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A critical role for dendritic cells in the formation of lymphatic vessels within tertiary lymphoid structures

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Abstract

Ectopic, or tertiary lymphoid aggregates often form in chronically inflamed areas. Lymphatic vessels, as well as high endothelial venules, form within these lymphoid aggregates, but the mechanisms underlying their development is poorly understood. Overexpression of the chemokine CCL21 in the thyroid of transgenic mice leads to formation of lymphoid aggregates containing topologically segregated T and B lymphocytes, dendritic cells and specialized vasculature, including Lyve-1^+/Prox-1^+ lymphatic vessels. In this report we show that adoptive transfer of mature $CD4^+$ T cells into animals expressing CCL21 in a RAG-deficient background, promotes the influx of host NK cells and DC into the thyroid and the formation of new lymphatic vessels within 10 days. This process is dependent on the expression of lymphotoxin ligands by host cells, but not by the transferred $CD4^+$ T cells. Ablation of host DC, but not NK cells, reduces the formation of new lymphatic vessels the thyroid. Taken together, these data suggest a critical role for $CD11c^+$ DC in the induction of lymphangiogenesis in tertiary lymphoid structures.

Keywords

Chemokines; transgenic/knockout mice; lymphatic vessels; dendritic cells; lymphotoxin; tertiary lymphoid organ

Introduction

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 antigens 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, Sjogren's syndrome and diabetes mellitus (11-16). Lymphatic vasculature is frequently observed within these aggregates.

Postnatal lymphangiogenesis is thought to occur primarily by sprouting of lymphatic endothelial cells from the preexisting lymphatic vessels. Recent evidence, however, suggests that circulating lymphatic endothelial progenitor cells also contribute to lymphangiogenesis,

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particularly in the setting of inflammation (17). In renal transplants, *de novo* lymphangiogenesis involves both incorporation of recipient-derived lymphatic progenitors, and preexistent endothelial cells (7, 18). 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 (19, 20). Other studies suggest that DC have a role in endothelial cell differentiation (21). DC that have migrated to the lymph node promote vascular growth by inducing $CD31^+$ endothelial cell proliferation (22, 23). A possible mechanism underlying the lymphangiogenesis in inflammation may be the production of the vascular growth factor VEGF-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 remain unclear, primarily because of the large number of variables in the models examined to date.

Expression of CCL21 in the thyroid induces formation of lymphoid follicles containing segregated T and B cell zones, which closely resembles those found in Hashimoto's thyroiditis (24, 25). Within these lymphoid follicles a vascular network comprised of lymphatic vessels and $PNAd^+$ HEVs is observed (10, 24). CCL21-induced formation of this differentiated vasculature results from stepwise recruitment and activation of $CD4^+$T cells and DC, with subsequent influx of additional cell subsets (10, 24, 25). Previous work from our group showed that LTβR signaling is involved in the formation of lymphatic vessels in these structures (10). The membrane-anchored heterotrimer LTα1β2 binds to LTβR (26). LTα-deficient mice ($LT\alpha^{ko/ko}$ mice), which lack both LTα homotrimer and LTα1β2, 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 LTα1β2-expressing cells recruited into the thyroid. Here we investigate the mechanisms underlying these responses and show that DC induce the development of a lymphatic network in tertiary lymphoid tissue induced by expression of CCL21.

Material and Methods

Mice

Mice expressing CCL21 under the control of the thyroglobulin promoter (TGCCL21 mice) were previously described (24). $RAG1^{ko/ko}$, $LT\alpha^{ko/ko}$, and CD11c-DTR mice were obtained from The Jackson Laboratory, ME. $Id2^{ko/ko}$ mice were a gift from Dr. Y. Yokota. 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 hour at room temperature. After washing, conjugated secondary Abs were added and then incubated for 35 minutes. The slides were then washed and mounted with Fluoromount-G (SouthernBiotech). Alternatively, tissues from adaptively transferred animals were fixed in 1.6% paraformaldehyde (Electron Microscopy Science) containing 20% sucrose for 20 hours 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 (145-2C11), -CD4 (L3T4) -CD11c (HL3) (e-bioscience), and anti-LYVE-1 antibody (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 antibodies 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 vessel 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 was calculated by averaging the area occupied by cells positive for Lyve-1 in the thyroid. The unpaired Student's t-test was used for statistical analysis.

Flow cytometry

Thyroid single-cell suspensions were prepared as previously described (24). Cells were stained with selected antibodies for 40 min on ice and analyzed in a FACSCanto cytometer (Becton Dickinson, Franklin Lakes, NJ). Directly conjugated antibodies against CD4 (GK1.1), CD11b (M17/70), NK1.1 (PK136) and, MHC II (M5/114.15.2), were obtained from e-Bioscience. Anti-CD11c antibody (HL3) was obtained from BD Bioscience. All flow cytometry were analyzed with FloJo software (Tree Star, Inc).

Cell purification and transfer

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). The resulting fraction contained >95% $CD4^+$ T cells. 1×10^6 $CD4^+$ T cells were resuspended in 100 μl of PBS and injected into the retro-orbital sinus of recipient animals.

DC ablation

For systemic DC depletion, RAGTGCCL21/CD11c-DTR transgenic mice were injected i.p. with diphtheria toxin (D-2918; Sigma-Aldrich) with the dose of 2 ng/g body weight on days 4 and 6 days after adoptive transfer of $CD4^+$ T cells. Efficient CD11c-GFP^+ cell depletion from the periphery blood was assessed by FACS analysis.

Antibody Blocking and Depletion

To deplete NK cells we used a purified anti-NK1.1 antibody (PK136, Bioxcell). This antibody was injected i.v. (200μg/animal) into RAGTGCCL21 mice on days -4, -1 and day 2 after adoptive transfer of T cells. Control mice received injections of the same amount of normal mouse IgG antibody (Bioxcell). Depletion of NK cells was confirmed at 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 using 2 μg of total RNA. Q-PCR was conducted in duplicates using 25 μg of reverse-transcribed cDNA and 0.4 μM of each primer in a 30 μL final reaction volume containing 1× SYBR Green PCR Master Mix (Applied biosystems). PCR cycling conditions were: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec, 60°C for 1 min. Relative expression levels were calculated

as 2ˆ(Ct Ubiquitin - Ct gene) (for details see ABI PRISM 7700 - User Bulletin #2) using Ubiquitin RNA as endogenous control. Primers were designed using Primer Express 2.0 software (Applied Biosystems). Primer sequences used were: ubiquitin (f = TGGCTATTAATTATTCGGTCTGCAT; r = GCAAGTGGCTAGAGTGCAGAGTAA), granzyme B (f = CCCAGGCGCAATGTCAAT; r = CCCCAACCAGCCACATAGC), LTα (f = CCAGGACAGCCCATCCACT; r = GTACCCAACAAGGTGAGCAGC) and LTβ (f = ACCTCATAGGCGCTTGGATG; r = ACGCTTCTTCTTGGCTCGC

Results

CD4+ T cells induce recruitment of host DC and NK cells into the thyroid of RAGTGCCL21 mice

We have previously shown that transfer of $CD4^+$ T cells into RAG mice expressing CCL21 in the thyroid (referred to as RAGTGCCL21 mice) leads to development of new lymphatic vessel and $PNAd^+$ HEVs (10, 25). To better understand the phenotype of the cells involved in this process, we transferred $CD4^+$ T cells to RAGTGCCL21 mice and examined the composition of the thyroid cell infiltrates 5 days after transfer by flow cytometry (Fig. 1). Single cell suspensions of thyroids from RAGTGCCL21 mice transferred with $CD4^+$ T cells were prepared as described (10). Leukocytes ($CD45^+$ cells) represented ~ 0.85% of all alive cells isolated from the thyroid of uninjected RAGTGCCL21 and approximately 2-3% of the cells in the thyroid of RAGTGCCL21 injected with $CD4^+$ T cells (not shown). The leukocytes that infiltrated the thyroids of the injected RAGTGCCL21 mice were donor $CD4^+$ T cells (9%) and CD11c+ cells (70%). Most of the DC 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 RAGTGCCL21 mice induced the migration of host DC and NK cells into the thyroid.

Lymphotoxin alpha expression by incoming T cells is not required for lymphangiogenesis in the thyroid

The lymphotoxin (LT)β receptor (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 TGCCL21 mice (10). LTα is constitutively expressed by lymphocytes and by cells of the myeloid lineage upon activation (27), suggesting that the cells infiltrating the thyroid could provide for the ligands necessary for formation of lymphatic vessels.

Lymphocytes are the major source of the LTα and LTβ in the spleens of naive mice (18). 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/animal). Ten days after cell transfer, thyroids were collected, sectioned, and stained with antibodies to Lyve-1 (to visualize lymphatic vessels) and CD45 (to visualize leukocytes). Thyroids from RAGTGCCL21 mice transferred with $CD4^+$ T cells obtained from $LT\alpha^{wt/wt}$ or from $LT\alpha^{ko/ko}$ mice were infiltrated by $CD45^+$ cells (Fig. 2B and 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 antibodies for Lyve-1, were prominent in the thyroid of animals injected with CD4+ T cells derived from $Lt\alpha^{wt/wt}$ and with $LT\alpha^{ko/ko}$ mice (Fig. 2B and 2C) but not in the thyroid of uninjected RAGTGCCL21 mice (Fig. 2A). To confirm

these observations we measured the lymphatic vessel area (LVA) in the thyroid of animals 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 $CD4^+/LT\alpha^{wt/wt}$ T cells led to a pronounced increase in the area occupied by lymphatic vessels in the thyroid (~2% of the total thyroid area). Transfer of $CD4^+/LT\alpha^{ko/ko}$ cells promoted changes in lymphatic area density that were similar to those elicited by transfer $CD4^+/LT\alpha^{wt/wt}$ 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.

Lymphotoxin alpha expression by host cells is required for lymphangiogenesis in the thyroid

Next we evaluated if 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/$LT\alpha^{ko/ko}$ 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/$LT\alpha^{wt/wt}$ and RAGTGCCL21/$LT\alpha^{ko/ko}$ recipient animals (1×10^6 $CD4^+$ cells/animal). Ten days after cell transfer, we collected thyroids, and processed them for histology. Sections were stained with antibodies to CD4 (to visualize T cells), to CD11c (to visualize DC) and to Lyve-1 (to visualize lymphatic vessels) (Fig. 3). Thyroids from RAGTGCCL21/$LT\alpha^{wt/wt}$ (Fig. 3A) and RAGTGCCL21/$LT\alpha^{ko/ko}$ (Fig. 3C) mice transferred with wt cells showed infiltrates composed of $CD4^+$ T cells and $CD11c^+$ DC. 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/$LT\alpha^{ko/ko}$ animals transferred with $CD4^+$ T cells showed reduced LVA (Fig. 3D) when compared to RAGTGCCL21/$Lt\alpha^{wt/wt}$ (Fig. 3B). The area occupied by lymphatic vasculature in the thyroid of RAGTGCCL21/$LT\alpha^{wt/wt}$ was ~2.3%. In contrast, the area occupied by lymphatic vasculature in the RAGTGCCL21/$LT\alpha^{ko/ko}$ 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 DC and NK cells migrate to CCL21 expressing thyroids after adoptive transfer of $CD4^+$ T cells. To determine if 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 antibody, which depletes NK1.1-positive cells in vivo. RAGTGCCL21 mice were treated with control or PK136 antibody (days -4 and -1) before $CD4^+$ T cell injection. Two days after $CD4^+$T cell transfer (1×10^6 cells), animals received another injection of control or PK136 antibody (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 antibody caused a dramatic reduction (10 fold) in the number of circulating NK cells when compared to the control group (Supplemental Figure 1A). To assess depletion of NK cells in the tissue, we examined the expression of the NK related molecule granzyme B, in the thyroid, by Q-PCR (28). Expression of granzyme B was markedly reduced in the thyroids of RAGTGCCL21 mice transferred with CD4+ T cells and treated with anti-NK

antibody when compared to the control group (Supplemental Figure 1B). These results indicate efficient depletion of NK cells in the thyroid of RAGTGCCL21 mice treated with PK136 antibody.

To assess the impact of NK cells in the formation of new lymphatic vessels, thyroids of RAGTGCCL21 mice transferred with $CD4^+$ T cells and treated with control or PK136 antibody were collected, sectioned and stained with antibodies to CD45 and LYVE-1. Analysis of the LVA showed that lymphangiogenesis was not impaired due to the lack 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 lacking the *Id2* gene lack Peyer's patches, 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* (25). To further evaluate the role of NK cells in lymphangiogenesis in the thyroid we crossed TGCCL21 mice to $Id2^{ko/ko}$ mice to generate TGCCL21/$Id2^{ko/ko}$ mice and analyzed the expression of Lyve-1 and Prox-1 in their thyroid by immunostaining. We observed that Lyve-1^+/Prox-1^+ lymphatic vessels were present within the lymphoid aggregates in the thyroid of TGCCL21/$Id2^{ko/ko}$ mice (Fig. 4D and 4F). The distribution of the lymphatic vasculature in TGCCL21/$Id2^{ko/ko}$ mice was similar to that observed in TGCCL21/$Id2^{wt/ko}$ mice (Fig. 4C and 4E). Together, these results indicate that NK cells are not required for formation of lymphatic vessels in the thyroid.

Depletion of DC inhibits lymphangiogenesis in the thyroid

To assess whether DC induces LTα-mediated lymphangiogenesis in the thyroid, we took advantage of a transgenic mouse model which allows the inducible ablation of all conventional CD11c DC (30). In these mice, the simian diphtheria toxin receptor (DTR) is expressed as a fusion protein with GFP under the transcriptional control of the CD11c promoter. Injection of diphtheria toxin (DT) leads to the ablation of all $CD11c^{high}$ DC 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/animal). Four days after transfer, a group of mice received PBS and a second group received two doses of DT (2n/g) every two days (Fig. 5A). Ten days after cell transfer, thyroids were collected, sectioned, and stained with antibodies 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, ten days after transfer (Fig. 5C). DC represented approximately 5.8% of $CD45^+$ cells in the blood of RAGTGCCL21-CD11c-DTR treated with PBS. The number of DC in circulation was reduced to 0.6% after DT treatment (Supplemental Figure 2A). Under these experimental conditions DT treatment did not affect the number of $CD4^+$ T cells in the periphery nor in the thyroid (Supplemental Figure 2B).

To determine if RAGTGCCL21/CD11c-DTR mice that received DT had impaired recruitment of $CD11c^+$ cells to the tissue, we performed immunostaining. The number of DC in the thyroid of RAGTGCCL21/CD11c-DTR mice that received DT (Fig. 5C) was markedly reduced when compared to that of animals injected with PBS (Fig. 5B). Next we evaluated whether DC ablation affected lymphatic vessel formation in the thyroid. RAGTGCCL21/CD11c-DTR mice transferred with $CD4^+$ T cells and treated with DT showed reduced Lyve-1^+ staining in the thyroid (Fig. 5E) when compared to animals treated with PBS (Fig. 5D). More importantly, the thyroid LVA of RAGTGCCL21/CD11c-DTR mice depleted of DC was significantly reduced when compared to that of PBS treated mice (Fig 5F). These results indicate that formation of new lymphatic vessels in the RAGTGCCL21 model requires the presence of DC in the tissue.

DC recruited into the thyroid express lymphotoxin ligands

The fact that both DC and lymphotoxin signaling are important for lymphangiogenesis suggests that DC recruited in the thyroid may affect the development of lymphatic vessels via production of lymphotoxin ligands. To test if DC express LTα we performed qPCR on DC ($NK1.1^{-}CD11c^{+}$) sorted from the spleen of $RAG^{ko/ko}/Lt\alpha^{wt/wt}$ (referred to $RLT\alpha^{wt/wt}$) and $RAG^{ko/ko}/Lt\alpha^{ko/ko}$ (referred to $RLT\alpha^{ko/ko}$) mice and performed qPCR for LTα. As shown in Fig. 5G, expression of LTα was absent in splenic DC purified from $RLT\alpha^{ko/ko}$ but present in DC from $RLT\alpha^{wt/wt}$ mice. These results indicate that LTα is expressed by DC. To test if LT ligands were expressed by DC we sorted $NK1.1^{-}CD11c^{+}$ cells from the thyroid and LNs ten days after injection CD4+ T cells and tested the expression of LTα and LTβ by Q-PCR. Expression of lymphotoxin ligands could be readily detected in DC infiltrating the thyroid. These findings document expression of lymphotoxin ligands in DC 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 $CD4^{+}$ T cells from circulation (24), clustering of T cells and DC, and the formation of HEVs (25) and lymphatic vessels in the thyroid (10). Here we report that lymphatic vessel formation under these conditions is dependent on DC recruited from the periphery.

To study the mechanisms of inflammatory lymphangiogenesis we used a reductionist model in which adoptive transfer of $CD4^{+}$ 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, DC and B cells in lymphangiogenesis (4, 5, 9, 19-22).

In our adoptive transfer model the cellular complexity in the site of lymphangiogenesis can be reduced to the endothelium, stroma, and to the incoming cells (T cells, DC and NK cells). Endothelial cells in the thyroid constitutively express LTβR and animals deficient of both LTβR and its ligand LTα1β 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 $CD4^{+}$ T into the thyroid of RAGTGCCL21 mice induces host DC and NK cell migration to tissue and the formation of new lymphatic vessel within 10 days. Membrane LTα1β is detected in T, B, and NK cells (18, 32), whereas LTβ mRNA is expressed by several DC subsets (33). In addition, LT ligands were also shown to be expressed by human DC (34). Thus, $CD4^{+}$ T cells, NK cells, and DC were plausible candidates for expressing LTβR-ligands in the thyroid. Our results show 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 DC. We reasoned that eliminating NK and DC 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 showed that the formation of new lymphatic vessels was not disturbed. Furthermore, as

shown here, lymphoid aggregates rich in lymphatic vessels are found in the thyroid of in the NK-deficient TGCCL21/Id2$^{ko/ko}$ mice (25, 29). As Id2-deficient mice also lack lymphoid tissue inducer (LTi) we conclude that the development of TLO and lymphangiogenesis occur independent of the presence of NK and LTi cells in this model.

In contrast to the results obtained with the 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 the elimination of DC abrogated a critical pathway in the genesis of new lymphatic vessels. DC are required for formation and maintenance of bronchus-associated lymphoid tissue (35, 36) and for ectopic lymphoid structures formed in atherosclerotic plaques (37). DC is also important for retention of B and T cells in the TLO, through a mechanism involving LTβ production (36). Our results show that DC can also contribute to vascular differentiation in tertiary lymphoid structures. Based on the findings described here we suggest a model in which the formation of new lymphatic vessels is dependent on the influx of CD11c^{+} DC into the thyroid. As shown by our group previously, subsequent to the entry of CD4^{+} T cells into the CCL21-expressing thyroids there is a marked up-regulation of DC-attracting inflammatory chemokines such as CCL2, CXCL10 and CXCL9 (25). These chemokines could originate from the incoming CD4^{+} T cells, from stromal cells, or from the endothelium, and facilitate recruitment of DC to the tissue. Thus, upregulation of DC-recruiting chemokines and activation of LTβR-signalling could be mechanistically important for the formation of new lymphatic vasculature in TLOs. Since LT ligands are critical for the development of lymphatic vessels and since DC express it, it is likely that LT expressed by DC is critical for the response observed here. The expression of lymphotoxin ligands by DC could act on LTβR-expressing endothelial cells, stromal cells or on incoming DC. 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 DC, and production of proinflammatory and lymphangiogenic factors). Delineation of the mechanisms triggered by DC influx into the tissue is likely to provide for the identification of novel therapeutic targets in lymphangiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard abbreviations

DC	Dendritic cells

DTR	diphteria toxin receptor
DT	diphteria toxin
HEV	high endothelial vessel
LTa	lymphotoxin alpha
LTβR	lymphotoxin beta receptor
LVA	lymphatic vessel area
PNAd	peripheral node addressin
qPCR	quantitative PCR

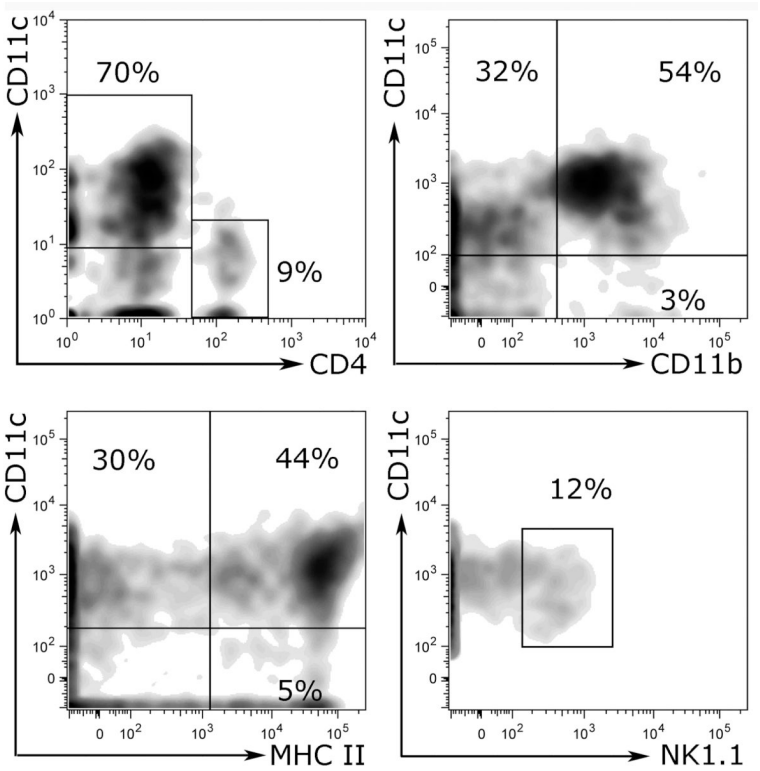

Fig. 1. CD4^{+}T cells induce recruitment of DC and NK cells to the thyroid of RAGTGCCL21 mice

Single cell suspensions of thyroids of RAGTGCCL21 mice transferred with CD4+ T cells for 5 days were stained with the indicated Abs. Cells were gated on the viable leukocyte population (CD45^{+}/PI^{-}). Results are representative of three independent experiments (n = 10-15 mice/experiment); PI, propidium iodide.

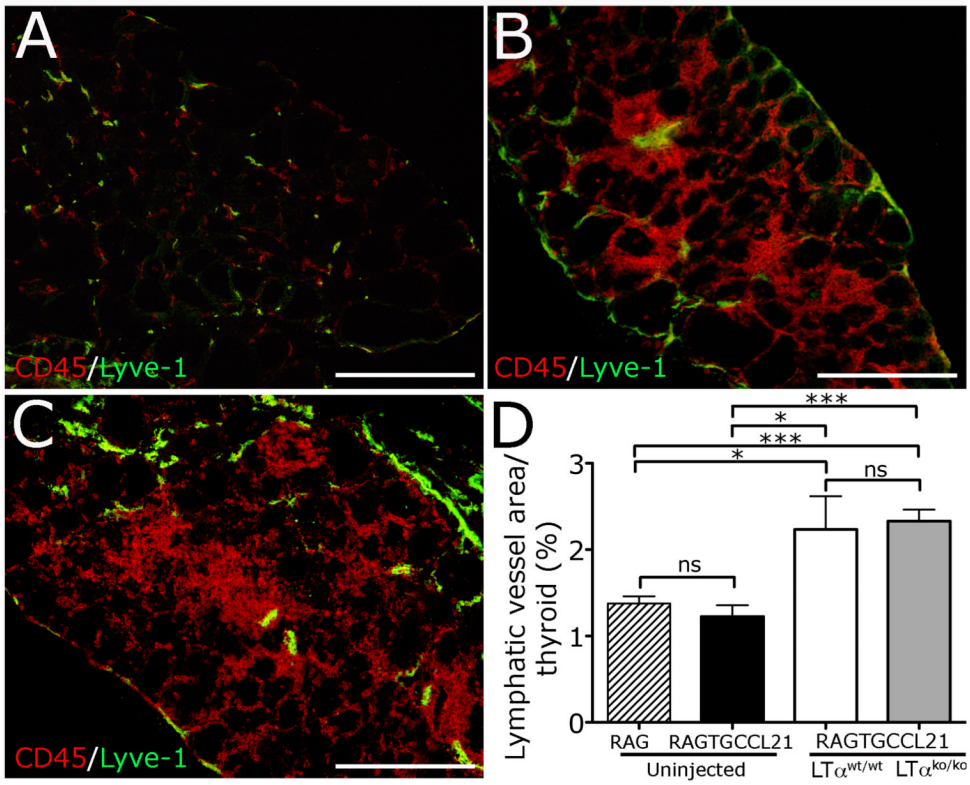

Fig. 2. LTα expression by incoming lymphocytes is not required for induction of lymphangiogenesis

(A) Thyroid of an uninjected RAGTGCCL21 mouse. (B) Thyroid of RAGTGCCL21 mouse injected with 1×10^6 $CD4^+/LT\alpha^{wt/wt}$ or (C) $CD4^+/LT\alpha^{ko/ko}$ T cells and stained with anti-CD45 and anti-Lyve-1 antibodies. Increased number of $CD45^+$ cells and Lyve-1^+ vessels were observed in the thyroid of mice injected with $CD4^+/LT\alpha^{wt/wt}$ (n = 3) and $CD4^+$ $LT\alpha^{ko/ko}$ (n = 10) cells ten days after injection (B and C, respectively). (D) Quantification of LVA in the thyroid of RAG (n = 5), RAGTGCCL21 without T cells (n = 4), and RAGTGCCL21 mice transferred with $CD4^+/LT\alpha^{wt/wt}$ (n = 3) or $CD4^+/LT\alpha^{ko/ko}$ (n = 10) T cells. Data are expressed as means ± SEM (n = 16-20 sections/mice, * p = 0.03, ** p = 0.004, *** p = 0.0001). Scale bars = 250 μm.

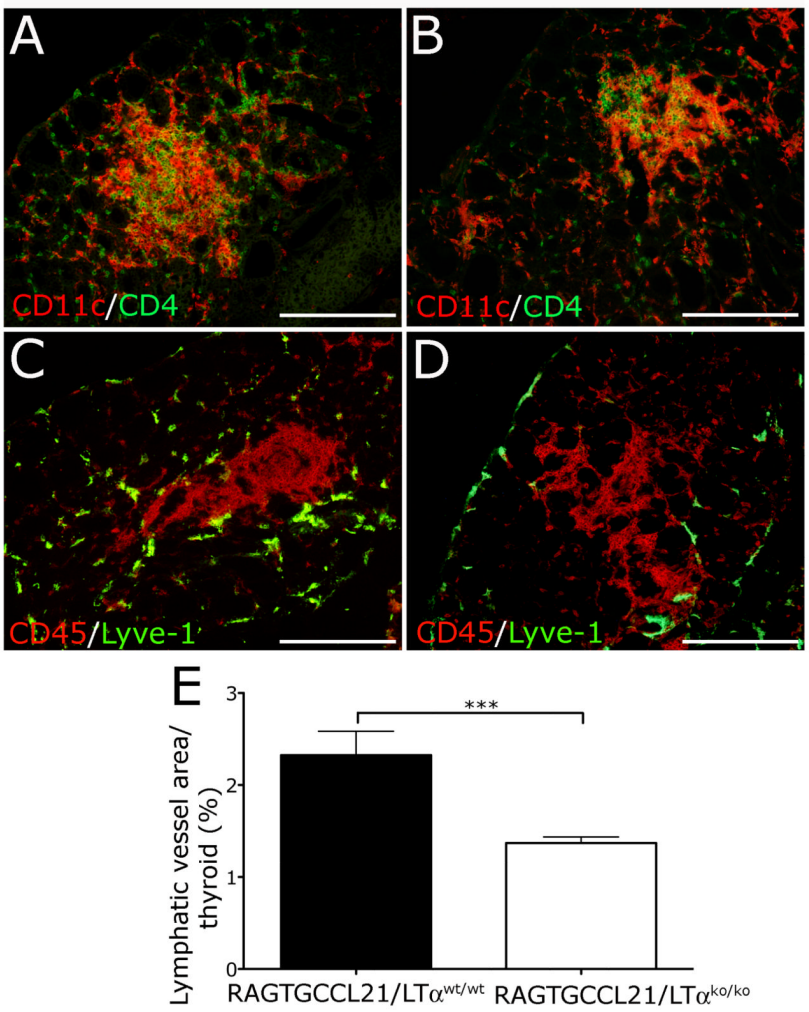

Fig. 3. Lymphotoxin expression by host cells is important for lymphangiogenesis in the thyroid

$CD4^+$ T cells were transferred into RAGTCCL21/$LT\alpha^{wt/wt}$ (A and C, *n* = 4) and RAGTGCCL21/$LT\alpha^{ko/ko}$ mice (B and D, *n* = 11). $CD4^+$ lymphocytes and DC were present in the thyroid of RAGTCCL21/$LT\alpha^{wt/wt}$ (A) and RAGTGCCL21/$LT\alpha^{ko/ko}$ mice (B). Lyve-1^+ lymphatic vessels were less frequent in the thyroid of RAGTGCCL21/$LT\alpha^{ko/ko}$ mice (D) when compared to RAGTCCL21/$LT\alpha^{wt/wt}$ mice (C). Shown are representative sections. (E) Quantification of LVA in the thyroid of RAGTGCCL21/$LT\alpha^{wt/wt}$ and RAGTGCCL21/$LT\alpha^{ko/ko}$ mice. LVA in the thyroid of RAGTGCCL21/$LT\alpha^{ko/ko}$ mice was significantly reduced when compared to that of RAGTCCL21/$LT\alpha^{wt/wt}$ mice. Data are expressed as means ± SEM (n = 20-25 sections/mice, *** p = 0.0002). Scale bars: 250 μm.

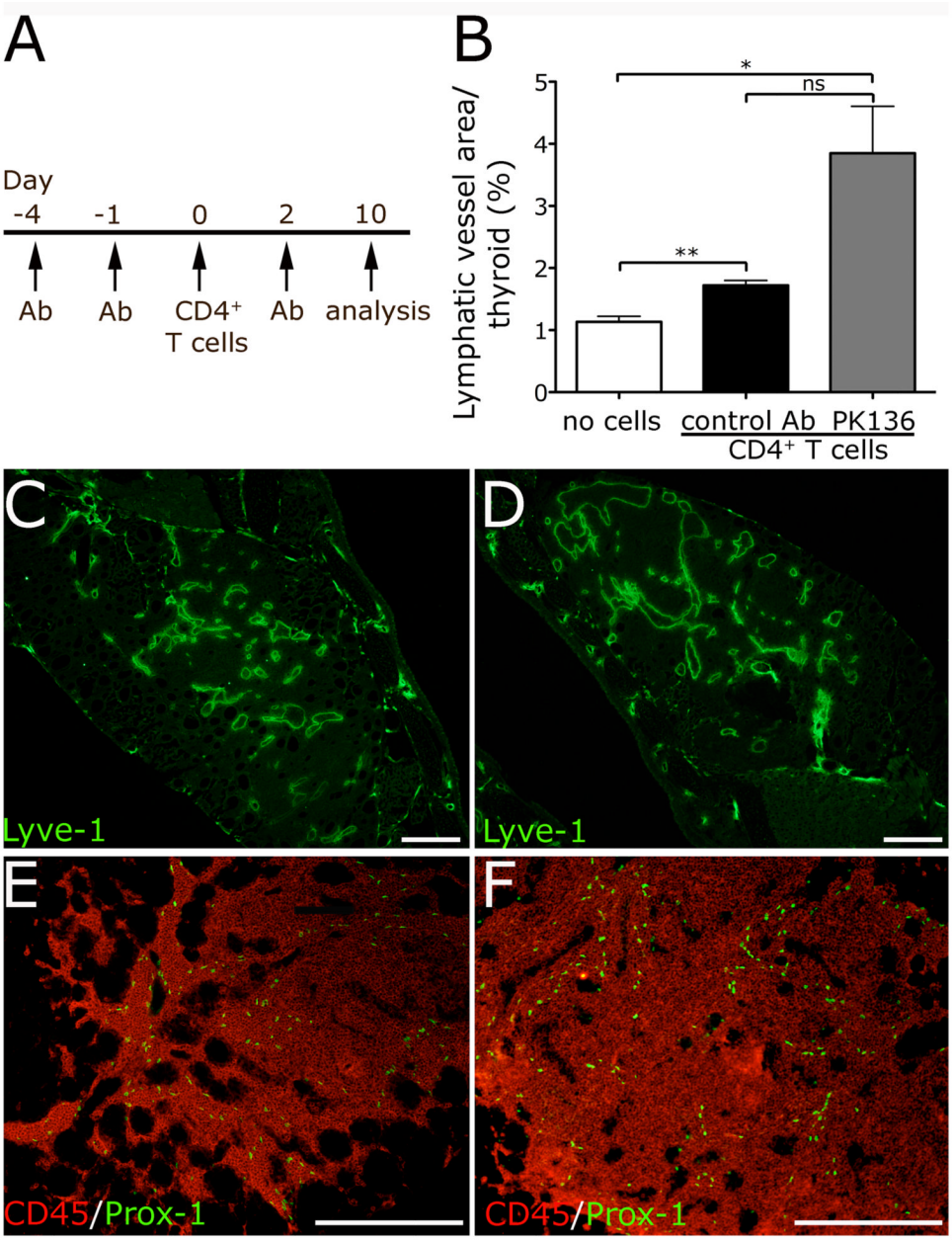

Fig. 4. NK cells are not required for lymphangiogenesis in the thyroid of RAGTGCCL21 or TGID2 mice

(A) RAGTGCCL21 mice received two i.v injections of control or PK136 antibody before adoptive transfer of $CD4^+$ T cells. A third dose of antibody was injected on day 2 after T cell transfer. (B) Quantification of LVA in the thyroid of uninjected RAGTGCCL21 mice (n = 3), or injected with $CD4^+$ T cells and treated with control (n = 4) or PK136 (n = 6) antibody. Data are expressed as means ± SEM (n = 14-20 sections/mice, * p = 0.04, ** p = 0.005). Lyve-1^+ and Prox-1^+ lymphatic vessels were present in the thyroid of TGCCL21/$Id2^{ko/wt}$ (C and E, respectively) and TGCCL21/$Id2^{ko/ko}$ (D and F, respectively) mice. Scale bars: 250 μm.

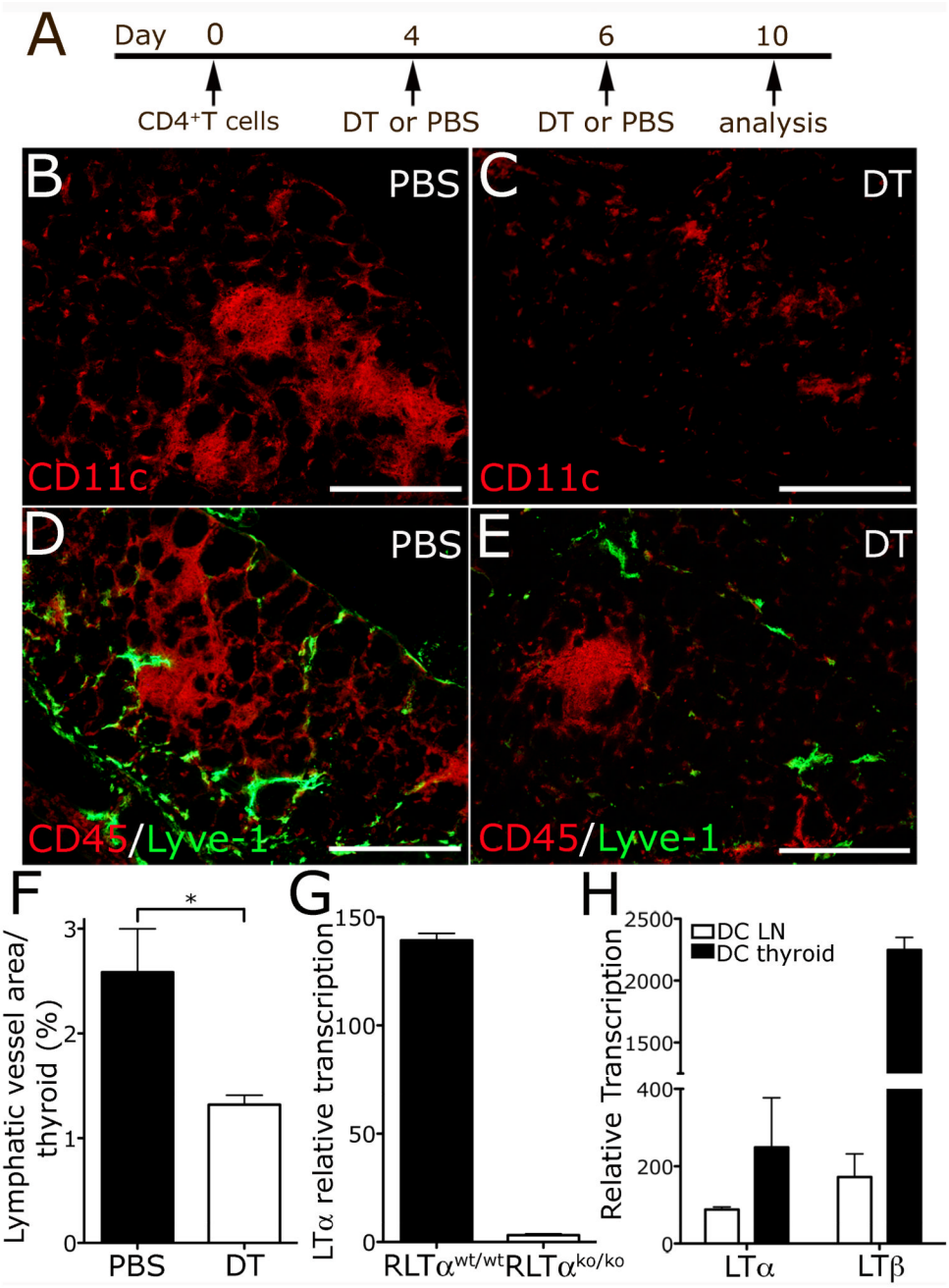

Fig. 5. Dendritic cells regulate the formation of lymphatic vessels in the thyroid

(A) RAGTGCCL21/CD11c-DTR mice were injected i.v. with 1×10^6 $CD4^+$ T cells. At days 4 and 6, mice were injected i.v. with 2ng/g of diphteria toxin (DT). The control group was treated with PBS. Immunostaining of the thyroids of animals injected with T cells was performed on day 10. (B) $CD11c^+$ in the thyroid of the RAGTGCCL21/CD11c-DTR mice that received PBS ($n = 7$). (C) $CD11c^+$ cells in the thyroid of mice treated with DT ($n = 6$). (D) Lyve-1^+ vessels in the thyroid of RAGTGCCL21/CD11c-DTR mice treated with PBS. (E) Lyve-1^+ vessels in the thyroid of RAGTGCCL21/CD11c-DTR mice treated with DT. (F) Quantification of LVA in the thyroid of RAGTGCCL21/CD11c-DTR mice treated with PBS ($n = 7$) or DT ($n = 6$). The lymphatic vessel area in the thyroid of RAGTGCCL211/ CD11c-DTR mice was significantly reduced when DC were ablated. Data are expressed as

means ± SEM (*n* = 16-20 sections/mice, * p = 0.02). (G) LTα mRNA expression in sorted $CD11c^{+}NK1.1^{-}$ cells from the spleen of $RLT\alpha^{wt/wt}$ and $RLT\alpha^{ko/ko}$ mice. (H) LTα and LTβ mRNA expression in sorted $CD11c^{+}NK1.1^{-}CD4^{-}$ cells from the LN and thyroid of RAGTGCCL21 mice transferred with $CD4^{+}$ T cells. Scale bars: 250μm.