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A High Endothelial Venule Secretory Protein, Mac25/Angiomodulin, Interacts with Multiple High Endothelial Venule-Associated Molecules Including Chemokines[1]

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We previously reported that mac25/angiomodulin (AGM), a 30-kDa secretory protein, is abundantly expressed in high endothelial venules (HEVs), which play a crucial role in lymphocyte trafficking to the lymph nodes and Peyer's patches. We report that mac25/AGM interacts preferentially with certain molecules that are expressed in or around HEVs. In particular, mac25/AGM interacted with not only the extracellular matrix proteins and glycosaminoglycans that are expressed in most blood vessels including HEVs, but also with some chemokines that are implicated in the regulation of lymphocyte trafficking, such as the secondary lymphoid-tissue chemokine (SLC; CCL21), IFN-γ-inducible protein 10 (IP-10; CXCL10), and RANTES (CCL5). The binding of mac25/AGM to SLC and IP-10 was dose-dependent and saturable. The binding to IP-10 could be inhibited by SLC but not by a non-mac25/AGM-binding chemokine, EBI1-ligand chemokine (ELC; CCL19). Interestingly, mac25/AGM failed to interact with 18 other chemokines, suggesting that it binds to certain chemokines preferentially. Immunohistochemical analysis indicated that mac25/AGM colocalizes at least partially with SLC and IP-10 at the basal lamina of HEVs. Upon binding with mac25/AGM, SLC and IP-10 retained all their Ca^{2+}-signaling activity in vitro, suggesting that mac25/AGM can hold and present chemokines in the basal lamina of HEVs. These results imply that mac25/AGM plays a multifunctional role, serving not only as an adhesion protein to interact with glycosaminoglycans and extracellular matrix proteins but also as a molecule to present chemokines so that lymphocytes extravasating through HEVs receive further directional cues subsequent to the luminal encounter with lymphoid chemokines. ***The Journal of Immunology,*** **2003, 171: 553–561.**

High endothelial venules (HEVs)[3] are specialized venules that allow rapid and selective lymphocyte trafficking from the blood into the lymph nodes and Peyer's patches (1). The endothelial cells of HEVs are characterized by their unique cuboidal morphology and discontinuous intercellular junctions (1–3), and are grossly distinct from the endothelial cells of other blood vessels in many respects. During the course of a search for novel genes expressed specifically in HEVs (4, 5), we identified mac25/angiomodulin (AGM) as a highly expressed secretory protein in mouse HEVs. Concurrently, Girard et al. (6) also reported a strong expression of mac25/AGM in the HEVs of human tonsils.

Mac25/AGM is a secreted protein of ~30 kDa that is identical with previously reported molecules, including insulin-like growth factor binding protein related protein-1 (IGFBP-rP1) (7–9), tumor-derived adhesion factor (10), which was later renamed AGM (11, 12), and prostacyclin-stimulating factor (13). Mac25/AGM contains two major domains (9), which include an amino-terminal cysteine-rich domain containing the IGFBP motif that is conserved among IGFBPs and a C-terminal Ig-like domain. Functionally, mac25/AGM has been implicated in various biological responses: it accumulates in the blood vessels of tumors (10), preferentially binds to extracellular matrix (ECM) components such as collagen type IV (10), promotes cell spreading of the human bladder carcinoma cell line ECV-304 on collagen type IV (10), and stimulates PGI_2 production, which is a potent vasodilator (13). It has been reported that mac25/AGM increases PGI_2 production in retinal endothelial cells, resulting in an increased retinal blood flow in vivo (14). In addition, mac25/AGM binds to heparan sulfate (HS) glycosaminoglycans (GAGs) (11, 12) and has been implicated in angiogenesis (10, 11).

HEVs express certain chemokines, such as the secondary lymphoid-tissue chemokine (SLC, CCL21) (15, 16) and EBI1-ligand chemokine (ELC, CCL19) (17), that can activate integrins on the cell surface of lymphocytes through a specific G protein-coupled receptor, CCR7. Whereas it has been suggested that chemokines function via binding to GAGs on the cell surface and/or the ECM proteins (18), it remains unclear how these lymphoid chemokines are retained in situ and how their concentration gradients are formed in the regions near HEVs. In this context, it is interesting to note that mac25/AGM, which can bind various humoral factors such as insulin-like growth factor (19, 20), localizes to the basal lamina of the venules in lymph nodes and Peyer's patches (21). We therefore thought it possible that mac25/AGM, by being strategically located in the basal lamina, could interact with certain lymphoid chemokines to hold them in situ and present them to lymphocytes at venules such as the HEVs.

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[3] Abbreviations used in this paper: HEV, high endothelial venule; AGM, angiomodulin; BLC, B lymphocyte chemoattractant; IGFBP-rP1, insulin-like growth factor binding protein-related protein-1; CS, chondroitin sulfate; ECM, extracellular matrix; ELC, EBI1-ligand chemokine; GAG, glycosaminoglycan; HS, heparan sulfate; HSBS, HS binding sequence; IP-10, IFN-γ-inducible protein 10; LN, laminin; SDF, stromal cell-derived factor; SLC, secondary lymphoid-tissue chemokine.

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To understand the biological role of mac25/AGM in HEVs, we investigated in the present study whether mac25/AGM interacts with molecules that are present within or in the vicinity of HEVs. We demonstrate that mac25/AGM interacts not only with the ECM proteins and GAGs that constitute the HEV microenvironment but also with certain chemokines that can alter the migratory behavior of lymphocytes. Our findings imply that mac25/AGM plays multiple roles at HEVs by interacting with various ECM proteins and GAGs around the HEVs and also by immobilizing chemokines that regulate lymphocyte migration.

Materials and Methods

Animals

All animal experiments were performed under the experimental protocol approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine. Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). They were reared under specific pathogen-free conditions and used at 8 wk of age.

Cells

The murine pre-B cell line L1.2 (22), kindly provided by Dr. E. C. Butcher (Stanford University, Stanford, CA), was cultured in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% (v/v) nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (RPMI-FCS). L1.2 cells that stably express human CCR7 (L1.2/CCR7) were generated as previously described (kindly provided by Dr. O. Yoshie; Kinki University School of Medicine, Osaka, Japan) (23) and cultured in RPMI-FCS containing 0.8 mg/ml geneticin (G418; Sigma-Aldrich, St. Louis, MO). The murine pre-B cell line 300.19 and its stable transfectant expressing CXCR3 (300.19/CXCR3) were kindly provided by Dr. B. Moser (University of Bern, Bern, Switzerland) and cultured in RPMI-FCS. 300.19/CXCR3 was cultured in the presence of 1.0 mg/ml geneticin (24).

Reagents

The myc- and his-tagged recombinant mouse mac25/AGM protein and polyclonal Abs against the recombinant mac25/AGM were produced as reported elsewhere (21). Mouse collagen type IV, mouse fibronectin, human collagen types I and III, and human vitronectin were purchased from Invitrogen (Carlsbad, CA). Human collagen types V and VI were purchased from Southern Biotechnology Associates (Birmingham, AL). Mouse collagen type II was from Elastin Products (Owensville, MO). Laminin (LN)-1, LN-8, and LN-10/11 were purified as reported previously (25, 26). HS derived from porcine intestinal mucosa was purchased from Sigma-Aldrich. Hyaluronic acid derived from human umbilical cord was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Other GAGs were purchased from Seikagaku Kogyo (Tokyo, Japan). Human stromal cell-derived factor (SDF)-1α, mouse IP-10, and mouse ELC were purchased from R&D Systems (Minneapolis, MN), and human B lymphocyte chemoattractant (BLC; CXCL13), human I-309 (CCL1), mouse RANTES (CCL5), and mouse SLC were from PeproTech (Rocky Hill, NJ). Mouse KC (CXCL1), mouse SDF-1α (CXCL12), and mouse BLC (CXCL13) were from DAKO Japan (Tokyo, Japan). Human fractalkine (CX3CL1) and mouse cutaneous T cell-attracting chemokine (CCL27) were kindly provided by Dr. O. Yoshie. Other human chemokines used in the present study were the same as previously described (27). MECA-79 mAb (28) was kindly provided by Dr. E. C. Butcher (Stanford University). Anti-his (C-term) and HRP-conjugated anti-myc Abs were purchased from Invitrogen. Anti-mouse SLC, biotinylated anti-mouse SLC, anti-mouse IP-10, biotinylated anti-mouse IP-10, and anti-human RANTES polyclonal Abs were purchased from PeproTech. Biotinylated anti-mouse ELC polyclonal Ab was purchased from DAKO Japan. HRP-conjugated goat anti-rabbit IgG (H&L) was purchased from American Qualex (San Clemente, CA). Synthetic peptides, designated as HS binding sequence (HSBS) peptide (GMECVKSRKRRKGKAGAAAG) (11) and shuffled HSBS peptide (AKGCRGAKEMKGARSVRGKA), were custom-synthesized by Sawady Technology (Tokyo, Japan).

Preparation of lipid-derivatized GAGs

The preparation of lipid-derivatized GAGs was conducted according to the method of Sugiura et al. (29) with some modifications. Briefly, the reducing end of each GAG was oxidized with iodine at room temperature. After the oxidization, the polysaccharides, dissolved in water, were passed through AG 50W-X8 Resin (H^+ form) (Bio-Rad Laboratories, Hercules, CA). After the aqueous solution was concentrated, the remaining water was removed by the addition of dimethylformamide followed by evaporation and allowed to stand at 4°C for 72 h. Dipalmitoyl-L-α-PE (Wako Pure Chemical, Osaka, Japan) was then added and the mixture was stirred at 60°C until a maximal level of GAG-PE was attained (6 h). The sample was precipitated with three equivalent volumes of ice-cold 95% (v/v) ethanol/1.3% (w/v) potassium acetate/0.2 mM EDTA on ice for 2 h, and the precipitate was dissolved in 0.2 M NaCl and applied to a TSK-gel Phenyl Toyopearl 650M column (Tosoh, Tokyo, Japan). The lipid-derivatized GAG was eluted with 30% methanol and stored at 4°C until use.

ELISA

The following different methods were used: Method 1) For the detection of mac25/AGM binding to immobilized ECM proteins, various ECM proteins (10 μg/ml) dissolved in PBS were immobilized on 96-well microtiter plates (Sumilon H; Sumitomo Bakelite, Tokyo, Japan) at 4°C (50 μl/well) overnight. The wells were blocked with 3% BSA and incubated with the recombinant mac25/AGM (15 μg/ml). Binding of recombinant mac25/AGM was detected with anti-myc-HRP mAb. To quantify the reaction, *o*-phenylenediamine was added, and the OD at 490 nm was read in a microtiter plate reader (InterMed, Tokyo, Japan); Method 2) For the detection of mac25/AGM binding to immobilized GAGs, various lipid-derivatized GAGs (10 μg/ml) dissolved in 30% methanol, were added to 96-well microtiter plates (50 μl/well) and dried at 65°C for 6 h. The wells were blocked with 3% BSA and incubated with recombinant mac25/AGM (15 μg/ml). The binding of mac25/AGM was detected as described above; and Method 3) For the detection of mac25/AGM binding to chemokines, recombinant mac25/AGM (1 μg/ml) was immobilized on 96-well microtiter plates at 4°C overnight (50 μl/well). The wells were washed with PBS and blocked with 3% BSA at room temperature for 2 h. Mouse IP-10, SLC, ELC, or human RANTES were added to the recombinant mac25/AGM-coated wells and incubated for 1 h. After washing, the wells were incubated with anti-IP-10, anti-RANTES, anti-SLC polyclonal Abs, or biotinylated anti-ELC polyclonal Ab in PBS containing 0.1% BSA and 0.05% Tween 20 for 1 h at room temperature. After washing, the wells were incubated at room temperature for 1 h with HRP-conjugated goat anti-rabbit IgG for the detection of IP-10, RANTES, or SLC, or with HRP-conjugated streptavidin (Zymed Laboratories, San Francisco, CA) for the detection of ELC. In competition experiments, the amount of IP-10 (0.1 μg/ml) binding to the immobilized mac25/AGM was examined in the presence or absence of various concentrations of SLC, ELC, or BLC. For inhibition experiments with peptides, the amount of SLC (0.03 μg/ml) binding to the immobilized mac25/AGM was examined in the presence or absence of the HSBS or shuffled HSBS peptide.

Dot blot analysis

The dot blot analysis was conducted as previously described (27) with some modifications. In brief, various concentrations of chemokines were spotted onto a Hybond C nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, U.K.). After blocking, the membrane was incubated with 1 μg/ml of the recombinant mac25/AGM at room temperature for 1 h. The bound mac25/AGM was detected with HRP-conjugated anti-myc Ab and the ECL Western blot detection reagents (Amersham Biosciences).

Immunoprecipitation

SLC (15 ng) or IP-10 (15 ng) were incubated with recombinant mac25/AGM (1.5 μg) at 4°C for 30 min and immunoprecipitated using anti-myc agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse IgG agarose beads (Santa Cruz Biotechnology). The immunoprecipitated material was solubilized in SDS sample buffer, electrophoresed, and blotted onto the polyvinylidene difluoride membrane. After blocking with 3% BSA, the filter was incubated with anti-mac25 polyclonal Ab, anti-SLC polyclonal Ab, or anti-IP-10 polyclonal Ab and further incubated with HRP-conjugated anti-rabbit IgG. The blot was developed using the ECL Western blot detection reagents.

Ca^{2+} mobilization assay

Ca^{2+} mobilization elicited by soluble chemokines was measured as previously described (27) with some modifications. In brief, L1.2, L1.2/CCR7, 300.19, and 300.19/CXCR3 cells were incubated at 37°C for 1 h with 2 μM fura 2-AM (Sigma-Aldrich) at 1.0×10^6 cells/ml in RPMI without FCS in the dark. The cells were washed twice and resuspended at 1.0×10^6 cells/ml in Ca^{2+}-, Mg^{2+}-containing PBS supplemented with 1% FCS. A 1.5-ml sample of cell suspension was placed in a cuvette and set into a

spectrofluorophotometer (RF1500; Shimadzu Scientific Instruments, Japan) with constant stirring. SLC (15 ng, for L1.2 and L1.2/CCR7 cells) and IP-10 (15 ng, for 300.19 and 300.19/CXCR3 cells) were preincubated with recombinant mac25/AGM (1.5 or 4.5 μg) for 1 h at room temperature and added to the cell suspension. The emission fluorescence intensity at 510 nm was measured upon excitation at 340 nm (F340) and 380 nm (F380). Data were presented as the ratio of F340 to F380 (R340/380). After each measurement, R340/380 in the presence or absence of excess calcium was determined by the subsequent addition of 100 μl of 1% Triton X-100 with or without 100 μl of 0.5 M EDTA.

Ca^{2+} mobilization elicited by chemokines immobilized on agarose beads via mac25/AGM was analyzed on an inverted fluorescence microscope equipped with a charge-coupled device camera. The data were acquired and quantified with an image acquisition software, AquaCosmos (Hamamatsu Photonics, Hamamatsu, Japan). Briefly, L1.2/CCR7 cells and 300.19/CXCR3 cells were immobilized onto poly-L-lysine-coated 96-well culture plates and incubated at 37°C for 45 min with 5 μM fura 2-AM in a buffer consisting of 20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 13.8 mM D-glucose, 2.5 mM probenecid (Sigma-Aldrich), 0.2% BSA, and 1% FCS. The cells were washed twice and subjected to stimulation with either a soluble chemokine or chemokine immobilized on agarose beads via mac25/AGM in the same buffer. The chemokine-immobilized agarose beads were then prepared. Either SLC (15 ng) or IP-10 (15 ng) was first incubated with recombinant mac25/AGM (1.5 μg) at 4°C for 30 min and then immunoprecipitated using anti-myc-conjugated agarose beads or mouse IgG agarose beads. The precipitated beads were washed and added to the cells. The intracellular Ca^{2+} level of individual cells was monitored by the fluorescence ratio at 340 and 380 nm.

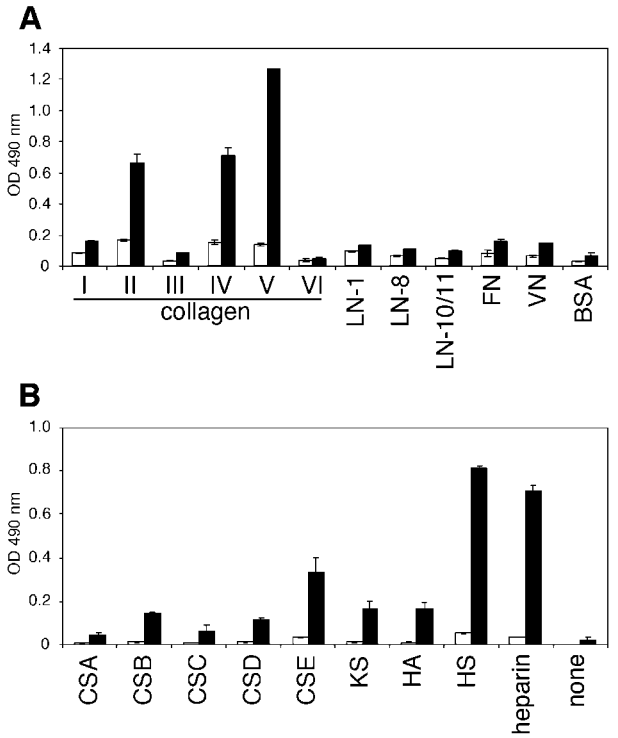

FIGURE 1. Binding of recombinant mouse mac25/AGM to various substrates. *A*, Binding of recombinant mac25/AGM to various ECM proteins. Microtiter plates (96-well) coated with various ECM proteins (10 μg/ml) were incubated with (■) or without (□) the recombinant mac25/AGM (15 μg/ml). The amount of bound mac25/AGM was determined by the binding of anti-myc-HRP Ab. The data represent the mean ± SD of triplicate determinations. LN, laminin; FN, fibronectin; VN, vitronectin. *B*, Binding of mac25/AGM to GAGs. Microtiter plates (96-well) coated with the lipid-derivatized GAGs (10 μg/ml) were incubated with (■) or without (□) recombinant mac25/AGM (15 μg/ml). The amount of bound mac25/AGM was determined by the binding of anti-myc-HRP Ab. The data represent the mean ± SD of triplicate determinations. CS, chondroitin sulfate; KS, keratan sulfate; HA, hyaluronic acid; HS, heparan sulfate.

Immunohistochemistry

Immunohistochemistry was performed as previously described (30). Briefly, cryosections of inguinal lymph nodes from nonimmunized mice fixed in acetone and then in 4% paraformaldehyde in PBS were incubated with FITC-conjugated anti-mac25 polyclonal Ab and biotin-conjugated anti-mouse SLC polyclonal Ab or biotin-conjugated anti-mouse IP-10 polyclonal Ab, then incubated with Alexa Fluor 594-conjugated streptavidin (Molecular Probes, Eugene, OR). To identify HEVs, serially cut sections were stained with biotin-conjugated MECA-79. To verify the specificity of the Abs, biotin-conjugated anti-mouse SLC polyclonal Ab and biotin-conjugated anti-mouse IP-10 polyclonal Ab were preincubated with recombinant mouse SLC and mouse IP-10, respectively, before applying them to the sections.

Results

Mac25/AGM binds to the ECM proteins and GAGs that are expressed near HEVs

Mac25/AGM is a secretory protein that localizes to the basal lamina of small blood vessels in the secondary lymphoid tissues (6), and a dense accumulation of this protein in the basal lamina bordering the endothelial cells is particularly prominent in the HEVs of lymph nodes (21) and Peyer's patches (data not shown). To understand the biological role of mac25/AGM in the basal lamina of HEVs, we first examined the ability of recombinant mac25/AGM to interact with various ECM proteins, some of which are expressed in the basal lamina of HEVs. Consistent with previous reports (10, 11), mac25/AGM bound to collagen type IV (Fig. 1*A*), which is a basement collagen abundantly expressed in the vascular basal lamina (31). The binding of mac25/AGM to collagen type IV was dose-dependent and reached a plateau at 20 μg/ml (data not shown). Mac25/AGM also bound to collagen type II and V, both of which are fibrillar collagens expressed ubiquitously (31), but not to other collagens, including types I, III, or VI (Fig. 1*A*). In contrast, mac25/AGM failed to interact with other ECM components

Table I. *Binding of mac25/AGM to human and mouse chemokines*

	Recombinant mac25/AGM Binding[a]	
	Human chemokines	Mouse chemokines
CXC chemokine		
ENA-78	–	
Gro α	–	– (KC)
IL-8	–	
IP-10	++	+
PF-4	–	
SDF-1α	–	–
BLC	–	–
C chemokine		
Lymphotactin	–	
CX3C chemokine		
Fractalkine	–	
CC chemokine		
MCP-1	–	
MCP-2	–	
MCP-3	–	
MIP-1α	–	
MIP-1β	–	
RANTES	+++	+
LARC	–	
PARC	–	
TARC	–	
ELC	–	–
SLC	+++	+++
I-309	–	

[a] The binding of mac25/AGM to each chemokine was detected using the recombinant mac25/AGM, anti-*myc*-HRP Ab, and ECL western blotting detection reagent as described in *Materials and Methods.* +++, >25 ng/spot detected; ++, >50 ng/spot detected; +, >100 ng/spot detected.

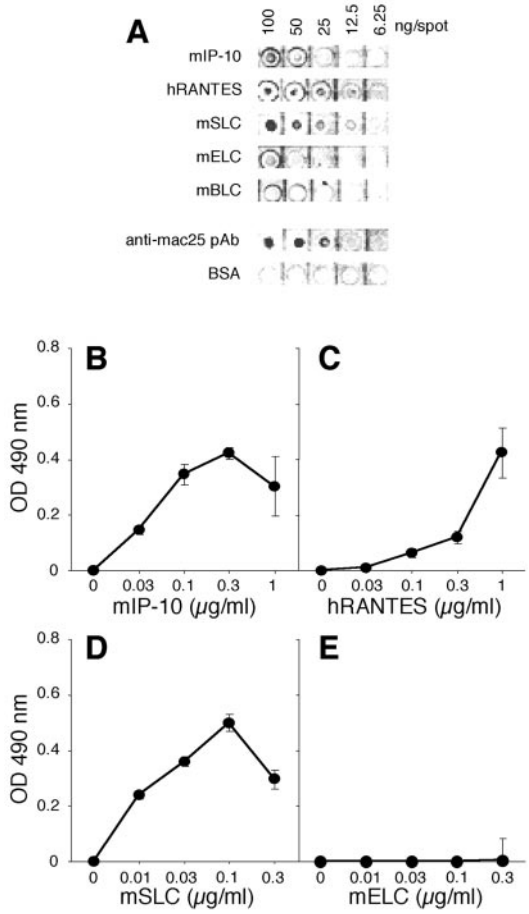

FIGURE 2. Binding of IP-10, RANTES, SLC, and ELC to mac25/AGM. *A*, Dot blot analysis. Various concentrations of chemokines were spotted onto a Hybond C nitrocellulose membrane, and the membrane was incubated with 1 μg/ml of the recombinant mac25/AGM. The bound mac25/AGM was detected as described in *Materials and Methods. B–E*, ELISA. Microtiter plates (96-well) coated with 1 μg/ml of recombinant mac25/AGM were incubated with the indicated concentrations of IP-10 (*B*), RANTES (*C*), SLC (*D*), or ELC (*E*). The amount of bound chemokine was determined by ELISA. The data represent the mean ± SD of triplicate determinations. The background binding was subtracted from the data shown in *B–E*.

such as fibronectin and vitronectin. In addition, it failed to bind various LN isoforms that are also expressed in the blood vascular basal lamina, including LN-8 and -10/11 (32–34). Thus, these results extend previous observations (11) and indicate that mac25/AGM interacts with restricted members of the ECM proteins that are expressed in the basal lamina of HEVs.

Because mac25/AGM has been reported to bind HS (12), we next examined the binding of mac25/AGM to various GAGs, some of which are localized to the region near HEVs. To this end, we used lipid-derivatized GAGs, which bind to plastic microwells efficiently and uniformly, and performed a direct binding assay using the recombinant mac25/AGM. As shown in Fig. 1*B*, mac25/AGM bound strongly to HS and heparin as previously reported (12), moderately to chondroitin sulfate (CS)-E, and weakly to CS-B, CS-D, keratan sulfate, and hyaluronic acid, and failed to interact significantly with CS-A or CS-C. Because HS and various CSs are expressed in the form of proteoglycans in the vascular structure (35), these results also indicate that mac25/AGM interacts with selected members of blood vessel-associated molecules, including HS and certain CSs.

Mac25/AGM can bind chemokines and support their functional activities

Because mac25/AGM has been implicated in growth factor binding (19, 20), we next investigated the ability of mac25/AGM to bind various soluble factors, such as cytokines, growth factors, and chemokines, that are expressed in the secondary lymphoid tissues. Preliminary experiments indicated that mac25/AGM failed to interact with various lymphokines but did interact strongly with certain chemokines (data not shown). We therefore examined the binding repertoire of mac25/AGM to chemokines in detail. Using dot blot analysis to detect the binding of mac25/AGM to chemokines that had been spotted on a nitrocellulose filter, it was found that mac25/AGM preferentially bound to certain CC chemokines (Table I), including RANTES and SLC as well as a CXC chemokine, IP-10 (Fig. 2*A*). These results were confirmed in a reverse type of binding assay in which chemokine binding to immobilized recombinant mac25/AGM was examined; this assay showed that mac25/AGM could bind to IP-10, SLC, and RANTES in a dose-dependent manner (Fig. 2, *B–D*) but little, if any, to ELC (Fig. 2*E*). The binding of IP-10 and SLC to mac25/AGM reached a maximal level at 0.3 μg/ml and 0.1 μg/ml of chemokine, respectively. The binding of IP-10 to mac25/AGM was significantly inhibited by SLC (Fig. 3*A*; *left panel*) and only slightly inhibited by ELC (Fig. 3*A*; *middle panel*) or BLC (Fig. 3*A*; *right panel*), indicating that mac25/AGM binds SLC and IP-10 using the same or a geographically very close region. In addition, as shown in Fig. 3*B*, the

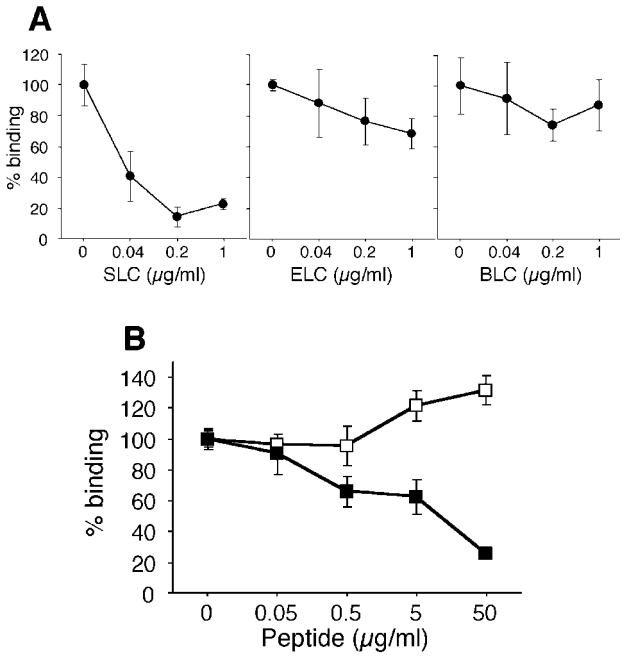

FIGURE 3. The competitive binding of IP-10 and SLC to mac25/AGM. *A*, Wells coated with recombinant mac25/AGM (50 ng/well) were incubated with IP-10 (0.1 μg/ml) in the presence of SLC, ELC, or BLC. The amount of bound chemokine was determined by ELISA. The data are expressed as percentages of the control value obtained in the absence of SLC, ELC, or BLC and represent the mean ± SD of triplicate determinations. Note that the IP-10 binding to mac25/AGM was significantly inhibited by SLC but not ELC or BLC. *B*, Inhibition of the binding of SLC to mac25/AGM by the HSBS peptide. The mac25/AGM-coated wells (50 ng/well) were incubated with SLC (0.03 μg/ml) in the presence of the HSBS peptide (■) or the shuffled HSBS peptide (□). The amount of bound chemokine was determined by ELISA. The data are expressed as percentages of the control value obtained in the absence of peptide and represent the mean ± SD of triplicate determinations.

binding of SLC to the immobilized mac25/AGM was inhibited in a dose-dependent manner by the HSBS peptide, which represents the HS binding region of mac25/AGM (11), but not by the shuffled HSBS peptide. Therefore, these results indicate that mac25/AGM binds SLC via its HS-binding site, located in the cysteine-rich domain in the amino-terminal region.

We then investigated whether mac25/AGM binding affects chemokine activities by examining the intracellular Ca^{2+} response elicited by SLC and IP-10 in the presence or absence of mac25/AGM. As shown in Fig. 4, *A* and *B*, and *B*, SLC and IP-10 induced a strong Ca^{2+} response in CCR7-transfected cells (L1.2/CCR7) and CXCR3-transfected cells (300.19/CXCR3), respectively, which was not affected at all by the addition of mac25/AGM at a molar ratio of 1:40–120, indicating that mac25/AGM can support chemokine activity upon interaction. As shown in Fig. 4*C*, it was confirmed that both SLC and IP-10 physically associate with mac25/AGM. Furthermore, as shown in Fig. 5, when L1.2/CCR7 cells and 300.19/CXCR3 cells were added with agarose beads coupled with mac25/AGM-SLC complex (Fig. 5*B*) and those beads coupled with mac25/AGM-IP-10 complex (Fig. 5*G*), respectively, only cells in contact with the beads showed an abrupt and transient rise of intracellular Ca^{2+} levels, similar to when these cells were stimulated with an appropriate chemokine in soluble form (Fig. 5, *A* and *F*). Neither cells that failed to make contact with the beads

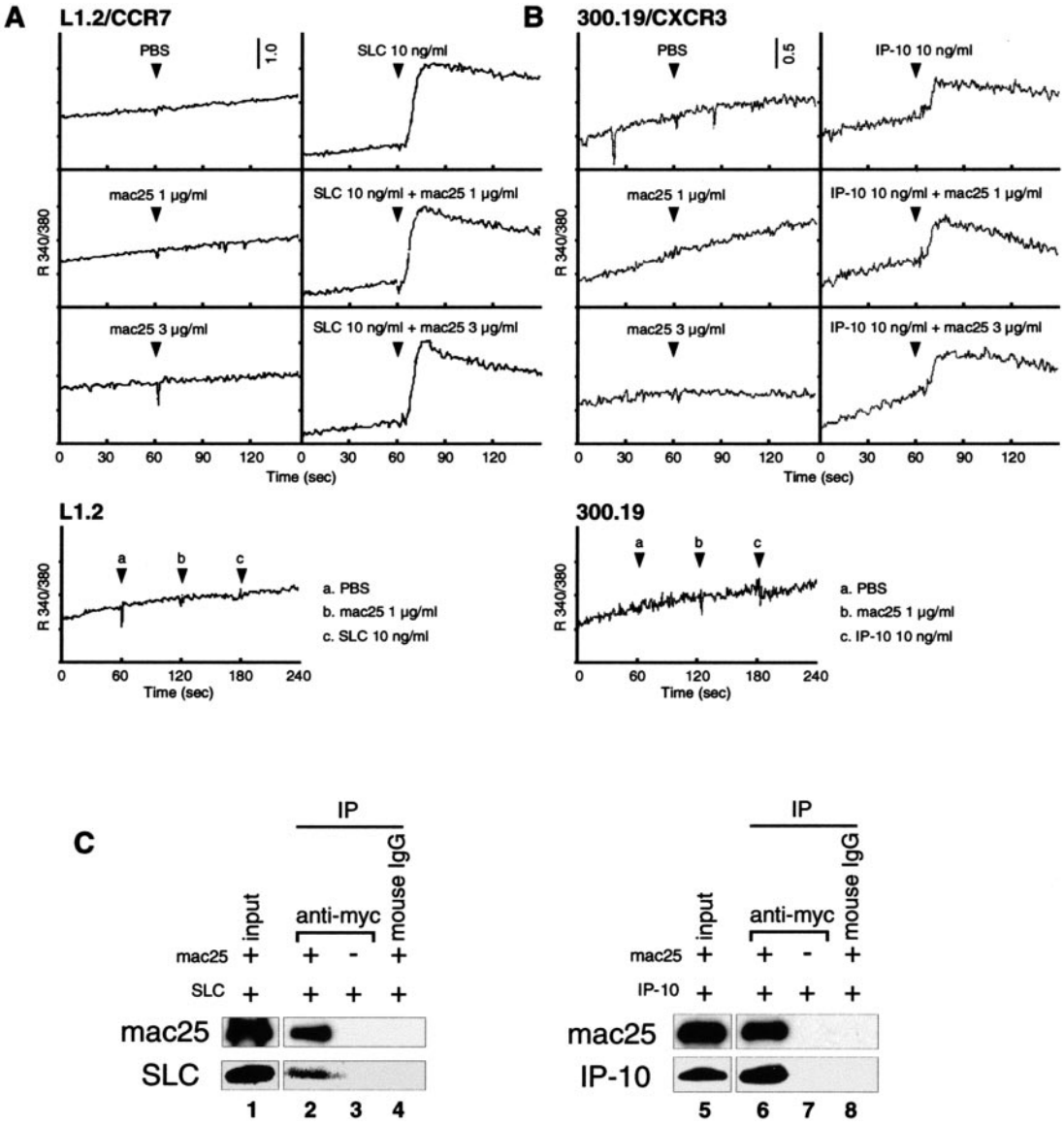

FIGURE 4. Effects of mac25/AGM on the intracellular Ca^{2+} mobilization induced by SLC or IP-10. L1.2 cells stably transfected with CCR7 (L1.2/CCR7) and the parental L1.2 cells (*A*), or 300.19 cells stably transfected with CXCR3 (300.19/CXCR3) and the parental 300.19 cells (*B*) were loaded with fura 2-AM and incubated with various stimulators. For the stimulation with the SLC-mac25/AGM complex (*A*) or IP-10-mac25/AGM complex (*B*), SLC or IP-10 was preincubated with the recombinant mac25/AGM for 1 h, respectively. The arrowheads indicate the time of application of the stimulator. The intracellular calcium concentration was monitored by measuring the fluorescence ratio (F340/F380). *C*, Immunoprecipitation of SLC-mac25/AGM complex (*left*) and IP-10-mac25/AGM complex (*right*). SLC (*lanes 2–4*) or IP-10 (*lanes 6–8*) was incubated with recombinant mac25/AGM and immunoprecipitated using anti-myc-conjugated beads (*lanes 2, 3, 6,* and *7*) or mouse IgG-conjugated beads (*lanes 4* and *8*). In *lanes 3* and *7*, BSA was used instead of recombinant mac25/AGM. The immunoprecipitates were electrophoresed and blotted using anti-mac25 polyclonal Ab (*upper panels*), anti-SLC polyclonal Ab (*left lower panels*), or anti-IP-10 polyclonal Ab (*right lower panels*). *Lane 1* represents the total input of SLC or mac25/AGM; *lane 5* represents the total input of IP-10 or mac25/AGM. Note that both SLC and IP-10 interacted with myc-tagged mac25/AGM and immunoprecipitated by anti-myc-conjugated beads but not control IgG-conjugated beads.

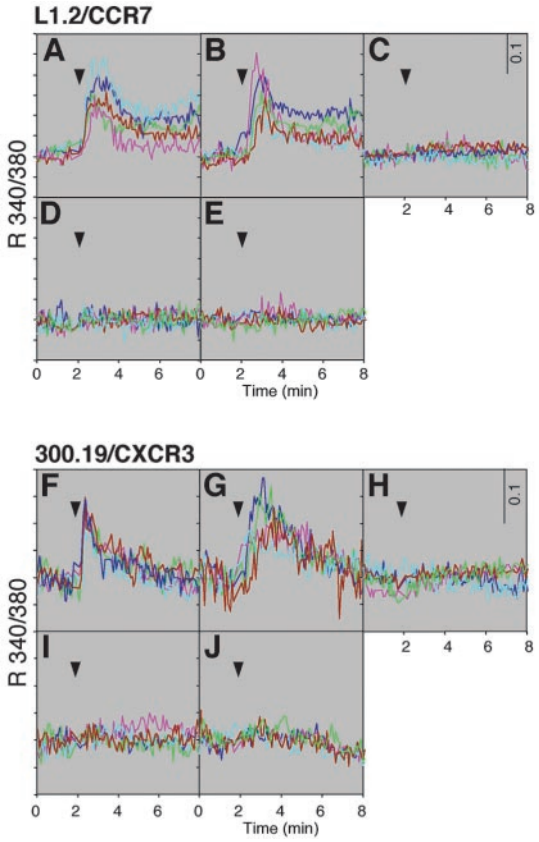

FIGURE 5. Ca^{2+} mobilizing activities of immobilized SLC-mac25/AGM and IP-10-mac25/AGM complexes. L1.2/CCR7 cells (*A–E*) and 300.19/CXCR3 cells (*F–J*) were loaded with fura 2-AM and incubated with various kinds of agarose beads that had been treated as described in Fig. 4*C*. The intracellular calcium concentration was monitored by measuring the fluorescence ratio at 340 and 380 nm. Five cells that were in contact with the agarose beads (*B*, *D*, *E*, *G*, *I*, and *J*) or the same number of cells that were not in contact with the beads (*C* and *H*) were chosen for recording. Each panel shows intracellular Ca^{2+} changes of five different cells (shown in five different colors individually) that occurred before and after the application of the stimulator. The arrowheads indicate the time of application of the stimulator. The stimulators are: SLC in soluble form (15 ng) (*A*); anti-myc-conjugated agarose beads that had been incubated with a mixture of SLC and mac25/AGM (*B* and *C*); anti-myc-conjugated beads incubated with SLC alone (*D*); control IgG-conjugated beads incubated with a mixture of SLC and mac25/AGM (*E*); IP-10 in soluble form (15 ng) (*F*); anti-myc-conjugated beads incubated with a mixture of IP-10 and mac25/AGM (*G* and *H*); anti-myc-conjugated beads incubated with IP-10 alone (*I*); control IgG-conjugated beads incubated with a mixture of IP-10 and mac25/AGM (*J*).

(Fig. 5, *C* and *H*) nor those added with the control beads (Fig. 5, *D*, *E*, *I*, and *J*) showed any significant changes of intracellular Ca^{2+} levels. These observations indicate that mac25/AGM can physically interact with SLC and IP-10 and can stimulate the chemokine receptor positive cells after complex formation with these chemokines. These results also imply that mac25/AGM expressed in the basal lamina of HEVs may have the ability to limit diffusion of chemokines and maintain them in an active state locally so that the chemokines can react with lymphocytes migrating through HEVs.

Because mac25/AGM binds collagen type IV, it was of interest to know whether mac25/AGM that had bound to collagen type IV can present chemokines. When mac25/AGM immobilized on collagen type IV was examined for chemokine binding, it was found unexpectedly that collagen type IV also binds chemokines such as SLC and IP-10 (data not shown), and because of this finding, we could not determine whether mac25/AGM can capture chemokines upon binding to collagen type IV. Therefore, although mac25/AGM is likely to bind certain chemokines upon its production at HEVs, it is uncertain whether mac25/AGM-collagen type IV complex can present chemokines.

Mac25/AGM colocalizes at least in part with SLC and IP-10 in HEVs

To investigate the possibility that mac25/AGM can interact with SLC, IP-10, and/or RANTES in vivo, we performed immunohistochemical analyses. As shown in Fig. 6, two-color immunofluorescence staining of frozen sections of the mouse inguinal lymph node clearly showed that both SLC (Fig. 6*C*) and IP-10 (Fig. 6*D*) were expressed in HEVs that were positive for the expression of mac25/AGM (Fig. 6, *A* and *B*) and the peripheral lymph node addressin (Fig. 6, *G* and *H*), whereas RANTES did not appear to be expressed (data not shown). The staining was almost completely abolished by preincubating the anti-SLC and anti-IP-10 Abs with SLC and IP-10, respectively (Fig. 6, *I* and *J*), indicating the specificity of the observation. In addition, there was a considerable overlap between the staining pattern of mac25/AGM and SLC (Fig. 6*E*) and of mac25/AGM and IP-10 (Fig. 6F*)*, indicating that these molecules are actually colocalized, at least in part, in the basal lamina of HEVs.

Discussion

Mac25/AGM is a unique multifunctional protein that is expressed abundantly in blood vessels in the secondary lymphoid tissues but not in blood vessels in normal nonlymphoid tissues (6). The expression in lymphoid tissues is particularly prominent in the HEVs (21) where a large-scale lymphocyte extravasation occurs continuously. In the present study, we demonstrated using a recombinant form of mac25/AGM that this protein could interact with several molecules that are found in the region near HEVs. Notably, mac25/AGM could bind specific chemokines, such as SLC, IP-10, and RANTES (Fig. 2), that have been implicated in the regulation of lymphocyte trafficking (36, 37). The following observations indicate that mac25/AGM binding to these chemokines is functionally significant. First, mac25/AGM bound to only certain chemokines and not to others we tested (epithelial-derived neutrophil attractant-78, growth-related gene product-α, IL-8, platelet factor-4, SDF-1α, BLC, lymphotactin, fractalkine, monocyte chemotactic protein-1, -2, and -3, macrophage inflammatory protein-1α and -1β, liver, pulmonary, and thymus activation-regulated chemokine, ELC, and I-309) (Table I), and the binding to SLC and IP-10 appeared to be dose-dependent and saturable (Fig. 2, *B* and *D*). Second, the binding pattern was reproducible irrespective of whether mac25/AGM or the chemokines were immobilized in the binding assay (Table I and Fig. 2). Third, mac25/AGM bound both human and mouse forms of SLC and RANTES (Table I). Fourth, the binding of mac25/AGM to IP-10 was strongly inhibited by SLC, which also binds mac25/AGM, and only slightly inhibited by ELC or BLC that binds little mac25/AGM (Fig. 3*A*). Finally, the binding of SLC to mac25/AGM was inhibited by the HSBS peptide, which represents the HS binding domain of mac25/AGM, but not by the shuffled HSBS peptide (Fig. 3*B*). Although the precise mechanism of how the HS-binding site in the basic region of mac25/AGM interacts with only certain chemokines remains unclear, mac25/AGM appears to colocalize at least in part with SLC and IP-10 in the HEVs' basal lamina or its vicinity (Fig. 6), implying that locally accumulated mac25/AGM in the basal lamina can function as a scaffold for SLC and IP-10 to regulate lymphocyte trafficking in HEVs. Given that mac25/AGM fully supported the Ca^{2+}-eliciting activities of SLC and IP-10 in vitro (Figs. 4 and

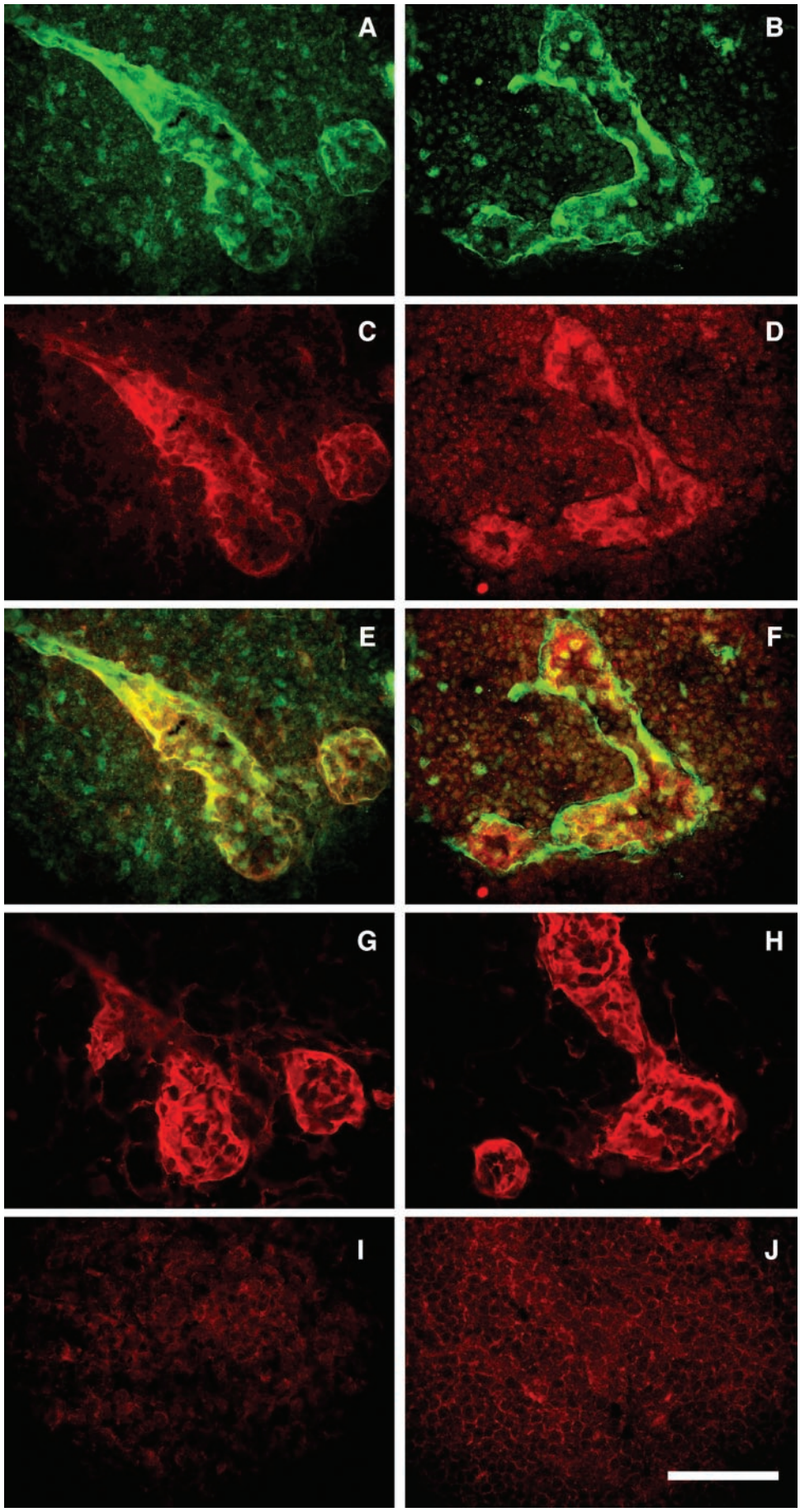

FIGURE 6. Colocalization of mac25/AGM with SLC and IP-10 in HEVs. Two-color staining of mouse inguinal lymph node cryosections with Abs against mac25/AGM (*A* and *B*) and SLC (*C*) or IP-10 (*D*). *E* and *F*, Merged views of *A* and *C* and *B* and *D*, respectively. To identify HEVs, serial sections were stained with MECA-79 (*G* and *H*). *I* and *J*, Staining with anti-SLC and anti-IP-10 Abs (1 μg/ml) in the presence of recombinant mouse SLC and mouse IP-10 (50 μg/ml), respectively. Four animals were used for each Ab (bar, 50 μm).

5), mac25/AGM is likely to help limit diffusion of the chemokines and maintain them in an active state in situ. An issue as to whether mac25/AGM can present chemokines after binding to collagen type IV could not be examined in the present study, because collagen type IV also appears to have a chemokine-binding ability. In this regard, it can be argued that SLC and IP-10 colocalized with mac25/AGM (Fig. 6) may at least in part be expressed in association with collagen type IV. Further study is required to resolve this issue.

Other than chemokines, mac25/AGM interacts with a few other molecules, such as certain ECM proteins and GAGs that may be expressed in the region near HEVs. As previously reported (10, 11), mac25/AGM bound to the basement collagen, collagen type IV. However, contrary to the previous reports, mac25/AGM was found to bind to some fibrillar collagens, such as collagen types II and V, that are expressed widely as ECM components in various tissues (Fig. 1*A*). This apparent discrepancy may be partly due to the fact that mouse mac25/AGM and collagens of the mouse and human origins were used in the present study, whereas human mac25/AGM and bovine collagens were used in the previous studies (10, 11). Among the GAGs examined, mac25/AGM showed robust binding to heparin and HS, as reported by others (12), and modest binding to CS-E (Fig. 1*B*). The binding of mac25/AGM to CS-E had been previously inferred from the in vitro observation that CS-E inhibited mac25/AGM binding to HS (12). The interaction of mac25/AGM with ECM proteins and GAGs may have significant bearing in the physiology of the secondary lymphoid tissues. Because HEVs are equipped with a conduit system that represents a rapid transport system of humoral factors and cells from afferent lymph (38), HEVs are continuously exposed to the outer environment, and hence are highly metabolically active (39) and subject to rapid and significant changes following antigenic stimulation (40). For such a dynamic structure to function coordinately with the surrounding environment, HEVs should have the ability to interact closely with ECM molecules and to respond to the cells and humoral factors present in the vicinity. The dense expression of ECM- and chemokine-binding molecules such as mac25/AGM in the basal lamina is certainly well-suited for this purpose. The ability of mac25/AGM to interact with HS and certain CSs may also have functional significance during the immune response or inflammation, given that HS and certain CSs such as CS-E can interact with L-selectin, P-selectin, and chemokines (41). HS and certain CSs are contained in mast cells and NK cells as secretory proteoglycans (35) and may be released extracellularly upon degranulation to bind mac25/AGM in the vascular basal lamina, which may in turn bind soluble selectins and chemokines that are released in inflammatory situations. The formation and accumulation of such molecular complexes may induce further changes in leukocyte trafficking into the secondary lymphoid tissues during the immune response. Detailed investigation of the interaction between mac25/AGM and these molecules in Ag-stimulated lymphoid tissues will elucidate this issue.

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