Inflammation-induced Uptake and Degradation of the Lymphatic Endothelial Hyaluronan Receptor LYVE-1*\textsuperscript{S}

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The hyaluronan receptor LYVE-1 is selectively expressed in the endothelium of lymphatic capillaries, where it has been proposed to function in hyaluronan clearance and hyaluronan-mediated leukocyte adhesion. However, recent studies suggest that hyaluronan homeostasis is unperturbed in LYVE-1-aorta mice and that lymphatic adhesion/transmigration may be largely mediated by ICAM-1 and VCAM-1 rather than LYVE-1. Here we have explored the possibility that LYVE-1 functions during inflammation and report that the receptor is down-regulated by pro-inflammatory cytokines. Using cultured primary lymphatic endothelial cells, we show that surface expression of LYVE-1 is rapidly and reversibly lost after exposure to tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) and TNF\(\beta\) via internalization and degradation of the receptor in lysosomes, coupled with a shutdown in gene expression. Curiously, internalization does not result in significant uptake of hyaluronan, a process that is largely insensitive to the novel LYVE-1 adhesion blocking monoclonal antibody 3A, and proceeds almost equally in resting and inflammation-activated lymphatic endothelial cells. Finally, we show that TNF can induce down-modulation of LYVE-1 in ex vivo murine dermal tissue explants and present evidence that the process occurs in vivo, in the context of murine allergen-induced skin inflammation. These findings suggest that LYVE-1 can function independently of hyaluronan and have implications for the use of LYVE-1 as a histological marker for lymphangiogenesis in human pathology.

LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1, is a 322-residue type I transmembrane glycoprotein that was first identified through its homology with the inflammatory leukocyte homing receptor CD44 (1, 2). In common with CD44, the extracellular domain of LYVE-1 contains a single cartilage Link module (1), the prototypic hyaluronan-binding domain conserved within all members of the Link superfamily (3). However, unlike CD44, which is widely expressed on cells of mesothelial, epithelial, and hematopoietic origin, LYVE-1 is almost entirely restricted to lymphatic endothelium, a property that makes it a powerful marker in studies of tumor lymphangiogenesis (4). Although the precise role of LYVE-1 is currently unclear, its mutually exclusive pattern of expression with that of CD44 suggests a distinct physiological function, specific to the lymphatic vasculature.

Hyaluronan (HA),\textsuperscript{3} the ligand common to both LYVE-1 and CD44, is a large, linear copolymer of D-glucuronic acid and N-acetyl-D-glucosamine (5) that is sequestered in tissues by matrix proteoglycans such as aggrecan, versican, and Link protein to form hygroscopic networks for CD44-mediated cell adhesion and migration (6–8). These HA networks are subject to constant turnover, initiated by proteolytic degradation and local uptake of glycosaminoglycan components in cells such as fibroblasts and macrophages. In addition, HA enters the lymphatics (9, 10), where it is transported in afferent lymph for degradation within the draining lymph nodes, liver, and spleen sinusoids via the high affinity HA receptor for endocytosis (HARE, aka Stabilin II, FEEL II) (11–14). This remote handling mechanism, which is responsible for catabolizing \(\sim 30\%\) of total body HA per day, is thought to be vital for protecting the tissues from prolonged exposure to short HA oligosaccharides with pro-angiogenic and pro-inflammatory properties (15, 16) that constitute danger signals for activating the immune system (17, 18). Previously, we hypothesized that LYVE-1 might participate in lymphatic HA metabolism, based on the capacity of the receptor to mediate specific, saturable binding and endocytosis of the glycosaminoglycan in transfected 293T human fibroblasts (19–21). However, in studies on LYVE-1\(-/-\) knock-out mice, we found that loss of the receptor had no significant effect on either serum or tissue HA levels and no obvious consequences for HA-mediated adhesion/migration events such as exit of skin dendritic cells through afferent lymph (4, 22, 23). A final anomaly is that we could not demonstrate binding of LYVE-1 to HA in normal lymphatic endothelial cells, most likely because of regulatory post-transla-
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tional modifications to the receptor (24). These various properties of LYVE-1 are reminiscent of the leukocyte HA receptor CD44, which is inactive by default in cells such as T lymphocytes and monocytes and binds HA only in response to inflammation or antigen receptor activation. This suggested to us that the function of LYVE-1, like that of CD44, might be more clearly manifest under inflammatory or other specific pathological conditions.

In the present study, we have investigated the effects of inflammation on LYVE-1 function both in vitro and in vivo. Rather than activating the receptor, we find that pro-inflammatory cytokines such as TNFα and TNFβ induce rapid internalization of LYVE-1 in lymphatic endothelial cells followed by terminal degradation in lysosomes. Moreover, we demonstrate that internalization does not trigger LYVE-1 to bind or transport HA to an appreciable extent, indicating that the process is not causally linked to HA metabolism. These results suggest that LYVE-1 does not function as an overt receptor for HA in either inflamed or uninflamed lymphatic endothelium and raise the possibilities of additional functions and/or further ligands for this abundant protein.

EXPERIMENTAL PROCEDURES

Cell Lines—Primary human dermal lymphatic endothelial cells (HDLEC) were derived from human dermal microvascular endothelial cells (PromoCell, Heidelberg, Germany) by selective outgrowth of LYVE-1⁺ve LEC as described previously (25). In addition, LEC were prepared from freshly resected human skin tissue samples by LYVE-1 mAb MACS® bead immunoselection as described recently (26). Cells in both cases were cultured in EGM-2 MV using plastic tissue culture flasks that had been precoated with gelatin (0.1%, Invitrogen). All experiments were performed using confluent cell cultures.

Cytokines and Growth Factors—All recombinant proteins were purchased from R&D Systems UK and used at the following concentrations: human IL-1α, 1 ng/ml; IL-2, 2 ng/ml; IL-4, 10 ng/ml; IL-6, 20 ng/ml; IL-8, 50 ng/ml; IL-13, 40 ng/ml; TNFα, 0.1–100 ng/ml (see “Results”); TNFβ, 100 ng/ml; transforming growth factor-β1, 10 ng/ml; secondary lymphoid chemokine, 25 ng/ml; epidermal growth factor, 50 ng/ml; fibroblast growth factor acidic, 500 ng/ml; fibroblast growth factor basic, 500 ng/ml; granulocyte colony-stimulating factor, 20 ng/ml; granulocyte-macrophage colony-stimulating factor, 50 ng/ml; interferon-γ, 100 ng/ml; MIP3α, 100 ng/ml; MIP3β, 25 ng/ml; Ang-2, 200 ng/ml; lipopolysaccharide, 1 μg/ml; VEGF-C, 100 ng/ml. Recombinant mouse TNFα was used at 100 ng/ml.

Antibodies—The rat monoclonal antibody C1/8 (IgG1, non-HA-blocking) and the mouse monoclonal antibodies 8C (IgG1, non-HA-blocking) and 3A (IgG1, HA-blocking) were generated using mouse and human LYVE-1 Fc as immunogen, respectively. The IgG fraction was purified from hybridoma supernatants using mouse and human LYVE-1 Fc as immunogen, respectively. The IgG fraction was purified from hybridoma supernatants using mouse and human LYVE-1 Fc as immunogen, respectively.

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Isotype-matched antibodies (mouse, rat, and rabbit IgG) were purchased from Sigma. Secondary antibodies Alexa Fluor 488 (green) and Alexa Fluor 568/594 (red) conjugates were purchased from Molecular Probes – Invitrogen.

Flow Cytometry—Cells were lifted with PBS-5 mM EDTA, suspended in FACS incubation buffer (PBS-5% fetal calf serum, 0.1% azide) and incubated for 30 min at 5°C with primary antibody followed by washing and further incubation with the appropriate Alexa Fluor® 488 goat conjugate and then analyzed on a flow cytometer (FACscalibur, BD Biosciences) using CellQuest software.

Immunofluorescent Antibody Staining of Cells and Tissues—For single/double immunofluorescent staining, cells grown in culture were fixed in paraformaldehyde-PBS, 4% (w/v). Appropriate primary antibodies were applied in PBS-5% fetal calf serum and incubated at 25°C for 30 min followed by washing and further incubation with the appropriate Alexa Fluor® secondary antibodies. Cells were fixed in paraformaldehyde-PBS, mounted in VECTASHIELD-DAPI (Vector Laboratories, Inc.), and viewed under a Zeiss Axiovert fluorescence microscope.

For whole-mount staining, tissue was fixed overnight at 4°C in parafomaldehyde (4% w/v in PBS, pH 7.4), blocked in PBS- Triton X-100 (0.3% w/v) supplemented with dried milk (3% w/v), and incubated overnight at 4°C with the appropriate primary antibodies. After washing, tissue was incubated with secondary antibodies for 2 h in the dark at 25°C and then mounted in VECTASHIELD and viewed on a confocal microscope (Radiance 2000; Bio-Rad Laboratories).

Assay for LYVE-1 Surface Shedding—HDLEC were cultured in 6-well dishes either alone or with TNFα or TNFβ for 24 h at 37°C followed by removal of the culture medium and clarification by centrifugation (9,000 × g, 5 min). Supernatants were assayed for shed LYVE-1 by applying them in triplicate to enzyme-linked immunosorbent assay wells precoated with rabbit anti-LYVE-1. Medium alone was used as a negative control, and LYVE-1-Fc soluble fusion protein was used as a standard, applied in duplicate. Bound LYVE-1 was detected using the mouse anti-LYVE-1 mAb 8C and a secondary horseradish peroxidase-conjugated antibody and substrate for measurement in a microplate reader at 490 nm.

Endothelial Cell Proliferation Assay—The proliferation rate of primary HDLEC was determined by a colorimetric assay that measured reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to the insoluble formazan product. Briefly, 40 μl of MTT solution (7.5 mg/ml in PBS) was mixed with 200 μl of culture medium and added to monolayers of endothelial cells cultured in 24-well dishes. After incubation for 1 h at 37°C, supernatants were discarded, and 200 μl of 0.4 M HCl in isopropyl alcohol was added to the monolayer. Cell lysates were centrifuged (4 min, 900 × g), and the resulting supernatants were analyzed in a plate reader (Bio-Rad Laboratories) at 590 nm.
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Northern Blot Hybridization—12 μg of total cellular RNA (RNaseasy, Qiagen) from HDLEC cultured ± TNFα was subjected to MOPS-formaldehyde agarose gel electrophoresis, transferred onto charged nylon membranes (Hybond N+; Amersham Biosciences), and hybridized at 65 °C overnight in ExpressHyb hybridization solution (Clontech) with DNA probes of either full-length LYVE-1 or ~600 bp of β-actin amplified by the primers ActinFwd (5′-AGGCATCCTACCCCTGAAGTAC-3′) and ActinRev (5′-TTGCAATGGTGATGACCTGGC-3′). Probes were radiolabeled with [α-32P]dCTP (Redi vue, Amersham Biosciences) by random oligonucleotide priming and Klenow polymerase (High Prime DNA labeling kit, Roche Applied Science). Blots were washed at high stringency (0.1× SSC, 0.1% SDS) before exposure to Kodak BioMax MR x-ray film.

Reverse Transcript-PCR—5 μg of total RNA isolated from HDLEC after various times in culture ± TNFα was extracted and purified using the RNaseasy RNA kit (Qiagen). First-strand cDNA syntheses were carried out by oligo(dT) priming and AMV reverse transcriptase (Cambio), according to the manufacturer’s instructions. LYVE-1 and β-actin cDNAs were PCR-amplified with either the LYVE-1 primers 269F (5′-GCCCTGAGGGCTGGGACTAAG-3′) and 764R (5′-CCCCAGCTCCTCATTTGGATG-3′) or the β-actin primers mentioned previously. Products were electrophoresed on 1.3% agarose gels, transferred to charged nylon membrane and hybridized at 42 °C overnight with either α-33P-labeled LYVE-1 or β-actin. Blots were washed at high stringency before autoradiography.

Subcellular Localization of Newly Synthesized or Internalized LYVE-1—To determine subcellular localization of newly synthesized LYVE-1, HDLEC were cultured in 8-chamber slides (Falcon), fixed in 4% (w/v) paraformaldehyde in PBS, and then washed with PBS and incubated in permeabilization buffer (10% (v/v) goat serum) for 20 min at room temperature. Cells were then dual-stained with LYVE-1 antibody and primary antibodies to either the endoplasmic reticulum marker PDI or the Golgi marker β-COP (45 min, room temperature) followed by the appropriate Alexa Fluor® secondary antibodies. To determine subcellular localization of newly internalized LYVE-1, HDLEC plated in 6-well dishes were incubated (3 h, 37 °C) with affinity-purified rabbit anti-human LYVE-1 polyclonal Ab (10 μg/ml) in the presence or absence of TNFα (10 ng/ml) followed by fixation in PBS, 4% (w/v) paraformaldehyde. After washing in PBS and permeabilization with saponin (described above), cells were stained with antibodies to either the early endosomal marker EEA-1 or the lysosomal marker CD63 followed by appropriate Alexa Fluor® secondary antibodies. In both cases, slides were washed, and the chambers were removed by soaking in 80% methanol prior to mounting in VECTASHIELD-DAP1 and viewing on a confocal microscope (Radiance 2000; Bio-Rad Laboratories).

Measurement of FI-HA Uptake by Primary HDLEC—Primary HDLEC plated in 8-chamber slides were preincubated (40 min, 37 °C) with HA-blocking mouse anti-human CD44 mAB BRC235 (50 μg/ml, included to block low levels of CD44 expression seen in cultured HDLEC (1, 26, 27)) and either the HA-blocking mouse anti-human LYVE-1 mAB 3A or control IgG1 (1–50 μg/ml), prior to the addition of Fl-HA (10 μg/ml) and reincubation with or without TNFα (10 ng/ml) for a further 1–12 h. Cells were then treated with papain (0.5 mg/ml) at 37 °C for 40 min to remove surface-bound HA (19) followed by quantitation of internalized Fl-HA by flow cytometry.

Animal Procedures—All animal procedures were carried out in accordance with UK Home Office regulations, under the appropriate personal and project licenses and with the prior approval of the Oxford Regional Ethical Committee.

Mouse Dermal Explants—Male BALB/c and C57Bl6 mice aged 8–10 weeks were euthanized by rising concentration of CO2, and the ears were removed, incubated in 5× penicillin-streptomycin on ice for 30 min, and then split into dorsal and ventral halves and floated split-side (dermis) down in RPMI 1640, 10% fetal calf serum, penicillin (1 units/ml), streptomycin (50 μg/ml), and 2 mM l-glutamine. Explants were cultured in a humidified atmosphere at 37 °C in 5% CO2 in the presence of recombinant murine TNFα, 100 ng/ml.

Oxazolone-induced Contact Hypersensitivity—Balb/c male mice aged 8–10 weeks were sensitized by topical application of 3% (w/v) oxazolone (4-ethoxymethylene-2 phenyl-2-oxazole-5-one; Sigma) in 95% aqueous ethanol to the shaved abdomen (50 μl per mouse). The following day, a further 100 μl of 2.5% w/v oxazolone was applied to the same site. On day 5, the dorsal surface of the left ear was challenged in each case by topical application of 0.8% oxazolone solution, 50 μl per ear, whereas the right ear (control) was treated with vehicle alone. Mice were sacrificed 3–24 h after challenge, and tissue was processed for staining as described above.

RESULTS

Inflammatory Cytokines Induce Rapid Loss of LYVE-1 in Primary Lymphatic Endothelial Cells—To investigate the possibility that LYVE-1 becomes functionally active during inflammation, we first assessed the effects of cytokines and growth factors on the levels of endogenous receptor in cultured primary HDLEC. To perform such studies, we initially used HDLEC derived from commercial mixed primary human dermal microvascular endothelial cells (HMVEC). The lymphatic endothelial phenotype of these cells has been reported recently but was again verified here by immunostaining for the sialomucin marker podoplanin (28), the lymphatic lineage-associated transcription factor Prox-1 (29), and the pan-endothelial marker CD31 in combination with fluorescence microscopy and flow cytometry. As shown by the data in Fig. S1, A and B, the HMVEC-derived LEC expressed high levels of each of these markers, in essentially identical fashion to LYVE-1 antibody-immunoselected HDLEC. Both HDLEC populations also displayed abundant, heterogeneous staining for LYVE-1 (Fig. S1 and Fig. 1, B and C), a characteristic feature of this receptor apparent from earlier studies (see e.g. Refs. 26 and 29). Preparations were routinely of 95% or greater purity as determined by podoplanin expression (Fig. 2E), and this phenotype was maintained throughout the course of the experiments reported here. Experiments were performed on confluent HDLEC at passage 12, when a predominantly lymphatic phenotype had been established.
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Incubation with the pro-inflammatory cytokines TNFα and TNFβ, however, resulted in virtually complete loss of LYVE-1 immunoreactivity (Figs. 1 and 2 and data not shown). In contrast, exposure to other cytokines including IL-1, IL-2, IL-4, IL-6, interferon-γ, transforming growth factor-β1, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, the CC chemokines secondary lymphoid chemokine (slc, CCL21), MIP3α stimulating factor, the CC chemokines secondary lymphoid chemokine (CCL19), and MIP3β (CCL20), and MIP3β (CCL19) or the lymphangiogenic/lymphangiogenesis-related growth factors VEGF-C (30), angiopoietin-2 (Ang-2 (31)), and fgf-two-dimensional (FGF-2) had relatively little effect on receptor levels, indicating that loss of LYVE-1 was unlikely to be a consequence of general cell activation or mitogenesis.

Further characterization of the response to TNFα showed that this was both potent and rapid, inasmuch as full down-regulation of LYVE-1 occurred at concentrations between 1 and 5 ng/ml (IC50 ~ 100 pg/ml, Fig. 2A), with a half-time (T1/2) in the region of 6 h (Fig. 2B). Down-regulation was also fully reversible, with complete replenishment of surface receptor occurring within 48 h of TNF withdrawal as assessed by flow cytometry (Fig. 2C). The selective loss of LYVE-1 in response to TNF was highlighted by comparative immunostaining with the lymphatic endothelial cellialoglycoprotein marker podoplanin, the levels of which remained stable throughout exposure to the cytokine (Fig. 2, D and E). Finally, the maintenance of full cell integrity by treated HDLEC was confirmed by trypan blue dye exclusion (which indicated greater than 90% viability) and by measurement of proliferation rate and capacity to form tubular structures in Matrigel™, both of which parameters were unaffected by short term (24–72 h) incubation with TNFα (data not shown).

Loss of LYVE-1 Protein Expression Is Accompanied by Disappearance of LYVE-1 mRNA—To gain further insight into the mechanism of LYVE-1 down-regulation, we next determined the effects of TNFα on LYVE-1 mRNA levels by Northern blotting of total HDLEC RNA. The results (Fig. 3A) show that the previously observed 1.8- and 2.8-kb LYVE-1 mRNA bands (1) are present only in untreated cells and are completely lost within 72 h of TNFα treatment. Indeed, analysis of mRNA levels at earlier time points using semiquantitative reverse transcription-PCR demonstrated that the loss of LYVE-1 mRNA is comparatively rapid, reaching completion within 12 h of cytokine exposure (Fig. 3B). Taken together, these results demonstrate that TNFα rapidly and reversibly down-regulates LYVE-1 expression in lymphatic endothelial cells at the level of either transcription or mRNA turnover.

LYVE-1 Is Internalized and Rapidly Degraded in Lysosomes—To study the fate of LYVE-1 in TNFα-treated HDLEC, we stained both intact and saponin-permeabilized cells with fluorescently labeled LYVE-1 mAb 8C for analysis by confocal microscopy. The results showed that the receptor shifted from a largely cell surface compartment to a predominantly intracellular, submembranous compartment within 3 h of cytokine exposure and that only trace levels could be detected after a further 45 h, consistent with terminal degradation (Fig. 4).

Some intracellular LYVE-1 was also detected in control untreated HDLEC. However, this had a different distribution to the internalized receptor, most likely corresponding to newly synthesized protein in the endoplasmic reticulum (see also Fig. 5A below). Moreover, the levels of soluble LYVE-1 detected within the culture supernatants of TNFα-treated HDLEC (0.9 ± 0.9 ng/ml; n = 3) were even less than those measured in untreated cells (11.8 ± 1.5 ng/ml; n = 3) as assessed by enzyme-linked immunosorbent assay, indicating that little if any of the TNF-induced down-regulation is mediated by surface shedding.

To further elucidate the pathway for LYVE-1 uptake and degradation, we studied the subcellular localization of bulk receptor in control and TNFα-treated HDLEC by dual immunofluorescent staining of saponin-permeabilized cells.
for discrete organellar markers followed by confocal microscopy (Fig. 5). Although the majority of intracellular LYVE-1 in untreated cells displayed a perinuclear staining pattern, co-localizing with the endoplasmic reticulum and Golgi markers PDI and β-COP, respectively (32), most of this was lost following exposure to TNFα (Fig. 5A). These findings are consistent with an early shutdown of de novo receptor biosynthesis by cytokine.

In a second approach, we tracked the fate of newly internalized LYVE-1 in HDLEC by tagging the receptor on the cell surface with LYVE-1 Ab prior to incubation with cytokine. In this case, we found a large proportion of the internalized recep-
tor co-localized with the early endosomal marker EEA-1 and the lysosomal marker CD63 in response to TNF treatment (Fig. 5B). In contrast, little if any LYVE-1 co-localized with either marker in mock-treated cells. These findings, together with our observations that LYVE-1 is relatively insensitive to the effects of inhibitors acting during biosynthesis (e.g. Tunicamycin), suggest that LYVE-1 has a low constitutive turnover rate when compared with certain other Link superfamily receptors such as Stabilin-1/CLEVER-1 in HDLEC (33) and that the mechanism of TNFα-triggered down-regulation operates through an inducible pathway of endocytosis and lysosomal degradation.

*Does Internalization of LYVE-1 Activate the Receptor for HA Uptake?*—As previously stated, the endogenous LYVE-1 in HDLEC appears to be functionally inactivated insofar as cultured HDLEC fail to display any binding of Fl-HA to the cell surface (not shown). Given the in vivo evidence that lymphatic vessels carry tissue HA for degradation in lymph nodes, we considered the possibility that TNF-induced internalization of LYVE-1 might act as a signal for the functionally quiescent receptor in HDLEC to bind and internalize hyaluronan for catabolism. To test this hypothesis, we quantitated uptake of Fl-HA in primary HDLEC by flow cytometry and determined the contribution of LYVE-1 in the process by taking advantage of a new monoclonal antibody 3A that blocks HA binding to the receptor. The potency of this novel mAb is demonstrated by its capacity to fully block Fl-HA binding to LYVE-1-transfected 293T cells, which in contrast to HDLEC express the receptor in constitutively active form (Fig. 6). Analysis of Fl-HA uptake by primary HDLEC revealed that these cells do engage in significant uptake of HA (Fig. 7). However, the extent to which TNF induced the process was relatively small when assessed in an independent experiment (mean fluorescence Fl-HA uptake 415 ± 5 control; 462 ± 8 TNFα, after 6 h). Furthermore, the effects of 3A blockade on TNF-induced HA uptake were only marginal even at time points beyond the 6–12-h period required for complete clearance of the receptor (Figs. 2B and 7). Indeed, these effects were similar in magnitude in untreated HDLEC in which the extent of LYVE-1 internalization is much lower (data not shown). In conclusion, these results show that LYVE-1 internalization does not contribute significantly to the process of HA internalization in HDLEC. Inflammation-induced turnover of LYVE-1 therefore seems unlikely to be a major stimulus for HA transport in such cells.

*TNFα Induces Down-regulation of LYVE-1 in Mouse Dermis ex Vivo*—Having established down-regulation of LYVE-1 by TNFα in primary HDLEC, we next asked whether a similar process occurs within the endothelium of lymphatic vessels ex vivo.
To address this issue, we prepared mouse dermal explants and assessed the expression of LYVE-1 within the endothelia of constituent lymphatic vessels (detected with podoplanin and CCL21/slc) both before and after 24-h incubation with TNFα/H9251.

Next, we looked for evidence of LYVE-1 down-regulation in vivo, in the context of the well characterized oxazolone-induced skin contact hypersensitivity response in mice. Sensitization and subsequent challenge with this agent is known to elicit a TNF-mediated inflammatory response that promotes lymph node trafficking of dendritic cells via afferent dermal lymphatics (34, 35). We assessed LYVE-1 expression in whole mount skin preparations from both inflamed and contralateral uninflamed ears 24 h after elicitation of inflammation. The strong staining for LYVE-1 visible on the branched network of blind-ended lymphatic vessels in unstimulated tissue was markedly reduced in the corresponding vessels of inflamed tissue as visualized with the LEC markers secondary lymphoid chemokine (slc/CCL21 (36)) and podoplanin (28, 37) (Fig. 8B), confirming the findings with tissue explants and underlining the fact that loss of LYVE-1 is an active process rather than the result of vessel necrosis or apoptosis.

Finally, to assess the kinetics of down-regulation, we prepared sections of dermis from the inflamed and contralateral
uninflamed ears at 3–24 h after oxazolone-challenge and subjected them to dual immunohistochemical staining for LYVE-1 and podoplanin followed by microscopic estimation of LYVE-1-positive vessel numbers. The results (Fig. 9 and Fig. S2) show that the percentage of lymphatic vessels expressing LYVE-1 fell by almost 70% between 6 and 18 h after oxazolone challenge and that by 24 h, only 30% of vessels had retained LYVE-1 expression. No such changes were seen with mock (vehicle only, no oxazolone)-challenged skin (data not shown).

**DISCUSSION**

The finding that LYVE-1 is down-regulated in inflammation has important consequences for understanding the biological function of the receptor. In recent studies using knock-out mice, we found that expression of LYVE-1 in lymphatic endothelium appears to be dispensable for both leukocyte trafficking and hyaluronan homeostasis, at least under normal physiological conditions. This led us to speculate that the true function of the receptor might only be manifest under conditions of stress such as inflammation, when immune activation signals an increase in leukocyte trafficking and matrix turnover. The rapid internalization and degradation of the receptor that we observed in response to inflammation was unexpected; however, it is still conceivable that LYVE-1 could function in cellular trafficking or HA metabolism under such conditions. For example, LYVE-1 could support inflammatory leukocyte translymphatic migration by engaging CD44-HA complexes (or other ligands, see below) on migrating cells (38, 39) as originally envisaged, e.g. if a proportion of the receptor were associated with the caveolar/vesiculovacuolar pathway. Indeed, this latter pathway contributes to transcellular leukocyte migration in HDLEC (40), and the capacity of LYVE-1 to engage CD44-HA complexes has already been demonstrated by plate binding assays in vitro (1). Clearly, however, our observations that native LYVE-1 is functionally inactivated in normal lymphatic endothelium argue against such a role. Furthermore, preliminary results from in vitro HDLEC transmigration assays (26) indicate that transit of leukocytes is unaffected by the addition of soluble LYVE-1 Fc.5 If LYVE-1 does have any role in leukocyte trafficking, it could for example be that of a gatekeeper that restricts leukocyte translymphatic migration under conditions of normal tissue homeostasis. Circumstantial evidence in support of this latter notion comes from the observation that LYVE-1 down-regulation occurs simultaneously with up-regulation of the endothelial adhesion molecules ICAM-1, VCAM-1, and E-selectin that were shown recently to mediate leukocyte transmigration in response to TNFα (26). Indeed, of some 121 transcripts that showed greater than 2-fold down-regulation (p < 0.1) in Affymetrix GeneChip® array analyses of TNFα-treated human LEC, LYVE-1 experienced the second greatest degree of reduction (14-fold) when compared with controls (26), (National Center for Biotechnology Information

5 L. A. Johnson and D. G. Jackson, unpublished.
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Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo, accession no. GSE6257). More detailed experiments comparing leukocyte transmigration of wild-type and LYVE-1−/− HDLEC will clearly be required to resolve this issue.

As regards extracellular matrix metabolism, a role for LYVE-1 is certainly attractive given the fact that HA turnover is particularly rapid in the skin (T1/2 24 h (41, 42), the tissue from which the HDLEC in our study were derived, and given that HA degradation in vivo is known to involve the lymphatics (9).

Again, however, the seeming paradox that LYVE-1 present in normal lymphatic endothelial cells does not bind HA argues against this role. Although our results provide the first indication that lymphatic endothelial cells do internalize HA, the process was shown to be equally efficient under both resting and TNF-stimulated conditions (i.e. in the presence and absence of LYVE-1 expression). Moreover, the insensitivity of TNF-stimulated HA uptake to the adhesion blocking mAb 3A rules out the remaining possibility that LYVE-1 becomes activated subsequent to internalization. We have not yet determined the mechanism(s) for uptake of HA by HDLEC, established the physiological significance of such uptake, or identified any of the receptors involved. Indeed, we cannot rule out the possibility that the process occurs by facilitated diffusion or pinocytosis rather than specific receptor-mediated endocytosis. However, the process is unlikely to involve the main receptor for HA catabolism in liver spleen and lymph node known as HARE/FEEL II/Stabilin II (11–14), as this molecule is not expressed in peripheral lymphatics. We could also exclude involvement of residual CD44 present in cultured LEC since uptake was insensitive to the CD44 HA-blocking mAb BRCI-235. It will be interesting to investigate the contribution of afferent lymphatic endothelium to HA catabolism, both in light of these findings and in light of recent preliminary observations that HDLEC display an active hyaluronidase activity.

The potent down-regulation of LYVE-1 by TNF contrasts markedly with the effects of the cytokine on CD44, which include transcriptional up-regulation via calmodulin/calmodulin kinase-II and AP-1 action (43), coupled with functional activation (i.e. induction of HA binding) via sulfation (44) and mitogen-activated protein kinase (MAPK) dependent activation of a membrane-bound sialidase activity (45, 46). Curiously, TNF-mediated LYVE-1 down-regulation also appears to involve an NFκB/AP-1-mediated pathway since preliminary experiments indicate that the IkB kinase inhibitor BAY-11-7082 can block LYVE-1 down-modulation in HDLEC by up to 70%.5 As already discussed, however, the mechanism for down-regulating LYVE-1, in contrast to that of CD44, is not coupled to functional activation. Thus, the physiological conditions that lead to functional activation of LYVE-1 remain enigmatic, and the likelihood exists that the “off” state of the receptor mediates a separate function. For example, it is conceivable that the sialylated sugar structures that block HA binding in LYVE-1 could function in adhesion, like those attached to CD44 glycoforms that bind the homing receptors L and E selectin (47–49). We are currently exploring these and other possibilities by probing for alternative ligands with fluorescent tagged LYVE-1 Fc dimers and higher order oligomers.

The studies reported in this report have examined the fate of LYVE-1 in a model of acute inflammation, and we have yet to extend the work to chronic inflammation, a condition that has long been known to provoke lymphangiogenesis (50). One recent study that utilized LYVE-1 as a marker to investigate mechanisms of lymphangiogenesis in chronic mycoplasma-induced airway inflammation appeared to indicate near normal levels of LYVE-1 expression within new lymphatics (51). However, these authors have subsequently observed that down-regulation of the receptor was apparent during the early stages 24–48 h after infection, prior to VEGF-C-mediated vessel hyperplasia.7 Indeed, the recovery of LYVE-1 expression at later time points is entirely consistent with earlier reports showing that TNFα levels fall soon after the initial stages of mycoplasma infection (52).

Finally, the capacity for down-regulation of LYVE-1 in inflammation may have implications for routine use of the receptor as a specific marker for lymphatic capillaries in clinical studies, in particular, tumor pathology. There is currently considerable interest in quantitating tumor lymphatic vessel density in light of growing evidence that tumor lymphangiogenesis may be predictive of early lymph node metastasis and could thus help oncologists optimize patient management (53–55). Given the fact that metastatic cancers have varying degrees of inflammatory infiltration, some LYVE-1 down-modulation might be envisaged within the tumor microenvironment that might lead to an underestimate in lymphatic vessel numbers. Indeed, studies in our laboratory already indicate LYVE-1 loss in tumor lymphatics of human squamous cell carcinomas,8 and other reports in the recent literature suggest similar findings in pancreatic and breast tumors (56–59). The use of LYVE-1 in combination with other markers of lymphatic endothelium in such studies may therefore be prudent.

REFERENCES

1. Banerji, S., Ni, J., Wang, S. X., Clasper, S., Su, I., Tammi, R., Jones, M., and Jackson, D. G. (1999) J. Cell Biol. 144, 789–801
2. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990) Cell 61, 1303–1313
3. Day, A. J., and Prestwich, G. D. (2002) J. Biol. Chem. 277, 4585–4588
4. Jackson, D. G. (2004) APNIS 112, 526–538
5. Meyer, K., and Palmer, J. (1989) J. Cell Biol. 107, 861–870
6. Knudson, C. B., and Knudson, W. (1993) FASEB J. 7, 1233–1241
7. Hardingham, T. E., and Fosang, A. J. (1992) FASEB J. 6, 861–870
8. Toole, B. P. (2004) Nat. Rev. Cancer 4, 528–539
9. Fraser, J. R., Kimpton, W. G., Laurent, T. C., Cahill, R. N., and Vakakis, N. (1988) Biochem. J. 256, 153–158
10. Fraser, J. R., and Laurent, T. C. (1989) CLRA Found. Symp. 143, 41–53
11. Zhou, B., Weigel, J. A., Fauss, L., and Weigel, P. H. (2000) J. Biol. Chem. 275, 37733–37741
12. Tamura, Y., Adachi, Y., Otsuga, J., Ohashi, K., Yahagi, N., Sekiya, M., Okazaki, H., Tomita, S., Lizuka, Y., Shimano, H., Nagai, R., Kimura, S., Tsujimoto, M., and Ishibashi, S. (2003) J. Biol. Chem. 278, 12613–12617
13. Weigel, J. A., Raymond, R. C., McGary, C. T., Singh, A., and Weigel, P. H. (2003) J. Biol. Chem. 278, 9808–9812
14. Harris, E. N., Kuyosseva, S. V., Weigel, J. A., and Weigel, P. H. (2007) J. Biol.

6 J. Espinosa-Fematt and D. G. Jackson, unpublished.
7 P. Baluk, personal communication.
8 L. A. Johnson, G. Ogg, and D. G. Jackson, unpublished.
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Chem. 282, 2785–2797

15. Noble, P. W. (2002) Matrix Biol. 21, 25–29

16. West, D. C., and Kumar, S. (1989) CIBA Found. Symp. 143, 187–201

17. Termeer, C. C., Hennies, J., Voith, U., Ahrens, T., Weiss, J. M., Prehm, P., and Simon, I. C. (2000) J. Immunol. 165, 1863–1870

18. Stern, R., Asari, A. A., and Sugahara, K. N. (2006) Int. J. Cell Biol. 85, 699–715

19. Prevo, R., Banerji, S., Ferguson, D. J., Clasper, S., and Jackson, D. G. (2001) J. Biol. Chem. 276, 19420–19430

20. Jackson, D. G. (2003) Trends Cardiovasc. Med. 13, 1–7

21. Jackson, D. G. (2004) Glycoforum www.glycoforum.gr.jp/science/hyaluronan/HA28/HA28E.html

22. Gale, N. W., Prevo, R., Espinosa-Fematt, J., Ferguson, D. J., Dominguez, M. G., Yancopoulos, G. D., Thurston, G., and Jackson, D. G. (2007) Mol. Cell Biol. 27, 595–604

23. Jackson, D. G., Prevo, R., Clasper, S., and Banerji, S. (2001) Trends Immunol. 22, 317–321

24. Nightingale, T., Banerji, S., and Jackson, D. G. (2005) in Hyaluronan Structure, Metabolism, Biological Activities, Therapeutic Applications (Balazs, E. A., and Hascall, V. C., eds) pp. 615–618, Vol. II, Matrix Biology Institute, Edgewater, NJ

25. Nisato, R. E., Harrison, J. A., Buser, R., Orci, L., Rinsch, C., Montesano, R., Dupraz, P., and Pepper, M. S. (2004) Am. J. Pathol. 165, 11–24

26. Johnson, L. A., Clasper, S., Holt, A., Lalor, P., Baham, D., and Jackson, D. G. (2006) J. Exp. Med. 203, 2763–2777

27. Anstee, D. J., Gardner, B., Spring, F. A., Holmes, C. H., Simpson, K. L., Parsons, S. F., Mallinson, G., Yousaf, S. M., and Judson, P. A. (1991) Immunology 74, 197–205

28. Breiteneder-Geleff, S., Soleiman, A., Kowalski, H., Horvat, R., Amann, G., Kriehuber, E., Diem, K., Weninger, W., Tschachler, E., Altitalo, K., and Kerjaschki, D. (1999) Am. J. Pathol. 154, 385–394

29. Wigle, J. T., and Oliver, G. (1999) Cell 98, 769–778

30. Jeltsch, M., Kaipanen, A., Joukov, V., Meng, X., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K., and Altitalo, K. (1997) Science 276, 1423–1425

31. Gale, N. W., Thurston, G., Hackett, S. F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M. H., Jackson, D. G., Sato, T., Suris, C., Campochiaro, P. A., Wieand, S. J., and Yancopoulos, G. D. (2002) Dev. Cell 3, 411–423

32. Harrison-Lavoie, K. J., Lewis, V. A., Hynes, G. M., Collison, K. S., Nutland, E., and Willisson, K. R. (1993) EMBO J. 12, 2847–2853

33. Prevo, R., Banerji, S., Ni, J., and Jackson, D. G. (2004) J. Biol. Chem. 279, 52580–52592

34. Grabbe, S., and Schwarz, T. (1998) Immunol. Today 19, 37–44

35. Cumberbatch, M., and Kimber, I. (1995) Immunology 84, 31–35

36. Sallusto, F., Schaeler, P., Loetscher, P., Schanien, C., Lenig, D., Mackay, C. R., Qin, S., and Lanzavecchia, A. (1998) Eur. J. Immunol. 28, 2760–2769

37. Kriehuber, E., Breiteneder-Geleff, S., Groeger, M., Soleiman, A., Schoppmann, S. F., Stingl, G., Kerjaschki, D., and Maurer, D. (2001) J. Exp. Med. 194, 797–808

38. Mummert, M. E., Mummert, D., Edelbaum, D., Hui, F., Matsue, H., and Takashima, A. (2002) J. Invest. Dermatol. 122, 846–847

39. Mummert, D. I., Takashima, A., and Mummert, M. E. (2004) J. Investig. Dermatol. 129, 126–130

40. Millan, J., Hewlett, L., Glyn, M., Toonre, D., Clark, P., and Ridley, A. (2006) Nat. Cell Biol. 8, 113–123

41. Tammi, R., Saamanen, A. M., Maibach, H. I., and Tammi, M. (1991) J. Invest. Dermatol. 97, 595–604

42. Tammi, R., Agren, U. M., Tuukkanen, A. L., and Tammi, M. (1994) Prog. Histochem. Cytochem. 29, 1–77

43. Misha, J. P., Misha, S., Gee, K., and Kumar, A. (2005) J. Biol. Chem. 280, 26825–26837

44. Maiti, A., Maki, G., and Johnson, P. (1989) Science 282, 941–943

45. Levesque, M. C., and Haynes, B. F. (1999) Cell Immunol. 193, 209–218

46. Gee, K., Kodlowski, M., and Kumar, A. (2003) J. Biol. Chem. 278, 37275–37287

47. Dimitroff, C. J., Lee, J. Y., Fuhlbrigge, R. C., and Sackstein, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13841–13846

48. Dimitroff, C. J., Lee, J. Y., Rafii, S., Fuhlbrigge, R. C., and Sackstein, R. (2001) J. Cell Biol. 153, 1277–1286

49. Burdick, M. M., Chu, J. T., Godar, S., and Sackstein, R. (2006) J. Biol. Chem. 281, 13899–13905

50. Pullinger, B. D., and Florey, H. W. (1937) J. Pathol. Bacteriol. 45, 157–170

51. Baluk, P., Tammela, T., Achen, M., Hicklin, D. J., Jeltsch, M., Petrova, T. V., Pytowski, B., Stacker, S. A., Yla-Herttuala, S., Jackson, D. G., Altitalo, K., and McDonald, D. M. (2005) J. Clin. Invest. 115, 247–257

52. Faulkner, C. B., Simecka, J. W., Davidson, M. K., Davis, J. K., Schoeb, T. R., Lindsey, J. R., and Everson, M. P. (1995) Infect. Immun. 63, 4084–4090

53. van Trappen, P. O., and Pepper, M. S. (2001) Gynecol. Oncol. 82, 1–3

54. Millan, J., Hewlett, L., Glyn, M., Toonre, D., Clark, P., and Ridley, A. (2006) Br. J. Cancer 94, 157–170

55. Witte, M. H., Jones, K., Wilting, J., Dicor, M., Selg, M., McHale, N., Grushenvald, J. E., and Jackson, D. G. (2006) Cancer Metastasis Rev. 25, 159–184

56. Rubbia-Brandt, L., Terris, B., Giostra, E., Dousset, B., Morel, P., and Pepper, M. S. (2004) Clin. Cancer Res. 10, 6919–6928

57. Stessels, F., Van den Eynden, G., Van der Auwera, I., Salgado, R., Van den Heuvel, E., Harris, A. L., Jackson, D. G., Colpaert, C. G., van Marck, E. A., Dirix, L. Y., and Vermeulen, P. B. (2004) Br. J. Cancer 90, 1429–1436

58. Van der Auwera, I., Colpaert, C. G., Fox, S. B., Turley, H., Harris, A. L., van Marck, E. A., Vermeulen, P. B., and Dirix, L. Y. (2004) Clin. Cancer Res. 10, 7965–7971

59. Van der Auwera, I., Cao, Y., Tille, J. C., Peeples, M. S., Jackson, D. G., Fox, S. B., Harris, A. L., Dirix, L. Y., and Vermeulen, P. B. (2006) Br. J. Cancer 94, 1–15