The selective distribution of LYVE-1-expressing endothelial cells and reticular cells in the reticulo-endothelial system (RES)

Miao ZHENG, Shunsuke KIMURA, Junko NIO-KOBAYASHI, and Toshihiko IWANAGA

Laboratory of Histology and Cytology, Department of Anatomy, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

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ABSTRACT

LYVE-1, a receptor molecule for hyaluronan, is expressed in the lymphatic endothelium, blood sinus endothelium, and certain macrophage lineages. The present immunohistochemical study revealed a broader distribution of LYVE-1 in vascular endothelial cells of the murine lung, adrenal gland, and heart as well as the liver and spleen. In addition, sinus reticular cells—including sinus-lining cells—in the medulla of the lymph node also intensely expressed LYVE-1. Ultrastructurally, immuno-gold particles for LYVE-1 were localized on the entire length of plasma membrane in all cell types. Most of these LYVE-1-expressing cells had previously been classified as the reticulo-endothelial system (RES) specialized for eliminating foreign particles. An LPS stimulation decreased the LYVE-1 expression in macrophages but elevated the expression at mRNA and protein levels in the liver and lung, major organs for the elimination of blood-born waste substances. LYVE-1-expressing endothelial cells in these organs participated in the endocytosis of exogenous particles, and the uptake ability was conspicuously enhanced by the LPS challenge. Although the expression of the degrading enzyme, hyaluronidase, was generally low in the LYVE-1-expressing cells, they were topographically associated with a dense distribution of macrophages possessing hyaluronidase activities in each tissue. These findings suggest that the LYVE-1-expressing cells might be involved in the uptake of hyaluronan and other waste products as well as foreign particles circulating in the blood and lymph while participating in the subsequent degradation in relay with adjacent macrophage populations.

LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1) has been identified as a reliable marker that is expressed predominantly in the lymphatic endothelium (5, 29). Both the chemical structure and function of LYVE-1 resemble those of a well-established hyaluronan receptor, CD44, which has a 41% similarity to LYVE-1 and is characterized as an inflammatory leukocyte homing receptor. CD44 is widely expressed in the epithelium, mesothelium,

mesenchymal cells, and cells of hematopoietic origin (22), while LYVE-1 has been shown in early studies to display a restricted distribution to the lymphatic endothelium and be completely absent from blood vessels, except for sinusoidal endothelial cells in the spleen (5). Subsequent studies have also detected the expression of LYVE-1 in sinusoidal endothelial cells using paraffin sections of the liver, spleen and lymph node of humans (1, 26). However, our preliminary immunohistochemical study using frozen sections from rodents found a broader distribution of LYVE-1 in the endothelium of other organs, including the lung, adrenal gland, heart, and adenohypophysis. In addition, the sinus reticular cells in the medulla of the lymph node intensely expressed LYVE-1. Interestingly, most of these belong

Address correspondence to: Dr. Toshihiko Iwanaga
Laboratory of Histology and Cytology, Hokkaido University Graduate School of Medicine, N15W7, Kita-ku, Sapporo 060-8638, Japan
Tel: +81-11-706-5033, Fax: +81-11-706-7151
E-mail: tiwanaga@med.hokudai.ac.jp

to the forgotten concept known as the reticulo-endothelial system (RES).

The RES, originally established by Aschoff in 1924 (4), was characterized by its higher activity to eliminate circulating exogenous substances and particles such as endotoxins and bacteria. The RES was historically replaced by the mononuclear phagocyte system (MPS) with authentic phagocytic activity by a research group of Van Furth *et al.* (38): the monocyte-macrophage lineage is the major cell type functioning in the elimination of pathogens and cell-debris, and there is no transition between macrophages and sinusoidal endothelial cells. However, some studies carried out in the 1990's reconfirmed that endothelial cells in hepatic sinusoids and reticular cells in the lymph node can vigorously incorporate the same vital staining dyes as Aschoff used (18). It has been shown that endothelial cells—especially hepatic sinusoidal endothelial cells, a representative RES member—comprise an important part of the innate immune system for eliminating foreign particles from the circulation. Smedsrød and his colleagues (32, 36, 37) have termed the endocytic endothelial cells as scavenger endothelial cells, leading us to resurrect the concept of the RES (39). A weak point in this effort to re-evaluate the RES is the lack of marker substances that can specify the RES members. In previous studies, we found that Src-Suppressed-C Kinase Substrate (SSeCKS), whose expression was induced by a lipopolysaccharide (LPS) challenge, was shared by some members of RES and was involved in the uptake of exogenous particles (31). However, the degree of its expression was less intense under normal conditions, and several non-RES cells also expressed SSeCKS (19).

In the present study, we examined the distribution of LYVE-1-expressing cells at light and electron microscopic levels, focusing on the topographical association with macrophages. We further observed the responses of LYVE-1-expressing cells in LPS-induced inflammation, including the elevated uptake of exogenous particles. The results confirm that LYVE-1 is a key molecule for a reconsideration of the RES concept.

MATERIALS AND METHODS

Animals and tissue sampling. Eight-week-old C57BL/6 and ddY mice of both sexes were used. For conventional microscopic examination, the mice were anesthetized by an intraperitoneal injection with sodium pentobarbital and then perfused with physiological saline through the heart, followed by 4% parafor-

maldehyde in 0.1 M phosphate buffer, pH 7.4. The liver, lung, spleen, lymph node, heart, adrenal gland, bone marrow, and hypophysis were dissected out and immersed in the same fixative for an additional 8 h. Lipopolysaccharide (LPS, 0.02 mg/20 g body weight) (E.coli055:B5; Sigma Aldrich, St. Louis, MO) was injected into the peritoneal cavity, and the organs were obtained at 4, 8, 24, and 48 h.

All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

Immunohistochemistry. The fixed tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections of 12 µm in thickness were mounted on poly-L-lysine-coated glass slides. After immersion in 0.01 M phosphate buffered saline (PBS) containing 0.3% Triton-X 100, the sections were pre-incubated with a normal donkey serum. Sections were incubated with a rabbit anti-mouse LYVE-1 antibody (AngioBio, Del Mar, CA) at a concentration of 1 µg/mL overnight, followed by incubation with Cy3-labeled donkey anti-rabbit IgG (1 : 200 in dilution; Jackson ImmunoResearch, West Grove, PA). For double immunofluorescence, sections were stained overnight with a mixture containing the anti-LYVE-1 antibody and rat anti-F4/80 antibody (Clone BM8, 1 µg/mL; BioLegend, San Diego, CA). The sites of antigen-antibody reactions were detected by incubation with a combination of Cy3-labeled anti-rat IgG (1 : 200 in dilution; Jackson ImmunoResearch) and AlexaFluor 488-labeled anti-rabbit IgG (1 : 100 in dilution; Invitrogen, Carlsbad, CA). Some of the immunostained sections were counterstained with SyTO 13 (SYTOX, Invitrogen) for observation of the nuclei. Stained sections were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan). The specificity of immunoreactions on sections was confirmed according to a conventional procedure, including absorption tests.

Silver-intensified immunogold method for electron microscopy. Frozen sections, 10–14 µm in thickness, were pretreated with normal donkey serum for 30 min, incubated with the rabbit anti-LYVE-1 antibody (1 µg/mL) overnight, and subsequently reacted with goat anti-rabbit IgG covalently linked with 1-nm gold particles (1 : 200 in dilution; Nanoprobes, Yaphank, NY). Following silver enhancement using

a kit (HQ silver; Nanoprobes), the sections were osmicated, dehydrated, and directly embedded in Epon. Ultrathin sections were prepared and stained with an aqueous solution of uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

In situ hybridization. Two non-overlapping antisense oligonucleotide DNA probes (45 mer in length) were designed for each mRNA of mouse LYVE-1, hyaluronidase 1, and hyaluronidase 2. Antisense probes were complementary to the following sequences: 421–465 and 1331–1375 of *Lyve-1* mRNA (accession number: NM_025989); 1831–1875 and 2651–2695 of *Hyaluronidase 1* mRNA (NM_177568); and 178–222 and 585–629 of *Hyaluronidase 2* mRNA (NM_001081143). The probes were labeled with ^{33}P -dATP using terminal deoxynucleotidyl transferase (Invitrogen). Fresh frozen sections, 14- μm -thick, were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed at 42°C for 10 h with a hybridization buffer containing ^{33}P -dATP-labeled oligonucleotide probes (10,000 cpm/mL). The sections were rinsed at room temperature for 30 min in 2 $\times$ SSC (1 $\times$ SSC: 150 mM sodium chloride, 15 mM sodium citrate) containing 0.1% *N*-lauroylsarcosine sodium, then rinsed twice at 55°C for 40 min in 0.1 $\times$ SSC containing 0.1% *N*-lauroylsarcosine sodium, dehydrated through a graded series of ethanol, and air-dried. Sections were exposed to X-ray film (BIOMAX MR Film; Carestream Health, Inc., New York, NY) or dipped in an autoradiographic emulsion (NTB-2; Kodak) at 4°C for 8–10 weeks. The hybridized sections used for autoradiography were counterstained with hematoxylin after development.

The *in situ* hybridization technique using the two non-overlapping antisense probes for each mRNA exhibited identical labeling in all the tissues examined. The specificity of the hybridization was also confirmed by the disappearance of signals upon the addition of an excess of an unlabeled antisense probe.

Non-radioisotope in situ hybridization. Non-isotope *in situ* hybridization was performed with the QuantiGene ViewRNA ISH Tissue Assay (Affymetrix, Santa Clara, CA). The preparation of frozen sections was as described above. Fresh sections were treated with a solution containing 4% paraformaldehyde in PBS for 20 min at room temperature. After three time wash with PBS, the sections were acetylated in

0.1 M triethanolamine (pH 8)/0.25% acetic anhydride for 10 min at room temperature. Subsequent hybridization processes were performed in accordance with the manufacturer's protocol. Specific oligonucleotide probe sets against *Lyve1* (catalog no. VB6-18604) and *Hyal2* (VB1-16724) were purchased from Affymetrix. After hybridization, slides were incubated with Fast Red substrate of alkaline phosphatase for 30 min at room temperature in the dark, rinsed with PBS, and then counter-stained with Gill's hematoxylin.

Quantitative RT-PCR. Total RNA was prepared using TRIzol (Life Technologies) from mouse liver and lung. First-strand cDNA synthesis was completed using ReverTra Ace (TOYOBO, Osaka, Japan). Quantitative PCR reactions were conducted in Rotor Gene 6000 equipment (Qiagen, Hilden, Germany) using KAPA SYBR Green Fast PCR kit (KAPA Biosystems, Woburn, MA). Data were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The following primers were used: *Gapdh*-sense 5'-AGGTCGGTGTGAACG GATTG-3', *Gapdh*-antisense 5'-TG TAGACCATGT AGTTGAGGTCA-3'; *Lyve1*-sense 5'-CAGCACAC TAGCCTGGTGTTA-3', *Lyve1*-antisense 5'-CGCCC ATGATTCTGCATGTAGA-3'.

Uptake of exogenous particles. To verify the uptake of exogenous particles by the liver and lung, LPS-treated and control mice were injected directly with fluorescent latex beads, 20 and 100 nm in diameter (Molecular Probes, Eugene, OR) or a physiological saline (control). The animals were anesthetized with halothane gas, and the 0.1 mL of latex bead solution diluted at 1 : 10 was injected into the inferior vena cava. Ten or 45 min later, the animals were perfusion-fixed under anesthesia, and the liver and lung were removed and immersion-fixed. Frozen sections were immunostained for LYVE-1 as mentioned above.

RESULTS

LYVE-1-expressing endothelial cells and reticular cells

Immunohistochemically, the hepatic sinusoidal endothelium as well as the lymphatics in the interlobular connective tissues stained positively for LYVE-1 in the liver (Fig. 1a), while arterial and venous endothelia were free from any positive immunoreaction. The sinusoidal endothelium in the middle zone of the hepatic lobules displayed a more intense, consistent immunoreactivity than that in the peri-portal and

Fig. 1 LYVE-1 immunoreactivities associated with macrophage distribution. Sections were stained with the anti-LYVE-1 antibody (green) and anti-F4/80 antibody (macrophage marker, red). Sinusoidal endothelium of the liver and vascular endothelium of the lung are immunoreactive for LYVE-1, while Kupffer cells and alveolar macrophages are stained positively for F4/80 (a, b). In the adrenal gland (c), the LYVE-1 antibody labels the vascular endothelium in the medulla and deep cortex. Two-direction arrows indicate the cortex (C) and medulla (M), respectively. In the red pulp of the spleen, LYVE-1-immunoreactive sinuses are embedded in an aggregation of F4/80-positive macrophages (d). The endocardium in the auricle (e) and reticular cells of sinuses in the medulla of lymph node (f) show a positive immunoreactivity for LYVE-1. WP, white pulp; C, cortex Bars 50 μ m

peri-central zones of the lobules (Supporting figure 1a, b). Vascular endothelial cells in the lung (Fig. 1b), the medulla and deep cortex of the adrenal gland (Fig. 1c), the red pulp of the spleen (Fig. 1d), and the atrium of heart (Fig. 1e) were all labeled with the LYVE-1 antibody. In the heart, endothelial cells of the endocardium were intensely immunoreactive, especially in the auricle, while positive staining of the endocardium in the ventricle was restricted to some secluded areas. The mesenteric lymph node possessed immunoreactive reticular cells within sinuses and sinus-lining cells in the medulla (Fig. 1f). These LYVE-1-expressing cells are generally classified as RES for eliminating foreign particles. Although endothelial cells in the pituitary gland and bone marrow have been listed as members of the RES, the pituitary endothelium was free of any LYVE-1 immunoreactivity, but the bone marrow displayed a significant expression in restricted numbers of sinusoidal endothelial cells (Supporting figure 1c). The immunostaining results suggest that LYVE-1 presents a novel marker substance for the RES—though not for all members of the RES.

Ultrastructural localization of LYVE-1

The immuno-gold method for electron microscopy labeled the cell membrane of LYVE-1-expressing endothelial cells in the liver (Fig. 2a), lung (Fig. 2b), the medulla of the adrenal gland (Fig. 2c), and spleen. Reticular cells in the lymph node were also heavily immunolabeled (Fig. 2d). The immuno-gold particles for LYVE-1 were localized along the entire length of the plasma membrane in all cell types, while the cytoplasm and nuclei escaped any significant labeling.

The LYVE-1-expressing cells are related to macrophages

In light of the LYVE-1 expression in the liver, lung, adrenal gland, spleen, and heart, we examined the relationship between endothelial cells and macrophages at the same regions. Results showed the LYVE-1-immunoreactive endothelial cells to be associated topographically with dense distributions of F4/80⁺ macrophages, such as Kupffer cells in the hepatic sinusoids (Fig. 1a), alveolar macrophages in the lung (Fig. 1b), macrophages within both the vascular lumen and parenchyma of the adrenal gland (Fig. 1c), macrophages in the red pulp of the spleen (Fig. 1d), and macrophages in the atrium (Fig. 1e). Also in the lymph node, the LYVE-1-immunoreactive reticular cells co-localized with macrophages in the medullary sinuses (Fig. 1f).

Reportedly, some macrophage lineages themselves

express LYVE-1. In a previous study (41), we found that F4/80⁺ macrophages in various connective tissues expressed LYVE-1, in contrast to a lack of LYVE-1 in F4/80⁺ macrophages within the parenchyma of visceral organs. Interestingly, none of macrophage populations mentioned above expressed LYVE-1, for example, macrophages residing in hepatic sinusoids and pulmonary alveoli.

LPS elevates LYVE-1 expression and the uptake activity of latex particles

We hypothesized that the function of LYVE-1-expressing cells may be the uptake and degradation of circulating particles and waste substances including hyaluronan, which is the most abundant glycosaminoglycan in the body and released under normal and pathological conditions. Thus, we first examined the uptake ability of LYVE-1-expressing cells. Since the uptake of fluorescent latex beads intravenously injected was scanty in the LYVE-1-expressing cells under normal conditions, we tried to stimulate LYVE-1-expressing cells by LPS injection into the peritoneal cavity of mice.

When we analyzed the mRNA expression of LYVE-1 by a quantitative RT-PCR in mice, the exposure to LPS for 4 h caused about a four fold increase in the LYVE-1 mRNA expression of the liver as compared with control livers (Supporting figure 2). In the lung, LPS administration also caused a significant increase in LYVE-1 at 4 h. The expression levels of LYVE-1 mRNA in these organs tended to decrease after 8 h but was still higher than the controls. X-ray film images of an *in situ* hybridization method confirmed elevated expressions of LYVE-1 mRNA at 4 h and 8 h after the LPS challenge in the lung (Fig. 3). There was a slight but significant change in LYVE-1 signals in the liver between LPS-injected mice and control mice. In accordance, the LYVE-1 immunoreactivity in the endothelial cells of the liver and lung increased in intensity at 24 and 48 h after the LPS challenge; longer exposure to LPS induced a more intense expression at a protein level (Fig. 4). There were no remarkable changes in the density or intensity of LYVE-1 immunoreactivity after the LPS injection in other tissues such as the spleen, heart, and adrenal gland (data not shown).

Next, the uptake of exogenous substances in the lung was examined by fluorescent latex beads of 20 nm and 100 nm in diameter being injected directly into the inferior vena cava (Fig. 5). In normal conditions, some alveolar macrophages slightly incorporated the latex particles of 20 nm, while no significant uptake was visible in the endothelial cells

Fig. 2 Immuno-gold method for electron microscopy. Gold particles proving the existence of LYVE-1 are distributed on the luminal and abluminal plasma membrane of endothelial cells in the liver (a), lung (b), and adrenal medulla (c). In the lymph node (d), the cell membranes of reticular cells are immunoreactive along the entire length. H, hepatocyte; L, lumen of lung capillaries; M, macrophage; N, nucleus Bars 2 μ m (a), 5 μ m (b–d)

of lung capillaries. The LPS challenge induced the trapping and uptake of abundant 20-nm latex particles by endothelial cells at 24 h. Latex particles appeared in the capillary wall at 10 min after the intravenous injection of particles and increased in number at 45 min (Fig. 5). However, large latex par-

ticles, 100 nm in size, accumulated only in macrophages but were not incorporated by endothelial cells, even in LPS-stimulated mice (data not shown).

Finally, we examined the activity for degrading hyaluronan by detecting hyaluronidases in murine tissues. Due to the lack of available antibodies against

Fig. 3 *In situ* hybridization analysis for LYVE-1 mRNA expression after LPS administration. Sections from the lung (upper panels) and liver (lower panels) of three animals at each time point (0, 4, and 8 h after LPS) were hybridized and exposed on an X-ray film. The expression of LYVE-1 mRNA in the lung increases in intensity with time following the LPS challenge. The liver shows a slight increase in mRNA expression in two of the three animals. Bar 5 mm

hyaluronidases, we tried to detect these enzymes by an *in situ* hybridization technique using ^{33}P -labeled DNA probes. We checked two major types of hyaluronidases: hyaluronidase 1 and 2 (16). Since these hyaluronidases showed the same expression pattern, we shall describe hyaluronidase 2 as a representative example. X-ray film images detected a significant expression of hyaluronidase 2 mRNA in the lymph node, liver, and lung with various intensities (Supporting figure 3), in addition to the kidney and small intestine under normal conditions. Because of the most intense expression of hyaluronidase 2 mRNA in the medulla of the lymph node, we compared the detailed expression pattern with that of LYVE-1 mRNA in this region. Both signals were concentrated in the medullary sinuses, but they did not appear to correlate at cellular levels (Supporting figure 4). The non-radioisotope *in situ* hybridization method further demonstrated that macrophages of large cell bodies expressed hyaluronidase 2 mRNA (Fig. 6a), in contrast to the LYVE-1 mRNA expression that was restricted to reticular cells with a scanty cytoplasm (Fig. 6b). Noteworthy, reticular cells with LYVE-1 mRNA expression were frequently in direct

contact with macrophages expressing hyaluronidase 2 mRNA, as shown in the same sections (Fig. 6 c, d).

DISCUSSION

The present study using mice revealed an extensive distribution of “lymphatics-dominant” LYVE-1 in the endothelium of several blood vessels as well as the sinusoidal endothelium of the liver and spleen. Ultrastructurally, LYVE-1 was localized at the plasma membrane of these endothelial cells on both luminal and abluminal (basal) sides. An LPS challenge induced a significant up-regulation of LYVE-1 expression in lung capillaries and hepatic sinusoids, with a concomitant elevation of uptake ability for 20 nm-latex particles. Since a predominant localization of hyaluronidases was restricted to adjacent macrophages, the degradation of hyaluronan may be completed in the relay of macrophages.

LYVE-1 is a novel marker for the reticulo-endothelial system (RES)

LYVE-1 has been believed to be a hyaluronan receptor molecule abundantly expressed in the endothelium of lymphatic vessels, with additional expressions in sinusoidal endothelia of the liver, lymph node, and spleen and in some macrophage lineages (1, 3, 21, 26, 41). However, we found a broader distribution of LYVE-1: vascular endothelial cells in the lung, adrenal gland, and endocardium. The present report is the first study using cryostat sections to detect LYVE-1 in the non-sinusoidal vascular endothelial cells. Interestingly, Gordon *et al.* (14) have documented positive staining for LYVE-1 in the cardiac endothelium and lung capillaries of mouse embryos, though they considered this reactivity an indicator of immature vessels. The sinus reticular cells as well as sinus-lining endothelium (especially medulla) of the lymph node also expressed LYVE-1, in agreement with previous studies in the human lymph node (1). In contrast to the reported positive staining of LYVE-1, Mouta Carreira *et al.* (26) failed to immunolabel the endothelium of splenic sinusoids in mouse strains. This failure may be explained partially by differences in strain species and sex in the immunoreactivity for LYVE-1, as we have experienced (Supporting figure 1e, f). The LYVE-1-expressing cells shown in the present study are largely classified as members of a previously-held concept, the RES (2, 4). Although the sinusoidal endothelial cells of the pituitary gland, nominated as a member of the RES, lacked the LYVE-1 immunoreactivity in the mouse, the pituitary gland of rats

Fig. 4 LYVE-1 immunoreactivity in controls and at 48 h after LPS administration. LPS stimulation elevates the LYVE-1 immunoreactivities in the vascular endothelium of the liver (a, b) and lung (c, d). Nuclei are stained with SYTOX green. C, central vein Bars 100 μ m

contained many LYVE-1-immunoreactive endothelial cells (Supporting figure 1d). The cellular distribution of LYVE-1 differs from that of another hyaluronan receptor molecule, CD44, which is absent in vascular endothelial cells but mainly localized in several types of epithelia and immune cells such as lymphocytes and macrophages (22). Stabilin-2 functions as an endocytic receptor removing hyaluronan from the blood (25, 28). Immunohistochemically, stabilin-2 is distributed in not only the sinusoidal endothelium of the liver, lymph node, spleen, and bone marrow, but also the epithelia of several organs (8). Our previous study demonstrated the selective up-regulation of Src-Suppressed-C Kinase Substrate (SSeCKS) by an LPS challenge in several RES members, including endothelial cells of the liver, lung, spleen, and pituitary gland (19). Unlike LYVE-1, the expression levels of SSeCKS were so low that its immunoreactivity in the endothelial cells of RES was undetectable under normal conditions. Thus, LYVE-1 shared by vascular endothelial cells, reticular cells, and mac-

rophages may be a more reliable and specific marker substance of the RES, even under normal conditions.

Uptake of exogenous substances by LYVE-1-expressing cells

LYVE-1 was initially regarded a key receptor responsible for the uptake and transport of hyaluronan in the lymph circulation (5, 29). However, subsequent studies using knock-out mice have revealed that the expression of LYVE-1 in the lymphatic vessels is dispensable for both the hyaluronan metabolism and leukocyte trafficking (13). The lack of significant alternations in hyaluronan homeostasis was explained by functional masking or silencing by a mechanism of sialylation, and the hidden functions might be released by a cytokine-stimulated membrane-bound sialidase activity (27). These findings led researchers to speculate that a critical function of the receptor might only appear under stress conditions such as inflammation, especially during early stages. Unexpectedly, pro-inflammatory cytokines

Fig. 5 Uptake of 20 nm fluorescent latex particles in the lung capillaries. The endothelium does not uptake latex particles under the control (a). When the animals were stimulated by LPS for 24 h, the capillary endothelium captures some latex particles 10 min after administration (b) and many particles 45 min after administration (c). Bars 20 μ m

Fig. 6 *In situ* hybridization analysis of hyaluronidase 2 and LYVE-1 mRNAs in the medulla of the lymph node. Signals for hyaluronidase 2 mRNA are seen in large cells with a rich cytoplasm and pale nucleus (a), while those for LYVE-1 mRNA are restricted to cells with a scanty cytoplasm (arrows in b). Counter-staining with hematoxylin. The same section as Fig. b was stained for F4/80, and observed under a confocal microscope for the dual detection of LYVE-1 mRNA and F4/80 immunoreaction (c, d). Arrows in b and c indicate the same reticular cells. Bars 20 μ m

such as TNFs induced the rapid internalization and subsequent degradation of LYVE-1 itself *in vitro*, and TNF-mediated inflammation markedly reduced

the staining of LYVE-1 in lymphatic vessels *in vivo* (17). Also, macrophages tended to show a similar response under systemic inflammation induced by

LPS (41). In contrast, the present study showed that LPS stimulation conversely increased the expression of LYVE-1 in the endothelial cells of the liver and lung, as demonstrated by quantitative RT-PCR and immunostaining. This means that the regulation of LYVE-1 expression—possibly also the functions—differ between lymphatics and blood vessels. In accordance with the elevated expression of LYVE-1, the uptake of latex beads was up-regulated by LPS in the endothelium of lung capillaries. Although the present study did not examine the endocytosis of hyaluronan-conjugated particles, studies concerning the drug delivery system focus on the effective uptake of hyaluronan-decorated particles by cancer cells which overexpress CD44, a LYVE-1 homologue (23, 34); the uptake of hyaluronan-conjugated platinum particles by carcinoma cells was about 50% lower with pre-treatment with the anti-CD44 antibody (7).

Modern methodology—including electron microscopic observation—has denied any phagocytotic activity in the RES except for macrophages. However, accumulating evidence has reconfirmed the uptake of denatured proteins, vital staining dyes, and small particles by the hepatic sinusoidal endothelium and lymph node reticular cells (18, 31). It is generally accepted that hepatic sinusoidal endothelial cells play a pivotal role in the clearance of blood-born waste macromolecules and particles by receptor-mediated endocytosis, whereas large particles are phagocytosed by Kupffer cells (37). Most researchers have not paid attention to the uptake of exogenous particles in the endothelium of the lung, adrenal gland, and heart, where we demonstrated the selective expression of LYVE-1 in the present study. However, historical studies have documented the uptake of proteins and particles in these organs: sinus endothelial cells of the adrenal gland were able to ingest denatured serum proteins (6, 40). The vascular endothelial cells of the rat adrenal gland were able to uptake ferritin from the blood stream, and the ferritin particles easily penetrated into the subendothelial spaces (9). The bone marrow sinusoidal endothelial cells endocytosed modified serum albumin in mice, rats, and rabbits (6, 30, 40). Information concerning the cardiac endothelium is limited regarding the uptake of circulating substances. The endocardial endothelial cells of fish have been shown morphologically to endocytose vigorously labeled collagen and hyaluronan; the uptake ability was higher in the atrium than in the ventricle (33, 35). Thus, the vascular endothelial cells of the RES serve as the most important site for the elimination of waste products and small particles in circulation,

while macrophages including Kupffer cells may play a minor part in the clearance of them circulating in the blood.

Topographical and functional relationship between LYVE-1-expressing cells and macrophages

Hyaluronan, a large glycosaminoglycan with molecular weights reaching the millions, is abundantly present as an extracellular matrix component. Tissue hyaluronan that has escaped from local metabolism enters lymphatic vessels and is transported to the lymph nodes, where as much as 80–90% of hyaluronan is degraded (10, 11). This finding is supported by the most intense expression of hyaluronidase mRNA appearing in the lymph node, as demonstrated by the present study. A small part of hyaluronan and/or fragments enter the blood circulation and are processed mainly in the hepatic sinusoidal endothelial cells (20). Accordingly, the LYVE-1 of the lymphatic endothelium was initially regarded a key receptor responsible for the uptake and transport of hyaluronan in tissues and lymph circulation. However, subsequent studies using knock-out mice showed that the expression of LYVE-1 in the lymphatic endothelium is not essential for the hyaluronan metabolism, as mentioned above. Stabilin-2 (also termed HARE) is considered the main receptor for endocytosis of hyaluronan, heparin, and chondroitin sulfates in the hepatic sinusoidal endothelium (15, 25). Although LYVE-1 may play an important role in the uptake of hyaluronan in the lymph nodes, stabilin-2 rather than LYVE-1 appears to be the primary receptor for lymphatic degradation (29).

Information on the cellular expression of the hyaluronan degrading enzymes is important for identification of the degradation sites. We confirmed the accumulated expression of hyaluronidases in the medulla of lymph node. Noteworthy, the expression of hyaluronidases there was restricted to macrophages but not reticular cells. Ultrastructurally, LYVE-1 is always localized in the plasma membrane but not in the cytoplasm and cell organelle, in contrast to its cytoplasmic localization in macrophagic cells, such as intracellular vesicles and vacuoles (21, 41). The dual membrane localization of LYVE-1 in the vascular endothelial cells is in agreement with early observations concerning LYVE-1 in the endothelium of lymphatics (29), suggesting transport via the cells (transcytosis) without intracellular degradation.

Our immunohistochemical data showed that the LYVE-1-immunoreactive cells were topographically associated with a dense distribution of macrophages in each tissue: Kupffer cells in the hepatic sinusoids,

alveolar macrophages in the lung, macrophages within both sinusoidal lumen and parenchyma of the adrenal gland, macrophages in the splenic red pulp, and macrophages in the auricular wall. Electron microscopy suggested that the phagocytosis of circulating particles in the adrenal gland and adenohypophysis was accomplished by the perivascular macrophages with processes passing through endothelial cells and extending into the lumen of the vessels (12, 24). In the lymph node, we were able to visualize the intimate relation of reticular cells with hyaluronidase-expressing macrophages. Taken together, these findings give credence to the notion that the LYVE-1-expressing RES cells may capture circulating hyaluronate and process it with relation to adjacent macrophages with intense hyaluronidase activities.

In conclusion, LYVE-1 is important for an evaluation of the RES as well as a marker substance, leading us to the resurrection of the RES. LYVE-1 expressed in the RES, especially in the vascular endothelium, may have different functions from that in lymphatic vessels.

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Supporting figure 1 Immunostaining of LYVE-1. (a, b) Immunostaining of the liver. A more intense reactivity for LYVE-1 is seen at the mid-regions of hepatic lobules. C: central veins, P: interlobular connective tissue. (c) LYVE-1 immunoreactivity in the bone marrow of a mouse. Sinus endothelial cells show LYVE-1 immunoreactivity with various intensities. (d) LYVE-1 immunoreactivity in the rat pituitary gland. (e, f) Immunostaining of the spleen of one male and one female mice of ddY strain. LYVE-1-immunoreactive sinuses are seen only in the female sample.

Supporting figure 2 Measurement of mRNA using quantitative RT-PCR shows the change in LYVE-1 mRNA expression after an LPS administration. The expression peaked at 4 h after the administration in both the liver and lung. Value at 0 h in the liver is set at 1 in the figure.

Supporting figure 3 In situ hybridization analysis for hyaluronidase 2 mRNA. An X-ray film image after hybridization using ^{33}P -labeled DNA probes displays a significant expression of hyaluronidase 2 mRNA in the medulla of the lymph node, liver, and lung. No signals are visible in the negative control.

Supporting figure 4 In situ hybridization analysis using for ^{33}P -labeled DNA probes. The expressions of LYVE-1 (a) and hyaluronidase 2 mRNA (c) are seen in serial sections from the lymph node. The same regions are observed under dark fields (b, d). Both signals are localized in the sinuses of the medulla.