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Selective Malformation of the Splenic White Pulp Border in L1-Deficient Mice

Shih-Lien Wang,* Michael Kutsche,† Gino DiSciullo,* Melitta Schachner,† and Steven A. Bogen**

Mononuclear cells enter lymphoid organs via two distinct routes. Although the blood vascular route has been a major focus of interest over the last decade, there is a paucity of information about mononuclear cell passage across sinusoidal walls. Specifically, mononuclear cells, such as macrophages, lymphocytes, and dendritic cells, travel from the site of Ag contact to a draining lymphoid organ. In the context of a lymph node (LN),3 mononuclear cells drain into the subcapsular sinus, enter the cortical sinuses, and then migrate into the lymphoid parenchyma by crossing the relatively poorly defined boundary of the sinusoidal lining. This lining comprises a single layer of flattened cells associated with collagen fibrils and extracellular matrix proteins, such as laminin (1, 2). Comparatively little is known about the physiological properties of this lining, such as the expression of adhesion molecules, mechanisms of locomotion of mononuclear cells along the lining surface, or whether the sinusoidal wall is porous or relatively impermeable to the free flow of macromolecules and cells (3).

In the spleen, the most analogous structure to the LN sinusoid is the marginal sinus. The marginal sinus lining cells are a thin layer of flattened cells that envelop the white pulp. These flattened cells form the inner layer of the marginal sinus, a space that communicates, directly or indirectly, with the blood vascular system (4–6). Ultrastructurally, the marginal sinus wall is more analogous to the LN sinusoidal lining cells than blood vascular endothelium. Namely, both types of sinusoidal lining cells associate with a similar type of protein matrix, do not assume the tall morphology often associated with postcapillary venules, and do not have a known intercellular junction capable of regulating fluid and solute flow. It is across this boundary that mononuclear cells must cross to enter the splenic white pulp.

We are particularly interested in identifying the underlying adhesion molecules that mediate structural features of sinusoidal linings. To this end, we have focused our attention on the L1 adhesion molecule. L1 is a 1260-aa-long Ig superfamily adhesion molecule (7). It has been implicated in several important neurobiological processes, including neurite outgrowth, neurite fasciculation, axon-Schwann cell interaction, myelination, neuronal cell migration, and synaptic plasticity (8–12). L1 acts homophilically and heterophilically (8, 13, 14). The importance of L1 in neuronal development is reflected by the fact that mutations in the human L1 gene lead to a group of neurological syndromes (15, 16).

Although L1 was initially identified in the nervous system, L1 is also expressed in nonneuronal tissues. Specifically, L1 is expressed by cells of hematopoietic origin (17, 18), intestinal epithelial cells (19), epithelium of the male urogenital tract (20), and other cells of epithelial origin (21). The functional role of L1 on these cells is largely unexplored. L1 has been implicated in an in vitro cell binding assay between lymphocytes and bend 3 endothelial cells, raising the possibility of a potential role in lymphocyte-endothelial cell interactions (22). It is also involved in kidney morphogenesis (23).

Previous data from our laboratory implicated the Ig superfamily adhesion molecule L1 in maintaining normal sinusoidal structure in LNs during immune hypertrophy (24). Specifically, we found that in vivo administration of an L1 mAb disrupted the normal remodeling of the cortical sinusoidal lining cells of LNs during an immune response. The L1 mAb did not disrupt static, quiescent sinusoidal lining cells. Rather, it interfered only with sinusoidal

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Abbreviations used in this paper: LN, lymph node; FDC, follicular dendritic cell; KO, knockout; MMM, marginal metallophilic macrophage; NRIGC, normal nonimmune rat IgG; RT, room temperature; DAB, 3,3′-diaminobenzidine; SLC, sinus lining cell; RC, reticular cell; MadCAM-1, mucosal addressin cell adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule-1.

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lining cells mediating the process of matrix remodeling induced by immune stimulation. A limitation with the in vivo L1 Ab study was that it required a xenogenic rat anti-mouse L1 mAb. Consequently, a mouse (host) anti-rat IgG immune response developed 10–14 days after L1 mAb administration. This model was therefore useful only in short term studies, such as acute hypertrophic responses to immune stimulation.

Because of the structural similarities between the splenic marginal sinus and LN cortical sinoids, we hypothesize that the splenic marginal sinus may also be reliant on the L1 adhesion molecule for structural integrity. To elucidate the role of L1 in the development and maintenance of the splenic marginal sinus and the white pulp boundary, we have studied lymphoid matrix development in an L1-null mutant mouse.

Materials and Methods

**Antibodies**

Normal nonimmune rat IgG (NRIGG) and the clone 324 rat anti-L1 mAb (IgG) were prepared and purified by HPLC as previously described (24).

**Animals**

Female L1 heterozygous mice of C57BL/6 background were generated as previously described (8). They were bred with either wild-type C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) or BALB/cByJ (Taconic Farms, Germantown, NY) males. The offspring were genotyped by PCR for detection of the L1 deletion as described (8). Male L1 knockout (KO) mice and their wild-type littermate controls were used at 8–15 wk of age. Normal 8- to 12-wk-old male BALB/cByJ mice were purchased from The Jackson Laboratory. Animals were housed at the Laboratory Animal Science Center, Boston University School of Medicine (Boston, MA), and cared for in accordance with the Institutional Animal Care and Use Committee of Boston University School of Medicine.

**Flow cytometry**

Splenocyte suspensions were made by dissociating the spleens through a 100-μm steel mesh. The RBC were lysed in Tris-buffered ammonium chloride (0.14 M NH₄Cl, 0.017 M Tris-HCl, pH 7.2) for 1 min at 37°C. Cells were resuspended in PBS at a concentration of 20 × 10⁶/ml. Splenocytes (1 × 10⁶/ml) were stained with anti-IgG-FITC, anti-CD8 PE (both from PharMingen), or anti-IgM-FITC (Serotec). Cell suspensions were incubated for 30 min incubation at RT.

Plastic embedding for light and transmission electron microscopy

Organs were immersed in Karnovsky’s half-strength fixative for 30 min and then cut with a razor blade into smaller pieces (~8 mm³). The tissue fragments were further fixed in Karnovsky’s half-strength fixative for ~1 day. Tissues were rinsed with 0.1 M sodium cacodylate with 5% sucrose, postfixed with 1% OsO₄, washed with 0.1% glutaraldehyde and 2% lead citrate, and then dehydrated through an ethanol gradient. The tissue was then embedded in Epon 812 and cured at 60°C overnight. Semithin sections (1 μm) were stained with 1% toluidine blue in 1% sodium borate. The 0.06-μm ultrathin sections were examined and photographed in an electron microscope (model 300, Philips Electronics, Eindhoven, The Netherlands).

**Immunochemistry**

For immunofluorescence studies, tissue sections were embedded in OCT (Miles, Elkhart, IN), snap frozen in liquid nitrogen, cryosectioned, and fixed for 10 min at 4°C. For the detection of L1, we used the tyramide signal amplification system (NEL Life Science, Boston, MA). Sections were incubated with rabbit anti-rat IgG Fab (0.1 mg/ml, a gift of Dr. Marie H. Kosco-Vilbois) for 30 min at room temperature (RT). This both hydrated the tissue sections and masked sites of nonspecific protein adsorption. We masked endogenous peroxidase activity by incubating the sections with 3,3′-diaminobenzidine (DAB) 0.6 mg/ml in 50 mM Tris buffer, pH 7.6, supplemented with 0.03% hydrogen peroxide for 20 min at RT. This treatment covers sites of endogenous peroxidase with DAB precipitate, physically encasing the enzyme to prevent further activity and thus effectively neutralizing the activity of the peroxidase enzyme. It also largely occludes the excitation and emission light (in a fluorescence detection system, which we used). We found this method to be more effective than quenching with high concentrations of hydrogen peroxide, especially because tyramide signal amplification is highly sensitive in detecting even trace levels of residual peroxidase activity. In the system that we describe, even if a small amount of peroxidase activity was left unquenched, there was no opportunity for confusion to arise. The brown DAB precipitate denoting endogenous peroxidase is easily distinguished on bright-field optics from the fluorescent signal identifying the location of Abs of interest.

For the single-color detection of all other Abs, the specific primary Abs were detected with a rabbit anti-rat IgG-FITC. The anti-B220 Ab was applied at a 10-fold lower than normal concentration (0.01 μg/ml). This ultralow Ab concentration can easily be detected using the highly sensitive tyramide signal amplification system (fluorescence signal) but is below the level of detection for the standard immunofluorescence detection system using Texas Red. Therefore, this helps ensure the fidelity of the two-color discrimination. After completing the first color (fluorescein) stain, we blocked immunoreactive sites on the donkey anti-rat IgG-biotin conjugate with NRIgG (60 μg/ml). This step blocks unoccupied Ig binding sites on the donkey anti-rat IgG-biotin conjugate reagent. Immunoreactive sites on the L1 rat IgG mAbs were blocked with a 40-min RT incubation of rabbit anti-rat IgG Fab fragment (50 μg/ml). The rabbit anti-rat IgG Fab was prepared by digestion with papain. Fab fragments were subsequently purified by HPLC. For the second color, sections were stained with anti-MADCAM-1 (4 μg/ml) for 40 min at RT. The anti-MADCAM-1 mAb was detected with donkey anti-rat-IgG-Texas Red and 30 min incubation at RT.

For immunocytochemical identification of T and B lymphocytes, T cells were stained with a combination of anti-CD4 and anti-CD8 mAbs, whereas B cells were stained with anti-B220 mAb. The anti-CD4 and anti-CD8 mAbs were detected with a rabbit anti-rat IgG-FITC. The anti-B220 mAb was detected with a donkey anti-rat-IgG-Texas Red. Because all the primary mAbs are of rat origin, the same previously described procedure modifications (above) were used for ensuring accurate two-color fluorescence discrimination.

For the double staining of laminin and marginal metallophilic macrophages (MOMA-1 Ab), tissue sections were initially stained with MOMA-1 Ab. The MOMA-1 Ab was detected with a donkey anti-rat IgG (Fab<sub>2</sub>)-biotin conjugate and visualized with avidin-FITC. Then, a rabbit anti-mouse laminin Ab was applied and detected with donkey anti-rabbit-Texas Red conjugate. The concentrations and incubation time for each were as previously specified.

For the detection of IgM<sup>hi</sup>IgD<sup>low</sup> marginal zone B cells, the sections were stained with anti-IgD and detected with donkey anti-rat-IgG-Texas Red. After incubation with NRIGG (60 μg/ml) to block the unoccupied Ig-binding sites on the donkey anti-rat-IgG-Texas Red reagent, the sections were stained with anti-mouse IgM-FITC.

For the detection of germinal center cells, tissue sections were stained with biotin-conjugated peanut agglutinin 1/100 (Vector Laboratories) and detected with avidin-FITC 1/1000 (also from Vector Laboratories).

For the single-color detection of all other Abs, the specific primary Abs were applied and detected with appropriate FITC-conjugated secondary Abs.
the double arrows outline the margin of white pulp. Opposing arrows depict the outer and inner boundaries of the marginal sinus. Opposing arrowheads depict the outer and inner boundaries of the marginal zone. The L1 KO section has a fragmented and discontinuous pattern of laminin staining (right). MS, marginal sinus; MZ, marginal zone; RP, red pulp; WP, white pulp. Bar, 50 μm.

### Immunization and ELISA

The mice were immunized with 5 × 10⁸ SRBC by i.p. injection on day 0 and then rechallenged with 3 × 10⁸ SRBC i.p. on day 18. Sera were collected by tail vein bleed on days 7, 14, and 25.

Soluble SRBC proteins were extracted using 0.5% Triton X-100 in 300 mM NaCl, 50 mM Tris-Cl. The SRBC protein extract was coated onto polyvinyl chloride (PVC) microtiter plates (Costar, Cambridge, MA) at 20 μg/ml overnight at 4°C. The plates were then blocked with 5% BSA for 1 h at RT. Between steps, the plates were washed 5–6 times with PBS, 0.2% Tween 20 (PBS/T). Serum, 50 μl, diluted either 1:30 or 1:100 in PBS/T, was incubated for 1 h at RT. Mouse anti-SRBC protein Abs were detected with an alkaline phosphatase-conjugated rabbit anti-mouse IgM or IgG (Sigma) after rinsing out unbound mouse serum from the PVC microtiter wells with PBS/T. The alkaline phosphatase-conjugated rabbit anti-mouse IgM or IgG was then incubated for 1 h at RT at a dilution of 1:1000 in PBS/T. Colorimetric development was performed with the alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma) and was read at 405 nm in a microplate reader (Bio-Tek Instruments, Winooski, VT).

### Results

#### Matrix abnormalities in L1 knockout (KO) spleen

As a first step in analyzing L1 KO mice, we examined peripheral lymphoid organs at necropsy. LNs and Peyer’s patches were unremarkable (data not shown). Splenic architecture, on the other hand, was abnormal. Specifically, there was a striking and selective abnormality at the red-white pulp border.

To generate a view of the splenic white pulp framework, we performed immunofluorescence microscopy on frozen sections of mouse spleen. We stained the spleen sections with a polyclonal rabbit anti-mouse laminin Ab. Because laminin is a component of the reticular matrix of the spleen, it outlines the margins of the white pulp and vasculature (28). As shown in Fig. 1 (left), the laminin staining of wild-type spleen outlines the white pulp margins as a smooth and continuous line. Laminin staining also outlines some fine matrix material in the marginal zone. As a result, the border of the marginal zone (MZ, arrowheads) and marginal sinus (MS, opposing arrows) can be clearly distinguished (Fig. 1, left). By contrast, the laminin staining pattern of the L1 KO spleen reveals an irregular, fragmented, and discontinuous white pulp border. The marginal sinus and marginal zone appear to be malformed as well (Fig. 1, right).

#### Malformation of the splenic marginal sinus in L1 KO mice

To elucidate the nature of this matrix abnormality, spleens from both wild-type and L1 KO mice were embedded in plastic and analyzed by light microscopy (Fig. 2) and transmission electron microscopy (Fig. 3). In a normal spleen, a flattened layer of sinus lining cells can be traced along the margin of the white pulp. The sinus lining cells have ovoid nuclei and elongated slender processes connecting to adjacent lining cells, forming a continuous lining (Fig. 2, left, and Fig. 3, top). In Fig. 2 (left), the double arrows identify the white pulp border. For the most part, it is comprised of elongated slender cytoplasmic processes. A single arrow (Fig. 2, left) identifies the cell body of a marginal sinus lining cell. In Fig. 3 (top), arrows denote the cytoplasmic processes of two flattened reticular cells. The arrow adjacent to the letters SLC identifies a sinus lining cell. The other arrow, adjacent to RC, denotes a reticular cell defining the outer boundary of the marginal sinus.

In contrast, the splenic marginal sinus lining cells in L1 KO mice were difficult to find. Marginal sinus lining cells in L1 KO mice did not regularly contact other sinus lining cells to form a continuous white pulp border. Rather, they displayed an abnormal stellate, rather than a flattened, linear cell shape. Their cytoplasmic processes were shorter, oriented in random directions, and often...
discontinuous from the adjacent sinus lining cells. These features are readily apparent in Fig. 2 (right) and Fig. 3 (bottom). In Fig. 2, the red and white pulp can be readily distinguished by the location of pale blue-staining erythrocytes. Arrows (“SLC”) identify misshapen sinus lining cells, and a series of arrowheads outline the presumed white pulp border. In L1 KO mice, this border is often difficult to identify. The marginal sinus is absent. One of the stellate-shaped sinus lining cells is shown at higher magnification in Fig. 3 (bottom) (“SLC”).

Normally, the marginal sinus is delimited by sinus lining cells of the white pulp and the opposing reticular cells adjacent to marginal zone. Few RBC are found in the marginal sinus (Fig. 2, left, and Fig. 3, top). However, in the L1 KO spleen, this anatomic distinction is absent. The reticular cells of the marginal zone are rare, and the marginal sinus is no longer distinguishable as a structure distinct from the marginal zone. As a result, RBC migrate right up to the white pulp border (Fig. 2, right, and Fig. 3, bottom). These data demonstrate that the splenic marginal sinus in L1 KO mice is abnormal. Moreover, the cellular abnormality spatially correlates with the abnormal pattern of laminin immunofluorescence staining as previously shown in Fig. 1.

L1 expression on sinus lining cells

We then examined whether L1 expression in wild-type mice spatially correlates with the abnormally formed marginal sinus lining cells in L1 KO mice. We expect that if the absence of L1 is the cause of the structural abnormality, then those cells at the white pulp border will likely express L1 under normal circumstances. By examining the splenic structure of normal BALB/cByJ mice, we localized L1 expression to the edge of the white pulp by immunofluorescence staining using an L1 mAb. L1" lining cells are present in the periphery of the white pulp, at the same approximate location as the marginal sinus (Fig. 4). We sometimes found that L1" staining almost completely circumscribed the entire white pulp (Fig. 4, left). However, most of the time, only part of the white pulp margin was stained (Fig. 4, right, and Fig. 5, top). L1 staining is denoted by double arrows at the white pulp periphery. We also noted intense staining around the central artery in the center of the white pulp. Two-color staining for tyrosine hydroxylase demonstrated that the periarteriolar staining was due to the expression of L1 by sympathetic neurons innervating the spleen (data not shown). However, such neurons did not innervate the marginal sinus. In addition, lymphocytes within the white pulp also weakly expressed L1 (Fig. 4, left). Low levels of lymphocyte L1 expression have been described previously (17).

The area of the marginal sinus/marginal zone contains a variety of specialized cell types. To pin down the precise location of L1 expression within this area, we performed colocalization studies using two-color immunofluorescence. L1 was stained using fluorescein while other cell type-specific markers were stained with Texas Red. Optical filters were used that do not allow spillover into the other respective color. Because the sinus lining cells of the
splenic marginal sinus are described to express MAdCAM-1 (29), we compared the localization of L1 and MAdCAM-1. Fig. 5 shows the staining pattern of L1, MAdCAM-1, and a two-color overlay of both (top, middle, and bottom, respectively). As seen in the bottom panel, L1 and MAdCAM-1 colocalize along the margin of the white pulp, indicating that the L1+ cells at the margin of white pulp are sinus lining cells. Moreover, these L1+ sinus lining cells sometimes expressed high levels of vimentin (data not shown), often associated with a mesenchymal, probably fibroblast-like origin (30). Marginal metallophilic macrophages (MMM) that express MOMA-1, situated at the margin of white pulp, did not express L1 (data not shown). A separate macrophage population in the marginal zone and red pulp areas that expresses CD11b also did not express L1 (data not shown).

These results indicate that L1 is expressed by MAdCAM-1+ sinus lining cells. Moreover, the location of L1 expression in wild type mice correlates with the location of the structural abnormalities (marginal sinus) in L1 KO mice.

**Cellular analysis of L1 KO spleens**

We then examined whether cell populations inside the white pulp or around the marginal sinus were affected by the malformation in the marginal sinus and sinus lining cells in L1 KO mice. The individual cell types were identified with cell type-specific Abs, either in situ or by flow cytometry. Because the abnormalities we found were concentrated in the marginal sinus and marginal zone, we examined two different cell populations known to inhabit this microanatomic region. Namely, we determined whether MMMs and marginal zone B lymphocytes were present and, if so, whether they are located in their normal anatomic location in L1 KO mice. As previously mentioned, there is a rim of MMMs situated at the margin of the white pulp, in the marginal zone. MMMs in L1 KO mice were examined by double staining of splenic sections with anti-laminin and MOMA-1 (Fig. 6, A and B). The laminin counterstain helps identify the white pulp border. In contrast to the wild-type mice (and as previously described), L1 KO mice display an irregular, poorly defined white pulp border (Fig. 6, A and B). Nonetheless (and similar to wild-type mice), L1KO mice demonstrate a rim of MMMs located along the white pulp border (Fig. 6, A and B). This finding suggests that L1 is not solely responsible for...
their localization in the marginal zone. Similarly, the location and the approximate number (as estimated using immunofluorescence microscopy) of IgM\(^{high}\)IgD\(^{low}\) marginal zone B cells in L1 KO mice were comparable with those of wild-type mice (Fig. 6, C and D). These cells are those that stain bright green (IgM) along the edge of the follicle in Fig. 6, C and D. B lymphocytes that coexpress high levels of IgM and IgD are stained orange, from the combination of green and red. These results indicate that cell subsets associated with the marginal zone are not noticeably affected by the splenic structural abnormality in L1 KO mice.

Table I summarizes our findings regarding follicle development and segregation of T/B lymphocytes in L1 KO mice. We find these parameters to be indistinguishable from wild-type mice. Follicular dendritic cells (FDC) and interdigitating dendritic cells were also present in their normal locations (B and T cell zones, respectively) and approximate number, as estimated visually. After immunization, germinal centers (highlighted using a peanut agglutinin-biotin conjugate) also developed normally in L1 KO spleens (data summarized in Table I). Flow cytometric analysis also showed that the major T and B lymphocyte subsets in the L1 KO spleen were present in proportions comparable with those of wild-type littermates (Table I).

**Immune response of L1 KO mice**

To test whether the immune response is impaired in L1 KO mice, we injected L1 KO mice and littermate controls with SRBC i.p. on days 0 and 18. The anti-SRBC IgM and IgG immune responses were measured by ELISA using extracted soluble SRBC protein as Ag. As shown in Fig. 7, there is no significant difference in the IgM and IgG titers of both primary and secondary responses. This result indicates that L1 is not required for a T cell-dependent immune response.

**Discussion**

In this report, we investigate the role of L1 in the development of normal splenic structure using L1-deficient mice. Using these mice, we were able to analyze the role of L1 in lymphoid organ development over longer time periods than otherwise possible using L1-specific mAbs. Therefore, these data provide a molecular/structural correlation, implicating L1 in the development of the white pulp border. Specifically, we conclude that L1 serves a critical and highly selective role in the development of the splenic marginal sinus. These data help define a poorly characterized sinusoidal boundary that defines the border of the splenic white pulp, and across which mononuclear cells transmigrate to enter the white pulp.

Our most important finding is that L1 serves an important role in the structural integrity of this microanatomic region. Specifically, L1 is expressed on the flattened sinus lining cells at the white pulp border. The congenital absence of L1 results in nearly a complete absence of the marginal sinus and disorganization of RCs in the marginal zone. There were obvious gaps in the white pulp boundary, as discerned by light and electron microscopy. Nonetheless, the white pulp mononuclear cells still maintained their cohesion to each other and the ability to exclude other blood cellular elements, such as erythrocytes and granulocytes. Although the boundary was damaged, there was still a distinction between the white and red pulp in L1 KO mice. These observations lead us to conclude that other factors besides the structural integrity of the boundary also regulate cellular traffic into the white pulp.

The marginal zone, and possibly the marginal sinus, are the major sites of termination for the branches of central arteriole (4–6). Consequently, an important function of this region is to selectively channel certain cell types into the white pulp. Namely, the direction of blood flow is toward the red pulp, as indicated by the left-facing arrow (Fig. 8). Representative blood cells such as erythrocytes and granulocytes are abundantly found in the red pulp and marginal zone. Selected mononuclear cells, on the other hand, migrate into the white pulp. As a result, the marginal sinus (the area immediately adjacent to the white pulp) is rich in mononuclear cells but has few other blood cells, such as erythrocytes or granulocytes. The mechanisms underlying this selective migration are poorly understood. One possibility is that most blood cells are passively steered along with the flow of blood toward the splenic red pulp. To enter the splenic white pulp, mononuclear cells probably must actively locomote, possibly under the influence of chemoattractant agents such as chemokines. If this hypothesis is correct, then partial defects in the reticular lining of the splenic white pulp might have minimal effect on the coalescence of mononuclear cells forming a white pulp.

What role does L1 serve in the development and maintenance of the normal integrity of the marginal sinus lining? We envision two likely possibilities. First, L1 may be involved in the adhesion of sinus lining cells to their associated reticular fibers. Laminin is a component of the extracellular matrix at the white pulp periphery (28) and is a known ligand for L1 (31). Second, L1 may be involved in the intercellular interaction between adjacent sinus lining cells. This interaction may be mediated by a homotypic interaction of L1 on adjacent sinus lining cells.

The biological significance of marginal sinus lining cells lies in the fact that it is the final physical barrier that mononuclear cells

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\(^a\) Single-color or two-color immunofluorescence microscopy were performed on splenic sections of both L1 KO mice and wild-type littermate controls. The specific cell subsets were stained with cell type-specific Abs as described in Materials and Methods. For all subsets, “normal” refers to both approximate quantity and tissue location.

\(^b\) For flow cytometric analysis, splenocytes from three L1 KO mice and three wild-type littermate controls (in C57BL/6 background) were stained with fluorochrome-conjugated mAbs. The numbers shown represent the mean ± SD.
must traverse before entering the white pulp (32). This “transmi-
gration” across the white pulp sinusoidal border is as yet poorly
characterized. Certain features suggest that novel mechanisms, dis-

tinct from those in blood vascular transmigration, are involved. For
example, the shear force and blood flow velocity in the marginal
sinus may be lower than those in postcapillary venules. This sup-
position is based on the fact that the marginal sinus and marginal
zone are relatively open spaces as compared with a postcapillary
venule. Consequently, it is likely that the initial rolling step, ne-

gotiating initial contact between a leukocyte and endothelium, is
absent in the marginal sinus.

Fig. 8 is a schematic representation of the microanatomy at
the red-white pulp border of wild-type mice. The white and red pulp
boundaries, marginal sinus, and marginal zone are delineated by
flattened, elongated reticular cells. These reticular cells comprise
the marginal sinus lining cells and reticular cells of the marginal
zone. They are distinct from endothelium, because they do not
assume a tall, activated morphology and do not express CD31
(platelet endothelial cell adhesion molecule-1) (PECAM-1), a
marker for endothelial cells. Also present at the red-white pulp
border are some rather unique cell types, including MMMs, a sub-
set of B lymphocytes expressing the IgM<sub>high</sub>IgD<sub>low</sub> immuno-

phenotype, and a subset of marginal zone macrophages. Of minor note
is that we observed the MMMs to be located just outside the white
pulp boundary. A previous description of MMMs placed them just
inside the white pulp boundary (5). This location was discerned
using two-color immunofluorescence; MOMA-1 Abs identified
MMMs whereas laminin identified the extracellular matrix of the
white pulp boundary.

Apart from L1, the only other well-characterized adhesion mol-

ecule expressed by marginal sinus lining cells is MAdCAM-1 (29).
MAdCAM-1 is also an Ig superfamily adhesion molecule. It is
expressed by mucosal venules and is responsible for the homing of
lymphocytes to Peyer’s patches and the intestinal lamina propria
(33, 34). Its function on marginal sinus lining cells is not known.
In this regard, L1 is the only adhesion molecule that is demon-
strated to directly serve a function in the development and pres-
ervation of the splenic white pulp boundary.

In this paper, we describe that L1 expression on marginal sinus
lining cells is somewhat heterogeneous. Namely, marginal sinus
lining cells do not express L1 all the time. We believe that L1 is
temporally regulated, in response to as yet unidentified signals.
One of these signals may the cytokine TNF-α. Pancook et al. (18)
identified TNF-α as capable of up-regulating L1 expression on
human dendritic cells. In addition, our previously published find-
ings suggest a temporal functional role for L1. We previously de-
scribed our findings that L1 antagonism (with an L1 mAb) dis-
rupted lymph node sinusoidal architecture, but only in LN

FIGURE 7. Immune response of L1 KO mice to SRBC. Four male L1
KO mice and their littermate controls (in a C57BL/6 × 129 background)
were immunized with 5 × 10<sup>8</sup> SRBC i.p. on day 0 and rechallenged with
3 × 10<sup>6</sup> SRBC i.p. on day 18. Sera were collected at days 7, 14, and 25.
The titers of anti-SRBC IgM (A) and anti-SRBC IgG (B) were determined
by ELISA. Mean ± SE is represented. For both IgM and IgG titers, there
are no statistically significant differences between the L1 KO mice and
littermate controls.

FIGURE 8. Schematic representation of mi-

croanatomy of white-red pulp border. PMN,
polymorphonuclear leukocyte.
undergoing remodeling after immune stimulation (24). Static, quiescent LN were not affected. Thus, L1 appears to be important during certain processes, such as matrix remodeling. L1 expression may therefore be temporally regulated, in response to immunological or inflammatory stimuli. We speculate that the heterogeneity in L1 expression among different marginal sinus lining cells may simply be due to variations in the basal rate of splenic white pulp remodeling.

The pattern of L1 expression on sinusoids that border lymphoid parenchyma suggests an analogous role to PECAM-1 (CD31) expression on vascular endothelium. Both interact in a homo- and heterotypic fashion, are expressed at low levels on hematopoietically derived cells, and are both in the Ig superfamily. PECAM-1 serves a role in the development and maintenance of vascular endothelium and leukocyte trafficking across vascular boundaries (35). Based on these similarities, it is possible that L1 may serve a similar role for mononuclear cell trafficking into the white pulp.

We found surprisingly little functional consequence of the structural abnormality in L1 KO mice. All major cell populations inside the white pulp and around the marginal sinus appeared to be present in normal proportions. Immune responses to SRBC also indicated that L1 was not required for a T-dependent immune response. Immune responses to a soluble Ag, keyhole limpet hemocyanin, were also not appreciably different in L1 KO mice (data not shown). The absence of clear functional consequences suggests that either 1) the structural abnormalities were not sufficiently severe so as to cause functional deficits or 2) humoral immune responses are not a sensitive indicator of abnormalities in the marginal sinuses.

In contrast to our studies in mice, the existence of the marginal sinus in the human spleen is controversial. For example, van Krieken et al. (36) reported finding no evidence for the presence of a human splenic marginal sinus. Schmidt et al. (37), on the other hand, used corrosion cast scanning electron microscopy to clearly demonstrate the presence of a marginal sinus in the human spleen. Interestingly, Steiniger et al. (38) also failed to find a human marginal sinus. They attributed the findings of Schmidt et al. to capillaries in the “peri follicular zone,” a zone that they claimed to be located between the marginal zone and the red pulp (38). In light of these contradictory conclusions over the very existence of a human splenic marginal sinus, it is not surprising that the function of the marginal sinus remains unclear. The identification of a new cell surface adhesion marker (L1) on marginal sinus lining cells may ultimately help contribute toward an understanding of the function of marginal sinus lining cells and thereby clarify this mystery.

Throughout our investigation, we noticed a varying degree of splenic structural abnormalities in L1 KO mice. Similar variability has been noticed in CNS abnormalities of L1 KO mice. Consistent with the previous report (8), the genetic background of L1 KO mice influences the resulting phenotype. The C57BL/6 background yielded few viable L1 KO offspring. However, the few L1 KO mice that were born in the C57BL/6 background demonstrated the most severe phenotypic abnormalities. By contrast, backcrossing of L1 female heterozygotes to a C57BL/6 × 129 F1 background yielded relatively normal proportions of L1 KO male offspring. However, we found few abnormalities in the lymphoid systems of these (C57BL/6 × 129 F1 background) L1 KO mice. These findings are reminiscent of the broad range of phenotypic abnormalities found in human genetic diseases involving L1 mutations (9, 16). The system is therefore probably multigenic, with at least two functionally redundant proteins. We hypothesize that other adhesion molecules may also likely contribute to the normal integrity of the structural features we described. Presumably, different strains of mice may potentially express varying levels of functionally redundant molecules. Without such redundancy, the L1-null genotype is lethal or nearly so. With increasing levels of redundant molecule(s), the L1-null genotype has little to no phenotypic effect. There is ample precedent for such functional redundancy, such as among the selectins in mediating initial contact between leukocytes and vascular endothelium.

We previously reported that administration of an L1 mAb during an immune response disrupted the normal remodeling of the fibroblastic reticular system in LNs. In these L1 KO mice, we did not find any obvious abnormalities in LNs. This discrepancy might be due to functional compensation by other, as yet unidentified, L1-like molecules in LNs of L1 KO mice. Several L1 homologues have been identified in the nervous system (9, 39, 40), but their expression outside the nervous system is largely unexplored. Moreover, our previous report utilized a distinct model involving acute hypertrophic responses after immunization in normal mice. It is possible that the sudden disruption of L1 function by L1-specific Abs in a normal mouse may have greater structural consequences than congenital absence of L1.

Several genetically targeted mutant strains of mice, notably the family of lymphotoxin-α, lymphotoxin-β, TNF, and their receptors, have been reported to have structural abnormalities in peripheral lymphoid organs (41). For example, the lymphotoxin-α KO mice developed structural abnormalities of the spleen, and a complete absence of LNs and Peyer’s patches (42). The TNF KO mice had a decreased number of Peyer’s patches and a defect in FDC development (43). We have observed that TNF and TNF receptor I/II KO mice have a similar irregularity of the white pulp border as that of L1 KO mice (data not shown). As expected, these mice also show an abnormal distribution of L1+ cells at the white pulp border. A naturally mutant mouse strain, aly/aly, also demonstrated severe developmental abnormalities in all peripheral lymphoid organs (44). Of particular interest, aly/aly mutant mice also had developmental abnormalities in the splenic marginal sinus (45). However, unlike all these other mutant strains, our L1 deficient mice had a selective defect on splenic marginal sinus development; no other structures were affected.

In summary, our findings indicate that L1 serves a crucial role in the proper development of the architecture at the white pulp border. The most notable defect resulting from this improper formation of white pulp lining was the near complete absence of the marginal sinus. These structural abnormalities correlate with the location of L1 expression in normal mice. By characterizing the molecular and functional features of the white pulp lining, we believe it may help shed light on the function of the splenic marginal sinus.

References


