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Production of two novel monoclonal antibodies that distinguish mouse lymphatic and blood vascular endothelial cells

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Abstract We produced two novel rat monoclonal antibodies (LA102 and LA5) to identify mouse lymphatic vessels and blood vessels, respectively. We characterized the two antibodies as to the morphological and functional specificities of endothelial cells of both types of vessels. The antibodies were produced by a rapid differential immunization of DA rats with collagenase- and neuraminidase-treated mouse lymphangioma tissues. LA102 specifically reacted with mouse lymphatic vessels except the thoracic duct and the marginal sinus of lymph nodes, but not with any blood vessels. In contrast, LA5 reacted with most mouse blood vessels with a few exceptions, but not with lymphatics. LA102 recognized a protein of 25–27 kDa, whereas LA5 recognized a molecule of 12–13 kDa. Neither antibody recognized any currently identified lymphatic or vascular endothelial cell antigens. Immunoelectron microscopy revealed that the antigens recognized by LA102 and LA5 were localized on both luminal and abluminal endothelial cell membranes of each vessel type. Interestingly, LA102 immunoreactivity was strongly expressed on pinocytotic or transport vesicle membrane in the cytoplasm of lymphatic endothelium. Besides endothelial cells, both

antibodies also recognized some types of lymphoid cells. Since, the LA102 antigen molecule is expressed on some lymphoid cells, it may play important roles in the migration of lymphoid cells and in some transport mechanisms through lymphatic endothelial cells.

Keywords Lymphatic vessel · Blood vessel · Endothelial cell · Biological marker · Monoclonal antibody

Introduction

Blood vascular and lymphatic systems both play essential roles in the local tissue microcirculation. However, despite its critical role in tissue fluid homeostasis, absorption of macromolecules or lipids, and migration of immune cells or malignant cells, the lymphatic system has not received nearly as much attention as the blood vascular system (Karpanen and Alitalo 2001; Hong et al. 2004; Jackson 2004; Zawieja 2005). In part this has been due to a lack of specific markers. For the proper understanding of lymphatic function, it is important to discriminate lymphatic vessels from blood vessels in any tissues and to characterize lymphatic endothelial cells in both physiological and pathological conditions (Swartz and Skobe 2001; Scavelli et al. 2004; Baluk et al. 2005; Ji 2005; Tammela et al. 2005). Furthermore, various vascular lineage-specific genes have been recently identified in both lymphatic and blood vascular endothelial cells in cultures (Hirakawa et al. 2003). The availability of monoclonal antibodies (mAbs) would therefore contribute greatly not only to immunohistochemical identification of lymphatic vessels particularly in mice, but also to understanding the molecular basis of their special functions. Recently, several useful markers for lymphatic vessels have been reported, such as LYVE-1, podoplanin, and Prox-1. LYVE-1 is a specific receptor for hyaluronic acid (Banerji et al. 1999). Podoplanin is a marker for renal podocytes and is also colocalized on lymphatic vessels (Breiteneder-Geleff et al. 1999; Schacht et al.

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2003). Prox-1 is another nuclear marker for developing lymphatic endothelial cells (Wigle and Oliver 1999; Wigle et al. 2002). However, most of the lymphatic markers reported so far have not been originally discovered on lymphatic vessels. It was only by accident that their empirical relationship with lymphatic vessels was realized (Banerji et al. 1999; Breiteneder-Geleff et al. 1999; Wigle and Oliver 1999; Scavelli et al. 2004). Furthermore, their immunohistochemical distribution is not strictly confined to lymphatic endothelial cells and shared with some blood vessel types (Partanen et al. 1999; Mouta Carreira et al. 2001; Jackson 2004; Scavelli et al. 2004).

In order to discover other lineage-specific markers of lymphatic origin, we sought to produce another novel monoclonal antibody directed to mouse lymphatic endothelium. However, there have been several technical problems in the production of specific monoclonal antibodies against lymphatic vessels. First, the source material of lymphatics as an antigen for immunization is difficult to obtain, particularly in small animals. Second, lymphatic specific antigens, if any, seem to have very weak immunogenicity compared with blood vessels, against which many good specific antibodies have been raised maybe due to their relatively strong immunogenicity. Lastly, good screening procedures for the selection of antibodies specific to lymphatics are currently not well established.

In the present study, to address these issues we applied several technical devices to obtain more confined specific antibodies to lymphatic vessels. We induced benign lymphangiomas in mice by intraperitoneal injection of Freund's incomplete adjuvant (Mancardi et al. 1999). This tumor model provides a good amount of relatively pure source material of lymphatic endothelium (Ezaki et al. 2004). Furthermore, we employed a mild enzyme treatment followed by the neuraminidase treatment to expose very minor hidden cell surface antigens (Toshimori et al. 1988), and used a rapid differential immunization protocol to overcome the possible weak immunogenicity of lymphatic endothelial cells. Here we report how we produced a rat mAb, LA102, reacting predominantly with mouse lymphatics, but not with any types of blood vessel. During the immunization, we also obtained another mAb, LA5, reacting selectively with most of peripheral blood vessels, but not to any type of lymphatic vessel. These novel antibodies may prove useful for further studies of the mouse vascular and lymphatic microcirculation. A brief report of the part of this study has been reported in a meeting proceedings (Morikawa et al. 2004).

Materials and methods

Animals

The DA rats and C57BL/6 mice were bred by sibling mating in our colonies at the Laboratory Animal Center for Experimental Research, Kumamoto University

Medical School (Kumamoto, Japan). Other strains (BALB/c, C3H/He, and CDF1) of mice, 6–8 weeks of age, were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were maintained in air-filtered clean rooms and fed with sterilized standard laboratory chow and water ad libitum. All handling and care of animals was approved by the Kumamoto University Animal Experiment Committee. The following experiments were conducted in accordance with the legislation of Institute of Laboratory Animals for Animal Experimentation at Tokyo Women's Medical University. Female DA rats, 3–4 months of age, and female C57BL/6 mice, 7–8 weeks of age, were used for the following antibody production. On use, all animals were killed by cervical dislocation under ether anesthesia.

Cell lines

Various mouse cell lines (Samelson and Schwartz 1983; Sakaguchi et al. 1988; Kuwahara et al. 1994) as A20 (BALB/c derived sIg⁺ B cell), X63 (BALB/c derived myeloma cell), K62 (T helper hybridoma cell: C57BL/6 × AKR/J), EL4 (C57BL/6N derived T lymphoma cell), 2B4 (T cell hybridoma cell: B10.A × AKR/J), and Ltk⁻ (C3H/An derived fibroblast L-cell line) were cultured in RPMI 1640 medium (Nissui Pharmaceutical CO., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (HI-FCS; Flow Laboratories, Rockville, USA), 5×10^{-5} M 2-mercaptoethanol (2-ME; Nakarai Chemicals, Kyoto, Japan), 2 mM L-glutamine (Wako Pure Chemical Industries, Osaka, Japan), 1 mM sodium pyruvate (Nakarai Chemicals), 3.75 g/l NaHCO₃, 10 mM HEPES (Dojin Chemical Laboratory, Kumamoto, Japan), 100 U/ml penicillin (Meiji Seika Ltd., Tokyo, Japan), and 0.1 mg/ml streptomycin (Meiji Seika Ltd.) at 37°C in a humidified atmosphere of 5% CO₂ in air.

Induction and development of lymphangiomas

Benign lymphangiomas were induced in C57BL/6 mice by two intraperitoneal (i.p.) injections of Freund's incomplete adjuvant (FIA: DIFCO Lab., Detroit, MI, USA) with a 2-week interval according to Mancardi et al. (1999). Briefly, FIA was mixed with an equal volume of calcium- and magnesium-free phosphate buffered saline (PBS; pH 7.2) to make an emulsion and 0.2 ml of the emulsion per mouse was injected i.p. each time. After 1–3 months from the last injection, the tumors developed in the peritoneal cavity, such as on the surface of the liver (Fig. 1a), diaphragm (Fig. 1b), abdominal wall, and omentum.

Immunization and production of rat monoclonal antibodies

The lymphangiomas were removed from the surface of the peritoneal cavity by peeling off with forceps from the

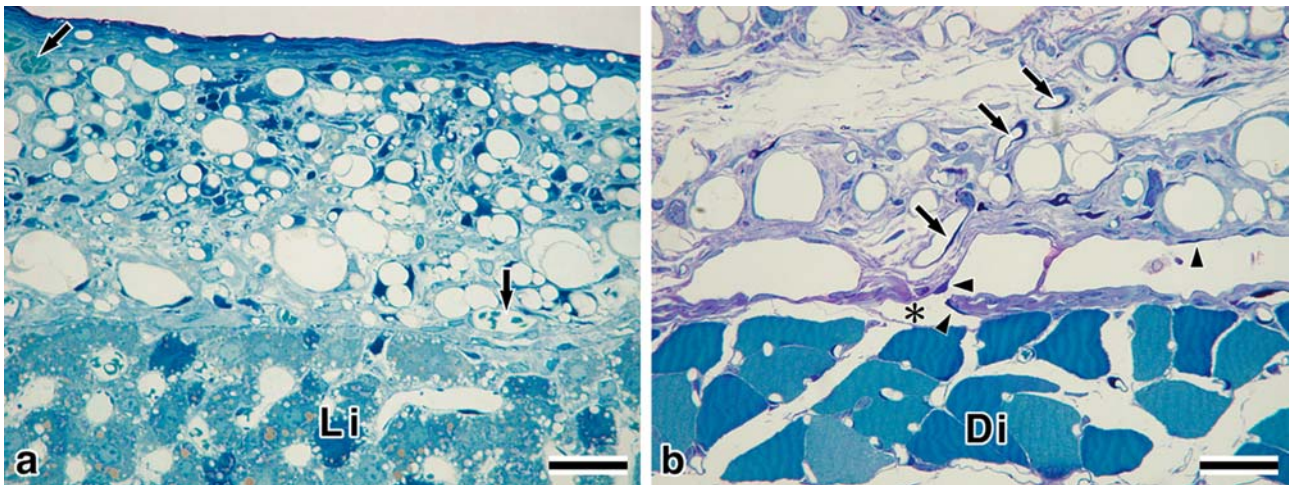


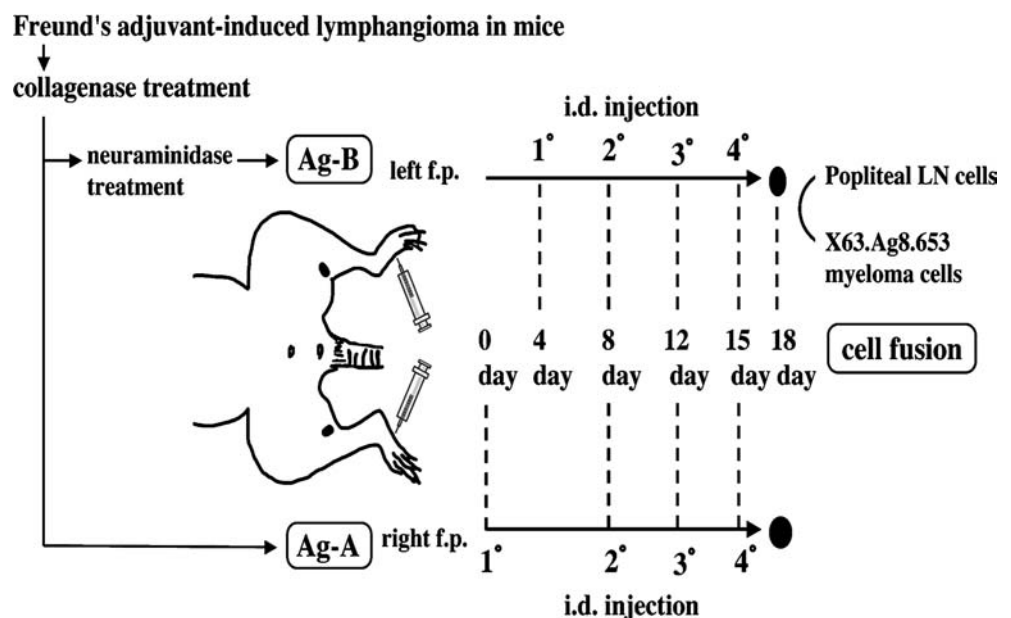
Fig. 1 Adjuvant-induced lymphangiomas used for immunization as an antigen source. Light microscopic views of semi-thin Epon-embedded tissue sections from lymphangiomas developed on the surface of the liver (a) and the abdominal side of diaphragm (b), showing typical cavernous or honeycomb-like structures of various

sizes. Note that a space of lymphatic sinus (asterisk; as recognizable also in Fig. 3a) on the surface of the diaphragm forms the further continuous spaces with endothelial cells (arrowheads) in the tumor tissue. Many blood capillaries (arrows) are also seen. *Li* liver, *Di* diaphragm. Scale bars: 30 μ m

tumor edge. The tumors were cut into small pieces and digested with collagenase D (2 mg/ml of PBS; Roche Diagnostics, Mannheim, Germany) at 37°C for 30 min. Debris or clots of the tissues were removed by sedimentation on ice and free cell suspensions were collected and washed twice with PBS by centrifugation at 200 g for 10 min at 4°C. The collagenase D-treated cell suspension (antigen source A) was divided into equal halves and the latter half was further digested with neuraminidase type V (1 mg/ml of PBS; Sigma Chemical CO., St. Louis, MO, USA) containing 1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF; Wako Pure Chemical Industries Ltd.) as a protease inhibitor at 37°C for 30 min. Debris was removed by

sedimentation on ice and the supernatant was then washed twice with PBS by centrifugation at 200 g for 10 min at 4°C. The final cell suspension (antigen source B) was used for the following immunization. For immunization of DA rats, we used a rapid differential immunization technique to facilitate efforts to raise antibodies specific for antigens that are poorly immunogenic and/or similar in structure or sequence to other proteins as illustrated in Fig. 2. Briefly, on the first day of immunization (Day 0), collagenase D-treated cells in PBS (antigen source A; $2-5 \times 10^6$ cells per rat) were mixed with an equal volume of Freund's complete adjuvant (FCA; DIFCO Lab.) for the first immunization. The mixture (200 μ l per rat) was intradermally

Fig. 2 Procedure for the rapid differential immunization protocol. DA rats received antigen source A (*Ag-A*) in the right footpads (*f.p.*) and antigen source B (*Ag-B*) in the left footpads by intradermal (*i.d.*) injection according to the time schedule as described in the text. After 18 days from the first *Ag-A* challenge, the left popliteal lymph nodes (*LN*) were removed and used for an immunized cell source of the subsequent cell hybridization with a fusion partner, X63.Ag8.653 myeloma cells



(i.d.) injected into right footpads. On Day 4, neuraminidase-treated cells in PBS (antigen source B; $2-5 \times 10^6$ cells per rat) were mixed with an equal volume of FCA and the mixture (200 μ l per rat) was injected i.d. into left footpads. On Day 8, the same antigen sources for each side were mixed with equal volumes of FIA and were injected i.d. into the footpads on the same side, respectively. The same immunization was repeated twice on Days 12 and 15. About 3 days after the last immunization, the left popliteal lymph nodes immunized with antigen source B were removed and teased with forceps to make single cell suspensions. The immunized lymph node cells were fused with X63-Ag8.653 myeloma cells as described previously (Ezaki et al. 1990). After fusion, cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical CO.) supplemented with 10% HI-FCS (Flow Laboratories), 5×10^{-5} M 2-ME (Nakarai Chemicals), 2 mM L-glutamine (Wako Pure Chemical Industries), 1 mM sodium pyruvate (Nakarai Chemicals), 3.75 g/l NaHCO_3 , 10 mM HEPES (Dojin Chemical Laboratory), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Meiji Seika Ltd.). The culture supernatants (total 144 wells) were initially screened on mouse tissue cryosections by immunohistochemical procedure as described below. Hybridomas from positive wells were cloned by limiting dilution (0.25 cells per well) in the presence of either DA rat thymocytes as feeder cells or human rIL-6. Two clones were selected and named LA102 that reacted to lymphatic vessels and LA5 that reacted to blood vessels, respectively. The immunoglobulin (Ig) isotypes of LA102 mAb (IgG2b, κ) and LA5 mAb (IgG2a, κ) were determined by a rat immunoglobulin isotyping ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA).

Labeling of blood vessels with tomato lectin

To discriminate blood vessels from lymphatic vessels, we used intravascular perfusion of tomato lectin as reported previously (Ezaki et al. 2001). Briefly, under anesthesia, mice were injected intravenously (i.v.) with FITC-conjugated tomato lectin (*Lycopersicon esculentum* lectin; Vector Laboratories Inc., Burlingame, USA, 100 μ g lectin per 100 μ l of buffered saline). The tomato lectin binds uniformly to the luminal surface of endothelial cells (Thurston et al. 1996), thus can label all blood vessels that have a patent blood supply. After 5 min, the chest was opened and the aorta was perfused via the left ventricle with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.2) for 5 min followed by PBS for 5 min at a pressure of 120 mm Hg.

Tissue preparation for morphological analyses

Tissue samples, including the tongue, small intestine, diaphragm, liver, mesentery, great omentum, spleen, mesenteric lymph nodes, heart, kidney, and other organs

were examined. After the perfusion, tissues were removed, cut into small pieces and then rinsed in PBS. The tissues were further immersed in PBS containing a graded series of sucrose (10, 15, and 20%) at 4°C for 6–18 h, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA), and then snap-frozen in liquid nitrogen. Cryostat sections (10–50 μ m thick) were made and placed on silane-coated glass slides and dried in air for at least 2 h. As whole-mount tissue samples, the mesentery and the diaphragm were directly spread onto silane-coated glass slides, air-dried and stored at -30°C as above until use.

For semi-thin Epon-embedded sections, various tissues were excised, cut into small blocks and fixed by immersion in 4% PFA in 0.1 M PB (pH 7.2) at 4°C for 24 h. After washing out the fixatives, tissues were dehydrated in a series of ethanol gradients, infiltrated with propylene oxide, and then embedded in Epon. Sections, 1 μ m-thick, were made and stained with 1% toluidine blue solution.

Immunohistochemistry

The distribution of staining for LA102 and LA5 was studied by the indirect immunostaining technique as described previously (Ezaki et al. 1995). After thawing and air-drying, cryosections were rehydrated in PBS, and then incubated first with 4% Block Ace blocking solution (Dainippon Seiyaku Ltd., Tokyo, Japan) to reduce nonspecific background staining. For the ordinary immunoenzymatic staining, tissue sections were incubated first with LA102 or LA5 mAbs for 2 h at room temperature (RT) or overnight at 4°C. The sections were further incubated with goat anti-rat immunoglobulins (Ig) F(ab')₂ fragments labeled with either horseradish peroxidase (HRP) or alkaline phosphatase (ALP) (GE Healthcare UK Ltd., Buckinghamshire, UK; 1:200 in PBS with 1% heat-inactivated normal mouse serum) for 1 h. The HRP reaction was developed at RT for 10–20 min in solutions of 10 mg of 3'-diaminobenzidine hydrochloride (DAB; Dojin Chemicals) in 30 ml PBS with 10 μ l of 30% H_2O_2 (HRP-DAB system). The ALP reaction was developed at RT for 10–20 min in a Vector blue reaction kit solution (ALP-Vector blue system; Vector Laboratories). Sections were fixed again with 1% glutaraldehyde in PBS at RT for 5 min, washed in distilled water and lightly counterstained with hematoxylin. Finally, the sections stained with HRP-DAB system was mounted in EUKITT (O. Kindler GmbH & CO., Freiburg, Germany) after dehydration, and the other sections stained with ALP-Vector blue system were mounted in Aquatex (Merck, Darmstadt, Germany) without dehydration.

For immunofluorescent staining, a Cy-3 conjugated goat anti-rat Ig antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary reagent. For double immunostaining, a rabbit anti-mouse LYVE-1 antibody (Angiobio, San Diego, CA, USA)

was used to compare with LA102 as a specific marker for the mouse lymphatic endothelial cells (Prevo et al. 2001). A Cy-3 conjugated goat anti-rabbit Ig antibody (Jackson ImmunoResearch) was used as its secondary reagent. All samples stained by the immunofluorescent method were examined with a Leica TCS-SL confocal laser-scanning microscope. The proper concentration of each antibody was predetermined beforehand.

Immunoelectron microscopy

After DAB reaction, the sections were fixed in 2.5% glutaraldehyde in PBS at 4°C for 1 h, subsequently in 2% osmium tetroxide in PB at room temperature (RT) for 1 h, dehydrated in a graded series of ethanol, and then embedded in an epoxy resin. Ultrathin sections, stained with lead citrate, were examined with a Hitachi H-7000 electron microscope.

Fluorescence-activated cell sorter (FACS) analyses

The immunoreactivities of LA102 and LA5 mAbs to mouse lymphoid cells were examined by immunofluorescence flow cytometry. Single cell suspensions of various lymphoid tissues were prepared by gentle teasing of tissues on stainless steel mesh (mesh size: about 250 μm). Cells ($1\text{--}3 \times 10^6$ cells) were washed in PBS containing 1% heat-inactivated fetal calf serum (PBS-FCS) and incubated with LA102 or LA5 monoclonal antibodies for 30 min on ice. Cells were washed in PBS-FCS twice and incubated with Alexa488-conjugated goat anti-rat IgG (H+L) (Invitrogen, Carlsbad, CA, USA) for 30 min at 4°C. For double staining, cells were further stained with biotin-labeled rat mAbs against B220 (CD45R) as a B cell lineage marker (RA3-6B2; BD Biosciences, Franklin Lakes, NJ, USA), CD3e as a T cell lineage marker (145-2C11; BD Biosciences), Mac-1 (CD11b) as a macrophage lineage marker (M1/70; BD Biosciences) in combination with PerCP-Cy5.5-conjugated streptavidin (BD Biosciences) were used. Cells stained were measured and analyzed by FACS Calibur (BD Biosciences) using CellQuest software. The data were expressed as cell frequency on the vertical line (linear scale) and fluorescence intensity on the horizontal line (logarithmic scale) in the single-color staining.

Isolation of cultured cell membrane fraction and Western blotting analyses

2B4 cells (4.5×10^7) and EL4 cells (2.5×10^7) were collected by centrifugation at 280g for 8 min at 4°C. The collected cells were resuspended in Tris-based buffer (20 mM Tris-HCl: pH 8.0, 5 mM CaCl_2 , 25 mM NaCl). Cells were sonicated, and then centrifuged at 200g for 1 min at 4°C. The supernatants were mixed with an equal volume of 60% (wt./vol.) sucrose in Tris-based

buffer to make up a cell lysate in 30% (wt./vol.) sucrose solution. Sucrose step gradients were made by the successive addition of 60, 50, 45, 40, and 30% (containing the cell lysate) into ultraclear centrifuge tubes. Gradients were ultracentrifuged in a HITACHI P28SA rotor (Hitachi, Tokyo) at 85,000g for 1 h at 4°C. Each sample of cells from the 40–45% sucrose fractions was collected in new tubes and diluted with Tris-based buffer to adjust the sucrose concentration to less than 10%. Then, these fractions were ultracentrifuged at 85,000g for 1 h at 4°C. The precipitate of cell membranes was analyzed by 15% SDS-polyacrylamide gel electrophoresis in an SDS-loading-buffer without 2-ME. Following semi-dry electrotransfer to nitrocellulose membranes (Advantec Ltd., Tokyo, Japan) and blocking with PBS containing 5% skim milk (Yukijirushi Ltd., Hokkaido, Japan) for 1 h at RT. The blots were incubated for 1 h with LA102 or LA5 at a final concentration of 1 $\mu\text{g}/\text{ml}$. After washing three times with PBS, the blots were incubated with a goat anti-rat IgG-HRP antibody (Jackson ImmunoResearch) at a dilution of 1:2,000 for 1 h and washed with PBS once again. The specific reaction was detected using the Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection Reagents (Amersham Biosciences, NJ, USA).

Results

Distribution of immunoreactivities of LA102 and LA5 mAbs in various microvascular beds

LA102 strongly recognized endothelial cells of almost all mouse lymphatic vessels, such as lymphatic capillaries in the diaphragm (Fig. 3a), in skin (Fig. 3b), and collecting lymphatics in the tongue (Fig. 3c). In lymph nodes (Fig. 3d), both afferent (Fig. 3e) and efferent lymphatic vessels (Fig. 3d, f) were strongly stained, however, the subcapsular marginal sinus endothelial cells were not evenly recognized. Namely, the sinus endothelial cells of the medullary side were recognized by LA102, whereas those of cortical marginal sinuses were clearly negative for LA102 (Fig. 3e, f). Furthermore, the thoracic duct as a large lymphatic trunk was not stained by this antibody (images not shown).

The immunoreactivity of LA102 was further compared with another monoclonal antibody, LA5, which was simultaneously obtained in the same immunization process and investigated as a control antibody for LA102. In the intestinal villi, LA102 reacted with the central lacteal (Fig. 4a), another typical lymphatic capillary, whereas LA5 only recognized the blood capillary networks (Fig. 4b) around the central lacteal. In the tongue, scattered lymphatics were recognized by LA102 (Fig. 4c), while overall vascular beds, including capillaries, small arteries, and veins, were stained by LA5 (Fig. 4d). Besides, LA5 recognized capillaries and small blood vessels in the brain (Fig. 4e) and in the heart

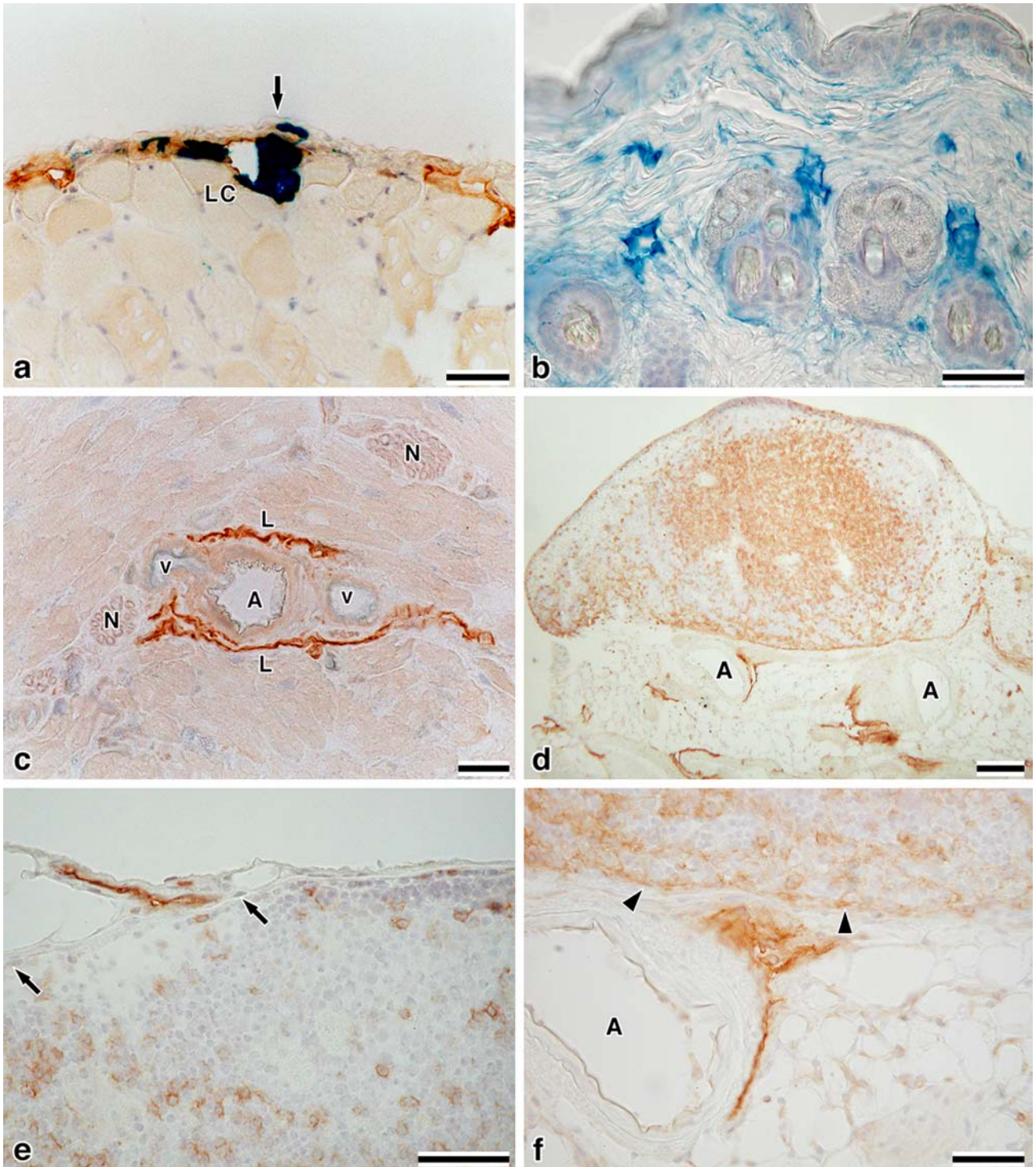


Fig. 3 Immunoenzymatic staining of various mouse tissues with mAb LA102. **a** The diaphragm from a mouse received an intraperitoneal injection of blue dyes (EM Blue; Toyo Ink MFG. CO., Ltd., Tokyo) 30 min prior to its sacrifice. Lymphatic capillaries (*LC*) or sinuses containing the absorbed blue dyes through the peritoneal mesothelium (*arrow*) are stained brown with HRP-DAB system. **b** The back skin. Lymphatic capillaries in the dermis are stained blue with ALP-Vector blue system. **c** The lymphatic vessels (*L*) in the muscle layer of tongue are stained brown with HRP-DAB system. **d** Lymphatic vessels around

mesenteric lymph nodes are stained brown with HRP-DAB system. Also note that lymphoid cells in the lymph nodes are also stained. **e** A higher magnification view of the outer cortical capsule of lymph nodes. An afferent lymphatic vessel is stained. Note that the outer lining cells of the marginal sinus (*arrows*) are not stained with LA102. **f** A higher magnification view of the hilum of lymph nodes. An efferent lymphatic vessel is stained. Note that the outer lining cells of the medullary sinus are also weakly stained (*arrowheads*, also see Fig. 3d). *A* artery, *N* nerve fibers, *V* vein. *Scale bars*: 20 μm (**a** and **e**), 50 μm (**b**, **e**, and **f**), and 100 μm (**d**)

(Fig. 4f). Interestingly, however, LA5 did not react with the endothelial cells of large blood vessels, such as the endocardium (Fig. 4f), the aorta, and the vena cava inferior. It did not recognize the liver sinusoids and

splenic sinuses, either. In general, LA5 reactivity was stronger in the arterial side than in the venous side. The overall immunoreactivity of the two antibodies to various vessels is summarized in Table 1. Interestingly, some

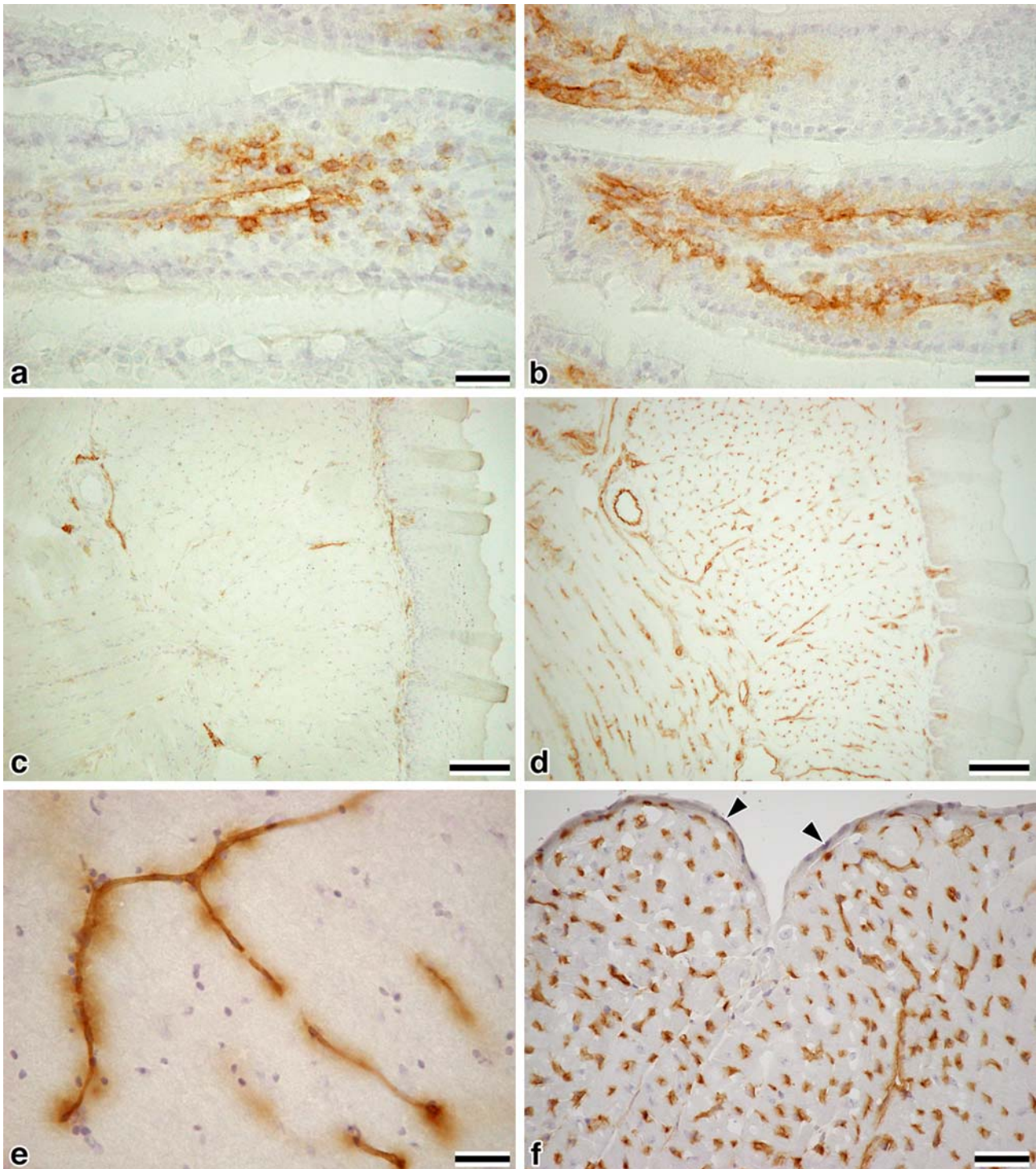


Fig. 4 Immunoperoxidase staining of the vasculature in various tissues with mAb LA102 and mAb LA5. Microvessels in small intestinal villi (**a** and **b**), the tongue (**c** and **d**), the brain (**e**), and the heart (**f**) are stained with mAb LA102 (**a** and **c**) and mAb LA5 (**b**,

d~**f**). LA5 recognizes blood vessels, whereas LA102 reacts only with lymphatic capillaries and collecting lymphatic vessels. *Scale bars:* 25 μm (**a** and **b**), 50 μm (**e** and **f**), and 100 μm (**c** and **d**)

Table 1 General immunoreactivity of LA102 and LA5 antibodies in various circulatory vessels

| Vessel types ^a | LA102 | LA5 |
|---|-----------------|-----|
| Lymphatic vessels | | |
| Lymphatic capillary (e.g. lacteals) | ++ ^b | - |
| Collecting lymphatic vessel | ++ | - |
| Afferent lymphatic vessel | ++ | - |
| Cortical marginal sinus of lymph nodes | - | - |
| Medullary marginal sinus of lymph nodes | ± | - |
| Efferent lymphatic vessel | ++ | - |
| Thoracic duct | - | - |
| Blood vessels | | |
| Endocardium | - | - |
| Large artery (e.g. aorta) | - | - |
| Small artery (e.g. in tongue) | - | ++ |
| Arteriole (e.g. in kidney) | - | ++ |
| Capillary (e.g. in diaphragm, tongue) | - | ++ |
| Sinusoids (e.g. in liver, spleen) | - | - |
| Venule (e.g. in Peyer's patches) | - | -/+ |
| Small vein (e.g. in kidney) | - | -/+ |
| Large vein (e.g. vena cava) | - | - |

^aLymphatic vessels are classified into four categories, such as lymphatic capillary, collecting vessels, lymph node sinus, and large trunk, whereas blood vessels are roughly divided from the arterial side to the venous side according to the blood circulation

^b++ strongly positive, + positive, ± weakly positive, - negative, and -/+ some are negative but others are positive

types of lymphoid cell were also stained by both LA102 (Fig. 3d) and LA5 mAbs as described later.

Antigen distribution patterns of LA102 mAb as revealed by the confocal laser scanning microscopy

The specificity of antigen distribution patterns of LA102 to the lymphatic vessels was confirmed by the simultaneous observation with blood vessels labeled with fluorescent tomato lectin. The lymphatic vessels in the tongue (Fig. 5a) and in the mesentery (Fig. 5b) were clearly distinguished from the local blood vessels. The lymphatic vessels of both surfaces of the diaphragm were also examined. The lymphatic vessels on the thoracic side (Fig. 5c) and on the abdominal side (Fig. 5d) were selectively stained without any colocalization of antigen as compared with the local blood vessels. These findings indicate that LA102 reacted predominantly with mouse lymphatic vessels, but not with any types of blood vessel. Furthermore, the reactivity of LA102 mAb to lymphatic vessels was also confirmed by the simultaneous observation with LYVE-1 distribution, which is a well-known marker for lymphatic vessel endothelial cells (Fig. 6). The LA102 positive lymphatic vessels in the diaphragm were also positive for LYVE-1, but not for blood vessels.

Localization of LA102 and LA5 antigens at the electron microscopic level

At the ultrastructural level the antigen recognized by LA102 was localized on both luminal and abluminal

sides of cell membrane of lymphatic endothelial cells. Furthermore, the antigen was highly condensed on pinocytotic or transport vesicle membrane in the cytoplasm of lymphatic endothelial cells (Fig. 7a).

LA5 antigen was also localized on the cell membranes of both luminal and abluminal sides of blood vessel endothelial cells (Fig. 7b), however, any clear association with vesicular membrane were not observed.

Reactivity of LA102 and LA5 mAbs to mouse lymphoid cells

Since, some lymphoid cells in tissue sections were also stained with LA102 and LA5 mAbs, we examined their reactivities to various cells from mouse lymphoid tissues and cell lines in cultures by flow cytometry (FACS analysis). LA102 recognized most thymocytes (approximately 94%) and about half of spleen cells (51.2%), but poorly responded to most bone marrow cells (Fig. 8). In the spleen, the majority of the LA102 positive cells were CD3⁺ T lymphocytes (Fig. 9). In addition to thymocytes, LA102 also reacted strongly with all T cell lines tested in this study, such as K62, EL4 (Fig. 10), and 2B4 (FACS data not shown). To a much less degree, however, LA102 recognized B cell lines (A20 and X63) and Ltk⁻ fibroblasts (Fig. 10). In contrast to LA102, LA5 strongly recognized about one third of bone marrow cells, but many fewer cells in the thymus and spleens (Fig. 8). The major populations of the LA5⁺ bone marrow cells were Mac-1⁺ macrophages, and some were B220⁺ B cells and CD3⁺ T cells to much less extent (Fig. 9). It also reacted with K62, EL4 (Fig. 10), and 2B4 (FACS data not shown), but little with the B cell lines (A20 and X63) and Ltk⁻ fibroblasts (Fig. 10).

Biochemical characterization of the antigens recognized by LA102 and LA5 mAbs

Since the FACS analyses revealed that both LA102 and LA5 mAbs recognized T lymphoma cell lines, the size of their antigen molecules expressed on EL4 or 2B4 cells was examined by Western blot analyses. LA102 and LA5 reacted with proteins of about 25–27 kDa and 12–13 kDa in size, respectively under nonreducing conditions (Fig. 11).

Discussion

The main aim of this study was to identify a novel lineage-specific marker for mouse lymphatic vessels and differentiate them from blood vessels in tissues. We produced a rat mAb, LA102 that only recognized lymphatic endothelial cells but not any types of blood vessel endothelium. During the same immunization protocol, we could also obtain another mAb LA5 that reacted

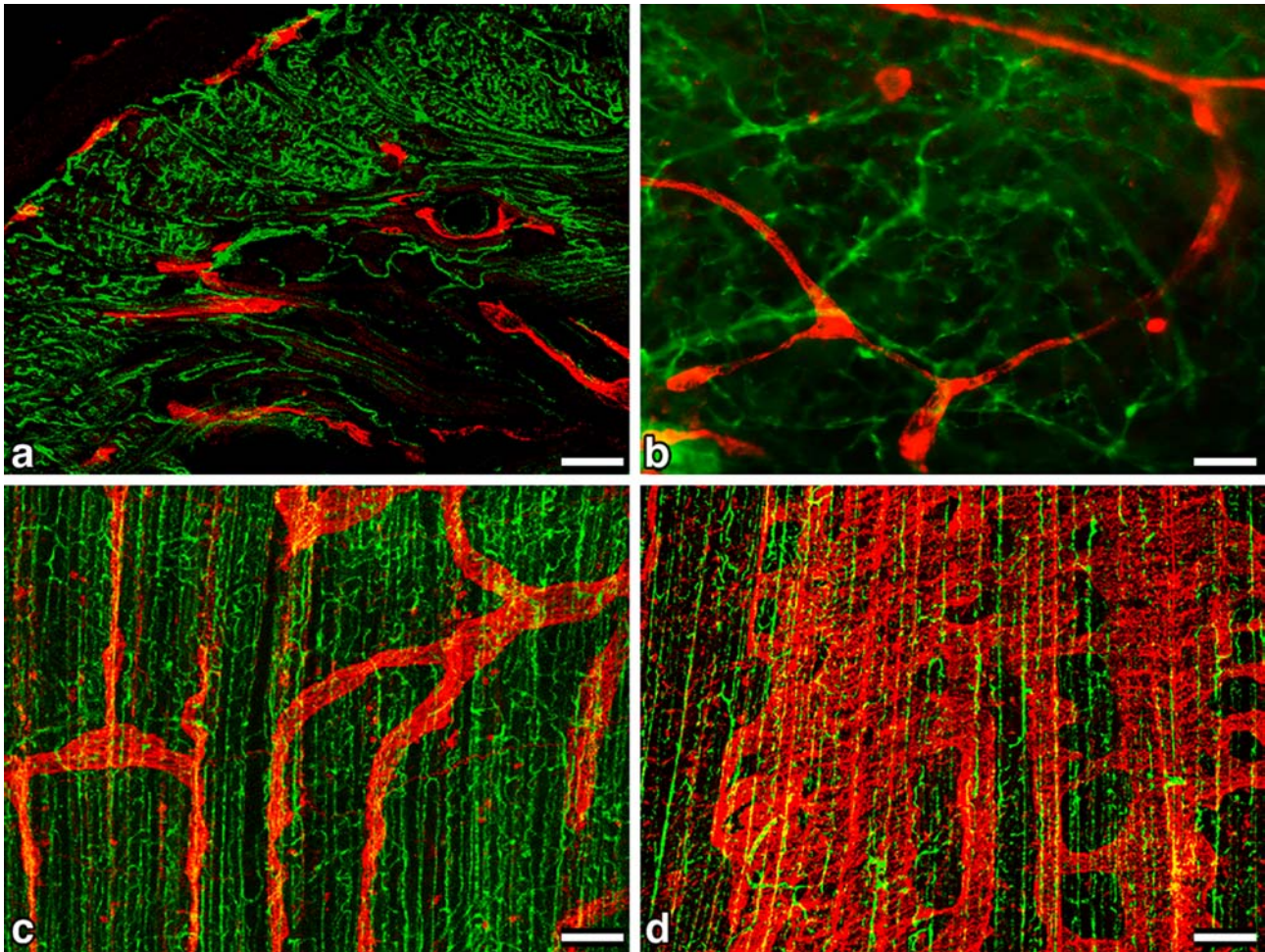


Fig. 5 Double immunofluorescent confocal scanning images of various tissues with LA102 and *L. esculentum* (tomato) lectin. Tissues from mice that had been perfused and labeled their blood vessels with FITC-labeled tomato lectin (green) were immunostained with mAb LA102 (red). Lymphatic capillaries and

collecting lymphatic vessels in the tongue (a) and in the mesentery (b) are clearly seen as an independent vascular system. The lymphatic vessels on the thoracic side (c) and on the abdominal side (d) of a diaphragm are distinguished from the blood vascular networks. Scale bars: 300 μm (a), 100 μm (b), and 150 μm (c and d)

with most blood vascular endothelial cells, but not with lymphatic endothelium.

In fact, it is difficult to obtain relatively pure lymphatic endothelial cells as an antigen source in rodents. Previously, we produced a mouse mAb, B27, against rat lymphatics using rat thoracic duct endothelial cells as the antigen source (Ezaki et al. 1990). Unfortunately, B27 cross-reacted with some types of blood vessels, perhaps due to the use of the thoracic duct as the immunogen. The thoracic duct, which is very close to veins in the topographical and developmental sense (Wilting et al. 2003). In the present study, we employed several technical devices to obtain more confined specific mAbs to lymphatic vessels. First, we used benign lymphangioma cells induced with adjuvant (Ezaki et al. 2004), because the benign tumors often overexpress their typical phenotypes of their normal counterparts (Mokhtar et al. 1984; Ezaki et al. 1991) and it is likely that benign lymphangiomas may follow this behavior (Xu et al. 2004b). The availability of large amounts of rela-

tively pure antigens from the lymphangioma for the immunization may have increased our chances of discovering a novel molecule from lymphatic endothelial cells. Second, the discovery of specific markers for lymphatic vessels has also been hampered by their weak immunogenicity. It has been generally much easier to get various specific markers for blood vessel endothelial cells, possibly due to their relatively strong immunogenicity. Furthermore, since lymphatic vessels may share many of the markers with blood vessels (Sleeman et al. 2001; Scavelli et al. 2004), many monoclonal antibodies raised against lymphatic vessels, including B27 mAb (Ezaki et al. 1990), cross-reacted with the other vessels. Therefore, we employed a mild enzyme treatment of the immunogen and a rapid differential immunization protocol for producing monoclonal antibodies in order to overcome the above fatal inconvenience of the lymphatic vessel system. The neuraminidase treatment was to expose very minor hidden cell surface antigens (Toshimori et al. 1988). Lastly, the rapid differential immunization

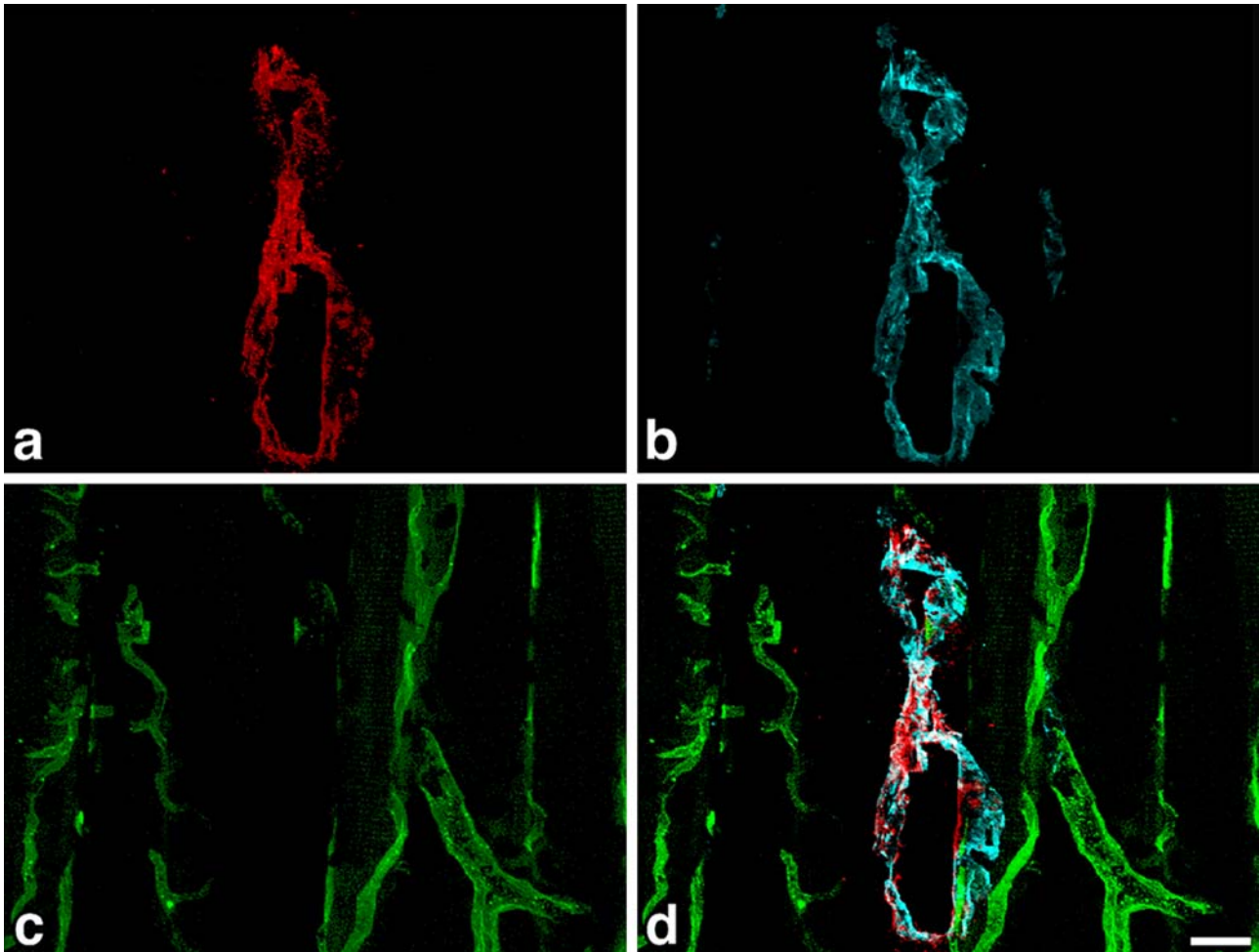


Fig. 6 Triple confocal immunofluorescent images of local microvascular segments with LA102, LYVE-1, and *L. esculentum* (tomato) lectin. The distribution of LA102 antigen (a), LYVE-1 (b), FITC-labeled tomato lectin (c), and their merged image (d) was

examined on a frozen section of diaphragm. Note that only the former two lymphatic markers are colocalized at the same vessel structure, but not in the surrounding blood vessels. Scale bars: 20 μ m

technique not only differentiated the unique or less-immunogenic antigens from common and strong antigens, but also reduced and saved the time for immunization to 18 days. The new immunization protocol would be very useful and lend itself greatly to discover various hidden markers or molecules that had never been isolated from tissues as antigen reservoirs.

The specificity of LA102 to the lymphatic system was confirmed by the double immunostaining with LYVE-1, as a typical lymphatic endothelial marker (Prevo et al. 2001), and by the simultaneous visualization of blood vessels labeled with tomato lectin. There was no colocalization of the LA102 antigen with lectin-labeled blood vessels. The triple staining with LA102, LYVE-1, and tomato lectin revealed that LA102 actually recognized only lymphatic endothelium, but not blood vessel endothelium. Furthermore, LA102 mAb did not react with large lymphatic trunks, such as the thoracic duct, but did strongly recognize the lymphatic vessels at the level from lymphatic capillaries to collecting lymphatic vessels. The data strongly suggest that LA102 mAb

recognizes characteristic lymphatic vessels, which play the important roles in the local microcirculation, rather than the large lymphatic trunks which are closely related to veins, as discussed above. In other words, LA102 antigen may reflect the typical phenotype of lymphatic vessels distinct from blood vessels. Although its functional significance at molecular level has yet to be established, the LA102 antigen localized on the cell membranes of both luminal and abluminal sides of lymphatic endothelium. Interestingly, the antigen was highly condensed on the pinocytotic or transport vesicle membrane in the cytoplasm of lymphatic endothelial cells, suggesting a possibility that this molecule may play an active role in the bidirectional membrane transport through the lymphatic endothelial cells. This possibility, however, needs to be further elucidated. Similar localization patterns of LYVE-1 in the lymphatic endothelial cells (Jackson et al. 2001), CD31 (Feng et al. 2004), and PAL-E molecule (Xu et al. 2004a) in the blood vessel endothelial cells have been reported in reference to the membrane transport. The FACS analyses indicated that

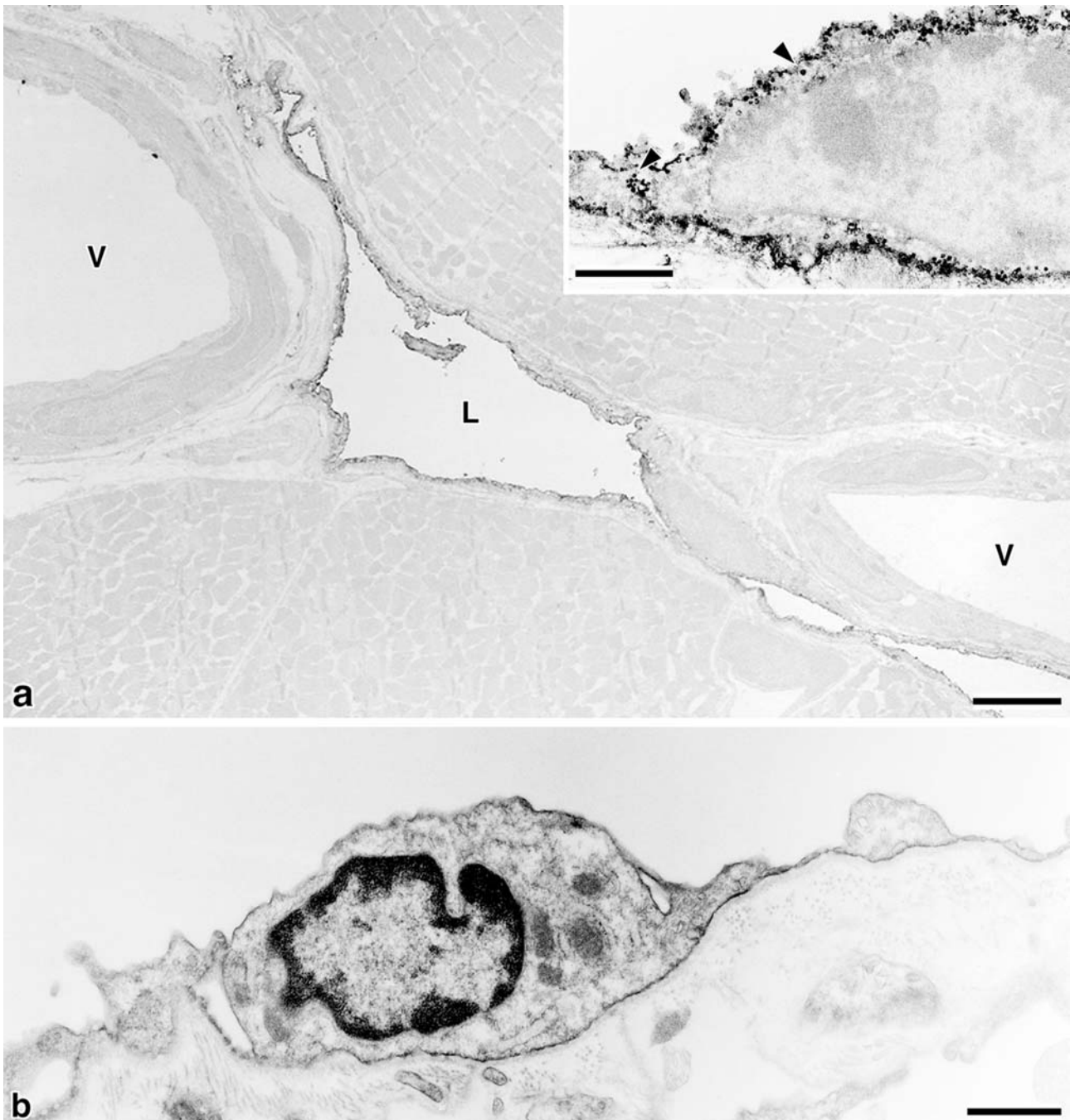


Fig. 7 Distribution of LA102 and LA5 antigens at the electron microscopic level. Electron micrographs of the immunoperoxidase staining of a collecting lymphatic vessel (*L*) in the mouse tongue (**a**: LA102) and the intestinal villus (**b**: LA5). Both LA102 and LA5 antigens are localized on the plasma membrane of both luminal

and abluminal surface of the endothelial cells. Note that the reaction products by LA102 mAb were often found to be concentrated on the pinocytotic vesicles or smooth surfaced vesicles (*arrowheads*). *V*: blood vessel (*venule*). Scale bars: 3.4 μm (**a**), 2 μm (*inset*), and 1 μm (**b**)

LA102 antigen localized at least on the outer surface of plasma membranes of the cells. In addition to lymphatic endothelial cells, LA102 was also expressed on various lymphoid cells, particularly on the cells in T cell lineage, such as thymocytes and some T lymphoma cell lines. Therefore, it might play an important role in the lymphoid cell migration and some transporting mechanisms through lymphatic endothelium. It has been reported

that LYVE-1 is also present on some macrophage populations (Jackson 2004) and CD31 (PECAM) on some mouse leukocytes and platelets (DeLisser et al. 1993). These molecules are very well known to mediate cellular migration and adhesion. The molecular size of LA102 antigen was demonstrated to be about 25–27 kDa under nonreducing conditions by Western blot analyses. As yet, no marker with a similar molecular size has been

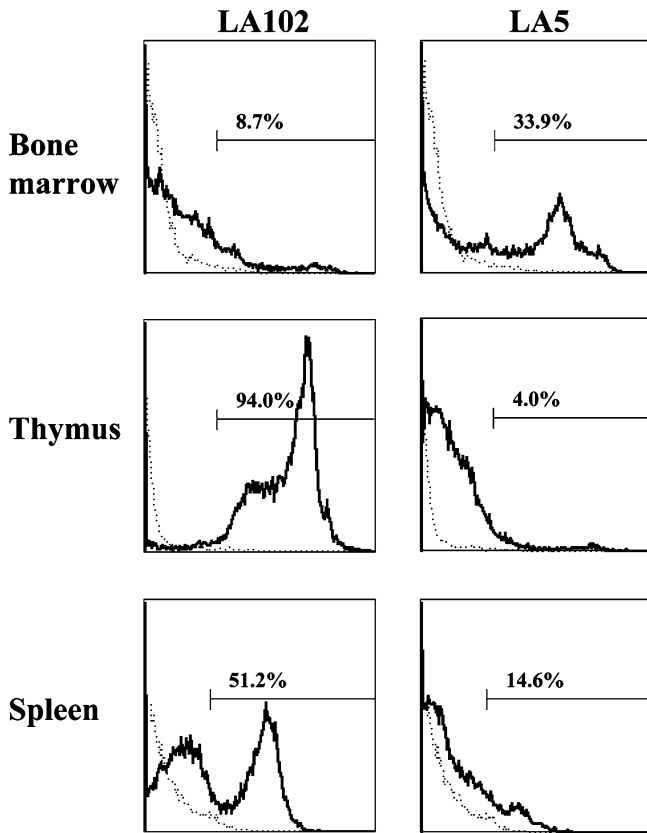


Fig. 8 Flow cytometer analyses of lymphoid cells from various organs. Lymphoid cells from bone marrow, thymuses, and spleens were stained with either LA102 (*left column*) or LA5 (*right column*) mAbs by the indirect immunofluorescence technique. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units and the linear cell frequency on the ordinate. Negative controls stained only with an FITC-labeled F(ab')₂ goat anti-rat Ig secondary antibody without primary mAbs were drawn by dotted lines

reported on the lymphatic endothelial cells as listed in Table 2. There was no cross-reactivity of LA102 mAb to the lymphatic vessels in other species examined in a preliminary study (Morikawa et al. 2004).

The other mAb, LA5, simultaneously obtained in the present study, recognized blood vessel endothelial cells with no cross-reactivity to lymphatic vessels. Immunohistochemical investigation revealed that LA5 reacted with most of the mouse blood vessels except sinusoid vessels in the liver and spleen, and large blood vessels including the aorta and endocardium. Like LA102 antigen, the LA5 antigen localized on the cell membranes of both luminal and abluminal sides of blood vessel endothelium as revealed by the immunoelectron microscopy. Furthermore, LA5 strongly recognized some populations of bone marrow cells (mainly Mac-1⁺ macrophages) and T lymphoid cells, suggesting that the antigen might also play a role in the cellular interaction between blood vessel endothelial cells and lymphoid cells. The molecular size of LA5 antigen was determined to be 12–13 kDa under nonreducing condition by the Western blot analyses. Although there have been many

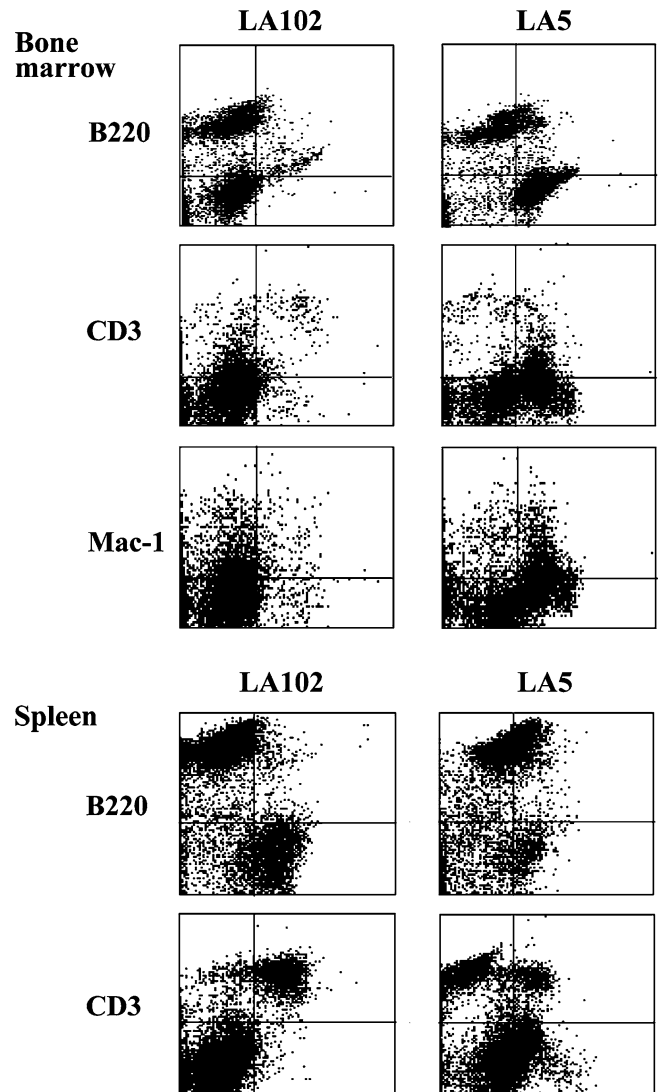


Fig. 9 Two-color flow cytometer analyses of bone marrow cells and spleen cells by double immunostaining. Cells from bone marrow and spleens were double stained with either LA102 (*left column*) or LA5 (*right column*) mAbs in combination with either of three cell lineage-specific antibodies, such as B220 for B cells, CD3 for T cells and Mac-1 for macrophages. Ten thousand stained cells were analyzed for the magnitude of green (FL1: LA102 or LA5) and red (FL2: B220, CD3 or Mac-1) fluorescence and the data were plotted and expressed as histograms. The cutoff points for the presence of the red and green fluorescence were predetermined by the medium control substituting for each mAb

markers reported as being specific for the blood vessel endothelial cells, LA5 antigen appears to be distinct from any of those previously-reported markers as listed in Table 2. This antibody is useful to identify small to medium sized blood vessels except liver sinusoids or splenic sinuses. The reason for the lack of staining in these sinusoid endothelia and some relatively large blood vessels including endocardium is unclear. However, this may also reflect their functional diversity of endothelial cells (Garlanda and Dejana 1997) from other blood vessels. For examples, LA5 reacted more strongly

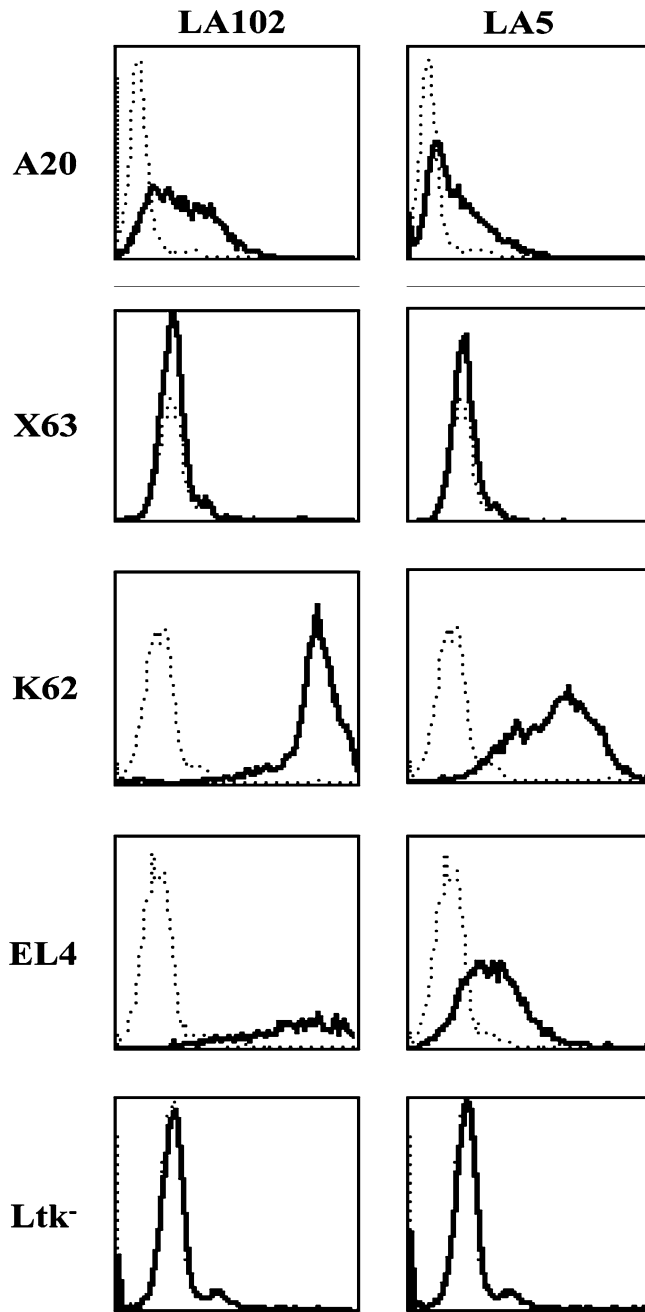


Fig. 10 Flow cytometer analyses of various mouse cell lines. Mouse B lymphoid cells (A20 and X63), T lymphoid cells (K62 and EL4) and a fibroblast line (Ltk⁻) were stained with either LA102 (*left column*) or LA5 (*right column*) mAbs by the indirect immunofluorescence technique. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units and the linear cell frequency on the ordinate. Negative controls stained only with an FITC-labeled F(ab')₂ goat anti-rat Ig secondary antibody without primary mAbs were drawn by dotted lines

with vessels on the arterial side than the venous side (Table 1), whereas PAL-E is entirely nonreactive with arterial endothelium in humans (Schlingemann et al. 1985) and MECA-32 reactivity is temporally and spatially correlated with the development of the vascular endothelium in the brain (Hallmann et al. 1995). The

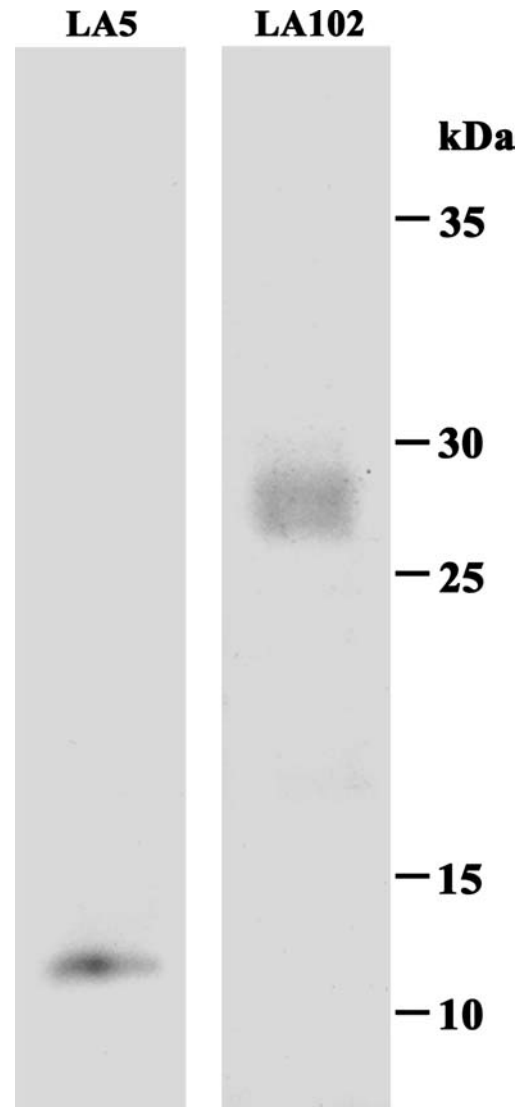


Fig. 11 Western blotting analyses of antigens recognized by mAbs LA102 and LA5. The molecular size of the LA102 and LA5 antigens expressed on EL4 or 2B4 cells was examined by Western blotting analysis. MAbs LA102 and LA5 reacted with proteins of 25–27 kDa and 12–13 kDa in size, respectively under nonreducing conditions

absence of OX-43 antigen expression in brain capillaries (Robinson et al. 1986) and capillary sprouts that invade avascular tissue during angiogenesis (Anderson et al. 2004) has been also reported. Recently, immunoreactivity to PAL-E in humans and MECA-32 in mice has been shown to correspond to immunoreactivity to rat PV1, the protein expressed in diaphragms of fenestrated capillaries and plasmalemmal vesicles/caveolae (Niemela et al. 2005; Stan 2005).

The immunostaining of various tissues with the novel mAbs may become a powerful tool to differentiate the local microcirculatory systems. Particularly, it is very important to identify lymphatic vessels and blood vessels in studies on lymphangiogenesis and angiogenesis under physiological and pathological conditions and during

Table 2 Molecular markers of lymphatic and blood vessel endothelial cells

| Markers | Species | Location | Molecular size (kDa) | Function | Representative references ^a |
|--------------------------|--------------|--------------|----------------------|--------------------------------|---|
| Lymphatic vessels | | | | | |
| LA102 | Mouse | Cell surface | 25–27 | ? | This study |
| LYVE-1 | Mouse, human | Cell surface | 60 | Hyaluronan receptor | Prevo et al. (2001), Banerji et al. (1999) |
| Podoplanin | Mouse | Cell surface | 43 | Adhesion? | Schacht et al. (2003) |
| VEGFR-3 (Flt4) | Mouse | Cell surface | 170 | VEGF-C receptor | Kaipainen et al. (1995) |
| Prox-1 | Mouse | Nuclear | 85–95 | Transcription factor | Wigle and Oliver (1999) |
| Foxc-2 | Mouse | Nuclear | 54–63 | Transcription factor | Petrova et al. (2004) |
| D2-40 | Human | Cell surface | 40 | Human podoplanin | Kahn et al. (2002), Schacht et al. (2005) |
| CD73 | Human | Cell surface | 69 | Adhesion, 5'-Nase | Thompson et al. (1989) |
| B27 | Rat | Cytoplasm | ? | ? | Ezaki et al. (1990) |
| Blood vessels | | | | | |
| LA5 | Mouse | Cell surface | 12–13 | ? | This study |
| CD31 | Mouse | Cell surface | 110–120, 130–140 | PECAM-1 | DeLisser et al. (1993) |
| CD34 | Mouse | Cell surface | 90, 105–120 | GlyCAM-2 | Baumhueter et al. (1993) |
| CD44 | Mouse | Cell surface | 80, 95, 130 | H-CAM | Lesley et al. (1993) |
| CD105 | Mouse | Cell surface | 90, 180 | TGF- β receptor | Gougos and Letarte (1990) |
| MECA-32 | Mouse | Cell surface | 50–55, 100–120 | Diaphragm protein (mouse PV-1) | Hallmann et al. (1995), Stan (2005) |
| PAL-E | Human | Cell surface | 60–120 | Diaphragm protein (human PV-1) | Schlingemann et al. (1985), Niemela et al. (2005) |
| OX-43 | Rat | Cell surface | 90 | ? | Robinson et al. (1986) |

^aOnly original or representative papers are listed

vasculogenesis. Studies are now underway to further characterize both of the newly identified antigens, which are not only distinct from, but are also of much lower molecular weight than previously identified blood vascular and lymphatic markers.

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