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Thymus cell antigen 1 (Thy1, CD90) is expressed by lymphatic vessels and mediates cell adhesion to lymphatic endothelium

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Abstract

The lymphatic vascular system plays an important role in inflammation and cancer progression, although the molecular mechanisms involved are poorly understood. As determined by comparative transcriptional profiling studies of *ex vivo* isolated mouse intestinal lymphatic endothelial cells versus blood vascular endothelial cells, thymus cell antigen 1 (Thy1, CD90) was expressed at much higher levels in lymphatic endothelial cells than in blood vascular endothelial cells. These findings were confirmed by quantitative PCR, and at the protein level by FACS and immunofluorescence analyses. Thy1 was also strongly expressed by tumor-associated lymphatic vessels, as evaluated in a B16 melanoma footpad model in mice. Blockade of Thy1 inhibited tumor cell adhesion to cultured mouse lymphatic endothelial cells. Importantly, treatment of human dermal microvascular endothelial cells with tumor necrosis factor or phorbol 12-myristate 13-acetate resulted in Thy1 upregulation in podoplanin-expressing lymphatic endothelial cells, but not in podoplanin-negative blood vascular endothelial cells. Moreover, adhesion of human polymorphonuclear and mononuclear leukocytes to human lymphatic endothelial cells was Thy1-dependent. Together, these results identify Thy1 as a novel lymphatic vessel expressed gene and suggest its potential role in the cell adhesion processes required for tumor progression and inflammation.

Keywords

Lymphatic endothelial cells; Thy1; cell adhesion

Introduction

Vertebrate organisms have developed two parallel vascular systems, the blood vascular and the lymphatic system, to cope with the need of transporting fluids, gases and cells throughout the body. While the blood vascular system has been intensely investigated for several decades, the lymphatic vascular system and its specific functions have only recently become the focus of intense scientific interest. The lymphatic vascular system is composed of a network of thin-walled capillaries and larger diameter collecting vessels that are responsible for draining interstitial fluids. Lymphatic vessels also absorb dietary fat and fat-soluble vitamins in the small intestine, and they participate in immune surveillance by

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transporting immune cells and antigens to draining lymph nodes [1]. Lymphatic vessels also play an important role in tumor metastasis spread [2], in chronic inflammatory disorders [3–5] and in transplant rejection [6–8], and lymphatic vessel defects are the underlying cause of congenital and acquired human lymphedema syndromes [4,9].

During the last decade, a growing number of lymphatic vessel-specific markers, including Prox1, LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) and podoplanin have been discovered [10–12], enabling investigations into lymphatic vascular development and function *in vivo*, and isolation of lymphatic endothelial cells for *in vitro* functional studies. Several transcriptional profiling studies have been performed in cultured human lymphatic endothelial cells (LECs) and blood vascular endothelial cells (BECs), revealing novel LEC-expressed genes such as hepatocyte growth factor receptor and coxsackie virus/adenovirus receptor [13–15]. Recently, two studies analyzed the gene expression of human dermal LECs and BECs from fresh tissue, identifying differences between the transcriptional programs of *in vitro* cultured cells and of cells obtained directly from their natural tissue environment [16,17]. The laboratory mouse is the most commonly used model to study normal, developmental and pathological lymphangiogenesis, including a large range of investigations in genetically engineered mice. However, mouse primary LECs are difficult to culture, and the lymphatic vascular gene expression profile in mice has remained unknown.

Therefore, we established a novel method for the specific *ex vivo* isolation of pure mouse LECs and BECs by fluorescence-activated cell sorting (FACS) from colon tissue. Expression analyses confirmed the lineage-specific expression of several previously known endothelial marker genes, but also identified previously unknown lymphatic-specific genes. One of these genes was thymus cell antigen 1 (Thy1, CD90), a cell membrane glycosylphosphatidylinositol-anchored glycoprotein whose expression has been reported on T cells, fibroblasts, neurons and a subset of hematopoietic cells with inter-species differences [18–20]. Thy1 expression was previously also reported on activated human microvascular endothelial cells *in vitro* and *in situ* in psoriatic skin lesions and in melanoma [21–23]. However, the identity of these endothelial cells has remained an open question. The Thy1 protein sequence contains an integrin binding, RGD-like sequence and has been found to bind to integrin α M β 2 on monocytes and integrin α V β 3 on melanoma cells and astrocytes [19,24,25].

In the present study, we found - by qPCR, flow cytometry and immunofluorescence analyses - that mouse LECs *in situ* and *in vitro* express high levels of Thy1. This was the case for the lymphatic vessels in many different organs. In studies of *in vitro* cultured human dermal microvascular endothelial cells (HDMEC), we found that Thy1 is specifically expressed by podoplanin-expressing lymphatic endothelial cells, but not by podoplanin-negative LECs upon *in vitro* activation. We also found that Thy1 expression by activated human LECs mediated immune cell adhesion to LECs. Importantly, Thy1 is also expressed on mouse tumor-associated lymphatic vessels and to a lesser extent on tumor-associated blood vessels, and we found that Thy1 plays an important role in the adhesion of tumor cells to mouse LEC monolayers. Thy1 was also expressed by lymphatic vessels in human tissue, in addition to activated blood vessels. Together, these results indicate that Thy1 plays a functional role in tumor metastasis and in the adherence of immune cells to lymphatic endothelium in inflammation.

Materials and methods

Ex vivo isolation of lymphatic endothelial cells by FACS

8 weeks old C57BL/6J mice, maintained under conventional conditions, were used to obtain colon tissue for *ex vivo* cell isolation as previously described [26]. Briefly, washed colon was cut in pieces and incubated at 37°C in 8 mg/ml collagenase IV (Invitrogen, Carlsbad, CA), 0.5 mg/ml DnaseI (Roche, Rotkreuz, Switzerland), and 5 mM CaCl₂ in PBS for 15 min. After passing through a cell strainer (BD Biosciences, Franklin Lakes, NJ), cell suspensions were centrifuged, resuspended and immunostained with allophycocyanin (APC)-conjugated rat anti-mouse CD31; fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD45.2 (BD Biosciences); hamster anti-mouse podoplanin (clone 8.1.1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) followed by anti-hamster phycoerythrin (PE)-conjugated secondary antibody (Invitrogen); and isotype control antibodies (BD Biosciences). FACS was performed using a FACSria and the FACSDiva software (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Animal experiments were approved by the Kantonales Veterinäramt Zurich.

RNA extraction and amplification

Colon endothelial cells were sorted into RLT Plus lysis buffer (Qiagen, Hilden, Germany), supplemented with β -mercaptoethanol for immediate cell lysis and RNA preservation. RNA was extracted with the RNeasy Plus Micro kit (Qiagen). Amplification of RNA was preformed using the Whole Transcriptome-Ovation Pico RNA Amplification System (NuGEN Technologies, San Carlos, CA).

T cell isolation and RNA extraction

CD4⁺ T cells were purified from dissociated and digested (Liberase and DNaseI solution, Roche) spleens of C57BL/6N mice by positive selection using CD4 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA was extracted from purified CD4⁺ T cells (>90% pure) using TRIzol reagent (Invitrogen) and treated with RQ1 RNase-free DNaseI (Promega, Madison, WI) and RNase-Inhibitor (Applied Biosystems, Foster City, CA). cDNA synthesis was performed using the High Capacity cDNA reverse transcription kit (Applied Biosystems).

Quantitative PCR

Amplified single-stranded cDNA obtained from the sorted endothelial cells was used to analyze CD34 and Prox1 mRNA levels with specific TaqMan assays (TaqMan Gene Expression Assays Mm00519286_m1 and Mm00435971_m1, Applied Biosystems) and the Taqman Gene Expression Master Mix (Applied Biosystems). Duplex reactions with beta-actin (4352341E, Applied Biosystems) as endogenous control were run under standard conditions (AmpliTaq Gold enzyme activation 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). For Thy1 expression analysis, a specific QuantiTect Primer Assay was used (QT00245287, Qiagen) with Power SYBR Green PCR Master Mix (Applied Biosystems), and PCR reactions were performed under standard conditions. 10 ng of cDNA per reaction were used for all qPCR experiments.

Induction of delayed-type hypersensitivity (DTH) reactions

DTH responses were induced in the ear skin of eight-weeks-old C57BL/6N female mice as described [5,27]. Animals were sacrificed and ears were dissected at 2 days after the oxazolone (4-ethoxymethylene-2 phenyl-2-oxazoline-5-one; Sigma, St. Louis, MO)

challenge, and inflammation was confirmed by measuring the ear skin swelling. Single-cell suspensions of ear skin were prepared as described [27]. FACS analysis was performed using the following antibodies: APC-conjugated rat anti-mouse CD31, peridinin chlorophyll protein (PerCP)-conjugated rat anti-mouse CD45 (BD Biosciences), PE-conjugated hamster anti-mouse podoplanin (clone 8.1.1, Angiobio, Del Mar, CA) and FITC-conjugated rat anti-mouse Thy1.2 (eBiosciences, San Diego, CA). Cells were analyzed on a FACSCanto (BD Biosciences) and data were processed with FlowJo software.

B16 mouse melanoma model

B16.F10-luc2 mouse melanoma cells (Caliper LifeSciences, Alameda, CA) were cultured in DMEM medium (Invitrogen) containing 10% FBS. 250,000 cells in 10 μ L PBS were injected into the right footpads of 9- to 11-week-old female C57BL/6N mice [28]. The tumors were allowed to grow for 21 days at which time they reached a diameter of approximately 6 mm. Tumors were excised from sacrificed mice and incubated in 6 mg/ml collagenase IV (Invitrogen) supplemented with 5 mM CaCl₂ for 45 min at 37 °C while rotating. The digestion mixture was passed twice through a 40 μ m cell strainer (BD Biosciences) while flushing with 2% FBS-PBS. Single-cell suspensions were centrifuged at 1100 rpm for 5 min at 4 °C and resuspended in FACS buffer. Cells were immunostained with the same antibodies as mentioned above for the skin single-cell suspensions. Additional B16 tumors were frozen in OCT for immunofluorescence analyses. Animal experiments were approved by the Kantonales Veterinäramt Zurich.

Tumor cell adhesion assay

LECs isolated from ‘immorto-mouse’ (H-2Kb-tsA58 mice [29]) skin were cultured at 37°C in 96-well black clear-bottom plates (Costar, Corning, NY) coated with 10 μ g/ml fibronectin (BD Biosciences) and collagen (Inamed, Fremont, CA), in DMEM/F-12 medium (Invitrogen) supplemented with 20% FBS, heparin (Sigma), and EC mitogen (AbD Serotec, Raleigh, NC). After 3 days, confluent LEC monolayers were incubated with 40 μ g/ml of blocking Thy1.2 antibody (clone 30-H12, BioLegend, San Diego, CA) or control IgG antibody in 0.2% BSA-supplemented DMEM medium for 1h at 37 °C. Mouse tumor cells (B16.F1 melanoma line kindly provided by S. Hemmi; C51 and CT26 colon carcinoma lines kindly provided by D. Neri) were cultured in 10% FBS-supplemented DMEM medium (Invitrogen). Calcein-AM (Sigma)-labeled tumor cells (30,000 per well) were applied on top of the LECs and incubated for 1h, after which non-adherent cells were removed by three PBS washes. Calcein-AM fluorescence was measured in a plate reader (Spectra Max Gemini EM, Bucher Biotec, Basel, Switzerland) and results were analyzed with an unpaired two-tailed t-test. AlphaV integrin subunit, beta3 integrin subunit (Biolegend), alpha M/CD11b integrin (BD Biosciences) and Thy1.2 antibodies were used for FACS analysis of B16. F1, CT26 and C51 tumor cells and immorto-mouse LECs harvested from culture with accutase (Invitrogen).

Histological analyses

Tissue was embedded and frozen in optimal cutting temperature compound (OCT; Sakura Finetek, Zoeterwoude, NE) and immunostains were performed as described [30]. Antibodies used were rabbit anti-mouse LYVE-1 (Angiobio), rat anti-mouse CD31 (BD Biosciences), anti-mouse Thy1.1 and anti-mouse Thy1.2 (eBiosciences), hamster anti-mouse podoplanin (clone 8.1.1), rabbit anti-human Thy1 (Sigma), rabbit anti-human LYVE-1 (Reliatech, Wolfenbuettel, Germany), mouse anti-human podoplanin (D2-40, Signet, Dedham, MA) and mouse anti-human von Willebrand factor (Dako, Glostrup, Denmark). Hoechst 33342 (Invitrogen) was used for nuclear stain. An Axioskop2 motplus microscope, AxioCam MRC digital camera and AxioVision Rel. 4.4 software (Zeiss, Jena, Germany) were used to acquire and process all of the images.

***In vitro* activation of human endothelial cells and leukocyte adhesion assays**

Human umbilical vein endothelial cells (HUVECs) (ScienCell, Carlsbad, CA), HDMECs, dermal LECs and BECs (PromoCell, Heidelberg, Germany) were cultured in EBM medium (Lonza) supplemented as described [15] in fibronectin (BD Biosciences)-coated dishes. Cells in passages 5–9 were used for the assays. Tumor necrosis factor (TNF) (1 ng/ml; R&D Systems, Minneapolis, MN) was added to 70% confluent cells for 24h. Phorbol 12-myristate 13-acetate (PMA) (Sigma) (20 ng/ml) was added to cells for 48h. Activated and control cells were harvested with accutase (Invitrogen). Antibodies used for FACS analysis were: PE-conjugated anti-ICAM-1 (BD Biosciences); anti-podoplanin (CellSciences, Canton, MA); anti-Thy1 (eBiosciences), and APC-conjugated and PE-conjugated secondary antibodies (Invitrogen). Peripheral blood mononuclear cells (MNC) and polymorphonuclear cells (PMNC) were obtained from buffy coats (Blutspende Zürich) of healthy donors. Fractions containing MNC and PMNC with erythrocytes were isolated by Ficoll density centrifugation according to the manufacturer's protocol (Ficoll-Paque PLUS, GE Healthcare, Sweden). The PMNC fraction was incubated with ACK buffer in order to remove erythrocytes. For FACS analysis of PMNC and MNC, a mouse anti-human CD11b antibody (Biolegend) was used. For adhesion assays, human LECs were seeded into 96-well black clear-bottom plates (Costar) and incubated with PMA for 48h. Prior to the addition of Calcein-AM (Sigma)-labeled MNC or PMNC (20,000 per well), LEC monolayers were preincubated with 10 µg/ml of a blocking anti-Thy1 antibody (clone BC9, kindly provided by Dr. A. Saalbach) or with 10 µg/ml of control IgG (Sigma) in 0.2% BSA-supplemented DMEM for 30 min at 37°C. Cells were allowed to adhere for 1h at 37°C. Thereafter, non-adherent cells were removed by two PBS washes. Calcein-AM fluorescence was measured in a plate reader (Spectra Max Gemini EM) and results were analyzed with an unpaired two-tailed t-test.

Results

Isolation of pure populations of mouse colon lymphatic and blood endothelial cells by high-speed cell sorting

To investigate the *in vivo* gene expression profile of mouse lymphatic versus blood vascular endothelial cells, we used FACS sorting of endothelial cells directly from mouse colon tissue. RNA from CD31⁺CD45[−]podoplanin[−] cells (BECs) and CD31⁺CD45[−]podoplanin⁺ cells (LECs) (Fig. 1A) was extracted and amplified and qPCR was performed. We found that in three matched pairs of LECs and BECs, the LEC lineage specific transcription marker Prox1 [12] was expressed up to 6,000 times higher in LECs, whereas CD34, an adhesion molecule shown previously to be highly expressed by blood vessels [15], was expressed up to 23,000 times higher in BECs (Fig. 1B). These results demonstrate the high purity and quality of the isolated cell preparations. Microarray analyses of the same RNAs revealed a number of vascular lineage-specific genes (data not shown).

Lymphatic vessels express high levels of Thy1 *in vivo*

One of the genes revealed by microarrays to be more strongly expressed by lymphatic endothelium was Thy1 (more than 300-fold higher expression in LECs than in BECs). Quantitative PCR analysis confirmed the microarray results and showed a more than 1,400 times higher expression of Thy1 mRNA by *ex vivo* isolated LECs than BECs (Fig. 2A). The higher Thy1 expression by LECs was consistent among matched LEC and BEC pairs isolated from four different mice. We next compared Thy1 expression levels in *ex vivo* isolated LECs, BECs and mouse T cells by qPCR and found that T cells expressed comparable levels of Thy1 as LECs (Fig. 2A and B). Because the *ex vivo* isolated LECs and BECs originated from mouse colon tissue, we next investigated whether the lymphatic-specific expression of Thy1 might be restricted to the colon. Therefore, double immunofluorescence stains for Thy1 and for the lymphatic-specific marker LYVE-1 were

performed on sections of mouse small intestine (Fig. 2C), mesentery (Fig. 2D) and heart (Fig. 2E). Thy1 co-localized with LYVE-1 on lymphatic vessels in mouse intestine and heart (Fig. 2C and E). In healthy mouse mesentery, Thy1 was not expressed by blood vessels which were identified by high expression levels of the pan-vascular marker CD31 (Fig. 2D, arrows), but by lymphatic vessels which were identified by lower levels of CD31 expression (Fig. 2D, asterisks). Consistent with previous reports [18], there were single cells expressing Thy1 (Fig. 2C, arrowhead), probably T cells, in the mouse intestine. We next investigated Thy1 expression in mouse skin by differential immunofluorescence analyses. We found that Thy1 is expressed in LYVE-1/podoplanin/CD31-positive mouse skin lymphatics but not in blood vessels (Fig. 3A). Additional studies in experimental delayed-type cutaneous hypersensitivity (DTH) reactions induced in the ear skin of mice revealed, by FACS analysis, that the high levels of Thy1 expression in mouse lymphatic vessels are not modulated by inflammation *in vivo* (Figure 3B).

Tumor-associated lymphatic vessels express Thy1

Thy1 was previously reported to be involved in binding of melanoma cells to activated human endothelial cells [22]. This prompted us to investigate the expression of Thy1 in a mouse model of melanoma where B16 tumor cells are implanted subcutaneously into the footpad [28]. Immunofluorescence stains of mouse B16 tumor serial sections revealed co-localization of Thy1 and CD31 (Fig. 4A, first row), indicating the existence of Thy1-positive tumor-associated vessels. To further characterize the Thy1-expressing vessel type in B16 tumors, we next performed differential immunofluorescence analyses for Thy1 and lymphatic vessel markers. Importantly, we found a clear co-localization of Thy1 and the lymphatic marker LYVE-1 (Fig. 4A second row), as well as Thy1 and the lymphatic marker podoplanin (Fig. 4A third row, arrowheads). Some podoplanin-negative vessel-like structures expressed Thy1 at lower levels, likely representing blood vessels (Fig. 4A, arrows). To quantitatively assess the expression levels of Thy1 in tumor-associated vessels, we next performed flow cytometry analysis of B16 tumor single-cell suspensions immunostained for CD31, podoplanin, the leukocyte marker CD45, and for Thy1 (Figure 4B). We found that CD31⁺CD45⁻podoplanin⁻ BECs expressed low levels (mean fluorescence intensity (MFI): 22-25) of Thy1, whereas CD31⁺CD45⁻podoplanin⁺ LECs expressed high levels (MFI: 576-596) of Thy1 (Fig. 3B). As expected, a subset (9–16%) of CD45⁺ cells was highly positive for Thy1 (MFI: 660-690), likely representing T cells (Figure 4B). We next investigated whether tumor cells might bind to lymphatic endothelium in a Thy1-dependent manner. Cultured LECs from mice expressing large T antigen under the control of a temperature-sensitive promoter ('immorto-mouse' [29]) expressed high levels of Thy1, as shown by flow cytometry (Fig. 4C). In contrast, B16 cells, as well as C51 and CT26 colon cancer cell lines were negative for Thy1 (Fig. 4C, Fig. S1).

We next preincubated mouse LEC monolayers with either Thy1-blocking antibody [31,32] or with control IgG and applied fluorescently labeled tumor cells on top of the monolayers. B16 cells, as well as the colon cancer lines C51 and CT26, adhered less strongly to LECs when Thy1 was blocked (Fig. 4D; **p*<0.05). To determine if the reduced tumor cell adhesion was specific to Thy1 inhibition, we analyzed by FACS the expression of Thy1 ligands in B16, C51 and CT26 cells. We found that all three tumor cell lines express both α V and β 3 integrins (Fig. 4E). In contrast, none of the tumor cell lines expressed the other known ligand of Thy1, integrin α M/CD11b (Fig. 4E). These results indicate that tumor-associated lymphatic vessels express high levels of Thy1 and that they might interact with tumor cells via Thy1 to serve as conduits for tumor metastasis to the sentinel lymph nodes.

Activated human lymphatic endothelium expresses Thy1

Previously, human dermal microvascular endothelial cells were shown to express Thy1 upon activation [25]. However, only a subset of activated cells expressed Thy1 and the character of these cells was not precisely defined [23]. To investigate the expression of Thy1 on different human endothelial cells, we analyzed by flow cytometry cultured primary human dermal LECs, HDMECs, dermal BECs and HUVECs activated with PMA or with TNF. We did not detect Thy1 expression in any of the untreated cells (Fig. 5A). Interestingly, only podoplanin⁺ cells, representing the dermal LECs and the LEC subset of HDMECs, expressed Thy1 after activation with PMA and, to a lesser extent, after activation with TNF (Fig. 5A). BECs and HUVECs that represent different blood vessel endothelial cell types did not express podoplanin and did not express Thy1 when activated. Activated LECs, HDMECs, BECs and HUVECs upregulated ICAM-1, an adhesion molecule present on inflammation-activated endothelial cells, to various extents (Fig. S2). These results clearly demonstrate that only human LECs upregulate Thy1 *in vitro* and that most likely the Thy1⁺ subset of HDMECs shown in earlier reports represented LECs.

To investigate whether Thy1 expressed by the activated human LECs might play a biological role, we next performed adhesion assays with leukocytes isolated from human peripheral blood. In the presence of a Thy1 blocking antibody, adhesion of both PMNC and MNC to PMA-activated LECs was significantly reduced (Fig. 5B). The extent of adhesion reduction in comparison with the control IgG treatment (PMNC: 34% reduction, **p=0.005, MNC: 23% reduction, *p=0.018) was proportional to the amount of cells expressing the Thy1 ligand CD11b in the blood leukocyte fraction. In the PMNC fraction, likely representing granulocytes, CD11b MFI was 3138 and in the MNC, fraction, likely representing monocytes, CD11b MFI was 1379, as determined by FACS analysis. These results indicate that the observed adhesion reduction was, at least in part, specific to the interaction of Thy1 with CD11b on leukocytes.

Previously, Thy1-positive vessels were found in human tumor tissue. Since these vessels were not characterized by the use of vascular differentiation markers, it remained unclear whether these vessels represented blood or lymphatic vessels [22]. Therefore, we next performed differential immunofluorescence analyses of human tissue samples for the expression of Thy1 and of lymphatic and blood vessel markers. In normal human skin, we found podoplanin-positive lymphatic vessels with low Thy1 expression; these vessels were negative for the blood vessel marker von Willebrand factor (Fig. 5C, asterisks). Importantly, in human prostate cancer tissue, podoplanin-positive lymphatic vessels with high Thy1 expression were found (Figure 5D, arrows). In addition, Thy1 was detected on several podoplanin-negative/Thy1-positive blood vessels and on surrounding fibroblasts (Figure 5D, arrowheads) indicating that in the activated human tissue, both blood and lymphatic vessels express Thy1.

Discussion

In the present study, we established and applied a novel protocol for the fast and efficient isolation of pure LECs and BECs directly from mouse colon tissue by high-speed cell sorting. By transcriptional analyses, we confirmed the expression of lymphatic lineage-specific genes such as Prox1 and podoplanin, and we identified Thy1, a gene previously not reported to be expressed by LECs, as a lymphatic vascular gene. Thy1 was originally discovered as a mouse pan T cell marker [33]. Since then, Thy1 has often been used for the separation of different populations of mouse lymphocytes [18,33]. The function of Thy1 on T cells has remained controversial due to the lack of an intracellular domain that might signal, but its ability to co-stimulate T cell activation [34]. Surprisingly, in spite of the intense Thy1 research in immunology, its high expression on lymphatic endothelial cells has

not been previously reported. Our results reveal, for the first time, that mouse LECs *in vivo* strongly express Thy1 with expression levels even higher than those of mouse T cells. We confirmed these findings both at the mRNA and the protein level, and found lymphatic-specific Thy expression in lymphatic vessels in several different mouse organs, indicating that Thy functions are not restricted to intestinal lymphatic vessels.

Thy1 was previously reported to have a role in cell-to-cell adhesion in neurons and activated endothelial cells [19,24,25]. Mediation of cell-to-cell adhesion is also an important biological function of lymphatic endothelium. Lymphatic vessels present a major conduit for metastatic cancer cells on their way to draining sentinel lymph nodes and, likely, to distant organs [2]. Increased tumor-associated and lymph node-associated lymphangiogenesis is correlated with enhanced cancer metastasis and poor prognosis [35–37], and the extent of lymph node metastasis is a major determinant for the staging and the prognosis of many human tumors [38]. Nonetheless, the exact molecular mechanisms that promote tumor spread via lymphatic vessels are still largely unknown, and the events governing tumor cell adhesion to lymphatic vessels are currently of great interest. Thy1 was previously suggested to participate in the binding of human melanoma cells to activated microvessels but the identity of these vessels remained unclear [24]. Our studies reveal that Thy1 is expressed by tumor-associated lymphatic vessels and to a lesser extent by blood vessels in mice, and that adhesion of several tumor cell types to LEC monolayers is significantly decreased when Thy1 is blocked. These findings indicate a possible novel role for Thy1 in cancer metastasis via lymphatic vessels. However, future studies are needed to address this issue in more detail.

The expression of Thy1 by human endothelial cells has remained rather controversial. Thy1 was found on CD31-expressing vessels *in situ* [21,22,25], but also in fibroblasts that closely surrounded the vessels [25]. Various results have been reported regarding the possible expression of Thy1 by cultured human endothelial cells, and it has remained unclear whether it is present on resting endothelial cells [21,23]. None of these reports investigated the endothelial cells for the expression of lymphatic vessel markers. Our analyses of Thy1 expression in resting and activated LECs, HDMECs, BECs and HUVECs provide the first evidence that *in vitro* Thy1 is expressed only by activated podoplanin-positive endothelial cells, thus cells of lymphatic vessels origin, but not by podoplanin-negative blood vascular endothelium. Importantly, we also found that Thy1 expressed on the surface of activated human LECs facilitates immune cell adhesion. These results have important implications for the increasing number of inflammatory diseases in which lymphatic vessels have been shown to play an important role [3,5,39].

Analyses of normal and neoplastic human tissues revealed that some, but not all, lymphatic vessels express Thy1, in contrast to the various mouse tissues studied where all of the lymphatic vessels were strongly Thy1 positive. Moreover, Thy1 expression on human endothelium is apparently activation-dependent both *in vitro* and *in vivo*, whereas Thy1 levels on mouse lymphatic vessels were not modulated by cell activation. In healthy mouse tissues, lymphatic vessels were the only vessel type expressing Thy1, whereas in mouse tumors, weakly Thy-1-positive blood vessels were also detected. In human prostate cancer tissue, we also found podoplanin-negative/Thy1-positive blood vessels. These findings are in line with the previously reported Thy1 expression on activated human blood vessels [22]. It is of interest that similar inter-species differences have also been found for Thy1 expression by T cells: Thy1 is a pan-T cell marker in mice, whereas it is almost completely absent from human T cells [18]. There are also other genes that have been found to be differently expressed by murine versus human lymphatic vessels, such as the Cocksackie- and adenovirus receptor [14]. The mechanisms of transcriptional gene control of the lymphatic

endothelium that lead to such inter-species differences will be of great interest for future studies.

In our study, PMA was the most potent inducer of Thy1 expression, in agreement with previous reports [21]. Phorbol esters are known to function by mimicking the natural ligand diacylglycerol and by binding directly to protein kinase C (PKC) [40]. A PKC specific inhibitor has previously been used to demonstrate that PMA-induced Thy1 expression is PKC-dependent [21]. Since PKC acts by phosphorylating potent activators of transcription, it will be of interest to further analyze the pathways that lead to Thy1 expression on human LECs in future studies.

Overall, our results reveal that lymphatic vessels express Thy1 in mouse and human tissues. Considering the important roles that the lymphatic vasculature plays in inflammatory disorders and tumor progression, the Thy1-dependent cell adhesion to lymphatic endothelium might provide a novel therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FACS	fluorescence activated cell sorting
LECs	lymphatic endothelial cells
BECs	blood endothelial cells
Thy1	thymus cell antigen 1
HDMEC	human dermal microvascular endothelial cells
HUVEC	human umbilical vein endothelial cells
PMA	phorbol 12-myristate 13-acetate
PKC	protein kinase C
MNC	mononuclear cells
PMNC	polymorphonuclear cells

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Figure 1. Pure populations of LECs and BECs were isolated by FACS from fresh mouse colon tissue

(A) Colon single-cell suspensions were immunostained for endothelial cell and leukocyte markers. CD31⁺CD45⁻ endothelial cells were further analyzed for podoplanin, and the podoplanin⁻ population was sorted as BECs and the podoplanin⁺ population was sorted as LECs. (B) RNA from sorted *ex vivo* populations of LECs and BECs was extracted and amplified, and qPCR for blood vessel (CD34) and lymphatic vessel (Prox1) marker genes showed a consistent differential expression across three matched pairs of LECs and BECs. Beta-actin was used for normalization of expression levels. Error bars show standard deviation.

Figure 2. Lymphatic vessel endothelium expresses high levels of Thy1

(A) By qPCR, we found more than 1000-fold higher Thy1 expression in *ex vivo* isolated LECs compared to matched BECs. This expression profile was consistent across matched pairs of LECs and BECs obtained from four different mice. (B) Thy1 expression levels were assessed by qPCR in T cells (CD4⁺), LECs and BECs. Beta-actin was used for the normalization of expression levels. Error bars show standard deviation. Immunofluorescence stains of mouse tissues revealed co-localization of Thy1 (green) with the lymphatic marker LYVE-1 (red) on lymphatic vessels in colon (C) and heart (E), and with the pan-endothelial marker CD31 (red) in the mesentery (D). Arrowhead depicts Thy1 positive single cells, probably T-cells. Arrows depict strongly CD31-positive blood vessels that are negative for

Thy1. Asterisks depict a lymphatic vessel with low CD31 expression that is positive for Thy1. Scale bars: 100 μ m, Hoechst nuclear stain (blue).

Figure 3. Lymphatic vessels in normal and inflamed mouse skin express high levels of Thy1
 (A) Mouse ear skin cryosections were immunostained for Thy1, the pan-endothelial marker CD31, and the lymphatic markers LYVE-1 and podoplanin. Thy1 was expressed by lymphatic vessels which expressed all three markers (arrowheads). LYVE-1 and Thy1 expression was analyzed on the same sections, while CD31 and podoplanin expression was analyzed on serial sections. Scale bars: 100 μ m, Hoechst nuclear stain (blue). (B) DTH reactions were induced in mouse ear skin and single-cell suspensions from normal and inflamed ear skin were analyzed by FACS. LECs (CD31+CD45–podoplanin+) expressed high levels of Thy1, whereas BECs (CD31+CD45–podoplanin–) expressed low levels of Thy1. A subset of CD45+ cells expressed high levels of Thy1. Thy1 levels in LECs and BECs were not modulated by inflammation. The experiments were repeated twice with comparable results.

Figure 4. Thy1 expressed by lymphatic endothelium might have a role in tumor cell adhesion
 (A) Serial sections of mouse foot pad-implanted B16 tumor contained intratumoral vessels (CD31 positive) that co-express Thy1 (green). Tumor sections were also stained for LYVE-1 and podoplanin, and a high degree of co-localization with Thy1 was found (arrowheads). Podoplanin-negative blood vessels were found to express low levels of Thy1 (arrows). Scale bars: 100 μ m, Hoechst nuclear stain (blue). (B) FACS analysis of single-cell suspensions of excised B16 tumors from two mice, 21 days after implantation, revealed that tumor LECs (CD31⁺CD45⁻podoplanin⁺) express high levels of Thy1, while tumor BECs (CD31⁺CD45⁻podoplanin⁻) express low levels of Thy1. A subset of CD45⁺ cells expresses high levels of Thy1. (C) Cultured mouse LECs, unlike the mouse melanoma cell line B16, express high levels of Thy1, as assessed by flow cytometry. (D) Pretreatment of mouse

LECs monolayers with Thy1-blocking antibody (30-H12) caused a reduction in the adhesion of tumor cells (B16 melanoma line, C51 and CT26 colon carcinoma lines) to lymphatic endothelium *in vitro* (* $p < 0.05$). Experiments were repeated 3 times and representative results are shown. (E) C51, CT26 and B16 cells were analyzed by flow cytometry for the expression of the αV , $\beta 3$ and $\alpha M/CD11b$ integrin subunits. The FACS plots were compared against the appropriate IgG control signal.

Figure 5. Podoplanin-positive primary human lymphatic endothelial cells express Thy1 when activated

(A) Human primary dermal lymphatic endothelial cells (LEC), dermal microvascular endothelial cells (HDMEC), dermal blood endothelial cells (BEC) and umbilical vein endothelial cells (HUVEC) were activated with PMA (48h) or TNF (24h) and analyzed for the expression of Thy1 by flow cytometry. The majority of LECs expressed Thy1 after PMA treatment. Only the podoplanin-positive subset of HDMECs (representing LECs) upregulated Thy1 when activated with PMA. Gates were set against the control IgG-stained cells. (B) Pretreatment of PMA-activated human LEC monolayers with a Thy1-blocking antibody (BC9) resulted in a significant reduction of adhesion of immune cells (PMNC and MNC) to lymphatic endothelium *in vitro* (* $p < 0.05$, ** $p < 0.001$). The experiments were repeated 3 times with comparable results. (C) Sections of normal human skin were immunostained for Thy1 and podoplanin (first row) and for Thy1 and vWF on serial sections (second row). Asterisks indicate a lymphatic vessel. Scale bar: 100 μ m. (D) Human prostate cancer sections were immunostained for Thy1 and podoplanin. Arrows point to a double positive lymphatic vessel. Arrowheads indicate a podoplanin-negative/Thy1-positive blood vessel. Scale bar: 50 μ m, Hoechst nuclear stain (blue).