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The β-Chemokine Receptor D6 Is Expressed by Lymphatic Endothelium and a Subset of Vascular Tumors

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The lymphatic vessels (lymphatics) play an important role in channeling fluid and leukocytes from the tissues to the secondary lymphoid organs. In addition to driving leukocyte egress from blood, chemokines have been suggested to contribute to leukocyte recirculation via the lymphatics. Previously, we have demonstrated that binding sites for several pro-inflammatory β-chemokines are found on the endothelial cells (ECs) of lymphatics in human dermis. Here, using the MIP-1α isoform MIP-1αP, we have extended these studies to further support the contention that the *in situ* chemokine binding to afferent lymphatics exhibits specificity akin to that observed *in vitro* with the promiscuous β-chemokine receptor D6. We have generated monoclonal antibodies to human D6 and showed D6 immunoreactivity on the ECs lining afferent lymphatics, confirmed as such by staining serial skin sections with antibodies against podoplanin, a known lymphatic EC marker. In parallel, *in situ* hybridization on skin with antisense D6 probes demonstrated the expression of D6 mRNA by lymphatic ECs. D6-immunoreactive lymphatics were also abundant in mucosa and submucosa of small and large intestine and appendix, but not observed in several other organs tested. In lymph nodes, D6 immunoreactivity was present on the afferent lymphatics and also in subcapsular and medullary sinuses. Tonsilar lymphatic sinuses were also D6-positive. Peripheral blood cells and the ECs of blood vessels and high endothelial venules were consistently nonreactive with anti-D6 antibodies. Additionally, we have demonstrated that D6 immunoreactivity is detectable in some malignant vascular tumors suggesting they may be derived from, or phenotypically similar to, lymphatic ECs. This is the first demonstration of chemokine receptor expression by lymphatic ECs, and suggests that D6 may influence the chemokine-driven recirculation of leukocytes through the lymphatics and modify the putative chemokine effects on the development and growth of vascular tumors. *(Am J Pathol 2001, 158:867–877)*

The system of lymphatic vessels is involved in transporting fluid and cells from the interstitial space, through lymph nodes, and ultimately back into the blood. By channeling lymphocytes and dendritic cells (DCs) from the periphery into the lymph nodes, lymphatics play an important role in the development of a functional immune response. Additionally, lymphatics may allow for dissipation of metastatic tumor cells away from the primary tumor site. Despite their paramount importance in homeostasis and disease, little is known about the biology of lymphatics. This has been due in part to the paucity of specific biochemical markers of lymphatic endothelium resulting in difficulties of enrichment and *in vitro* culture. The recent identification of VEGFR-3/Flt4 (a receptor for VEGF-C), LYVE-1 (hyaluronan receptor), and podoplanin (a membrane glycoprotein of unknown function) as specific markers of adult lymphatic endothelial cells (ECs) may, along with experiments showing adhesion molecule expression by these cells, herald the beginning of a new era in studies on lymphatic endothelium.[1–7]

Migration of leukocytes and their coordinated encounters are crucial for efficient inflammatory and immune responses. For example, in lymph nodes, naïve T lymphocytes attracted from blood via the high endothelial venules (HEVs) interact with antigen-presenting cells, such as DCs, that have migrated from the peripheral tissue via the lymphatics.[8,9] Chemokines, members of the

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family of structurally related chemotactic cytokines, have been implicated in driving and directing these tightly choreographed leukocyte movements.[8–12] Chemokines are subclassified according to the relative position of conserved cysteine residues:[10–12] the members of the most numerous CC or β-chemokine subfamily have the first two cysteines adjacent, whereas in the CXC or α-chemokines the first two cysteines are separated by a single amino acid. CX_3C and C chemokine subfamilies are characterized by the first two cysteine residues being separated by three intervening amino acids, and the absence of the first and third cysteine, respectively. Although chemokines are classically viewed as leukocyte chemoattractants, they have emerging roles in cell proliferation control, embryogenesis, development, and neuronal function.[13–15] Of particular relevance here, is their role in EC biology in which chemokine interaction with ECs contributes to the pathophysiological processes as diverse as angiogenesis, vascular remodeling, and the transcytosis and surface presentation of chemokines during leukocyte transmigration.[14,16,17] Classically, chemokine receptors are members of the G-protein-coupled heptahelical receptor superfamily.[8–12] In addition to these, other molecules bind chemokines specifically, with high affinity and can either produce a functional response or modify chemokine action. These molecules include glycosaminoglycans (most notably heparan sulfate), α2-macroglobulin, virally-encoded secreted proteins, and virally-encoded heptahelical viroceptors.[12,18–21]

To examine the location and specificity of chemokine binding sites in skin, we have previously used an *in situ* binding analysis of several chemokines using intact pieces of normal human skin.[22] This revealed that in addition to chemokine receptors on resident leukocytes, chemokines show specific saturable binding to the ECs of postcapillary and collective venules, and also afferent lymphatics. Binding cross-competition studies showed that venular EC display a chemokine binding fingerprint identical to Duffy antigen/receptor for chemokines (DARC) that indeed is expressed on postcapillary and collective venules, and also on HEVs.[23–25] DARC is unusual in that it acts as a promiscuous receptor for α- and β-chemokines, and additionally, is currently classified as nonsignaling because of the absence of data demonstrating signal transduction or any cellular response on ligand binding.[26,27] Surprisingly, the profile of chemokine binding to the lymphatic endothelium was different from venular ECs:[22] RANTES, monocyte chemotactic proteins (MCPs)-1 and -3, but not macrophage inflammatory protein-1α (MIP-1α) or interleukin (IL)-8, showed saturable and cross-competable binding. Recently, we have cloned a human chemokine receptor, named D6, that we demonstrated is able to bind with high affinity to a wide array of pro-inflammatory β-chemokines including RANTES, MCP-1, and MCP-3, but not human MIP-1α, despite very high-affinity binding of murine MIP-1α to murine and human D6.[28,29] Thus, D6, but not any other currently known chemokine receptor, exhibits ligand specificity similar to the receptor identified on lymphatic endothelium. Just like DARC, human D6 does not transduce detectable signals on ligand binding when expressed in heterologous cell lines.[28] Although the function of D6 still remains uncertain, it is likely to bear more functional similarity to DARC than to other classical chemokine receptors.

More recently, we have resolved the anomaly of human D6 high-affinity binding of murine, but not human, MIP-1α.[30] In all of the above experiments, one particular isoform of human MIP-1α was used, namely MIP-1αS (or LD78α). However, an alternative nonallelic isoform exists called MIP-1αP (or LD78β). These genes are nearly identical with the notable exception that MIP-1αP encodes a proline residue at position 2 of the mature protein, whereas in MIP-1αS it is a serine.[31,32] This proline at position 2 dramatically alters the properties of MIP-1αP, now allowing its high-affinity binding to D6.[30] MIP-1αP is therefore viewed as the human functional homologue of murine MIP-1α.

Here, we have capitalized on these data to further demonstrate that the chemokine receptor on lymphatic vessels bears ligand specificity similar to D6. We have used the *in situ* binding assay to demonstrate specific binding of radioiodinated MIP-1αP to these vessels. Moreover, we have raised monoclonal antibodies against human D6 and demonstrate D6 immunoreactivity on dermal lymphatics, confirmed as such by staining serial sections with antibodies against podoplanin, a lymphatic EC marker.[4] In addition, *in situ* hybridization has been used to show that D6 mRNA is expressed by lymphatic ECs in the dermis. Subsequent analysis of sections taken from a panel of tissues showed D6 immunoreactivity on lymphatics in the gastrointestinal tract and lymphatic sinuses within the parenchyma of secondary lymphoid organs. Furthermore, we have used the anti-D6 antibodies to demonstrate the presence of D6 immunoreactivity in a subset of vascular tumors. The possible functional roles of D6 in the biology of lymphatic endothelium and vascular tumorigenesis are discussed.

Materials and Methods

Antibodies

The production of mouse monoclonal anti-D6 antibody is detailed below. Anti-podoplanin antibody was prepared as described recently.[4] Control murine IgGs and rabbit Ig were purchased from Sigma (Poole, UK) and DAKO (Glostrup, Denmark), respectively. Fluorescein isothiocyanate-coupled goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA).

In Situ *Binding Assay*

MIP-1αP was labeled with ^{125}I using Iodogen (Pierce, Rockford, IL) as previously described.[33] *In situ* ligand-binding analysis with this labeled protein on skin pieces was performed as detailed before.[22] In brief, intact viable skin was removed in the process of elective reductive surgery on informed consent. Immediately on removal the skin was cubed into 1 to 2 mm^3 pieces by a sharp scalpel blade. Five to six cubes were incubated with ~10 pg and

100 pg ^{125}I-MIP-1αP (specific radioactivity, ~10 μCi/μg) in 0.2 ml of Hanks' balanced salt solution supplemented with 0.1% bovine serum albumin and 20 mmol/L HEPES for 1 hour at room temperature on a platform shaker (200 rpm). The pieces were washed three times for 10 minutes in 2 ml of the buffer on the platform shaker and then fixed overnight in 4% buffered paraformaldehyde. Afterward the pieces were washed, dehydrated in increasing concentrations of ethanol, embedded in paraplast (Monoject Scientific, Athy, Ireland), and 5-μm-thin sections cut. These were deparaffinized, rehydrated, air-dried, and coated with K2 nuclear track emulsion (Ilford, Mobberley, UK). Slides were exposed at 4°C in the dark for 4 to 12 weeks and then developed with D-19 developer (Kodak-Pathe, Paris, France), fixed (Unifix, Kodak-Pathe), and counterstained with hemalaun. Slides were studied and photographs taken under a BX 60 Olympus microscope equipped with a DP10 digital camera (Olympus Austria, Vienna, Austria).

Generation of Anti-Human D6 Monoclonal Antibodies

Monoclonal antibodies to human D6 were raised using techniques described elsewhere.[34] Briefly, human D6 was expressed in the murine cell line L1.2 and this transfectant was used to immunize C57BL/6 mice. Fusion of spleen and myeloma SP2/0 was performed following standard procedures and the hybridoma supernatants were screened by flow cytometry for positively staining the D6 transfectant. Specificity of the antibodies was tested by staining wild-type L1.2 or Chinese hamster ovary (CHO) cells and L1.2 or CHO cells transfected with other human chemokine receptors. CHO cell transfectants were tested using immunohistochemistry as outlined below. To assess L1.2 transfectants, ~10^6 cells were incubated with 50 μl of hybridoma supernatant for 30 minutes at 4°C, washed, then similarly incubated with goat anti-mouse IgG (fluorescein isothiocyanate-coupled), and subsequently assessed by fluorescence-activated cell sorting (FACS). Supernatants from several clones that specifically stained the D6 transfectant were selected, further subcloned by limiting dilution, expanded, and tissue culture supernatant collected. Specific D6 immunoreactivity was again confirmed and each antibody titrated to give a maximal differential between D6 transfectants and untransfected controls. One antibody (4A5) was purified using a Protein-G column. Purified antibody and antibody supernatants were stored at −20°C until use.

Immunohistochemistry

Normal human skin was removed during elective plastic surgery on informed consent. The skin samples were snap-frozen in liquid nitrogen-chilled isopentane. 5-μm-thin serial frozen sections were cut onto glass slides coated with 3-(triethoxysilyl)-propylamin (TESPA; Sigma Chemical Co., St. Louis, MO). The sections were fixed for 10 minutes in acetone at room temperature and thereafter were kept at −20°C for up to 8 weeks until the staining procedure.

The frozen serial skin sections were rehydrated and incubated with anti-D6 mouse monoclonal or anti-podoplanin rabbit polyclonal antibodies. The bound primary antibodies were detected by sequential incubations with alkaline phosphatase-conjugated goat anti-rabbit and rabbit anti-mouse antibodies (for podoplanin and D6, respectively) and an alkaline phosphatase-anti-alkaline phosphatase staining kit (DAKO), according to the manufacturer's instructions. To control the specificity of antibody binding, mouse and rabbit IgG and Ig control antibodies (DAKO) were used at equimolar concentrations. Each immunostaining protocol was performed on skin from at least three different donors.

All of the other immunohistochemistry was done using 5-μm-thin paraffin sections cut from blocks obtained from the archives of the Department of Pathology, University of Glasgow. The sections were deparaffinized in Histoclear (Fisher Scientific, Loughborough, UK), rehydrated through decreasing concentrations of ethanol and then pure water, and incubated for 10 minutes in 3% hydrogen peroxide. To unmask the antigens, slides were either microwaved for 10 minutes in 1 mmol/L ethylenediaminetetraacetic acid (pH 8) (for D6) or for 15 minutes in 10 mmol/L citrate buffer (pH 6) (for podoplanin). After blocking with serum, avidin, and biotin, the appropriate primary antibodies were added and the sections left for 45 to 60 minutes at room temperature. The bound antibodies were detected using the Vector avidin-biotin-peroxidase detection system and Nova-Red substrate (Vector Laboratories, Peterborough, UK), according to the manufacturers instructions. Sections were fixed with neutral buffered formalin, counterstained with hematoxylin (Surgipath Europe Ltd., Peterborough, UK), dehydrated through increasing concentrations of ethanol, cleared in Histoclear, and mounted in Histomount (Fisher Scientific). The staining was evaluated by at least two independent examiners and photographed with an Olympus BX60 microscope.

In Situ *Hybridization*

Adjacent 7-μm frozen sections of formaldehyde-fixed human skin were either stained with anti-podoplanin antibodies as above, or used for *in situ* hybridization as follows. Sections were rehydrated, immersed in 100 mmol/L glycine-phosphate-buffered saline, permeabilized in 0.3% Triton X-100, and treated with Proteinase K (20 μg/ml) for 15 minutes at 37°C in 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.4 buffer. After fixing in 4% paraformaldehyde at 4°C, sections were acetylated in TE buffer containing 0.25% acetic anhydride. Prehybridization and hybridization were done in buffers supplied by Novagen (Madison, WI). Sense and antisense D6 probes were generated from linearized human D6 cDNA plasmid using T7 or SP6 polymerases in combination with digoxigenin RNA-labeling mix (Roche Molecular Biochemicals, Mannheim, Germany). Transcripts were subjected to alkaline hydrolysis and 100- to 200-bp fragments purified.

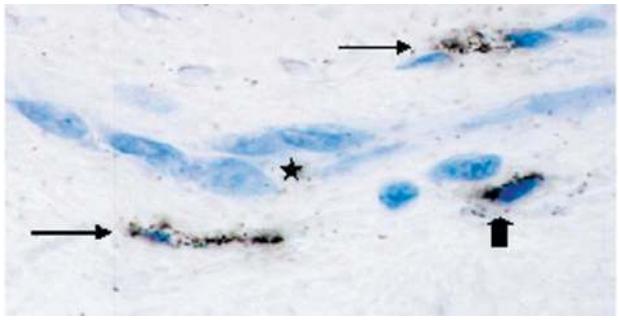

Figure 1. *In situ* binding of radioiodinated MIP-1αP to lymphatic vessels in normal human skin. Cubes of viable human skin were incubated with ^{125}I-MIP-1αP, sectioned, and the bound protein visualized as outlined in Materials and Methods. Tissue-bound ^{125}I-MIP-1αP appears as black autoradiographic grains. Sections were counterstained with hemalaun. **Thin arrows** mark vessels with clear morphological features of lymphatics. **Thick arrow** indicates a resident dermal cell also labeled with ^{125}I-MIP-1αP. The **asterisk** marks an unlabeled blood vessel. Original magnification, ×1,400.

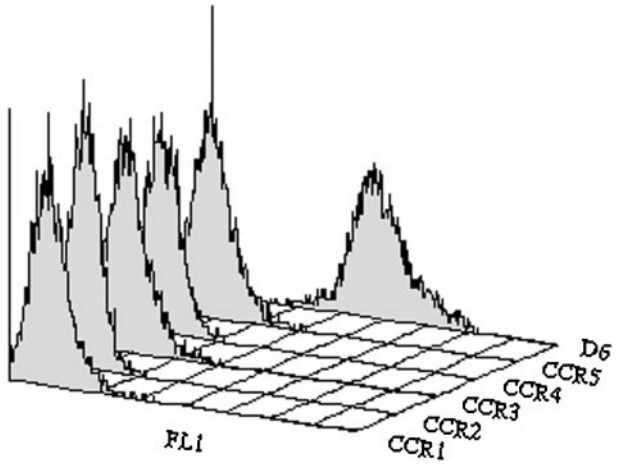

Figure 2. FACS analysis of chemokine receptor transfectants with anti-D6 antibodies. Approximately 10^6 L1.2 cells stably expressing human chemokine receptors CCR1–5 or D6 were incubated with anti-D6 antibodies (50 μl of hybridoma supernatant from clone 4A5), and then antibody-containing complexes detected using a fluorescein isothiocyanate-coupled goat anti-mouse IgG and FACS analysis.

Probes were added to the hybridization buffer to 100 ng/ml, denatured, hybridized for 16 hours at 42°C, and the sections then washed in several changes of 1× standard saline citrate at 37°C, treated for 30 minutes with RNase A (10 μg/ml), and washed extensively at 42°C with 0.1× standard saline citrate. Pre-incubation for 1 hour in blocking solution (150 mmol/L NaCl, 100 mmol/L Tris, pH 7.5, 1% rabbit serum, 1% goat serum, 1% sheep IgG, 0.15 Triton X-100), was followed by 2 hours in blocking solution containing alkaline phosphatase-conjugated sheep anti-DIG. After washing extensively, sections were equilibrated in AP buffer (100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$, 1 mmol/L levamisole) and BCIP/NBT solution applied in AP buffer. Color development was done for 16 hours at room temperature in the dark.

Results

In Situ *Binding of ^{125}I-MIP-1αP in Viable Human Skin*

Our previous *in situ* binding experiments[22] had demonstrated the presence on lymphatic endothelium of a chemokine receptor to which RANTES, MCP-1, and MCP-3 (but not MIP-1αS and IL-8) bind and fully cross-compete for each others binding. To our knowledge D6 is the only chemokine receptor that can bind RANTES and MCP-1 but not IL-8 or MIP-1αS. This prompted us to investigate the possible D6 expression on lymphatic ECs.

To further assess the specificity of lymphatic chemokine receptor, we performed *in situ* ligand-binding experiments on human skin using ^{125}I-MIP-1αP, a MIP-1α isoform, which unlike the previously studied MIP-1αS,[22] binds to D6 with high affinity.[30] As shown in Figure 1, MIP-1αP bound selectively to the EC lining of lymphatics and some resident dermal cells but weakly, if at all, to vascular ECs. Thus, our current results, together with our previous findings,[22,30] suggest the presence of chemokine receptors with ligand specificity identical to D6 on the surface of dermal lymphatic ECs. The almost complete cross-competition of RANTES binding by MCP-1 and *vice versa*[22] excludes the significant involvement of specific chemokine receptors, eg, CCR-2 and CCR-5 in chemokine *in situ* binding to lymphatic ECs.

Generation and Analysis of Monoclonal Anti-Human D6 Antibodies

To further examine tissue distribution and confirm lymphatic EC expression of D6, a panel of monoclonal antibodies was generated against cells transfected with the human D6 gene (see Materials and Methods). Supernatants from hybridomas were screened by immunohistochemistry and FACS analysis for binding to D6 expressed on heterologous cell lines. Several supernatants contained D6 binding antibodies (Figure 2). One clone (4A5) appeared to have the highest titer and was further purified on a protein G column. None of the antibody supernatants tested was able to block ^{125}I-MIP-1α binding to D6 transfectants, or detect human D6 in D6 transfectant cell extracts using Western blotting (not shown), suggesting that they recognize conformational epitopes outside the ligand-binding domain. Clones were tested for their ability to bind to a panel of cell lines stably transfected with individual human chemokine receptors [CCR1–5 (Figure 2), CXCR1–3 (not shown)] and no cross-reaction was observed. Furthermore, using FACS analysis, the 4A5 monoclonal did not bind to any cell types from peripheral blood, including permeabilized cells or cells fixed before antibody staining. Human D6 transfectants and the Jurkat T cell line that expresses the D6 gene, both bound the 4A5 antibody in FACS analysis (data not shown). Moreover, we have not observed staining by 4A5, or other clones, of cells within blood vessels in any of the tissue sections that we have screened (see below). These results effectively exclude cross-reaction with a wide array of chemokine receptors known to be expressed on peripheral blood cells. Also, importantly,

these data show that D6 expression on, or within, peripheral blood cells is below the level of detection with our monoclonal antibodies. These results are in accordance with gene expression analysis that has shown that D6 mRNA is of low abundance in peripheral blood samples.[28] However, it remains a possibility that hemopoietic cells express this protein if they are subjected to the appropriate stimulation or activation.

Monoclonal Anti-D6 Antibodies Stain Lymphatic ECs in Human Skin

Next, we used the mouse monoclonal anti-D6 antibodies in immunohistochemical analysis of frozen and paraffin-embedded sections of human skin. All of the anti-D6 antibody clones tested stained ECs lining thin-walled channels within the dermis that had the morphological appearance of afferent lymphatics (Figure 3; A, B, and D). Blood vessels corresponding to different segments of the circulatory tree were immunonegative in all sections tested (Figure 3B and not shown). In some skin sections, both frozen and paraffin-embedded, a few individual cells scattered within the lower and more rarely upper dermis showed D6 immunoreactivity (Figure 3B, insets). These cells were not associated with either blood or lymphatic vessels and their identity is currently under investigation. In these experiments as in all of the subsequent analyses, immunohistochemistry using anti-D6 antibodies was performed alongside controls on adjacent sections using no primary antibody and equimolar isotype-matched irrelevant control antibody that showed no specific staining (not shown). The stainings presented are representative of that seen in several sections from skin from at least five different individuals.

D6-Immunoreactive Dermal Structures Express Podoplanin Immunoreactivity

To confirm the identity of the D6-immunoreactive ECs as lymphatic, we stained the adjacent serial sections of human skin with antibodies against podoplanin, a known lymphatic EC marker.[4] As shown in Figure 3, C and D, all of the vessels in skin that expressed D6 contained podoplanin immunoreactivity. However, podoplanin expression was considerably broader than D6 with many podoplanin immunoreactive vessels being D6-negative (not shown). This suggests that only a subset of lymphatics in skin expresses D6. The exact fingerprint of D6 chemokine specificity observed in the *in situ* binding to lymphatics, and the D6 immunoreactivity of lymphatic ECs, together strongly imply that D6 is specifically expressed on lymphatic endothelium.

Lymphatic ECs Express D6 mRNA

Next, sections of skin were examined for expression of D6 mRNA using *in situ* hybridization. Although sense D6 probes produced no specific hybridization (Figure 4A), antisense D6 probes hybridized to ECs lining vessels in the dermis, both in regions near the epidermis (Figure 4B) and also deeper within the dermis (Figure 4B, inset). These vessels had the morphological appearance of lymphatics. To confirm this, serial sections were either hybridized with antisense D6 probes or subjected to immunohistochemistry using anti-podoplanin antibodies (Figure 4; C, D, E, and F). ECs lining the superficial (Figure 4C) and the deep dermal (Figure 4E) vessels that hybridized effectively to the antisense D6 probe also showed podoplanin immunoreactivity (Figure 4, D and F). ECs lining blood vessels, such as the one seen in Figure 4, E and F, did not hybridize to the antisense D6 probe and showed no podoplanin immunoreactivity. These observations provide further evidence that lymphatic ECs express D6, and strongly suggest that the anti-D6 antibodies are not cross-reacting with other antigens on these cells.

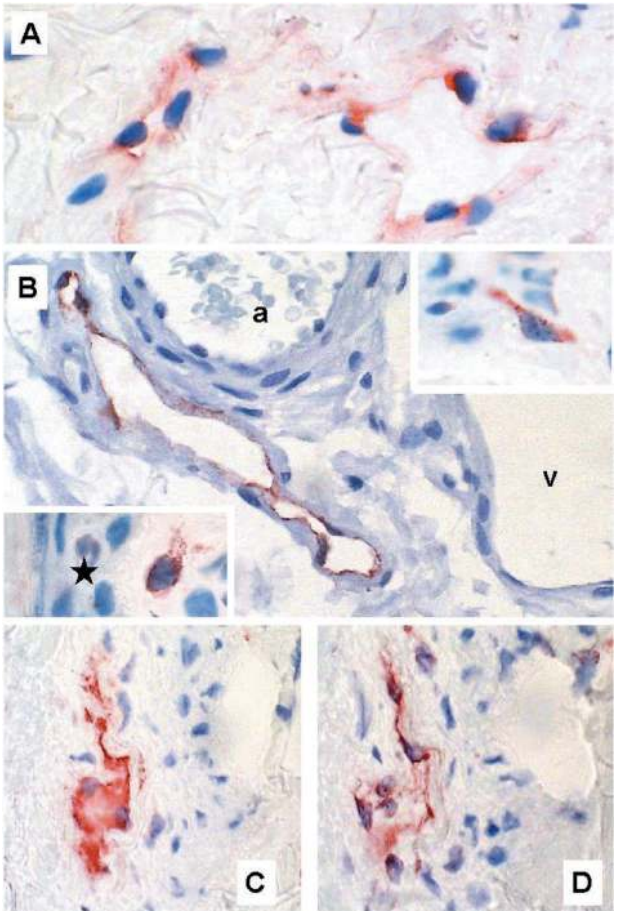

Figure 3. D6 immunoreactivity on lymphatic ECs in skin. Frozen sections of human skin immunostained with anti-D6 antibodies (**A** and **D**) or anti-podoplanin antibodies (**C**). In **B**, D6 immunoreactivity was detected on paraffin sections. Antibodies are visualized using an alkaline phosphatase-anti-alkaline phosphatase kit producing a red stain; all of the sections are counterstained with hemalaun. **A:** ECs lining two lymphatic capillaries in upper dermis display D6 immunoreactivity. Original magnification, ×2,000. **B:** Large collective lymphatic is stained by anti-D6 antibody, "a" marks an artery, "v" a vein, both immunonegative. Original magnification, ×900. Two **insets** contain resident non-ECs that show D6 immunoreactivity (both original magnifications, ×1,400). The **asterisk** indicates the epidermis. **C** and **D** are taken from adjacent serial sections and show a lymphatic vessel with podoplanin and D6 immunoreactivity, respectively. Original magnifications, ×1,200.

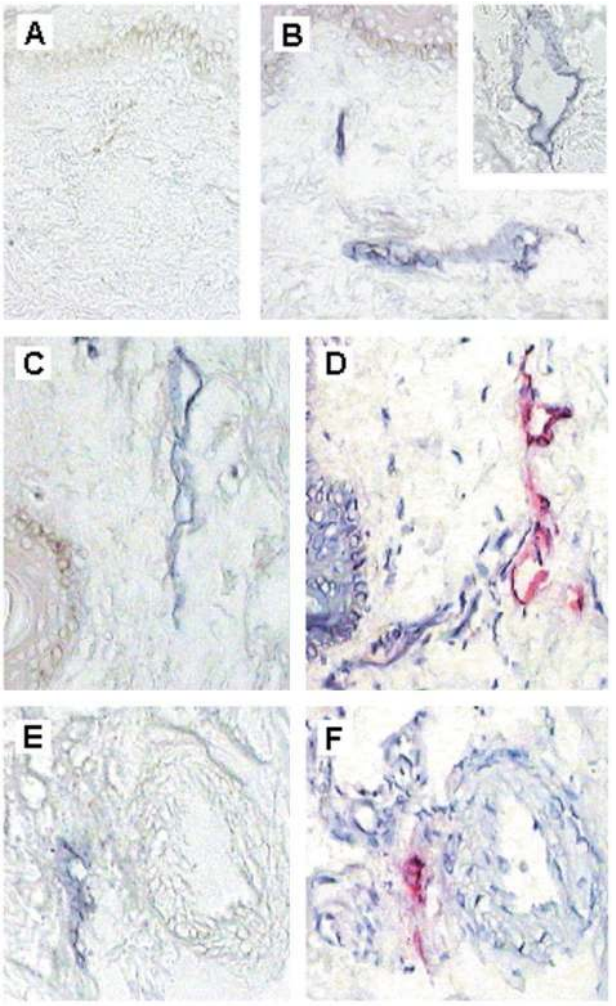

Figure 4. D6 mRNA expression by lymphatic ECs in the skin. Images taken from sections of human skin hybridized to DIG-labeled sense (**A**), or antisense (**B, C**, and **E**), D6 RNA and visualized with alkaline phosphatase-conjugated anti-DIG antibodies and BCIP/NBT solution. The presence of hybridized probe is indicated by a blue stain. **D** and **F** are sections adjacent to those used in **C** and **E**, respectively, and are immunostained with anti-podoplanin antibodies, visualized with an alkaline phosphatase-anti-alkaline phosphatase kit producing a red stain, and counterstained with hemalaun. **A–D:** Images taken from the upper dermis, with epidermis visible. **B** (**inset**), **E** and **F:** Images of deep dermal lymphatic vessels. Original magnifications: ×240 (**A–D**); ×100) (**E** and **F**).

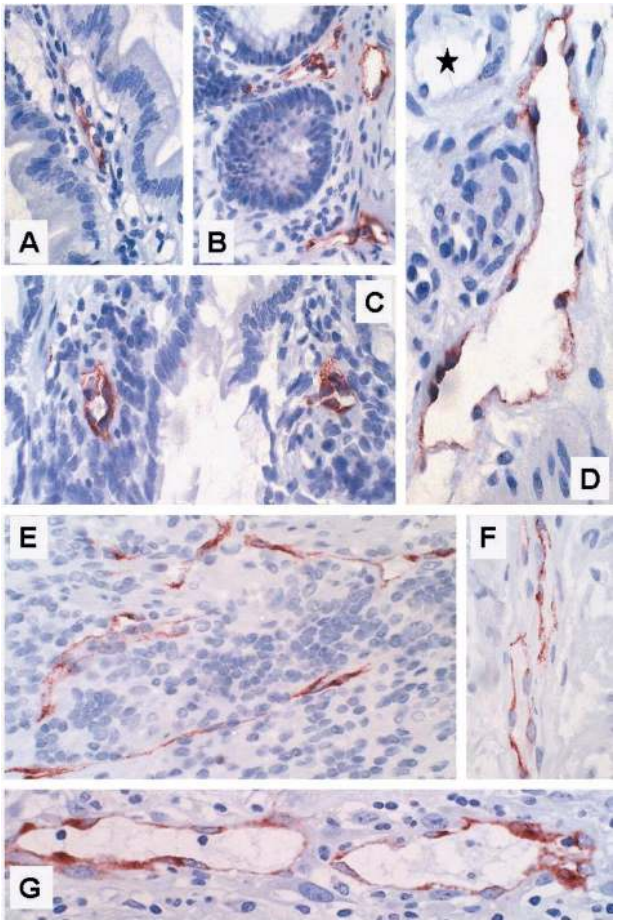

Figure 5. D6-immunoreactive lymphatic vessels in the gut. Paraffin-embedded sections of the gut immunostained with anti-D6 antibodies visualized using an avidin-biotin-peroxidase detection system and a peroxidase substrate producing a red stain. Representative D6 immunoreactivity associated with lymphatics in a longitudinal section of a small intestinal villus (**A**), lamina propria mucosae of the colon (**B**), a cross-section of two villi in the large intestine (**C**), muscular layer of the colon (**D**), lymphoid follicle in the lamina propria (**E**), lamina muscularis in normal appendix (**F**), and a patient with acute appendicitis (**G**). Original magnifications: ×400 (**A**), ×280 (**B**), ×530 (**C**), ×600 (**D**), ×440 (**E**, **F**, and **G**). The **asterisk** in **D** marks an immunonegative blood vessel. All sections counterstained with hematoxylin.

D6 Is Expressed by the Lymphatics in the Gut

Next we embarked on a search for D6-immunoreactive lymphatics in a panel of normal human tissues. As shown in Figure 5, anti-D6 antibodies stained abundant lymphatics in the wall of large and small intestine and appendix. Small lymphatics located in the villi of small and large intestine and in the lamina propria mucosae of colon (Figure 5; A, B, and C) and large collective lymphatics located in the muscular layer (Figure 5D) displayed D6 immunoreactivity. However, not all of the lymphatics identified as such by their podoplanin immunoreactivity were D6-positive (not shown). In appendix, a network of D6-positive lymphatics was observed in the lymphoid tissue of the lamina propria (Figure 5E) and in the lamina muscularis externa (Figure 5F). As observed in skin sections (Figure 3), blood vessels of different caliber representing different segments of circulatory tree were consistently D6-negative (eg, Figure 5D). Isotype control antibody showed no specific staining in any of the tissues examined (not shown). However, in the gut one anti-D6 clone out of four tested, weakly immunostained additional structures, eg, the enterocytes lining the lumen (not shown). The lack of the epithelial immunoreactivity by all of the other anti-D6 monoclonal antibody clones tested (all stained the afferent lymphatics) strongly suggests that enterocyte staining may be because of cross-reactivity with an unrelated epitope. All of the observations were on normal tissue from at least three different individuals; D6-immunoreactive lymphatics were also seen in a section from an acute appendicitis specimen (Figure 5G). Anti-D6 antibodies failed to reveal the presence of D6-immunoreactive ECs in paraffin sections of heart, kidney, liver, skeletal muscle, brain, cerebellum, pancreas, prostate, and thyroid, although in some tissues (eg, lung,

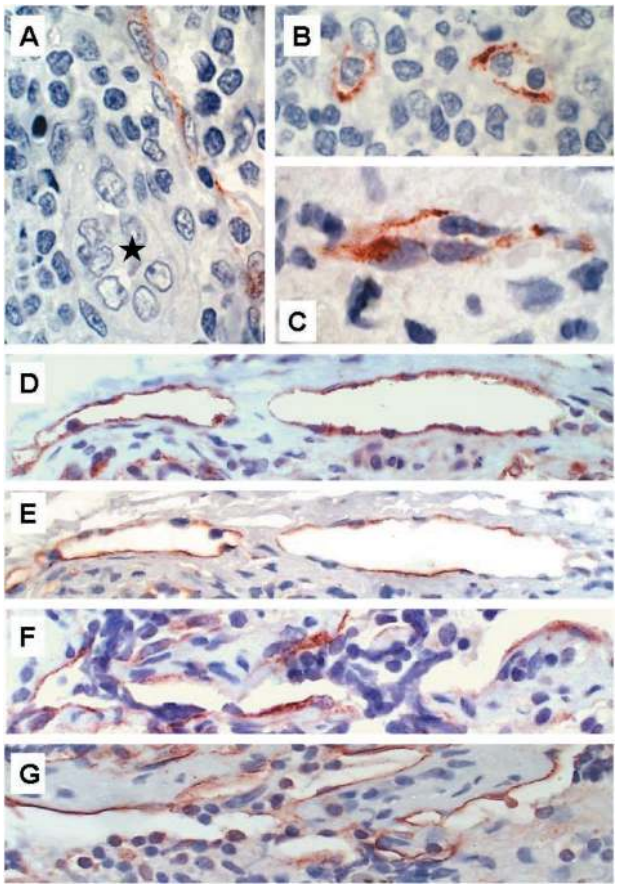

Figure 6. D6 immunoreactivity in secondary lymphoid organs. Representative images of paraffin-embedded sections from secondary lymphoid organs, immunostained with either anti-D6 (**A–D** and **F**), or anti-podoplanin (**E** and **G**), antibodies. Immunoreactivity is visualized using an avidin-biotin-peroxidase detection system and a peroxidase substrate producing a red stain; all sections counterstained with hematoxylin. **A** and **B:** D6 immunostaining in tonsils. **Asterisk** marks an HEV (both original magnifications, ×1,000). **C:** D6-immunoreactive vessel in red pulp of a spleen (original magnification, ×1,250). **D–G:** Lymph node. Adjacent sections showing subcapsular (**D** and **E**) and medullary (**F** and **G**) sinuses of the lymph node stained with anti-D6 (**D** and **F**) or anti-podoplanin (**E** and **G**) antibodies. Original magnifications: ×720 (**D–G**).

liver, and placenta) D6 immunoreactivity was seen in cells that were not lymphatic ECs. The identity of these cells is being investigated.

In conclusion, whereas in several organs known to contain lymphatics (eg, heart and liver) no D6-immunoreactive vessels could be detected, skin and gut, both tissues in intimate contact with external environment, contained D6-immunoreactive lymphatics.

D6 Immunoreactivity in Secondary Lymphoid Organs

Because of the presence of D6-immunoreactive channels within the lymphoid tissue of the appendix, we also examined sections from other secondary lymphoid organs (ie, tonsils, spleen, and lymph nodes), for the presence of D6-immunoreactive structures. The parafollicular areas of the tonsils contained abundant D6-immunoreactive sinus-like channels (Figure 6, A and B); HEVs were D6-negative (Figure 6A). The white pulp of spleen contained no D6-immunoreactive structures, whereas in one out of four different spleens tested, D6-immunoreactive vessels were observed in the red pulp (Figure 6C).

In the lymph nodes, afferent lymphatics entering the nodes were D6-positive (not shown) suggesting, together with the findings in skin and gut, that afferent lymphatics may express D6 throughout their entire length. Also, cells lining the subcapsular and medullary sinuses displayed strong D6 immunoreactivity (Figure 6, D and F). Again, no blood vessel ECs were D6-immunoreactive in any secondary lymphoid tissue, including cells lining HEVs. Using the anti-podoplanin antibodies on adjacent sections from lymph nodes and tonsils revealed that D6-immunoreactive vessels also expressed podoplanin-immunoreactivity (Figure 6, E and G, and not shown), but additionally, there were lymphatic sinuses detected that reacted with the anti-podoplanin antibodies only (not shown).

D6 Is Expressed by a Subset of Vascular Tumors

There has been considerable debate concerning the origin and differentiation of the aberrant ECs seen in malignant vascular tumors, ie, whether they are blood vessel EC-like or lymphatic EC-like. Recently, the lymphatic EC markers podoplanin and VEGFR-3 have been shown in some vascular tumors.[1,2,4,35] As D6-immunoreactive ECs are exclusively lymphatic, we reasoned that D6 immunoreactivity might be expressed in the vascular tumors that show lymphatic origin or differentiation. As an initial assessment, we screened a panel of 15 vascular tumors predominantly from the skin for the expression of D6 immunoreactivity, and by using adjacent sections, for the expression of podoplanin immunoreactivity. As shown in Table 1, there was considerable variation in the presence of these two markers. Examples of vascular tumors stained with the D6 antibodies are shown in Figure 7. In general, those tumors that expressed podoplanin also displayed, on a variable proportion of their cells, D6 immunoreactivity. The same 11 tumor samples showed some immunoreactivity with both antibodies, although in only three of these cases D6 immunoreactivity was present in almost all of the cells of the tumor (Figure 7; A, B, and D): podoplanin immunoreactivity was expressed on 100% of the tumor cells in these three cases plus a further four samples (Figure 7, E and F, and Table 1). In fact, podoplanin immunoreactivity was consistently seen to be expressed on a higher percentage of the tumor cells than D6. Also, the intensity of the staining with the D6 antibodies varied between samples: six samples showed intense immunoreactivity, three stained only weakly, and two tumors contained populations of cells that expressed different levels of D6 immunoreactivity (Table 1). These results further support the observations made by others,[1,2,4,35] showing that vascular tumors often express markers of lymphatic ECs. However, there is considerable heterogeneity between vascular tumor samples with respect to the distribution and levels of expression of these markers.

Table 1. D6 and Podoplanin Immunoreactivity in 15 Vascular Tumors

	Number	Sex	Age	Tumor and localization	% Observed positive tumor cells	
					D6	Podoplanin
1.	P99/9205	F	29	AS, skin	100 strong	100
2.	P98/10612	M	40	AS, larynx	100 strong	100
3.	166/93	M	64	AS, skin	40 strong	80
4.	3731/95	M	N/A	AS, skin	40 strong	100
5.	10151/99	M	78	AS, skin	50 strong	100
6.	P89/0542	N/A	N/A	EHE, skin	100 strong	100
7.	8703/94	M	96	AS, skin	20 mixed	80
8.	7652/95	M	37	AS, heart	10 weak	40
9.	P96/4967	F	72	AS, skin	20 weak	100
10.	3485/93	M	86	AS, skin	10 weak	80
11.	13260/95	F	31	AS, vagus nerve	5 mixed	100
12.	P99/1256	F	56	AS, skin	0	0
13.	P97/8908	M	53	HE, shoulder bone	0	0
14.	P98/9946	F	67	EHE, liver	0	0
15.	P96/6947	F	72	AS, skin	0	0

Serial sections were stained with anti-D6 or anti-podoplanin antibodies, and staining was assessed in terms of the percentage of cells within the tumor that were stained. In the case of D6, the intensity of the staining was also assessed and is presented as "strong," "weak," or "mixed" (when strong and weak stained cells were seen in the same section). M, male; F, female; N/A, not available; AS, angiosarcoma; EHE, epithelioid haemangioendothelioma; HE haemangioendothelioma.

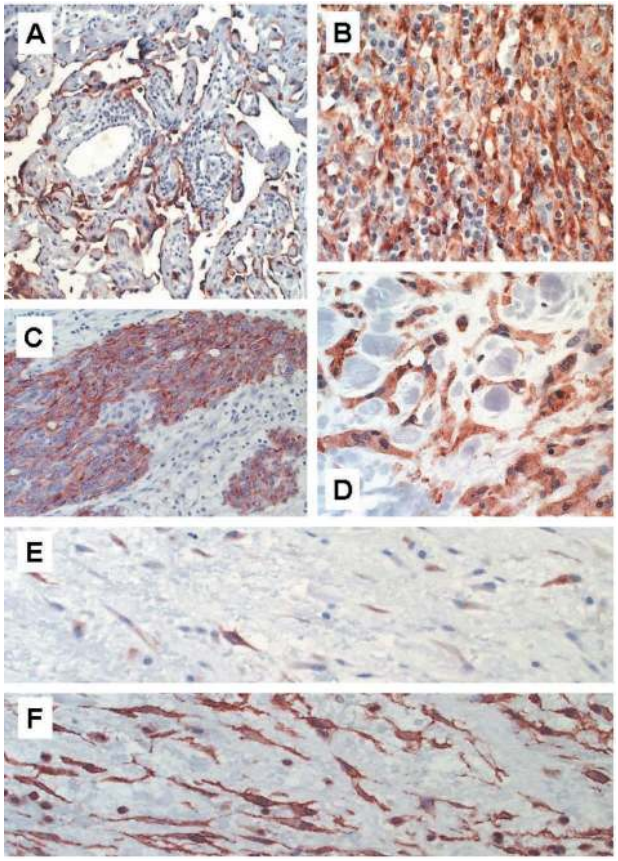

Figure 7. D6 immunoreactivity in vascular tumors. Paraffin-embedded sections of vascular tumors stained with anti-D6 antibodies (**A–E**), or anti-podoplanin antibody (**F**) with immunoreactivity visualized using an avidin-biotin-peroxidase detection system and a peroxidase substrate producing a red stain. All sections counterstained with hematoxylin. **A, B, C, D**, and **E** show tumors listed in Table 1 as numbers 1, 2, 3, 6, and 11, respectively. **F** shows a section adjacent to **E** stained with anti-podoplanin antibody. Only a small proportion of podoplanin-positive cells is D6-positive. Original magnifications: ×140 (**A** and **C**); ×300 (**B**); ×400 (**D**, **E**, and **F**).

Discussion

The mechanisms that control the growth and function of lymphatic vessels are poorly understood, and only recently have proteins specifically expressed by lymphatic ECs been identified. Thus, the demonstration that VEGFR-3/Flt4 (a receptor for VEGF-C), LYVE-1 (a hyaluronan receptor), and podoplanin (function currently unknown) are present on lymphatic ECs has represented a major advance in the study of these cells that may ultimately lead to new insights into the biology of lymphatic vessels.[1–4] Here, we add an additional molecule to the list of proteins expressed by lymphatic but not vascular ECs, the β-chemokine receptor D6. Moreover, the expression of this receptor on only a subset of lymphatics suggests the existence of functional heterogeneity within the lymphatic vasculature.

Using *in situ* binding assays we have demonstrated previously that a receptor for pro-inflammatory β-chemokines is expressed on lymphatic ECs in the skin.[22] This receptor exhibited ligand-binding specificity akin to that observed with the β-chemokine receptor D6. Monoclonal antibodies raised against the receptor, and antisense D6 RNA probes, specifically stained ECs in the skin that were also stained, in serial sections, with antibodies against the lymphatic EC marker podoplanin. D6-immunoreactive lymphatic ECs were also seen in the mucosa and other wall layers of the gut, in afferent lymphatic vessels entering lymph nodes and in lymphatic sinuses within secondary lymphoid tissue. We did not observe D6 immunoreactivity on ECs lining blood vessels of any caliber in any of the sections tested. The specificity of D6 expression described here is intriguing and, even in the absence of functional data, suggests a role for this molecule in lymphatic EC biology, possibly regulating leukocyte trafficking through these vessels, or the development and growth of lymphatic channels themselves.

Lymphocytes and DCs leave the peripheral tissues and migrate via the afferent lymphatics into the draining

lymph nodes. This requires leukocytes to find the afferent lymphatics, enter them, and rapidly advance through them. Although traditionally all three of these migration steps have been considered passive, they may be influenced by chemokines. The ability of several β-chemokines to bind D6 on the lymphatic ECs may enable or disable the chemokine control of leukocyte trafficking through the afferent lymphatics. One can envisage a number of possible functions for D6 on lymphatic vessels in lymphocyte and DC trafficking. The range of potential alternatives is particularly broad because currently the cellular function of D6 is unclear. However, the promiscuity, nonsignaling status and EC expression of D6 make it tempting for us to group it together with DARC into a functionally related subclass of chemokine receptors.

We are considering the following functional outcomes for D6-chemokine interaction on the lymphatic EC surface that may be relevant for leukocyte trafficking.

First, the chemokines may be sequestered and neutralized by D6 ("D6 the chemokine sink" hypothesis). This function is reminiscent of the suggested role of erythrocyte DARC.[27] Pro-inflammatory β-chemokines that bind to D6 would be neutralized, but other non-D6-binding chemokines such as SLC (R. Nibbs, unpublished observation), a chemokine that can be expressed by lymphatic ECs[36] and likely to be involved in constitutive lymphocyte and DC trafficking,[9] would remain free to interact with their target cells. It is also possible that neutralization of the inflammatory β-chemokines by D6 within and around the lymphatic vessels prevents the chemokine-induced firm adhesion of leukocytes to the lymphatic ECs. An anti-adhesion mechanism of this type may allow the passive lymph flow to carry the leukocytes that entered lymphatics into the lymph node.

Second, the chemokines may remain fully functional on D6 binding and be presented to leukocytes ("D6 the chemokine presenting molecule" hypothesis), a role also suggested for DARC on venular ECs.[17] This putative function for D6 would allow tissue-derived pro-inflammatory β-chemokines to drive leukocyte migration into the lymph node (assuming the expression of the appropriate chemokine receptors by the pertinent cell populations) and thus contribute to the immune processing of the inflammation. Conversely, firm adhesion of D6 ligand-responsive leukocytes to lymphatic ECs may actually prevent their migration into the lymphatic vessels and retain them in the tissue. Only those cells no longer responsive to D6 ligands, such as mature DCs, would be able to enter the lymph nodes via the D6-expressing lymphatics.

Third, D6 may influence the transport of chemokines across the lymphatic ECs ("D6 the chemokine transporter" hypothesis). This may be analogous with chemokine transcytosis in abluminal to luminal direction by venular ECs[17] that allows tissue-derived chemokines to cross the endothelium, a function possibly involving DARC.[17] Recently, the transport of chemokines from skin via lymphatics into the draining lymph node has been described,[37] and chemokines are readily detectable in the afferent lymphatics of larger experimental animals.[38] It is tempting to speculate that these chemokines form a component of the afferent lymph that can alter the structure or function of the draining node.[39] Indeed, intravital microscopy has shown that, at least in the case of secondary lymphoid tissue chemokine, lymphatic chemokine transport allows for the "remote control" of cell recruitment into the lymph node via the HEV, as the peripheral tissue-derived SLC can be presented on the surface of these cells.[37] Although SLC, the chemokine used in this study, does not bind D6, the demonstrable ability of several pro-inflammatory chemokines to rapidly reach the lymph node after their intradermal injection (A. Rot, unpublished observation) may involve D6-mediated chemokine transport. Conversely, it is also possible that in its putative role as a "chemokine sink" (see above), D6 inhibits the lymphatic transport of the chemokines, thus preventing their "remote control" of lymph node function. A careful analysis of the lymphatic transport of D6-binding and -nonbinding chemokines to the lymph node may provide support for these alternative functions of D6.

Although we can speculate about D6 functions on afferent lymphatics, it is more difficult to discuss the putative function of D6 expressed on the sinuses and channels within tonsils and lymph nodes, as the molecular mechanism of leukocytes trafficking through these, eg, in the process of exiting the secondary lymphoid tissues, has not been thoroughly investigated.

Extensive experimental work and a combination of *in vivo* and *in vitro* approaches will be required to establish which of the several alternative hypotheses described above best reflects the function of D6 on the lymphatic ECs.

We currently favor a role for D6 in some aspect of chemokine-driven leukocyte trafficking. However, bearing in mind that chemokines mediate cellular chemotaxis, plus the observations that chemokines can induce EC migration and regulate angiogenesis,[14,16,40,41] it cannot be excluded that D6 is involved in regulating lymphangiogenesis during development and/or tissue regeneration, or some other aspect of lymphatic EC biology, by outside-in signaling through D6 on ligand binding. Lymphangiogenesis is poorly understood, although a number of potential regulatory molecules have been identified:[42–45] for example, the development of the lymphatic vasculature is aberrant in mice lacking the transcription factor Prox1,[42] whereas the ligand for VEGFR-3, VEGF-C, induces lymphatic vessel hyperplasia when transgenically expressed in mouse skin[45] and may play a role in lymphatic vessel regeneration in healing wounds.[43] It remains to be seen if D6 and its chemokine ligands contribute to developmental or regenerative lymphangiogenesis.

We have shown here that D6 expression is restricted to subsets of lymphatic vessels. Although podoplanin is expressed on a large proportion of these vessels in a wide variety of tissues, D6 immunoreactivity is restricted to a selection of vessels in the gut, skin, and secondary lymphoid tissue. These results suggest phenotypic heterogeneity among lymphatic vessels, an observation not without precedent as the expression of certain adhesion molecules differs between lymphatic vessels.[5–7] It is not

known whether this reflects differences in the maturation status of the lymphatic ECs, or is a consequence of the environment in which an individual lymphatic vessel finds itself. It is interesting to note that examination of the murine D6 promoter sequence suggests that it is likely to be responsive to a range of inflammatory stimuli (R Nibbs, unpublished observation). Inflammation-regulated expression of D6 would be in keeping with the fact that the chemokines bound by D6 are expressed predominantly during inflammatory reactions. Lymphatic vessels draining inflamed sites, or tissues exposed to the external environment, may have different functional requirements in terms of molecule and/or cell movement, and this may be in part regulated by D6 presentation, sequestration, or transport of pro-inflammatory β-chemokines. Finally, it is an interesting possibility that D6-immunoreactive lymphatic vessels in lymphoid tissue are the termini of D6-immunoreactive lymphatic vessels in the adjacent draining tissue, such that expression of this phenotype is present along an entire branch of the lymphatic vasculature. Inductive signals transported within the afferent lymph, akin to those that are required to maintain the properties of ECs lining HEVs,[39] may likewise be necessary to maintain lymphatic vessel EC phenotypes. These questions need to be addressed by further experimentation.

Anti-D6 antibodies are likely to be of use in immunohistochemically identifying a subset of lymphatic ECs. We have also used them here to examine D6 immunoreactivity in vascular tumors. Our experiments with a small group of vascular tumors show 11 out of 15 with detectable D6 immunoreactivity. The same 11 samples also stained with the podoplanin antibodies. In most cases, within a tumor section, there was only expression of these lymphatic EC markers in a proportion of the cells, with podoplanin consistently expressed in a larger proportion of the cells than D6. Also, there was considerable variation in the expression level of D6 between and within tumor samples. Although D6 and podoplanin immunoreactivity may indicate that the tumor is derived entirely, or at least in part, from the lymphatic endothelium, similar experiments by others suggest that this simple interpretation may not be valid and that much ectopic transcription is seen in these tumors.[1,2,4,35] VEGFR-3/Flt4 for instance, which is restricted to lymphatics in normal adult tissue, is reported to be widely expressed in vascular tumors that have a clear blood vessel EC origin.[35] Its presence in vascular tumors is certainly not indicative of their derivation from lymphatic ECs, and in fact, VEGFR-3/Flt4 can be expressed by blood vessel ECs during development and tumor angiogenesis.[46,47] Similarly, podoplanin expression in vascular tumors was often coincident with the expression of blood vessel EC markers.[4] In each case, as with D6, expression of the marker was often seen only in a proportion of the tumor cells.[1,2,4,35] These experiments suggest that rather than simply acting as an indicator of the source of the tumor, the expression of lymphatic EC markers may be because of the process of cellular transformation, or the cytokine milieu of the tumor, aberrantly up-regulating their expression. Whether lymphatic EC proteins are involved in the development of vascular tumors is uncertain, but the presence of D6 could conceivably alter a tumor's response to locally produced chemokines, or affect leukocyte infiltration of the tumor. An examination of the role of D6 in vascular tumorigenesis awaits further insight into the biological function of this receptor.

In summary, we have shown that the β-chemokine receptor, D6, can be expressed by lymphatic ECs and within a subset of vascular tumors. Further experiments will shed light on the function of D6 and how this impinges on the biology of the lymphatic vasculature and leukocyte trafficking.

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