**Sulfation-dependent Recognition of High Endothelial Venules (HEV)-Ligands by L-Selectin and MECA 79, an Adhesion-blocking Monoclonal Antibody**

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**Summary**

L-selectin is a lectin-like receptor that mediates the attachment of lymphocytes to high endothelial venules (HEV) of lymph nodes during the process of lymphocyte recirculation. Two sulfated, mucin-like glycoproteins known as Sgp50/GlyCAM-1 and Sgp90/CD34 have previously been identified as HEV-associated ligands for L-selectin. These proteins were originally detected with an L-selectin/Ig chimera called LEC-IgG. GlyCAM-1 and CD34 are also recognized by an anti-peripheral node addressin (PNAd) mAb called MECA 79, which blocks L-selectin-dependent adhesion and selectively stains lymph node HEV. The present study compares the requirements for the binding of MECA 79 and LEC-IgG to HEV-ligands. Whereas desialylation of GlyCAM-1 and CD34 drastically reduced binding to LEC-IgG, this treatment enhanced the binding of MECA 79 to HEV-ligands. In contrast, the binding of both MECA 79 and LEC-IgG to GlyCAM-1 and CD34 was greatly decreased when the sulfation of these ligands was reduced with chlorate, a metabolic inhibitor of sulfation. Because MECA 79 stains HEV-like vessels at various sites of inflammation, recognition by L-selectin of ligands outside of secondary lymphoid organs may depend on sulfation. In addition to their reactivity with GlyCAM-1 and CD34, both MECA 79 and LEC-IgG recognize an independent molecule of ~200 kD in a sulfate-dependent manner. Thus, this molecule, which we designate Sgp200, is an additional ligand for L-selectin.
L-selectin (14, 19), consistent with the finding that in vivo or in vitro desialylation of HEV markedly reduces lymphocyte attachment (20, 21). Although fucosylation has not been directly demonstrated as an essential modification of these ligands, its importance is strongly suspected based on the observation that sialyl Lewis X, although a very weak inhibitor/ligand (19, 22, 23) for L-selectin, is superior to 3' sialyl lactose (neu5Aca2 → 3Galβ1 → 4Glc) (19), which differs only in its lack of fucose. Also, an essential role for fucose has been established for the myeloid ligands of both P- and E-selectin (24), which possess calcium-type lectin domains that are highly homologous to that of L-selectin (8, 9). Finally, HEV express binding sites for E-selectin (25), CSLEX (PLN) addressin (i.e., MECA 79) that stains lymph node addressin in organ culture (44).

Additional interest in the sulfation of HEV-ligands has derived from the knowledge that several sulfated glycoconjugates, although completely lacking in sialylation, exhibit specific ligand activity for L-selectin (28-33). A direct role for the sulfation of GlyCAM-1 was first demonstrated using chlortetracycline, a highly selective metabolic inhibitor of sulfation (34). Undersulfated GlyCAM-1, produced in organ culture of lymph nodes with chlorlate present, shows dramatically reduced binding to L-selectin. Direct analysis of the GlyCAM-1 carbohydrates has been undertaken with the objective of defining a minimal recognition determinant that accommodates the requirements for sialylation, fucosylation, and sulfation (35-37).

An independent approach to the identification of HEV-ligands for L-selectin has relied on the use of a mAb strategy. Streeter et al. (38) described an anti-peripheral lymph node (PNL) addressin mAb (i.e., MECA 79) that stains lymph node HEV and blocks L-selectin-dependent lymphocyte attachment both in vitro and in vivo. In contrast, the luminal staining of HEV in Peyer's patches is weak, consistent with the lesser role of L-selectin at this site (38, 39). The function-blocking activity of MECA 79 has recently been extended to HEV in human lymph nodes and tonsils (40). By Western blotting or radioiodination of immunoprecipitated material, MECA 79 reacts with a complex set of proteins in both human tonsils and mouse lymph nodes (41, 42). It is important to note that GlyCAM-1 and CD34, as isolated with LEC-IgG from mouse lymph nodes, carry the epitope for MECA 79 (14). Moreover, the set of MECA 79-precipitated bands (detected by radioiodination) from lymph nodes of young mice includes components at ~50 and ~90 kD as well as five additional species (42) (see Discussion). The isolated complex of MECA 79-reactive proteins (PNL addressin [PNAd]) is able to support specific lymphocyte attachment via L-selectin in a sialidase-sensitive manner (41). The occurrence of the MECA 79 epitope on multiple components has suggested that it might represent a carbohydrate posttranslational modification closely associated with the determinants that confer binding activity for L-selectin (41). Although the MECA 79-defined modification has clearcut functional significance, as adduced from the function-blocking activity of the antibody and the adhesion-promoting activity of the PNAd complex, the biochemical nature of the epitope has heretofore not been examined.

In this study, we show that both L-selectin and MECA 79 mAb recognize the same set of [H3Gal-labeled and [35S]SO4-labeled glycoproteins from lymph nodes. Furthermore, we show that as for L-selectin, recognition of these HEV-ligands by MECA 79 is dependent on sulfation.

Materials and Methods

Materials. LEC-IgG was provided by Dr. L. Lasky and Dr. S. Watson (Genentech Inc., South San Francisco, CA) and immobilized on protein A-Sepharose 4B (Zymed Laboratories, Inc., South San Francisco, CA) at 10 mg/ml of gel. The rabbit polyclonal antisera to CAM02 against an internal peptide (peptide 2) of GlyCAM-1 has been described (15). Rabbit antiserum directed against recombinant murine CD34 (16) was a gift of Dr. L. Lasky and Dr. S. Baumhueter. Rabbit antibodies and preimmune serum were covalently cross-linked with dimethylpimelimidate (43) to protein A-Sepharose after saturating the protein A with serum. MECA 79 mAb (rat IgM) and a control rat IgM mAb (OZ42), described previously (38), were immobilized to a substitution level of 2 mg/ml gel on anti-rat Ig Sepharose (Sigma Chemical Co., St. Louis, MO). CAM02-peptide (CKEPSIFREELISKD) was synthesized by Dr. C. Glabe of the University of California (Irvine, CA). Limax flavus agglutinin was purchased from Calbiochem-Novabiochem (San Diego, CA). Recombinant Aleuria aurantia agglutinin (AAA) was a gift of Dr. A. Kobata and Dr. T. Endo (University of Tokyo, Tokyo, Japan). Both lectins were immobilized on CNBr-activated Sepharose 4B (Sigma Chemical Co.) at a level of 2 mg lectin/ml gel (44).

Metabolic Labeling of Murine Lymph Nodes. Murine axillary, brachial, cervical, and mesenteric lymph nodes from five ICR mice were split into two equal parts and labeled in parallel in organ culture (0.5 ml RPMI 1640, 4 h, 37°C), each containing 0.5 mCi/ml d-[6-3H]galactose (Du Pont-NEN, Boston, MA). In one of the two cultures, sulfate content was depleted to 1/10 of normal and 10 mM sodium chloride was added as an inhibitor of sulfation. For labeling with [35S]SO4, lymph nodes from five mice were labeled in organ culture (4 h, 37°C) in 1 ml RPMI 1640 with sulfate depleted to 1/10 normal and Na2[35SO4] (ICN, Irvine, CA) added at 1 mCi/ml.

Immunoprecipitations. To generate cell lysates, labeled tissues from control or chlorate-treated cultures were washed with 1 ml Dulbecco's PBS and then homogenized in 1 ml of PBS with 2% Triton X-100 supplemented with leupeptin (10 μg/ml), pepstatin A (10 μg/ml), aprotinin (0.28 U/ml trypsin inhibitor), benzamidine (0.75 mM), and PMSS (1 mM). Homogenates were centrifuged (5 min, 10,000 g) and supernatants precleared by 18 h incubation with 50 μl preimmune Ig-agarose. The supernatants were then immunoprecipitated sequentially (4 h/immunoprecipitation) with 10 μl of CD34 Ab-agarose followed by 20 μl of CAM02 Ab-agarose. The immunoprecipitates were washed 5× with 1.5 ml of PBS containing 0.25% Triton X-100 and then boiled (5 min) in 0.4 ml of this buffer. For reprecipitation, each antigen preparation was split into four 100 μl aliquots. Three of the aliquots were incubated in parallel (4 h) with 2.5 μl LEC-IgG matrix (+ 2 mM CaCl2), 5 μl MECA 79-agarose, or 5 μl Limax flavus agglu-
tinitin-agarose. The fourth aliquot was boiled after addition of 5 μl 3x concentrated reducing SDS-sample buffer. Matrices were washed 4x with 0.5 ml of PBS containing 0.25% Triton X-100 and boiled in reducing SDS-sample buffer (150 μl final) for SDS-PAGE.

Lysates were also directly precipitated with LEC-IgG or MECA 79. Lysates prepared as above were precleared with 50 μl protein A-agarose and then precipitated with 10 μl of LEC-IgG matrix or alternatively were precleared with 10 μl OZ42-agarose plus 40 μl protein A-agarose and then precipitated with 10 μl of MECA 79-agarose. Precipitates were washed 5x with 1.5 ml of PBS 0.25% Triton X-100. The LEC-IgG matrix was eluted with 100 μl Tris-buffered saline (TBS; 10 mM Tris-Cl, 150 mM NaCl, pH 7.4) containing 5 mM EDTA, whereas the MECA 79 matrix was eluted by boiling in 100 μl of reducing SDS-sample buffer.

Exoglycosidase Digestions of GlyCAM-1. Conditioned medium was collected from the organ cultures above (cultured in the presence or absence of chlorate), and clarified by brief centrifugation (10,000 g, 5 min). After preclearing with 50 μl protein A-Sepharose, the conditioned medium was immunoprecipitated with 25 μl CAM02 Ab-agarose. The beads were washed 5x with 1.5 ml of TBS and eluted with 1 mg/ml of CAM02-peptide in TBS (200 μl). 10 μl aliquots of the eluate were reacted with 25 μl of Artrobacter ureafaciens sialidase (Calbiochem-Novabiochem) alone or with the sialidase combined with 5 μU of Streptomyces sp142 α(1→3/4) fucosidase (Takara Shuzo Co., Berkeley, CA) in 50 mM Na-cacodylate (pH 6.0), 0.05% BSA, 0.05% NaN₃, and 0.125% Triton X-100 (final volume 50 μl) for 48 h at 37°C. A control sample was incubated in parallel (48 h, 37°C) in buffer only. Each of the digests and the control sample were then split into four equal (12.5 μl) parts, of which three were diluted to 300 μl with PBS containing 0.1% BSA, 0.25% Triton X-100, and 0.02% NaN₃, while the fourth was boiled in reducing SDS-sample buffer (100 μl final). The three aliquots were then reprocipitated with 10 μl of either MECA 79-agarose, Limax flavus agglutinin-agarose, or AAA-agarose. Matrices were washed 4x with 0.5 ml of PBS containing 0.25% Triton X-100 and boiled in reducing SDS-sample buffer (100 μl final) for SDS-PAGE.

SDS-PAGE. SDS-PAGE (10% polyacrylamide gels) was carried out as described by Laemmli (45).

Results

We first examined the possible contribution of sialylation and fucosylation to the MECA 79 epitope on GlyCAM-1. GlyCAM-1 was metabolically labeled with [3H]galactose in lymph node organ cultures and was isolated from conditioned medium with CAM02, a polyclonal antibody directed to an internal peptide sequence of GlyCAM-1 (15). To remove sialic acid, the isolated glycoprotein was treated with a broad spectrum sialidase from A. ureafaciens. As shown in Fig. 1 and Table 1, desialylation was effective, since the treated glycoprotein completely lost its reactivity with Limax agglutinin, a lectin with broad specificity for sialic acid (44). Desialylated GlyCAM-1 retained its ability to bind to MECA 79 mAb, as seen by visual inspection of the gel (Fig. 1). In fact, quantitation by densitometry revealed significantly increased (~70%) binding of MECA 79 to desialylated GlyCAM-1 as compared with normal GlyCAM-1.

To remove fucose, isolated GlyCAM-1 was treated with a combination of Artrobacter sialidase and Streptomyces α(1→3/4) fucosidase. We previously demonstrated that this fucosidase removes ~70% of total fucose from desialylated GlyCAM-1 but is inactive on sialylated GlyCAM-1 (36). We confirmed that sialidase/fucosidase-treatment of [3H]galactose-labeled GlyCAM-1 removed a substantial fraction of fucose, as shown by the 89% reduction in binding to AAA (Table 1, Fig. 1), a fucose-specific lectin (46). MECA 79 binding to sialidase/fucosidase-treated GlyCAM-1 was reduced 26% relative to its binding to sialidase-treated GlyCAM-1 (Table 1, Fig. 1). In an independent experiment, a reduction of 35% was observed.

The possible importance of sulfate to the MECA 79 epitope was investigated using chlorate as a metabolic inhibitor of sulfation. We previously devised conditions that suppress the sulfation of GlyCAM-1 and other macromolecules within lymph nodes by ~90% without affecting the overall sialylation or fucosylation of GlyCAM-1 or altering the pattern of protein synthesis within the nodes (34). As shown in Fig. 2, undersulfated [3H]galactose-labeled GlyCAM-1 showed undiminished binding to CAM02 or to Limax flavus agglutinin. The decreased mobility of undersulfated GlyCAM-1 on SDS-PAGE may reflect the strong contribution of sulfation to the overall charge of the glycoprotein. In correspondence with previous results obtained with other metabolic

![Figure 1](image-url)

Figure 1. Effects of sialidase and sialidase/fucosidase treatments of GlyCAM-1 on binding to MECA 79. GlyCAM-1, labeled with [3H]galactose and isolated with CAM02 antibody, was either not treated (normal), digested with Artrobacter sialidase (desialylated), or digested with combined Artrobacter sialidase and Streptomyces α(1→3/4) fucosidase (desialylated/fucosidylated). Each GlyCAM-1 preparation was divided into four equal aliquots, of which three were subjected to precipitation with AAA, a lectin with specificity for fucose moieties; Limax flavus agglutinin (LFA), a lectin with specificity for sialic acid moieties, and MECA 79 (M79). The precipitates and the fourth aliquot of each preparation containing the total CAM02-reactive GlyCAM-1 (T) were run on a 10% SDS gel. The gel was analyzed by autoradiography.
### Table 1. Binding of MECA 79 mAb to GlyCAM-1 after Treatments with Sialidase and Fucosidase

| Fraction          | Untreated       |          | Sialidase      |          | Sialidase/α(1→3/4) Fucosidase |          |
|-------------------|-----------------|----------|----------------|----------|-------------------------------|----------|
|                   | Amount          | Percent  | Amount         | Percent  | Amount                        | Percent  |
| MECA 79-bound     | 2194            | 30       | 2695           | 52       | 1719                          | 38       |
| Limax-bound       | 5491            | 74       | 0              | 0        | 3                             | 0        |
| AAA-bound         | 7096            | 96       | 5095           | 99       | 501                           | 11       |
| Total input (T)   | 7363            | 100      | 5150           | 100      | 4569                          | 100      |

The data presented in the table were obtained by densitometry (Molecular Dynamics, Sunnyvale, CA) of the autoradiograph depicted in Fig. 1. In this analysis, the region of the autoradiograph limited at the upper end by the 66-kD marker and at the lower end by the 31-kD marker was divided into twelve parallel segments containing the GlyCAM-1 bands of the 12 lanes in the gel. The amount of GlyCAM-1 in each lane (given in arbitrary units) was obtained by integration of the optical density over the entire respective segment with subtraction of the background. Each value is given as a percentage of total GlyCAM-1, with total (T) defined as the amount of CAM02-reactive GlyCAM-1 in the respective sample.

Sulfation dependence was also investigated with [3H]galactose-labeled CD34 isolated from cultured lymph nodes (Fig. 3). Undersulfation did not affect the recognition of CD34 by either the anti-CD34 Ab or Limax agglutinin. However, the binding of undersulfated CD34 to LEC-IgG or to MECA 79 was almost completely abrogated. Again, the MECA 79 and LEC-IgG reactive species occurred in a higher Mr subset of the total CD34, as defined by precipitation with the polyclonal CD34 Ab.

To directly compare components from lymph node that were reactive with MECA 79 and LEC-IgG, a detergent lysate...
Figure 4. Parallel precipitations from lymph node lysate with MECA 79 and LEC-IgG. Lymph nodes (peripheral and mesenteric) were labeled in organ culture with $[^{35}S]$SO₄. Equal aliquots of the detergent lysate were precipitated with beads conjugated to Ig control (a mixture of CD4-IgG and rat IgM), MECA 79, LEC-IgG, anti-CD34, or anti-GlyCAM-1 (CAM02). The precipitates were electrophoresed on a 7.5% SDS gel and the gel was analyzed by autoradiography. The double arrowheads denote the bands corresponding to Sgp200, CD34/Sgp90, and GlyCAM-1/Sgp50 whereas the single arrowhead denotes the ~170-kD component. In the LEC-IgG precipitate, the CD34 component is somewhat compressed due to the presence of unlabeled LEC-IgG at ~90 kD.

of $[^{35}S]$SO₄-labeled lymph nodes was subjected to precipitation with the two reagents. Both MECA 79 and LEC-IgG precipitated three major bands at ~50, ~90, and ~200 kD (Fig. 4). The first two components comigrated with GlyCAM-1 and CD34/Sgp90, as shown by precipitation with monoclonal Abs. The ~200-kD component was apparently distinct, since it was not precipitated by either CAM02 or CD34 Ab (Fig. 4). A relatively minor band at ~170 kD was also precipitated by both LEC-IgG and MECA 79 but not by CAM02 or CD34 Ab.

To examine the sulfate dependency of precipitation, lymph nodes were labeled in organ culture with $[^{3}H]$galactose in the presence or absence of chlorate. Detergent lysates from these cultures were reacted in parallel with LEC-IgG and MECA 79. From control lymph nodes, each precipitated the same four bands, corresponding to CD34, GlyCAM-1, and the ~200 and ~170 kD components (Fig. 5). Culture of lymph nodes in chlorate dramatically reduced or eliminated the ability of both LEC-IgG and MECA 79 to recognize GlyCAM-1, CD34, and the ~200-kD component (Fig. 5). In contrast, the recognition of the ~170-kD component from chlorate-treated cultures was largely preserved. Besides the four components that were precipitated in common by the two reagents, all of which were relatively diffuse in character, a number of sharp bands were precipitated by MECA 79 (Fig. 5). These bands were judged to be nonspecific components, since they were not reproducibly present in repeat experiments.

Discussion

Our analysis revealed that several $[^{35}S]$SO₄ and $[^{3}H]$galactose-labeled glycoproteins within lymph node lysates were precipitated in common by MECA 79 and LEC-IgG i.e., GlyCAM-1, CD34, ~200 kD, and an additional component at ~170 kD. Previous analyses of MECA 79-reactive components, employing radiiodination of immunoprecipitated material, detected bands corresponding in molecular weight to these four components as well as species at 115, 75, and ~60 kD (42).

The ~60-kD molecule is of particular interest, because it constitutes a subset of MadCAM-1, a novel glycoprotein possessing both Ig-like domains and a mucin domain (42,
lymph nodes and peripheral lymph nodes of young mice ready detectability in radioiodination and Western blotting IgG in our metabolic radiolabeling studies, as opposed to its detectability of this component with MECA 79 and LEC-IgG, we were unable to detect an obvious ~60 kD component with either [35S]SSO4 as the metabolic label. The dominance of GlyCAM-1 in the 50–60 kD range may have obscured a weak MAdCAM-1 band. In fact, in separate studies using MECA 367 mAb (47) to precipitate MAdCAM-1 from [35S]SSO4-labeled mesenteric lymph nodes, we confirmed that MAdCAM-1 incorporated label, although weakly (Veals, S., and S. Hemmerich, unpublished observations). The limited detectability of this component with MECA 79 and LEC-IgG in our metabolic radiolabeling studies, as opposed to its ready detectability in radiodination and Western blotting analyses (42), could be due to a low content of sulfate and galactose and/or a slow metabolic turnover. Similar considerations can be invoked to explain the absence of the 75- and 115-kD components in our analysis.

Our experiments demonstrate that LEC-IgG and MECA 79 both react with a major lymph node component at ~200 kD. Because of the evidence implicating this component as an independent HEV-associated ligand for L-selectin (summarized below), we designate it as Sgp200. A similar component was identified in the previous analyses carried out with MECA 79 (42). Also, a molecule of ~190 kD was detected in rat lymph nodes with a rat L-selectin/Ig chimera (50) and a ~250 kD component was detected in human tonsilar HEV with a antibody directed to a complex form of sialyl Lewis X (51). Sgp200 closely corresponds in behavior to GlyCAM-1 and CD34. Desialylation of Sgp200 severely reduced its interaction with LEC-IgG, while allowing its binding to MECA 79 (data not shown). However, the binding of Sgp200 to both LEC-IgG and MECA 79 was dependent on sulfation. Sgp200 did not appear to be an aggregate of either GlyCAM-1 or CD34, since antibodies against these ligands failed to precipitate it. Because the LEC-IgG staining of lymph nodes is largely restricted to HEV (13), we expect that Sgp200, like GlyCAM-1 and the L-selectin reactive form of CD34, will also be localized to HEV.

It is noteworthy that mucin domains are present within the three molecularly characterized glycoproteins (i.e., GlyCAM-1, CD34, and MAdCAM-1) that can function as ligands for L-selectin. Additionally, a recently described myeloid ligand for P-selectin has a large mucin-like domain (52). It seems quite plausible that the multivalent presentation of carbohydrate chains, inherent in this structural organization, is an important feature in defining the avidity of these ligands. We therefore, predict that Sgp200 will also be mucin-like. A major challenge for the future will be to determine the physiologic roles of the different L-selectin ligands.

The distribution of the MECA 79 epitope on multiple components has suggested that it represents a posttranslational modification (41). Since the antibody functionally blocks ligand sites for L-selectin, the most obvious possibility was that the epitope consists of a carbohydrate or modification of a carbohydrate. We have shown here that the sialylation of GlyCAM-1 and CD34 is not required for MECA 79 recognition, although it is essential for the avid interaction of each with L-selectin. In contrast to sialic acid, fucose appears to contribute positively to the MECA 79 epitope. We observed a ~30% reduction of MECA 79 binding to GlyCAM-1 following ~70% removal of fucose. It remains to be seen whether more complete defucosylation would result in a complete loss of the epitope. The most striking finding of the present study is that sialation is critical for the MECA 79 epitope. Undersulfated GlyCAM-1, CD34, and Sgp200, created by the use of chlorate, were not recognized by MECA 79. Furthermore, as previously shown for GlyCAM-1 and now extended to CD34 and Sgp200, ligand activity for L-selectin was dramatically reduced by undersulfation.

The exact nature of the MECA 79 epitope remains to be defined. In our previous work, we identified the major sulfated mono- and disaccharides of GlyCAM-1 as Gal-6-SO4, GlcNAc-6-SO4, (SO4-6)Galβ1→4GlcNAc and Galβ1→4(SO4-6)GlcNAc (36). Furthermore, we showed (37) that a major capping group of the GlyCAM-1 O-linked chains is 6'-sulfated, sialyl Lewis X, i.e., Siaα2→3(SO4-6)Galβ1→4(Fucα1→3)GlcNAc. The finding that MECA 79 reacts only with a subset of GlyCAM-1 and CD34, as described also for MAdCAM-1 (42), suggests that the epitope for this mAb may be complex. Future work will be directed at defining this epitope in terms of the sulfated and fucosylated constituents that are present within the O-linked chains of GlyCAM-1.

MECA 79 blocks L-selectin dependent lymphocyte binding to HEV in mouse lymph nodes and in human tonsils and lymph nodes (38, 40). Thus, the sulfation requirement for recognition of PNAd by this antibody, reinforces the previous demonstration that sulfation is essential for HEV-ligand activity (34). It is important to note that MECA 79 staining is induced on venular endothelium in many chronic inflammatory settings, including multiple cutaneous lesions, rheumatoid synovium, lymphocytic thyroiditis, and inflammatory bowel diseases in human (40, 53); a DTH site in sheep (54); inflamed pancreatic acinar tissue in a IL-10 transgenic mouse model (55); inflamed pancreas in an IFN-γ transgenic mouse model (56); and spontaneously inflamed pancreatic islets and salivary glands in the nonobese diabetic (NOD) mouse (57, 58). In the case of NOD mice, expression of the MECA 79 epitope was shown to be associated with the expression of functional ligands for L-selectin (57). It is also noteworthy that strong metabolic incorporation of [35S]SSO4 has been seen, not only in HEV of rodent and human lymph nodes (27, 59), but also in HEV-like vessels that are induced at sites of chronic inflammation in both rodent and human (59, 60). Taken together, these findings raise the possibility that sulfation, in addition to being essential for the function of HEV-ligands in secondary lymphoid organs, is also required for recognition of L-selectin ligands on blood vessels at sites of chronic inflammation.
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