Lymphatic Endothelial Murine Chloride Channel Calcium-Activated 1 Is a Ligand for Leukocyte LFA-1 and Mac-1

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Lymphatic Endothelial Murine Chloride Channel Calcium-Activated 1 Is a Ligand for Leukocyte LFA-1 and Mac-1

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The lymphatic circulation mediates drainage of fluid and cells from the periphery through lymph nodes, facilitating immune detection of lymph-borne foreign Ags. The 10.1.1 mAb recognizes a lymph endothelial Ag, in this study purified by Ab-affinity chromatography. SDS-PAGE and mass spectrometry identified murine chloride channel calcium-activated 1 (mCLCA1) as the 10.1.1 Ag, a 90-kDa cell-surface protein expressed in lymphatic endothelium and stromal cells of spleen and thymus. The 10.1.1 Ab-affinity chromatography also purified LFA-1, an integrin that mediates leukocyte adhesion to endothelium. This mCLCA1-LFA-1 interaction has functional consequences, as lymphocyte adhesion to lymphatic endothelium was blocked by 10.1.1 Ab bound to endothelium or by LFA-1 Ab bound to lymphocytes. Lymphocyte adhesion was increased by cytokine treatment of lymphatic endothelium in association with increased expression of ICAM-1, an endothelial surface protein that is also a ligand for LFA-1. By contrast, mCLCA1 expression and the relative contribution of mCLCA1 to lymphocyte adhesion were unaffected by cytokine activation, demonstrating that mCLCA1 and ICAM-1 interactions with LFA-1 are differentially regulated. mCLCA1 also bound to the LFA-1-related Mac-1 integrin that is preferentially expressed on leukocytes. mCLCA1-mediated adhesion of Mac-1– or LFA-1–expressing leukocytes to lymphatic vessels and lymph node lymphatic sinuses provides a target for investigation of lymphatic involvement in leukocyte adhesion and trafficking during the immune response.


T

he lymphatic system has a central role in immune sur-

veillance and in the adaptive immune response (1, 2).

Lymph drains from the periphery through lymphatic vessels into lymph nodes (LNs) to present Ags to lymphocytes that enter LNs via high endothelial venules (HEVs) (3, 4). Lymphatic vessels also transport immune cells including mast cells, neutrophils, or dendritic cells from the periphery to draining LNs as a rapid response to infection (5–7). Lymphocytes and other leukocytes also increase their adhesion and transmigration through the HEVs and accumulate within LNs to mount an adaptive immune response to infection. Similar adhesion mechanisms could mediate leukocyte trafficking through the lymphatic system and through vascular HEVs (1). However, little is known yet about the involvement of lymphatic endothelium in regulation of leukocyte trafficking through the lymphatic system in homeostasis or during immune responses (1). The development of Abs against lymphatic endothelial markers such as lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and Prox-1 (8, 9) now allows detailed investigation of the contributions of the lymphatic system to immune functions.

The entry of lymphocytes into LNs from the bloodstream via HEVs involves an adhesion and transmigration pathway that has been extensively characterized and serves as the model of endothelial/immune system cross talk (reviewed in Refs. 10–12). For example, naive lymphocytes entering peripheral LNs from the bloodstream express selectin that recognizes sialyl Lewis X modifications on CD34 or glycosylation-dependent cell adhesion molecule-1 glycoproteins expressed on the HEV, resulting in lymphocyte slowing and rolling along the HEV. Lymphocyte LFA-1 then binds to endothelial ICAM-1 to arrest rolling and promote leukocyte adhesion to the HEV. LFA-1 binding activity is increased by inducers such as CCL21 chemokine or PMA treatment of lymphocytes, whereas ICAM-1 on HEVs is induced by cytokines such as TNF-α to strongly increase the affinity of this adhesive interaction (10, 13). This regulated process is critical for cytokine activation of the immune response to accelerate leukocyte entry into LNs. LFA-1 is expressed on B and T lymphocytes, macrophages, and neutrophils (14). Leukocyte arrest on HEVs can also be promoted by α4β1 integrin adhesion to endothelial VCAM-1. This mechanism is particularly important for leukocyte entry into the bone marrow (BM), although it makes some contribution to lymphocyte trafficking via LN HEVs (15).

Upon arrest from rolling, lymphocytes transmigrate through HEV endothelium to enter the LN parenchyma by transcellular or paracellular mechanisms involving a number of proteins including ICAM-1, junction adhesion molecule-A, and CD99 (11, 12). Leukocytes then migrate to the B or T cell regions, where they encounter soluble Ag or APCs (4, 16). In the absence of immune stimulation, lymphocytes transit through lymphatic sinus endo-
thelium to exit LNs via the lymphatic sinuses within 1 d (17). Lymphatic endothelium expresses ICAM-1 and VCAM-1 (18, 19) so that lymphatic sinuses of the LN could also potentially mediate adhesion and/or transmigration of immune cells by the same LFA-1– and α4β1-dependent mechanisms used by HEVs. A recent study found that ICAM-1 and VCAM-1 mRNA are upregulated 8- and 214-fold after TNF-α and treatment of primary murine lymphatic endothelium, respectively, suggesting that lymphatic endothelium can regulate leukocyte trafficking during the immune response (20). LFA-1 is primarily expressed in Tand B cells so that it is the major ligand for ICAM-1–mediated lymphocyte adhesion to endothelium. Mac-1 is an LFA-1–related integrin that is preferentially expressed on leukocytes including granulocytes, dendritic cells, and macrophages, which also binds to ICAM-1 to promote adhesion and migration of leukocytes through blood vessels (10). Thus far, studies of lymphatic endothelial ICAM-1 interaction with Mac-1 suggest a key role in macrophage adhesion and migration (21). ICAM-1– and Mac-1–mediated adhesion to lymphatic endothelium also can suppress dendritic cell maturation (22). Much remains to be learned about the involvement of these and other adhesion molecules in leukocyte trafficking through the lymphatic system during the immune response.

Our interest in leukocyte–lymphatic endothelial adhesion interactions arose from our studies of the 10.1.1 mAb, which recognizes an Ag expressed on the surface of lymphatic endothelium (23). The 10.1.1 Ab was generated by immunizing hamsters with murine thymic stromal cells to obtain an Ab recognizing specific cell types in lymphoid organs (24). The 10.1.1 Ab recognizes lymphatic vessels in the skin and colon, intestinal lacteals, and lymphatic sinuses of LNs, suggesting that it is widely expressed on lymphatic endothelium (23). The expression of 10.1.1 on lymphatic vessels and LN lymphatic sinuses overlaps with that of other lymphatic endothelial markers, Prox-1, podoplanin, and LYVE-1, making it a useful marker of lymphatic endothelium (23, 25). In the thymus, the 10.1.1 Ag is expressed on stromal medullary epithelium, and it is concentrated at sites of thymocyte contact, suggesting that this Ag functions in adhesion (24). In this study, affinity chromatography was used to purify the Ag recognized by the 10.1.1 Ab as a first step to identify and characterize its function in the lymphatic system and in lymphoid organs. The 10.1.1 Ag was discovered to be a lymphatic endothelial surface ligand for LFA-1 and Mac-1, which is a major mediator of lymphocyte adhesion to lymphatic endothelium.

**Materials and Methods**

**Mice and Abs**

C57BL/6J wild-type mice (The Jackson Laboratory, Bar Harbor, ME) were housed in sterile microisolator rooms under specific pathogen-free conditions. Experimental methods involving animals were approved by Fred Hutchinson Cancer Research Center Animal Care and Use Committee (Seattle, WA).

Syrian hamster Abs used were 10.1.1 (24) andhamster IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Rat Abs used were CD18 (clone YTS213.1, Upstate Biotechnology, Lake Placid, NY), CD18 (clone M18/2), CD11a (M17/4), CD11b (M17/0), and F4/80 (BM3; BD Pharmingen, San Diego, CA); MECA-32 (SP2/0, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), LYVE-1 (ALY7, eBioscience, San Diego, CA), and rat IgG (Sigma-Aldrich, St. Louis, MO) were used. Secondary goat Abs were anti-Syrian hamster HRP (Jackson Immunoresearch Laboratories, anti-rat HRP (Southern Biotechnology Associates, Birmingham, AL), anti-hamster IgG Alexa 568, and anti-rat IgG Alexa 658.

**Purification of 10.1.1 Ag**

Lysate was prepared from 14 C57BL/6J mouse spleens by Dounce homogenization (B pestle) in 20 ml lysis buffer (1% Triton X-100, 50 mM Tris [pH 7.4], 0.3 M NaCl, 0.5 mM EDTA, 10 mM iodoacetamide, and Complete Protease Inhibitor [Boehringer Mannheim, Indianapolis, IN]), incubation on ice 30 min, and centrifugation at 4000 × g for 10 min. The supernatant was brought to 1% sodium deoxycholate and centrifuged at 43,000 × g for 40 min. Nonspecific Syrian hamster IgG (Jackson Immunoresearch Laboratories) and protein G-Sepharose–purified 10.1.1 Ab were linked to cyanogen bromide-activated Sepharose 4B (Sigma-Aldrich) for Ab-affinity chromatography (26). Sheep lyses (100 mg protein) were first passed through 0.5-mmlong-hamster-IgG-Sepharose in wash buffer (0.14 M NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.01 M Tris [pH 8]), bound to 0.5 ml 10.1.1 IgG-Sepharose, washed with stringent buffer (0.5 M NaCl, 0.5% Nonidet P-40, and 0.05 M Tris HCl [pH 8]), and Ag was then eluted with 2% SDS. Fractions were subjected to SDS PAGE on 7% Tris-acetate NUPAGE gels (Invitrogen, Carlsbad, CA) followed by Coomassie blue staining.

**Protein identification by in-gel digestion and mass spectrometry**

Gel bands were excised, denatured with 60% methanol, reduced with 10 mM DTT at 60˚C for 1 h, and alkylated with 50 mM iodoacetamide at room temperature in the dark for 30 min. Methanol concentration was reduced to 20% by adding ammonium bicarbonate (50 mM), and proteins were digested with trypsin (Promega, Madison, WI) at 37˚C for 6 h at a protein-to-enzyme ratio of 50:1 (w/w), dried in a SpeedVac (Savant SC110; Thermo Fisher Scientific, Pittsburgh, PA), and resuspended in 50 mM NH4HCO3. Peptides (12 pmol) were injected for liquid chromatography-tandem mass spectrometry analysis in a nano-LC system interfaced with a linear ion trap mass spectrometer (Thermo Electron, San Jose, CA). Instrument settings included spray voltage of 1.5 kV, ion transfer in the presence of 3% isopropanol, and an ion trap receiver potential of 200˚C, and collision gas pressure of 1.3 Torr. Voltages across capillary and quadrupole lenses were tuned for optimal signal intensity using the +2 charge state ion of angiotensin I (m/z 649). For each liquid chromatography-tandem mass spectrometry analysis, a mass spectrometry (MS) survey scan (m/z 400-1600) was followed by three data-dependent MS/MS scans. Database searches were performed using Sequest (Thermo Electron, San Jose, CA) and ProteinProphet (Applied Maths, Austin, TX). The search engines included the mouse proteome and known proteins. Test searches were first performed against the National Cancer Institute human sequence database using Comet (Bethesda, MD). Database search results were validated using PeptideProphet cutoff >0.95, and peptides were then assigned a protein identification using ProteinProphet.

**Tissue culture and transfection**

293, M1, and EL-4 cells (American Type Culture Collection, Manassas, VA) were cultured as recommended. SV-LEC cells were grown in DMEM plus 10% FBS at 37˚C (27). The 293 cells were transfected with murine chloride channel calcium-activated (mCLCA)1, mCLCA2 (28), murine angiotatin [mAST (29)], or pcDNA (Invitrogen) by calcium phosphate treatment (30) and harvested after 48 h.

**Immunoprecipitation and immunoblotting**

For immunoprecipitation, lyses were prepared in lysis buffer (Figs. 2, 4A, 4B). For mixed lysate immunoprecipitations, cells were lysed in low stringency buffer (1% Nonidet P-40, 0.05% Triton X (pH 8)) with Complete Protease Inhibitor mixture, and 10 mM iodoacetamide and protein concentration measured by Bradford assay before mixing 800 μg EL-4 or 4 mM lysates with 800 μg 293 cell lystate. Lyses were preclotted with Protein G beads (Amersham Biosciences, Piscataway, NJ) four times, bound to 25 μl Ah-coated protein G beads at 4˚C 1.5 h, washed four times in buffer (0.5% Nonidet P-40, 1 M NaCl, 0.0005% EDTA, 0.05% Triton X-100, 0.5 M NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.01 M Tris pH 8), and resuspended in nonreducing SDS sample buffer (0.25M Tris pH 6.8), 50% glycerol, 10% w/v SDS, 0.005% w/v bромонедол blue). For immunoblotting, cells or tissues were prepared for SDS-PAGE by lysing in non-reducing SDS sample buffer, pre-electrophoresis on 7% SDS-PAGE gels, and immunoblotting.

**Immunostaining**

The 293 cells were plated on glass coverslips in six-well plates (Corning Glass, Corning, NY) at 4 × 104 cells/well for 6 h. Cells were stained prepared using tissues frozen in Tissue-Tek OCT compound (Sakura, Torrance, CA). Samples were fixed in –20 ºC acetone 10 min, air dried 15 min, and fixed in phosphate-buffered formalin for 10 min, followed by immunostaining and mounting in ProLong Gold (Invitrogen). For transmission electron microscopy, mice were perfused with 0.1 M cacodylate buffer (pH 7.4) containing 4% paraformaldehyde and 1 mM CaCl2. Floating tissue sections (50–100-μm thick) were sequentially reacted with primary and secondary Abs, then reacted to demonstrate peroxidase activity and embedded for transmission electron microscopy. Sections were viewed and photographed with a Philips 201 electron microscope (Philips, The Netherlands).
RT-PCR

RNA was purified using TRIzol (Invitrogen), reverse transcribed, and PCR-amplified using 0.3 mM forward and reverse primers (mCLCA-1 forward primer: 5′-GGAGCAGGCCTTCCATGTC-3′ and reverse primer: 5′-GGAGCCGCTTTTCACTTCT-3′; and mCLCA-2 forward primer: 5′-GGACGGCTTTCTACATGTCTAG-3′ and reverse primer: 5′-TCGTGAGACCACCTTCTTTCCCTG-3′) (32). PCR amplification was performed at 95°C 5 min, followed by 32 cycles of 95°C, 64°C, and 72°C for 30 s each.

Lymphocyte adhesion assay

SV-LEC cells (10,000 cells/well) or MS1 cells (15,000 cells/well) were plated on 96-well plates. Cells were untreated or pretreated with murine TNF-α (10 ng/ml; eBioscience) and IL-1β (5 pg/ml; PeproTech, Rocky Hill, NJ) for 24 h. Lymphocytes were dissociated from spleens of C57BL/6 mice with a plunger, and passed through a 200-μm nylon filter, followed by RBC lysis and removal of B lymphocytes and macrophages by passage through a nylon wool column, resulting in >80% pure T cells (33). The cells were labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) fluorescent dye (Molecular Probes, Eugene, OR) and were sometimes activated with 10 ng/ml PMA (Sigma-Aldrich) for 30 min at 37°C. Lymphocytes or endothelium were sometimes pretreated with Abs for 30 min before the 30-min adhesion assay using 130,000 lymphocytes/well (34). Plates were rinsed five times and read using a Fluoroskan Ascent fluorometer (Thermo LabSystems, Beverly, MA). Percent bound lymphocytes were determined by comparison with serially diluted input lymphocytes.

Results

The Ag recognized by the 10.1.1 Ab is expressed on the surface of lymphatic endothelial cells. To identify and characterize the function of this protein, we used 10.1.1 Ab-affinity chromatography to purify the corresponding Ag from spleen, where it is expressed at high levels. Mouse spleen lysates were passed through a non-specific hamster IgG-agarose column, bound to a 10.1.1 Ab-agarose column, and eluted with SDS. SDS-PAGE purification of the eluted fractions consistently identified several protein bands (Fig. 1), which were subjected to in-gel trypsin digestion and mass spectrometric analysis. The strongly bound protein band 1 at 90 kDa in fraction 5 (Fig. 1) included two distinct proteins: mCLCA1, a 90-kDa glycoprotein, and LFA-1β (CD18), a 94-kDa glycoprotein, both detected by multiple high-confidence peptide identifications (Table I). The 94-kDa LFA-1β protein is preferentially eluted in early fractions, whereas the 90-kDa mCLCA1 protein required higher SDS concentrations for elution (Fig. 1). Sequencing of the 180-kDa band 2 in fraction 2, identified LFA-1α (CD11a), the major dimerization partner of LFA-1β (26). Thus, 10.1.1 Ab-affinity chromatography copurified mCLCA1 and the LFA-1 heterodimer.

The mCLCA1 gene encodes the 10.1.1 Ag

The 10.1.1 Ab-affinity chromatography purified several candidates to encode the lymphatic endothelial Ag. The 10.1.1 Ag and mCLCA1 are both glycosylated membrane proteins migrating at ~90 kDa (24, 28), so that mCLCA1 was the first candidate we examined. In this paper, we present several lines of evidence that lead us to conclude that mCLCA1 is the 10.1.1 Ag. mCLCA1 is a member of the CLCA gene family, which encodes six membrane-associated or integral membrane proteins in mice (reviewed in Refs. 35, 36). Most CLCA gene family members are thought to function in calcium-dependent chloride ion transport, perhaps by interacting with chloride channel proteins. Initial characterization suggested that mCLCA1 is also involved in tumor cell survival and metastasis (37, 38). Otherwise, little is known yet about the functions of mCLCA1 or other CLCA gene family members.

Immunoprecipitation was used to test whether the 10.1.1 Ab recognizes the mCLCA1 gene product. The 10.1.1 Ab immunoprecipitates a 90-kDa protein from spleen lysates, whereas hamster IgG does not, as revealed by immunoblotting with 10.1.1 Ab (Fig. 2A). The 10.1.1 Ab also immunoprecipitated the slightly smaller ~85-kDa mCLCA protein exogenously expressed from 293 cells, whereas hamster IgG did not (Fig. 2A). This size discrepancy is likely due to glycosylation, as N-glycosidase digestion of the spleen lysate reduces the molecular mass of 10.1.1 Ag by ~5 kDa (24 and data not shown). As an additional negative control, pcDNA-mAST–transfected cells producing the unrelated mAST protein did not immunoprecipitate any protein (29). These findings that the 10.1.1 Ab specifically recognizes mCLCA1 were confirmed by direct immunoblotting experiments. The 10.1.1 Ab detects a protein of ~90 kDa in spleen and a smaller 85-kDa protein in mCLCA1-transfected 293 cells, but not in pcDNA-mAST–transfected cells (Fig. 2B). These studies demonstrate that the 10.1.1 Ab specifically recognizes the mCLCA1 gene product.

The mCLCA1 gene was recently duplicated in rodents to generate the closely related mCLCA2 gene (39). Although these genes are 95% identical in amino acid sequence, mCLCA2 shows a distinct pattern of tissue expression in the thymus and involuting mammary gland (32, 39). We tested whether the 10.1.1 Ab recognizes these closely related proteins by immunofluorescent staining of 293 cells transfected with mCLCA1, mCLCA2, or control pcDNA expression plasmids. The 10.1.1 Ab recognizes both mCLCA1 and mCLCA2 on the cell surface and also overexpressed Ag trapped in cytoplasmic vesicles surrounding DAPI-stained nuclei (Fig. 3A). The cell surface expression of mCLCA1 and mCLCA2 resembles that of the 10.1.1 Ag on lymphatic endothelium (23).

The expression of mCLCA1 and mCLCA2 mRNA was compared in different lymphoid organs and cell types by RT-PCR using gene-specific primers (Fig. 3B). mCLCA1 mRNA is the major species expressed in LNs and spleen, whereas mCLCA2 is preferentially expressed in thymus (Fig. 3C) in agreement with previous mRNA expression surveys (32). mCLCA1 is the major species in the SV-LEC cell line (Fig. 3D), which is a lymphatic endothelial isolate expressing the lymphatic markers Prox-1 and LYVE-1 (26). The mCLCA1 mRNA is also the major species expressed in MS1 endothelial cells (Fig. 3D), which were isolated from SV40 TAg-transformed pancreatic islets (40). Taken together, these findings are consistent with the mCLCA1 protein encoding the 10.1.1 Ag of lymphatic vessels and LN lymphatic sinuses (23). In the thymus, mCLCA1 and mCLCA2 are likely restricted to...
stromal medullary epithelium, based on previous 10.1.1 Ab immunostaining of these cells (24).

The spleen selectively expresses mCLCA1, with very little mCLCA2 expression (Fig. 3C). We performed immunostaining with 10.1.1 Ab to identify the cells expressing mCLCA1. The red pulp of the spleen immunostained with 10.1.1 Ab, whereas white pulp was negative (Fig. 3E). The major cellular components of the red pulp are venous sinuoids, blood cells, and specialized stromal cells. Transmission electron microscopy demonstrated that the major 10.1.1 Ab-positive cells are stromal cells with irregular processes (Fig. 3F), distributed among leukocytes and RBCs (R). This electron-dense reaction product was due to specific 10.1.1 Ab immunoprecipitated by 10.1.1 Ab but not by hamster IgG (Fig. 4A blotting with CD18 Ab. The 94-kDa CD18 subunit was specifically CD18 subunit of LFA-1, as detected by SDS-PAGE and immunoprecipitate spleen lysates, to test whether this copurifies the test whether mCLCA1 specifically binds to the CD11a and CD18 subunits of LFA-1 (24) for each other. Second, immunoblotting of immunoprecipitates prepared using CD18 Abs or a CD11a Ab isolated the 90-kDa 10.1.1 Ab (F4/80) did not (Fig. 4A), indicating that the mCLCA1–LFA-1 interaction is distinct from the ICAM-1–LFA-1 interaction. Immunoprecipitation and immunoblotting were used to test whether mCLCA1 specifically binds to the CD11a and CD18 subunits of LFA-1 in vitro. First, the 10.1.1 Ab was used to immunoprecipitate spleen lysates, to test whether this copurifies the CD18 subunit of LFA-1, as detected by SDS-PAGE and immunoblotting with CD18 Ab. The 94-kDa CD18 subunit was specifically immunoprecipitated by 10.1.1 Ab but not by hamster IgG (Fig. 4A), supporting the idea that CD18 and mCLCA1 have a strong affinity for each other. Second, immunoblotting of immunoprecipitates prepared using CD18 Abs or a CD11a Ab isolated the 90-kDa 10.1.1 Ag, whereas immunoprecipitation with an isotype control IgG2a,κ Ab (F4/80) did not (Fig. 4B). These findings confirm that the CD11a and CD18 LFA-1 subunits both bind to the 10.1.1 Ag under stringent conditions, no matter which Ab was used for the initial immunoprecipitation.

Finally, the mCLCA1 protein was directly tested for its ability to bind to LFA-1. mCLCA1 or control angiotatin were expressed from pcDNA plasmids in 293 cells, and EL-4 murine thymic lymphoma cells were used as a source of LFA-1 (26). Lysates were prepared using CD18 Abs or a CD11a Ab isolated the 90-kDa mCLCA1 gene product from mCLCA1-transfected 293 cells (Fig. 4C), but not from mAST-transfected cells, demonstrating that mCLCA1 is the 85–90-kDa protein that specifically binds to LFA-1.

mCLCA1 mediates lymphocyte adhesion to lymphatic endothelium

Our finding that mCLCA1 strongly binds to LFA-1 in vitro suggests that this interaction could contribute to lymphatic endothelial adhesion to lymphocytes in vivo. Functional assays were developed to test whether mCLCA1 is involved in LFA-1–mediated binding of lymphocytes to lymphatic endothelium. Purified splenic lymphocytes were labeled with fluorescent BCECF dye, bound to endothelial cells under static conditions, and washed extensively before fluorometer measurement of bound cells (34). The lymphocyte preparations were >80% T cells, with the remainder B cells and macrophages (33). SV-LEC and MS1 endothelial cells were used for these assays, as they both express mCLCA1 (Fig. 3D).

Two treatments were tested to optimize lymphocyte binding to lymphatic endothelium. First, PMA treatment activates LFA-1 to increase lymphocyte adhesion to vascular endothelium (13). PMA treatment of lymphocytes increased their binding to SV-LEC lymphatic endothelial cells by 50% and to MS1 endothelial cells by 322% (Fig. 5A). Lymphocytes were therefore PMA-treated in subsequent experiments to maximize LFA-1 adhesion activity. Second, cytokines such as TNF-α and IL-1β increase the affinity of HEV endothelium for lymphocytes by increasing the expression of adhesive proteins including the LFA-1 binding partner ICAM-1 (19, 20). TNF-α/IL-1β pretreatment of SV-LEC cells for 48 h increased their lymphocyte binding activity by 48% (hamster IgG samples; Fig. 5B) and increased MS1 endothelial cell binding by 92% (data not shown). These findings demonstrate that treatments known to enhance lymphocyte adhesion to vascular endothelium exert similar effects on lymphatic endothelial cells.

The involvement of mCLCA1 in lymphocyte adhesion to lymphatic endothelium was assessed by incubating endothelial cells with 20 μg 10.1.1 or control hamster Ab before lymphocyte adhesion.

The number of unique peptides identified and percent coverage of the full-length protein are given.

### Table I. Summary of mass spectrometry peptide identification from SDS-PAGE–purified gel bands 1–3 depicted in Fig. 1

<table>
<thead>
<tr>
<th>Gel Band No.</th>
<th>Molecular Mass (kDa)</th>
<th>Identification</th>
<th>No. of Unique Peptides</th>
<th>Coverage (%)</th>
</tr>
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<td>1</td>
<td>90</td>
<td>mCLCA1</td>
<td>40</td>
<td>38</td>
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<td>94</td>
<td>LFA-1β</td>
<td>32</td>
<td>44</td>
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<tr>
<td>3</td>
<td>94</td>
<td>LFA-1β</td>
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The involvement of mCLCA1 in lymphocyte adhesion to lymphatic endothelium was assessed by incubating endothelial cells with 20 μg 10.1.1 or control hamster Ab before lymphocyte adhesion.

**FIGURE 2.** The 10.1.1 Ab recognizes mCLCA1. A, Immunoprecipitation of spleen or transfected 293 cell lysates with 10.1.1 or hamster Abs, followed by SDS-PAGE and immunoblotting with 10.1.1 Ab. The 90-kDa species is detected after 10.1.1 Ab immunoprecipitation of lysates from spleen, and a slightly smaller protein is detected in immunoprecipitates from 293 cells transfected with mCLCA1, but not from control mAST-transfected 293 cell lysates. Hamster IgG does not immunoprecipitate these proteins. B, SDS-PAGE and immunoblotting with 10.1.1 Ab identifies a 90-kDa protein in spleen lysates and a slightly smaller protein in 293 cells transfected with pcDNA-mCLCA1 plasmid (293-mCLCA1), but not with pcDNA-mAST plasmid (293-mAST). Blots were reprobed with Ab to detect 42-kDa actin as a loading control.
This Ab concentration was selected as titration experiments indicated that this concentration gave maximal inhibition of lymphocyte binding (data not shown). The 10.1.1 Ab blocked 27% of PMA-treated lymphocyte adhesion to control SV-LEC cells (Fig. 5B). 10.1.1 Ab inhibition of adhesion remained at 28% when SV-LEC were activated by TNF-α/IL-1β treatment (Fig. 5B), indicating that mCLCA1 adhesion activity is constitutive, whereas some other molecule such as ICAM-1 mediates cytokine-induced lymphocyte binding activity. In fact, RT-PCR demonstrated no change in mCLCA1 mRNA levels in SV-LEC or MS1 cells after cytokine treatment (Fig. 3D). 10.1.1 immunostaining and flow cytometry confirmed that mCLCA1 surface expression on SV-LEC cells was not affected by cytokine treatment (Fig. 6A). In contrast, surface ICAM-1 expression was strongly induced by TNF-α/IL-1β treatment (Fig. 6B), in agreement with previous findings that cytokine treatment induces ICAM-1 and increases lymphatic endothelial adhesiveness (18, 19). Taken together, these findings demonstrate that mCLCA1 contributes to lymphatic en-
dothelial adhesion in a constitutive manner, which is distinct from cytokine-induced LFA-1–ICAM-1 interactions.

The contributions of LFA-1, ICAM-1, and 10.1.1 to lymphocyte adhesion were compared in Ab-blocking experiments, using mAbs with demonstrated neutralizing activity in this assay (34, 41). TNF-α/IL-1β treatment of the SV-LEC cells was used to maximize ICAM-1 binding activity. The LFA-1 (CD18) Ab showed the strongest effect to block 55% of lymphocyte adhesion to SV-LEC (Fig. 5C), in agreement with previous reports that LFA-1 is a major mediator of lymphocyte adhesion to endothelium (42). The 10.1.1 Ab significantly blocked 32% of the binding in these assays, whereas neutralizing ICAM-1 Ab (43) reduced binding by only 16% (Fig. 5C). This ICAM-1 Ab preparation is biologically active, however, as it efficiently bound to SV-LEC cells (Fig. 6B).

These findings demonstrate that mCLCA1 significantly contributes to lymphocyte adhesion activity of SV-LEC cells.

Ab inhibition experiments using TNF-α/IL-1β–treated MS1 endothelial cells also demonstrated that mCLCA1 is a major contributor to lymphocyte adhesion. LFA-1 Ab treatment significantly blocked 67% of the adhesion, whereas 10.1.1 Ab treatment significantly decreased lymphocyte binding to MS1 cells by 49% (Fig. 5D). Again, the ICAM-1 Ab showed a smaller effect to decrease binding by only 34% (Fig. 5D). Thus mCLCA1–LFA-1 adhesion predominates over ICAM-1–mediated adhesion in both endothelial cell lines, even under conditions in which ICAM-1 adhesion activity is stimulated by cytokine treatment.

mCLCA1 binds to the LFA-1–related leukocyte integrin Mac-1

ICAM-1 binds not only to LFA-1 but also to the related leukocyte integrin Mac-1 (44). mCLCA1 could therefore potentially also bind to Mac-1. Mac-1 was not detected in our Ab-affinity purification (Fig. 1); however, the spleen contains much more LFA-1 than Mac-1 (26). We therefore used BM as an enriched source of Mac-1–expressing leukocytes, to test whether mCLCA1 also binds to Mac-1. BM lysates were incubated with lysates of mCLCA1- or control pcDNA-transfected 293 cells and immunoprecipitated with Mac-1 Ab or nonspecific rat IgG (Fig. 7A). Immunoprecipitates of lysates of BM with 293 cells transfected with pcDNA detected a nonspecific band but not the larger mCLCA1 species (Fig. 7A). As a loading control, similar yields of the Mac-1 subunit of CD18 were detected.

FIGURE 5. mCLCA1 contributes to lymphatic endothelial cell adhesion to lymphocytes. A, PMA activation increases binding of BCECF-labeled splenic lymphocytes to SV-LEC or MS1 endothelial cells in static adhesion assays. B, TNF-α/IL-1β treatment of SV-LEC cells increases PMA-treated lymphocyte adhesion. The 10.1.1 Ab inhibits lymphocyte adhesion to control or TNF-α/IL-1β–treated endothelium to the same extent, relative to hamster IgG-treated samples. C, 10.1.1 Ab but not hamster IgG blocked PMA-treated lymphocyte adhesion to TNF-α/IL-1β–treated SV-LEC. LFA-1 Ab blocked lymphocyte adhesion to SV-LEC cells, whereas ICAM-1 Ab or nonspecific rat IgG did not significantly block binding. D, 10.1.1 Ab but not hamster IgG blocked lymphocyte adhesion to MS1 cells. LFA-1 Ab blocked lymphocyte adhesion to MS1 cells, whereas ICAM-1 Ab or nonspecific rat IgG did not significantly block binding. Two-tailed unpaired t test analysis identified assays showing statistically significant inhibition. Standard errors are shown. \( p < 0.02; \) \( **p < 0.005; \) \( ***p < 0.0001.\)
in each immunoprecipitate (Fig. 7B). These findings demonstrate that mCLCA1 specifically binds to Mac-1, so that it could potentially regulate the interaction of Mac-1—expressing leukocytes with lymphatic endothelium. The ability of mCLCA1 to bind both Mac-1 and LFA-1 is a feature shared with ICAM-1, suggesting that lymphatic endothelial mCLCA1 and ICAM-1 could potentially compete for binding to leukocytes expressing LFA-1 or to Mac-1, as depicted in Fig. 7C.

Discussion

Our purification and characterization of the 10.1.1 Ag identified the 90-kDa glycosylated surface protein mCLCA1. mCLCA1 is expressed at high levels in lymphatic endothelium and in specialized stromal cells of lymphoid organs, matching the tissue-restricted expression of the 10.1.1 Ag. mCLCA1 may contribute to multiple cellular functions in addition to its LFA-1 and Mac-1 binding activity. Previous studies demonstrated that mCLCA1 binds to β4 integrin in vitro (38) and that it can promote hematogenous metastasis (37). In addition, mCLCA1 influences calcium-dependent chloride channel conductance by an unknown mechanism (36). Lymphatic endothelial cells show enriched expression of a variety of ion transporters relative to vascular endothelium, and lymphatic vessels are active in transendothelial fluid and vesicle transport (45), so that mCLCA1 could potentially contribute to these specialized lymphatic endothelial functions. Some or all of these diverse activities could contribute to mCLCA1 functions in lymphatic vessels and lymphoid organs.

The 10.1.1 Ab-affinity chromatography identified a strong interaction of mCLCA1 with the leukocyte surface molecule LFA-1. This finding was initially surprising, as ICAM-1 is assumed to be the major binding partner of LFA-1 on lymphatic endothelium, as it is on vascular endothelium (1). Our adhesion assays using neutralizing Abs demonstrated that LFA-1 is the major determinant of lymphocyte adhesion to lymphatic endothelium, in agreement with previous studies of vascular endothelium (46, 47). Interestingly, the 10.1.1 Ab blocked lymphocyte adhesion more effectively than the ICAM-1 Ab in both SV-LEC and MS1 cells, suggesting that mCLCA1 is the major LFA-1 binding partner in lymphatic endothelium.

mCLCA1 is expressed on lymphatic vessels, where it could function in LFA-1–dependent leukocyte adhesion during trafficking from the periphery, and on LN lymphatic sinuses, where it could contribute to leukocyte adhesion within LNs. The 10.1.1 Ab-affinity chromatography copurified LFA-1 but not ICAM-1, suggesting that the mCLCA1–LFA-1 interaction does not permit ICAM-1 binding. In this case, lymphatic endothelial mCLCA1 and ICAM-1 could compete for LFA-1 binding activity, as depicted in Fig. 7C. ICAM-1 is strongly induced by TNF-α/IL-1β treatment of lymphatic endothelium, whereas mCLCA1 is not affected by these cytokines, so that the relative contributions of ICAM-1 and mCLCA1 to leukocyte adhesion to lymphatic endothelium could vary during an immune response. However, our adhesion assays demonstrated a major role of mCLCA1 in LFA-1–mediated adhesion even after ICAM-1 expression was activated by cytokine treatment, suggesting that mCLCA1 is a major mediator of leukocyte adhesion to lymphatic endothelium during inflammation.

The 10.1.1 Ab recognizes native and denatured mCLCA1 and also exhibits neutralizing activity, making it a useful reagent for identification and characterization of murine lymphatic endothelium, including lymphatic vessels and LN lymphatic sinuses (23). Importantly, mCLCA1 is expressed on the cell surface, facilitating flow cytometric purification of lymphatic endothelial cells with the 10.1.1 Ab (48). Only a few markers of lymphatic endothelium have been developed thus far, and no single marker identified thus far is specific to lymphatic endothelium. Prox-1 is a transcription factor that is expressed in several organs in addition to lymphatic vessels (49). Podoplanin is a membrane protein expressed on a variety of cell types including lymphatic endothelium (50), whereas LYVE-1 is a membrane protein expressed in lymphatic and tumor vascular endothelium and macrophages that is downregulated by cytokines (20, 51). Thus far, mCLCA1 appears to be constitutively expressed in LNs or in lymphatic endothelium (23). mCLCA1-specific reagents should therefore be useful to identify lymphatic endothelium alone or in combination with these other lymphatic markers in normal or inflamed organs.

The mCLCA1 and mCLCA2 genes resulted from a recent gene duplication event in rodents, so that these closely related proteins likely have similar functions (39). However, mCLCA1 and mCLCA2 show very different tissue expression patterns (32). mCLCA2 is preferentially expressed in the thymus, presumably in 10.1.1 Ab-positive thymic stromal epithelium that was used as the Ag for 10.1.1 hybridoma production (24) and also in the involuting mammary gland (32). mCLCA2 could potentially also function in LFA-1 or Mac-1 binding and leukocyte adhesion in these tissues, as it is so similar to mCLCA1 in sequence (95% amino acid identity (39)). Interestingly, the thymic medullary epithelial 10.1.1 Ag (predominantly mCLCA2) is induced by IL-1β treatment (24), whereas we find that lymphatic endothelial mCLCA1 is not IL-1β responsive, suggesting complexity in regulation of the expression of these genes. The 10.1.1 Ag is selectively expressed on thymic stromal epithelium at sites of thymocyte binding (24), which could involve LFA-1–mCLCA2 adhesion. The human gene CLCA family member most
closely related to mCLCA1/2 at the amino acid sequence level is human CLCA3 (69% identical), a cell surface protein of unknown function (36). Further studies will be required to determine whether human CLCA3 performs similar lymphatic endothelial adhesion functions in humans.

The mCLCA1 and ICAM-1 proteins share no regions of amino acid sequence similarity, even though they both show a strong affinity for LFA-1 and Mac-1. Distinct Ig-like domains of ICAM-1 mediate binding to LFA-1 and Mac-1, or the same mCLCA1 domain could bind to the CD18 subunit shared by the LFA-1 and Mac-1 heterodimers. Biochemical investigations will be required to identify the sites of mCLCA1 interaction with LFA-1 and Mac-1 for comparison with those of ICAM-1. The ability of mCLCA1 and ICAM-1 to independently bind to LFA-1 or to Mac-1 increases the complexity of regulation of leukocyte adhesion to lymphatic endothelium.

Our finding that mCLCA1 binds to Mac-1 suggests that mCLCA1 contributes to adhesion of Mac-1–expressing granulocytes, dendritic cells, NK cells, or macrophages to lymphatic endothelium. Mac-1 and ICAM-1 interaction is important for adhesion and migration of lymphocytes within blood vessels (10), so that mCLCA1 could serve similar functions to regulate trafficking in the lymphatic system. The expression of mCLCA1 in stromal cells of the splenic red pulp could also potentially mediate adhesion of resident granulocytes or other leukocytes abundant in red pulp. Mac-1 mediates peritumoral macrophage efflux to lymphatics during resolution of inflammation (21), which could also potentially involve mCLCA1–Mac-1 interaction. Thus, Mac-1–mediated adhesion to mCLCA1 could potentially impact a variety of leukocyte interactions with lymphatic endothelium, thymic stroma, or splenic red pulp stroma.

The exact role of mCLCA1 interaction with LFA-1 and Mac-1 in regulation of lymphocyte and leukocyte adhesion and trafficking in vivo remains to be defined. Immunotherapeutic drugs blocking LFA-1 or ICAM-1 activity can inhibit abnormal lymphocyte activation and pathology in chronic inflammatory or autoimmune diseases including rheumatoid arthritis, psoriasis, and multiple sclerosis (11, 52). Our discovery that LFA-1–mCLCA1 interaction is distinct from the LFA-1–ICAM-1 interaction makes it a target for therapeutic manipulation of pathological immune responses. Similarly, the mCLCA1–Mac-1 interaction provides a target to block undesirable leukocyte adhesion and trafficking. Further investigation of the functional significance of lymphatic endothelial or lymphoid organ stromal mCLCA1 adhesion to Mac-1– or LFA-1–expressing leukocytes will also provide insight to the trafficking of immune cells through the lymphatic circulation and lymphoid organs.

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Disclosures

B.U.P. holds a patent on CLCA1 gene sequences. There has been a discovery filed on the 10.1.1 Ab by A.G.F.

References