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A Critical Role for Dendritic Cells in the Formation of Lymphatic Vessels within Tertiary Lymphoid Structures

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Lymphatic vessels are necessary for the maintenance of tissue fluid and protein balance (1). They also play an essential role in initiating the immune response, by directing leukocytes and Ags from tissues to the lymph nodes (2). Recent studies have described the formation of new lymphatic vessels in several inflammatory conditions, including corneal inflammation (3, 4), renal transplant rejection (5–7), inflammatory bowel disease (8), and chronic airway inflammation (9). We have recently shown that new lymphatic vessels are also formed within organized lymphoid aggregates present in autoimmune thyroiditis (10). Similar lymphoid aggregates are found in chronic inflammatory diseases such as rheumatoid arthritis, Sjögren’s syndrome, and diabetes mellitus (11–16). Lymphatic vasculature is frequently observed within these aggregates.

Postnatal lymphangiogenesis is thought to occur primarily by the sprouting of lymphatic endothelial cells from the pre-existing lymphatic vessels. Recent evidence, however, suggests that circulating lymphatic endothelial progenitor cells also contribute to lymphangiogenesis, particularly in the setting of inflammation (17). In renal transplants, de novo lymphangiogenesis involves both incorporation of recipient-derived lymphatic progenitors and pre-existent endothelial cells (7, 17). These lymphatic endothelial precursors appear to originate from the bone marrow, but their exact identities have not been established. More recently, lymphangiogenesis has been described in draining lymph nodes following immunization and shown to be dependent on the entry of B cells into the node (18, 19). Other studies suggest that dendritic cells (DCs) have a role in endothelial cell differentiation (20). DCs that have migrated to the lymph node promote vascular growth by inducing CD31+ endothelial cell proliferation (21, 22). A possible mechanism underlying the lymphangiogenesis in inflammation may be the production of vascular endothelial growth factor C (9). Together these studies suggest that inflammation-induced lymphangiogenesis involves complex interactions between the vascular endothelium and cells of the immune system. However, the nature of these interactions remains unclear, primarily because of the large number of variables in the models examined to date.

Expression of CCL21 in the thyroid gives rise to formation of lymphoid follicles containing segregated T and B cell zones, which closely resemble those found in Hashimoto’s thyroiditis (23, 24). Within these lymphoid follicles, a vascular network composed of lymphatic vessels and peripheral node addressin-positive high endothelial vessels (HEVs) is observed (10, 23). CCL21-induced formation of this differentiated vasculature results from stepwise recruitment and activation of CD4+ T cells and DCs, with subsequent influx of additional cell subsets (10, 23, 24). Previous work from our group showed that lymphotxin β receptor (LTβR) signaling is involved in the formation of lymphatic vessels in these structures (10). The membrane-anchored heterotrimer LTα1β2 binds to LTβR (25). Lymphotxin α (LTα)-deficient mice (LTα<sup>ko/ko</sup> mice), which lack both LTα homotrimer and LTαβ2, and LTβR-deficient CCL21-transgenic mice do not develop lymphatic vessels within the thyroid aggregates. These results suggest that lymphangiogenesis is triggered by ligation of LTβR by LTαβ2-expressing cells recruited into the thyroid. In this article, we investigate the mechanisms underlying these responses and show that DCs promote the development of a lymphatic network in tertiary lymphoid tissue induced by expression of CCL21.

Materials and Methods

Mice

Mice expressing CCL21 under the control of the thyroglobulin promoter (TG/CCL21 mice) were previously described (23). RAG<sup>1<sub>ko/ko</sub></sup>, LT<sup>α<sub>ko/ko</sub></sup>, and CD11c-diphertheria toxin receptor (DTR) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Id2<sup>ko/ko</sup> mice were a gift from Dr. Y. Yokota (Department of Molecular Genetics, School of Medicine, Mount Sinai School of Medicine, New York, NY). Id2<sup>ko/ko</sup> mice were a gift from Dr. Y. Yokota (Department of Molecular Genetics, School of Medicine, Mount Sinai School of Medicine, New York, NY).
University of Fukui, Fukui, Japan). All mice were housed under specific-pathogen-free conditions in individually ventilated cages at the Mount Sinai School of Medicine Animal Facility. All experiments were performed following institutional guidelines.

**Immunostaining**

Tissue samples were embedded in OCT buffer (Sakura) and snap frozen in 2-methylbutane (Merck) chilled in dry ice. Cryostat sections (8 μm) were fixed in acetone, blocked, and incubated with primary Abs in a humidified atmosphere for 1 h at room temperature. After washing, conjugated secondary Abs were added and then incubated for 35 min. The slides were next washed and mounted with Fluoromount-G (Southern Biotech). Alternatively, tissues from adaptively transferred animals were fixed in 1.6% paraformaldehyde (Electron Microscopy Science) containing 20% sucrose for 20 h at 4°C. Images were captured using a Nikon fluorescence microscope and processed using Adobe Photoshop version 7.0.

Primary Abs used were anti-CD45 (30-F11), -CD3 (I45-2C11), -CD4 (LJT4), -CD11c (HL3) (eBioscience), and -LYVE-1 Ab (Abcam). Secondary Abs used were Alexa Fluor 488 and 594 goat anti-rat IgG, Alexa Fluor 594 goat anti-rat IgM, Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes); Cy3 goat anti-Armenian hamster and Cy5 goat anti-rat (Jackson ImmunoResearch Laboratories).

**Morphometric analysis of lymphatic vasculature**

Thyroids of recipient mice were collected, sectioned, and stained with Abs to CD45 (to visualize leukocytes) and Lyve-1 (to visualize lymphatic vessels). The whole thyroid was sectioned, and 16–25 representative sections were used for image analysis. The area occupied by lymphatic vessels in the thyroid was quantified using the Volocity software (Invision). This software identifies signal by thresholding key intensity values. The thyroid area and the area occupied by Lyve-1 vessels were determined. The lymphatic vessel area (LVA) was calculated by averaging the area occupied by cells positive for Lyve-1 in the thyroid. The unpaired Student t test was used for statistical analysis.

**Flow cytometry**

Thyroid single-cell suspensions were prepared as previously described (23). Cells were stained with selected Abs for 40 min on ice and analyzed in a FACScanto cytometer (BD Biosciences, Franklin Lakes, NJ). Directly conjugated Abs against CD4 (GK1.5), CD11b (M17/70), NK1.1 (PK136), and MHC II (M5/114.15.2) were obtained from eBioscience. Anti-CD11c Ab (HL3) was obtained from BD Biosciences. All flow cytometry studies were analyzed with FloJo software (TreeStar).

**Cell purification and transfer**

A single-cell suspension of splenocytes from C57BL/6 mice was prepared and incubated with anti-CD4 beads (Miltenyi Biotec). Cells were then washed and passed through a magnetic cell-sorting column (Miltenyi Biotec). Alternatively, tissues from adaptively transferred animals were washed and passed through a magnetic cell-sorting column (Miltenyi Biotec). The resulting fraction contained >95% CD45+ T cells. A total of 1 × 10^6 CD4+ T cells were resuspended in 100 μL PBS and injected into the retro-orbital sinus of recipient animals.

**DC ablation**

For systemic DC depletion, RAGTCGCL21/CD11c-DTR transgenic mice were injected i.p. with diphtheria toxin (DT; D-2918; Sigma-Aldrich) at a dose of 2 ng/g body weight on days 4 and 6 after adoptive transfer of CD4+ T cells. Efficient CD11c-GFP+ cell depletion from the peripheral blood was assessed by FACS analysis.

**Ab blocking and depletion**

To deplete NK cells, we used a purified anti-NK1.1 Ab (PK136; Bioxcell). This Ab was injected i.v. (200 μg per animal) into RAGTCGCL21 mice on days −4, −1, and 2 after adoptive transfer of T cells. Control mice received injections of the same amount of normal mouse IgG Ab (Bioxcell). Depletion of NK cells was confirmed on the day of the experiment by flow cytometric analysis of the peripheral blood.

**Analysis of mRNA expression**

Total RNA was extracted from the thyroid, using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed with 2 μg total RNA. Quantitative PCR (qPCR) was conducted in duplicates, using 25 μg reverse-transcribed cDNA and 0.4 μM each primer in a 30-μL final reaction volume containing 1 × SYBR Green PCR Master Mix (Applied Biosystems). PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and of 60°C for 1 min. Relative expression levels were calculated as 2^(-ΔΔCt) (for details see ABI PRISM 7700—User Bulletin #2), using ubiquitin RNA as endogenous control. Primers were designed with Primer Express 2.0 software (Applied Biosystems). Primer sequences were as follows: ubiquitin (f = 5′-TGGCTTATATTATTGCTGCTAC-3′; r = 5′-GCCAAGTGGCTTAGTGCAAGTAA-3′), granzyme B (f = 5′-CCCCAGGCGCAATGTCAAT-3′; r = 5′-CCCCAACCAGCCACCATAGC-3′), LTF (f = 5′-CCAGGAGCCAGCCCATCCACT-3′; r = 5′-GGTACCAACAAAGTGGAGCAGC-3′), and LTB (f = 5′-ACCTCATAGGGCTGTTGATG-3′; r = 5′-AGGCTTCTTCTTGGGCTGC-3′).

**Results**

CD4+ T cells induce recruitment of host DCs and NK cells into the thyroid of RAGTCGCL21 mice

We have previously shown that transfer of CD4+ T cells into RAG mice expressing CCL21 in the thyroid (referred to as RAGTGGCL21 mice) leads to development of new lymphatic vessels and peripheral node addressin-positive HEVs (10, 24). To better understand the phenotype of the cells involved in this process, we transferred CD4+ T cells to RAGTCGCL21 mice and examined the composition of the thyroid cell infiltrates 5 d after transfer by flow cytometry (Fig. 1). Single-cell suspensions of thyroids from RAGTGGCL21 mice transferred with CD4+ T cells were prepared as described (10). Leukocytes (CD45+ cells) represented ~0.85% of all live cells isolated from the thyroids of un.injected RAGTGGCL21 and ~2–3% of cells in the thyroids of RAGTGGCL21 injected with CD4+ T cells (not shown). The leukocytes that infiltrated the thyroids of the injected RAGTGGCL21 mice were donor CD4+ T cells (9%) and CD11c+ cells (70%). Most of the DCs expressed the myeloid marker CD11b+ and were mature (expressed high levels of MHC II). Finally, 12% of the CD11c+ cells in the thyroid expressed the NK1.1 marker, indicating their NK origin. Thus, adoptive transfer of CD4+ T cells into RAGTGGCL21 mice induced the migration of host DCs and NK cells into the thyroid.

![FIGURE 1. CD4+ T cells induce recruitment of DCs and NK cells into the thyroid of RAGTGGCL21 mice](http://www.jimmunol.org/Downloaded/from/2017/10/4.png)
**LTα expression by incoming T cells is not required for lymphangiogenesis in the thyroid**

The LTβR-signaling pathway is critical for the development of lymphatic vessels in the thyroid gland in response to CCL21 expression (10). We have previously shown that LTβR is expressed by endothelial cells, HEV, and lymphatic vessels in the thyroid of TGCGCL21 mice (10). LTα is constitutively expressed by lymphocytes and by cells of the myeloid lineage upon activation (26), suggesting that cells infiltrating the thyroid could provide for the ligands necessary for lymphatic vessel formation.

Lymphocytes are the major source of LTα and LTβ in the spleens of naive mice (27). Thus, we first asked if LT production by T cells was important for vessel differentiation in the thyroid of RAGTGCCL21 animals. CD4+ T cells were sorted from the spleens of wild-type and LTα-deficient animals and injected into RAGTGCCL21 animals (1 × 10^6 CD4+ cells per animal). At 10 d after cell transfer, thyroids were collected, sectioned, and stained with Abs to Lyve-1 (to visualize lymphatic vessels) and CD45 (to visualize leukocytes). Thyroids from RAGTGCCL21 mice transferred with CD4+ T cells obtained from LTα^wt/wt or from LTα^ko/ko mice were infiltrated by CD45^+ cells (Fig. 2B, 2C). These infiltrates were composed of CD4+ T cells and host CD11c^+ cells (not shown), indicating that the absence of LTα expression on T cells did not affect their capacity to migrate to CCL21-expressing thyroids. We then evaluated whether absence of LTα production by incoming T cells affected the de novo formation of lymphatic vessels. Lymphatic vessels, stained with Abs for Lyve-1, were prominent in the thyroid of animals injected with CD4+ T cells derived from LTα^wt/wt and from LTα^ko/ko mice (Fig. 2B, 2C), but not in the thyroid of uninjected RAGTGCCL21 mice (Fig. 2A). To confirm these observations, we measured the LVA in the thyroids of lymphocytes in each experimental group by computer-assisted morphometric analysis of digital images (Fig. 2D). The total area occupied by lymphatic vasculature in the thyroid of RAG and RAGTGCCL21 mice was similar (~1% of total thyroid area) (Fig. 2D), confirming our previous results that expression of CCL21 in the thyroid does not induce new lymphatic vessel growth in the absence of T cells (10). As expected, transfer of CD4+/LTα^wt/wt T cells led to a pronounced increase in the area occupied by lymphatic vessels in the thyroid (~2% of total thyroid area). Transfer of CD4+/LTα^ko/ko cells promoted changes in lymphatic area density that were similar to those elicited by transfer CD4+/LTα^wt/wt T cells. These results indicate that LTα production by T cells is not directly involved in de novo formation of lymphatic vessels in this model.

**LTα expression by host cells is required for lymphangiogenesis in the thyroid**

Next we evaluated whether LTα expression by host cells played an important role in lymphatic vessel differentiation. To accomplish this, we generated LTα-deficient RAGTGCCL21 animals (referred to as RAGTGCCL21/LTα^ko/ko mice). Using an approach similar to that described above, we sorted CD4+ T cells from the spleen of wild-type mice and injected them into RAGTGCCL21/LTα^wt/wt and RAGTGCCL21/LTα^ko/ko recipient animals (1 × 10^6 CD4+ cells per animal). At 10 d after cell transfer, we collected thyroids and processed them for histologic examination. Sections were stained with Abs to CD4 (to visualize T cells), to CD11c (to visualize DCs), and to Lyve-1 (to visualize lymphatic vessels) (Fig. 3). Thyroids from RAGTGCCL21/LTα^wt/wt (Fig. 3A) and RAGTGCCL21/LTα^ko/ko (Fig. 3B) mice transferred with wild-type cells showed infiltrates composed of CD4+ T cells and CD11c+ DCs. These results indicate that lack of LTα production by host cells did not impair the capacity of leukocytes to migrate to CCL21-expressing thyroids.

Despite the presence of leukocytes in the thyroid, RAGTGCCL21/LTα^ko/ko animals transferred with CD4+ T cells showed reduced LVA (Fig. 3D) when compared with RAGTGCCL21/LTα^wt/wt animals (Fig. 3C). The area occupied by lymphatic vasculature in the thyroid of RAGTGCCL21/LTα^wt/wt was ∼2.3%. In contrast, the area occupied by lymphatic vasculature in the RAGTGCCL21/LTα^ko/ko animals was ∼1.3% (Fig. 3E), a value similar to that seen in the thyroid of RAGTGCCL21 mice that did not receive CD4+ T cells (Fig. 2D). These results indicate that LTα expression by host cells is required for CD4 T cell-induced lymphangiogenesis in the thyroid.

**Depletion of NK cells does not inhibit lymphangiogenesis in the thyroid of RAGTGCCL21 mice**

We have shown in Fig. 1 that host DCs and NK cells migrate to CCL21-expressing thyroids after adoptive transfer of CD4+ T cells. To determine whether LTα production by NK cells is involved in lymphatic vessel differentiation, we depleted NK cells from RAGTGCCL21 mice. To do so, we injected RAGTGCCL21 mice with anti-NK1.1 monoclonal PK136 Ab, which depletes NK1.1-positive cells in vivo. RAGTGCCL21 mice were treated with control or PK136 Ab (days −4 and −1) before CD4+ T cell injection. At 2 d after CD4+ T cell transfer (1 × 10^6 cells), animals received another injection of control or PK136 Ab (Fig. 4A). On day 10 the peripheral blood was collected and stained with CD49b to assess NK cell depletion in the periphery. Treatment of RAGTGCCL21 with anti-PK136 Ab caused a dramatic reduction (10-fold) in the number of circulating NK cells when compared with the control group (Supplemental Fig. 1A). To assess depletion of NK cells in the tissue, we examined expression of the NK-related molecule granzyme B, in the thyroid, by qPCR (28). Expression of granzyme B was markedly reduced in the thyroids of RAGTGCCL21 mice transferred with CD4+ T cells and treated...
with anti-NK Ab when compared with the control group (Supplemental Fig. 1B). These results indicate efficient depletion of NK cells in the thyroid of RAGTGCCL21 mice treated with PK136 Ab.

To assess the impact of NK cells in the formation of new lymphatic vessels, thyroid of RAGTGCCL21 mice transferred with CD4+ T cells and treated with control or PK136 Ab were collected, sectioned, and stained with Abs to CD45 and LYVE-1. Analysis of the LVA showed that lymphangiogenesis was not impaired by depletion of NK cells in the thyroid (Fig. 4B). These results suggest that NK cells do not contribute significantly to lymphatic vessel differentiation in the thyroid.

Animals missing the Id2 gene lack Peyer’s patches and lymph nodes, and have a severe defect in the production of NK cells (29). We have previously shown that CCL21-driven formation of lymphoid aggregates does not require Id2 (24). To further evaluate the role of NK cells in lymphangiogenesis in the thyroid, we crossed TGCCCL21 mice to Id2−/− mice to generate TGCCCL21/Id2−/− mice, and analyzed the expression of Lyve-1 and Prox-1 in their thyroid by immunostaining. We observed that Lyve-1- and Prox-1- lymphatic vessels were present within the lymphoid aggregates in the thyroid of TGCCCL21/Id2−/− mice (Fig. 4D, 4F). The distribution of the lymphatic vasculature in TGCCCL21/Id2−/− mice was similar to that observed in TGCCCL21/Id2+wt/mice (Fig. 4C, 4E). Together, these results indicate that NK cells are not required for formation of lymphatic vessels in the thyroid.

**Depletion of DCs inhibits lymphangiogenesis in the thyroid**

To assess whether DCs induce LTα-mediated lymphangiogenesis in the thyroid, we took advantage of a transgenic mouse model that allows the inducible ablation of all conventional CD11c+ DCs (30). In these mice, the simian DTR is expressed as a fusion protein with GFP under the transcriptional control of the CD11c promoter. Injection of DT leads to the ablation of all CD11c+DCs, but not of NK cells (31). RAGTGCCL21 mice were repeatedly crossed to CD11c-DTR mice to generate RAGTGCCL21/CD11c-DTR mice. RAGTGCCL21/CD11c-DTR mice were injected with CD4+ T cells (1 × 10^6 CD4+ cells per animal). At 4 d after transfer, a group of mice received PBS and a second group received two doses of DT (2 ng/g) every 2 d (Fig. 5A). At 10 d after cell transfer, thyroids were collected, sectioned, and stained with Abs to CD11c, CD45, and LYVE-1. DC depletion was assessed in the peripheral blood by flow cytometry (Supplemental Fig. 2), and in the thyroid, by immunostaining, 10 d after transfer (Fig. 5C). DCs represented ~5.8% of CD45+ cells in the blood of RAGTGCCL21/CD11c-DTR animals treated with PBS. The number of DCs in circulation was reduced to 0.6%...
DCs recruited into the thyroid express LT ligands

The fact that both DC and LT signaling are important for lymphangiogenesis suggests that DCs recruited in the thyroid may affect the development of lymphatic vessels via production of LT ligands. To test whether DCs express LTα, we performed qPCR on DCs (NK1.1+CD11c+) sorted from the spleens of RAG<sup>ko/ko</sup> RLT<sup>a/wt</sup> (referred to RLTα<sup>a/wt</sup>) and RAG<sup>ko/ko</sup>/LTα<sup>ko/ko</sup> (referred to RLTα<sup>ko/ko</sup>) mice, and performed qPCR for LTα. As shown in Fig. 5G, expression of LTα was absent in splenic DCs purified from RLTα<sup>ko/ko</sup>, but present in DCs from RLTα<sup>a/wt</sup> mice. These results indicate that LTα is expressed by DCs. To test whether LT ligands were expressed by DCs, we sorted NK1.1+CD11c+ cells from the thyroid and lymph nodes 10 d after injection of CD4<sup>+</sup> T cells and tested the expression of LTα and LTβ by qPCR. Expression of LT ligands could be readily detected in DCs infiltrating the thyroid. These findings document expression of LT ligands in DCs recruited into the thyroid after adoptive transfer of T cells.

Discussion

Our previous studies have shown that expression of CCL21 by thyrocytes promotes recruitment of CD4<sup>+</sup> T cells from circulation (23), clustering of T cells and DCs, and formation of HEVs (24) and lymphatic vessels in the thyroid (10). In this article, we report that lymphatic vessel formation under these conditions is dependent on DCs recruited from the periphery.

To study the mechanisms of inflammatory lymphangiogenesis, we used a reductionist model in which adoptive transfer of CD4<sup>+</sup> T cells promotes development of lymphatic vessels in the thyroid of RAGTGCCL21 mice. Our results indicate that the development of the lymphatic vasculature is dependent on the recruitment of peripheral cells by CCL21, rather than on the direct activity of CCL21 on the vasculature. The LVA in the thyroid is similar in both RAG and RAGTGCCL21 mice prior to the transfer of T cells, and the recruitment of cells from the peripheral blood precedes the growth of new lymphatic vessels in the thyroid. These findings strongly suggest a role for bone marrow-derived cells in the formation of new lymphatic vessels and are in agreement with other studies reported in the literature, implicating macrophages, DCs, and B cells in lymphangiogenesis (4, 5, 9, 18–21).

In our adoptive transfer model, cellular complexity in the site of lymphangiogenesis can be reduced to the endothelium, stroma, and incoming cells (T cells, DCs, and NK cells). Endothelial cells in the thyroid constitutively express LTβR, and animals deficient in both LTβR and its ligand LTαβ show reduced number and size of lymphatic vessels in the thyroid (10). As LTβR-mediated signaling is involved in lymphangiogenesis, we were therefore interested in determining the nature of the LT-producing cells that trigger the development of new lymphatic vasculature. Entry of CD4<sup>+</sup> T cells into the thyroid of RAGTGCCL21 mice induces host DC and NK cell migration to tissue and the formation of new lymphatic vessels within 10 d. Membrane LTαβ is detected in T, B, and NK cells (27, 32), whereas LTβ mRNA is expressed by several DC subsets (33). In addition, LT ligands were also shown to be expressed by human DCs (34). Thus, CD4<sup>+</sup> T cells, NK cells, and DCs were plausible candidates for expressing LTβR ligands in the thyroid. Our results indicate that LTα production by incoming T lymphocytes is not required for lymphatic vessel formation but that its expression by host cells is critical for lymphangiogenesis. We have shown that vessels in the thyroid were
significantly decreased in LTα-deficient host mice (10). Having ruled out a role for T cells in the process, we investigated the role of host-derived NK cells and DCs. We reasoned that eliminating NK cells and DCs would block LTβR signaling in the thyroid and, consequently, the genesis of new lymphatic vessels. Experiments in which T cells were injected into NK-depleted RAGTGCCL21 animals revealed that the formation of new lymphatic vessels was not disturbed. Furthermore, as shown in this article, lymphoid aggregates rich in lymphatic vessels are found in the thyroid of LTα-deficient TGCL21/Idx<sup>ko</sup>ko</sup> mice. As Id2-deficient mice also lack lymphoid tissue inducer cells (29), we conclude that the development of tertiary lymphoid organs (TLOs), and in this model, lymphangiogenesis occurs independently of the presence of NK and lymphoid tissue inducer cells.

In contrast to the results obtained with NK cell depletion, DC depletion markedly inhibited the lymphangiogenesis induced by T cells. These results were similar to those obtained in the LTα-deficient hosts, suggesting that elimination of DCs abrogated a critical pathway in the genesis of new lymphatic vessels. DCs are required for formation and maintenance of BALt (35, 36) and for ectopic lymphoid structures formed in atherosclerotic plaques (37). DCs are also important for retention of B and T cells in the TLO, through a mechanism involving LTβ production (36). Our results show that DCs can also contribute to vascular differentiation in tertiary lymphoid structures. On the basis of findings described in this article, we suggest a model in which the formation of new lymphatic vessels is dependent on the influx of CD11c<sup>+</sup> DCs into the thyroid. As shown by our group previously, subsequent to the entry of CD4<sup>+</sup> T cells into CCL21-expressing thyroids is a marked-up regulation of DC-attracting inflammatory chemokines, such as CCL2, CXCL10, and CXCL9 (24). These chemokines could originate from the incoming CD4<sup>+</sup> T cells, from stromal cells, or from the endothelium, and facilitate recruitment of DCs to the tissue. Thus, upregulation of DC-recruiting chemokines and activation of LTβR signaling could be mechanistically important for the formation of new lymphatic vasculature in TLOs. Because LT ligands are critical for the development of lymphatic vessels and because DCs express the ligands, it is likely that LT expressed by DCs is critical for the response we observed. The expression of LT ligands by DCs could act on LTβR-expressing endothelial cells, stromal cells, or incoming DCs. These interactions could allow for differentiation of lymphatic vessels directly (via activation of LTβR on endothelial cells) or indirectly (via activation of LTβR on stromal cells or incoming DCs, as well as production of proinflammatory and lymphangiogenic factors). Delination of the mechanisms triggered by DC influx into tissue is likely to aid in the identification of novel therapeutic targets in lymphangiogenesis.

**Disclosures**

The authors have no financial conflicts of interest.

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