it is conceivable that the HS may regulate DC trafficking in a CCL21 independent manner via mechanisms that were not captured by our model system, for example via tissue fluid drainage.

In conclusion, we show that prevention of the HS production at the source of the CCL21 production does not prevent handing over of the CCL21 chemokine to the mesenchymal HSs (Figure 4), which are necessary for the interstitial CCL21 anchoring and gradient (4). To our knowledge, earlier studies showing a necessary role for HSs in growth factor/morphogen gradient formation have deleted HS production in all the cell-types or a
clone of all the cells. Here, we have been able to delete HSs only at the source of CCL21 production, leaving HSs at the site of the gradient (interstitium) intact (Figure 4). These results highlight the significance of the microenvironment on CCL21 binding and show that not the molecular identity but rather the presence of any diffusion limiting interactions at the lymphatic capillary is sufficient for regulation of CCL21 gradient shape and decay length.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Austrian Federal Ministry of Science, Research and Economy (identification code: BMWF-66.018/0005-II/3b/2012).

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AUTHOR CONTRIBUTIONS

KV and MS conceptualized the study. KV and CM performed the experiments. KV and RH analyzed the data. KV wrote the manuscript with contributions from MS. All authors contributed to the article and approved the submitted version.

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