

Original Article

# Expression of mucosal addressin cell adhesion molecule-1 on the reticular framework between white pulp and the marginal zone in the human spleen

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The antigenic heterogeneity of the reticular framework of the white pulp and marginal zone is well documented in the human adult spleen. Immunostaining of  $\alpha$ -smooth muscle actin characterizes the heterogeneity of the reticular framework of the white pulp and marginal zone. In the human spleen, the blood cells flow in an open circulation. T and B lymphocytes flow out from the arterial terminal, and migrate in the reticular framework. Homing of lymphocytes to lymphoid tissues is regulated by selective interactions between cell surface homing receptors and tissue vascular addressins at sites of lymphocyte recruitment from the blood. In the present study, mucosal addressin cell adhesion molecule-1 was selectively expressed on  $\alpha$ -smooth muscle actin-positive reticular framework. The reticular framework may function in lymphocyte homing and segregation into the periarteriolar lymphoid sheath, lymph follicle and marginal zone.

**Keywords:** human spleen; white pulp; marginal zone; reticular framework; MAdCAM-1

## INTRODUCTION

The spleen is a complex filter interposed in the blood stream and plays an important role in immune defense against blood-borne antigens. In the spleen, the white pulp (WP) and marginal zone (MZ) function as the immunological active compartment. The WP consists of the periarteriolar lymphoid sheath (PALS) and the lymph follicle (LF). The PALS is a thymus-dependent area in which T lymphocytes predominate.<sup>1,2</sup> Although the LF and MZ are B lymphocyte areas,<sup>1,3</sup> the cellular composition of the MZ comprises intermediate-sized B lymphocytes, and the immunophenotype of these lymphocytes is different from that of the B lymphocytes in the mantle zone of the LF.<sup>3-6</sup> Reticulum cells and reticulin fibers form the basic reticular framework of the WP and MZ.<sup>7</sup> A distinct microenvironment is formed in the reticular framework of the WP and MZ. Among several stromal cells in the lymphoid tissues, the dendritic cells are thought to provide a microenvironment specific for lymphoid subclasses. Interdigitating cells (IDCs) and follicular dendritic cells (FDCs) are found in the T and B lymphocyte areas, respectively.<sup>8,9</sup> The spleens of humans and rodents have an open circulation.<sup>10-12</sup> The splenic arteries terminate in the splenic cord and the MZ, and blood flows out from the arterial terminal. During intrasplenic migration, T and B

lymphocytes are segregated and selectively sorted in the reticular framework of the PALS, LF and MZ according to the respective distinct microenvironments. Dendritic cells play a role in the homing mechanisms of T and B lymphocytes in each area.

A heterogeneous population of reticulum cells was observed immunohistochemically in the WP of rat and mouse spleens.<sup>13,14</sup> The distribution of extracellular matrix protein in the reticular framework is also heterogeneous in the T- and B-lymphocyte areas of the WP.<sup>15</sup> Our previous reports revealed that the reticular framework of the PALS, LF and MZ in the human adult spleen is specialized into heterogeneous components as in the rat and mouse spleen, and immunostaining of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) characterizes the heterogeneity of the reticular framework of the WP and MZ.<sup>7,16</sup> In the human fetal spleen, the reticular framework of the PALS, LF and MZ is also heterogeneous, and the development of the heterogeneity is related to the ontogeny of the PALS, LF and MZ.<sup>16</sup> The heterogeneity of the reticular framework of the PALS, LF and MZ may induce the segregation of the lymphoid subclasses.

Lymphocyte homing to lymphoid tissues is regulated by selective interactions between cell surface homing receptors and tissue vascular addressins at sites of lymphocyte recruitment from the blood. Mucosal addressin cell adhesion

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molecule 1 (MAdCAM-1) regulates lymphocyte homing to gut-associated lymphoid tissue (GALT) and is expressed on endothelial cells in GALT. Recent studies reported expression of MAdCAM-1 in the spleen. The reticular mesh localized between the MZ and WP expresses MAdCAM-1 in the mouse and human spleens, and plays essential roles in lymphocytic homing and the compartmentalization mechanism in the WP.<sup>17,18</sup>

In the present study, human spleens were examined using immunohistochemistry, electron microscopy and confocal laser scanning microscopy. The aim of the present study was to investigate the expression and localization of MAdCAM-1 on the reticular framework with special reference to the heterogeneity of the reticular framework.

## MATERIALS AND METHODS

### Spleen specimens

Eight surgically resected human spleens were obtained from splenectomies for gastric cancer (5 cases), hemangioma (1 case), traumatic rupture (1 case) and hereditary spherocytosis (1 case). In the case of hemangioma, non-tumorous regions were examined. The patient ranged from 15 to 77 years old. The materials were fixed in 10% buffered formalin solution for two days. Tissue blocks were cut from the fixed materials, dehydrated in a graded ethanol series, and then embedded in paraffin. Serial sections were prepared for hematoxylin-eosin, silver staining (Gomori's method) and immunohistochemical examination. Tissue blocks were also embedded in Tissue-Tek OCT Compound (Sakura Finetek Japan, Tokyo, Japan), snap-frozen in liquid nitrogen-cooled isopentane and stored at -80 °C for immunohistochemistry.

### Electron microscopy

For transmission electron microscopy, tissue blocks, measuring 1 x 1 x 1 mm, were prepared from the spleens. They were fixed in 1% glutaraldehyde solution. After postfixation in 1% osmium tetroxide solution and dehydration in a graded

ethanol series, they were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, then examined using a transmission electron microscope (H7650, Hitachi Ltd., Tokyo, Japan).

### Immunohistochemistry

The antibodies used in the present study are summarized in Table 1. Commercially available staining kits (Histofine SAB-PO and SAB-AP, Nichirei, Tokyo; Dako APAAP kit, Dako, Denmark) were used. An antigen retrieval method was applied in the immunohistochemistry protocol.<sup>19</sup>

**Paraffin sections:** After deparaffinization, the sections were immersed in methanol-H<sub>2</sub>O<sub>2</sub> solution for 30 min to block endogenous peroxidase activity. Non-specific binding of antibodies was blocked by incubation with normal goat or rabbit serum for 15 min at room temperature. The sections were incubated with primary antibodies overnight, and then treated using the streptavidin biotin complex (SABC) method. Each step was followed by repeated washing in phosphate-buffered saline (PBS) adjusted to pH 7.4. Coloration was developed in 3-amino-9-ethylcarbazole solution. The sections were counterstained with hematoxylin.

For double immunostaining, the first antigen was visualized in brown as described above. After washing with PBS, the alkaline phosphatase anti-alkaline phosphatase (APAAP) method was applied to the second antigen, which was visualized in blue with the aid of alkaline phosphatase by incubating sections in 1 mg/ml of naphthol AS phosphate (Sigma, St. Louis, MO), 1 mg/ml of Fast blue BB salt (Sigma) and 1 mM Levamisole (Sigma) in 0.05 M propandiole buffer, pH 9.75.

**Cryostat sections:** Six-micrometer-thick sections were mounted on neoprene-coated slides, dried at room temperature and fixed in acetone at 4 °C for 10 min. Sections were then immunostained in the same manner as for paraffin sections.

**Confocal laser scanning microscopy:** For immunofluorescent double staining, acetone-fixed cryostat sections were prepared and immunostained using the SABC method. The

**Table 1.** Antibodies used for immunohistochemistry

Antibodies	Reactivity	Working dilution	Antigen retrieval*	Source
CD45RO (UCHL1)	T lymphocytes	1:100	-	DakoCytomation, Glostrup, Denmark
CD20 (L26)	B lymphocytes	1:100	-	DakoCytomation, Glostrup, Denmark
Ki-M4P	Follicular dendritic cells	1:1,000	PK	Prof. H. J. Radzun, Goettingen, Germany
$\alpha$ -smooth muscle actin	$\alpha$ -smooth muscle isoform of actin	1:100	-	DakoCytomation, Glostrup, Denmark
Type IV collagen	Type IV collagen	1:100	H	DakoCytomation, Glostrup, Denmark
Laminin	Laminin	1:100	H	DakoCytomation, Glostrup, Denmark
Fibronectin	Fibronectin	1:100	H	DakoCytomation, Glostrup, Denmark
Tenascin	Tenascin	2 mg/ml	-	LAB VISION, Westinghouse Drive Fremont, CA, USA
Podoplanin	Podoplanin	1:100	H	Dako Cytomation, Glostrup, Denmark
MAdCAM-1	MAdCAM-1	1:400	-	Dr. H. Ohtani, Dept. of Pathology, Tohoku University Graduate School of Medicine

\*PK; proteinase K, H; heat treatment

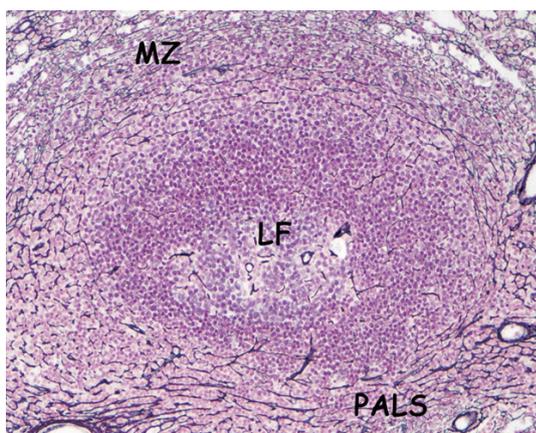
first antigen was labeled with FITC-conjugated streptavidine (Dako) and the second antigen with Texas Red-conjugated streptavidine (Vector Lab., Burlingame, CA, USA). The slides were imaged using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany).

**Immunoelectron microscopy:** The procedure for pre-embedding labeling was used as described previously, with some modifications. Cryostat sections were incubated with a fixative containing 1% glutaraldehyde and 4% paraformaldehyde in Tris-buffered saline (TBS). After blocking with 10% normal goat serum in TBS, the sections were incubated with the primary antibody for 24 h at 4°C and followed by colloidal gold-conjugated secondary antibody for 2 h at room temperature. After washing with TBS, immunolabelled sections were post-fixed with 1% glutaraldehyde and 1% osmium tetroxide solution, dehydrated in a graded ethanol series and embedded in Epon. Ultrathin sections were stained with uranyl citrate and examined using an electron microscope (H7650, Hitachi Ltd., Tokyo).

## RESULTS

### Reticular framework of the WP and MZ

On the sections treated by silver impregnation, the basic reticular framework of the WP and MZ was well observed in all cases (Fig. 1). However, the number and spatial orientation of reticulin fibers differed between the WP and MZ. In the PALS, several layers of reticulin fibers ran parallel to the central artery, which exhibited a stratiform pattern on longitudinal sections and circumferential pattern on cross-sections. The distribution of reticulin fibers was sparse in the LF. A few layers of reticulin fibers bordered the LF and MZ. The fine reticulin fibers were anastomosing and forming a mesh structure in the MZ.



**Fig. 1.** Silver impregnation of the reticular framework in the WP and MZ

In the PALS, several layers of reticulin fibers run parallel to the central artery, and exhibit a stratiform pattern on longitudinal sections. In the LF, the distribution of reticulin fibers is sparse. A few layers of reticulin fibers border the LF and MZ. The fine reticulin fibers are anastomosing and form a mesh structure in the MZ. x 100.

### Immunohistochemistry of the reticular framework

The WP was clearly bordered by  $\alpha$ -SMA-positive reticulum cells (Fig. 2a, b). They were connected by their cytoplasmic processes in places. At the border of the LF and MZ, a few layers of  $\alpha$ -SMA-positive reticulum cells were found. The distribution of  $\alpha$ -SMA-positive reticulum cells in the WP was unique and characteristic. Double immunostaining of  $\alpha$ -SMA and UCHL-1 or L26 revealed that  $\alpha$ -SMA-positive reticulum cells were selectively distributed in the PALS and formed its reticular framework (Fig. 2a). At the perifollicular region, T-lymphocytes were found between  $\alpha$ -SMA-positive reticulum cells. Silver impregnation and immunostaining of the  $\alpha$ -SMA revealed that  $\alpha$ -SMA-positive reticulum cells ensheathed the reticulin fibers (Fig. 2c).

In the LF,  $\alpha$ -SMA-positive reticulum cells were not found (Fig. 2b). The mesh of FDCs was observed in the germinal center (Fig. 2d). In places, the reticulin fibers were involved in the mesh of the FDCs and encased by the cytoplasm of the FDCs (Fig. 2e). The reticulin fibers of the WP and MZ were immunostained for type IV collagen, laminin and fibronectin. However, tenascin was found in reticulin fibers of the PALS and MZ (Fig. 2f).

In the MZ, a mesh structure was formed by  $\alpha$ -SMA-positive reticulum cells (Fig. 2a, b), which covered the fine reticulin fibers.

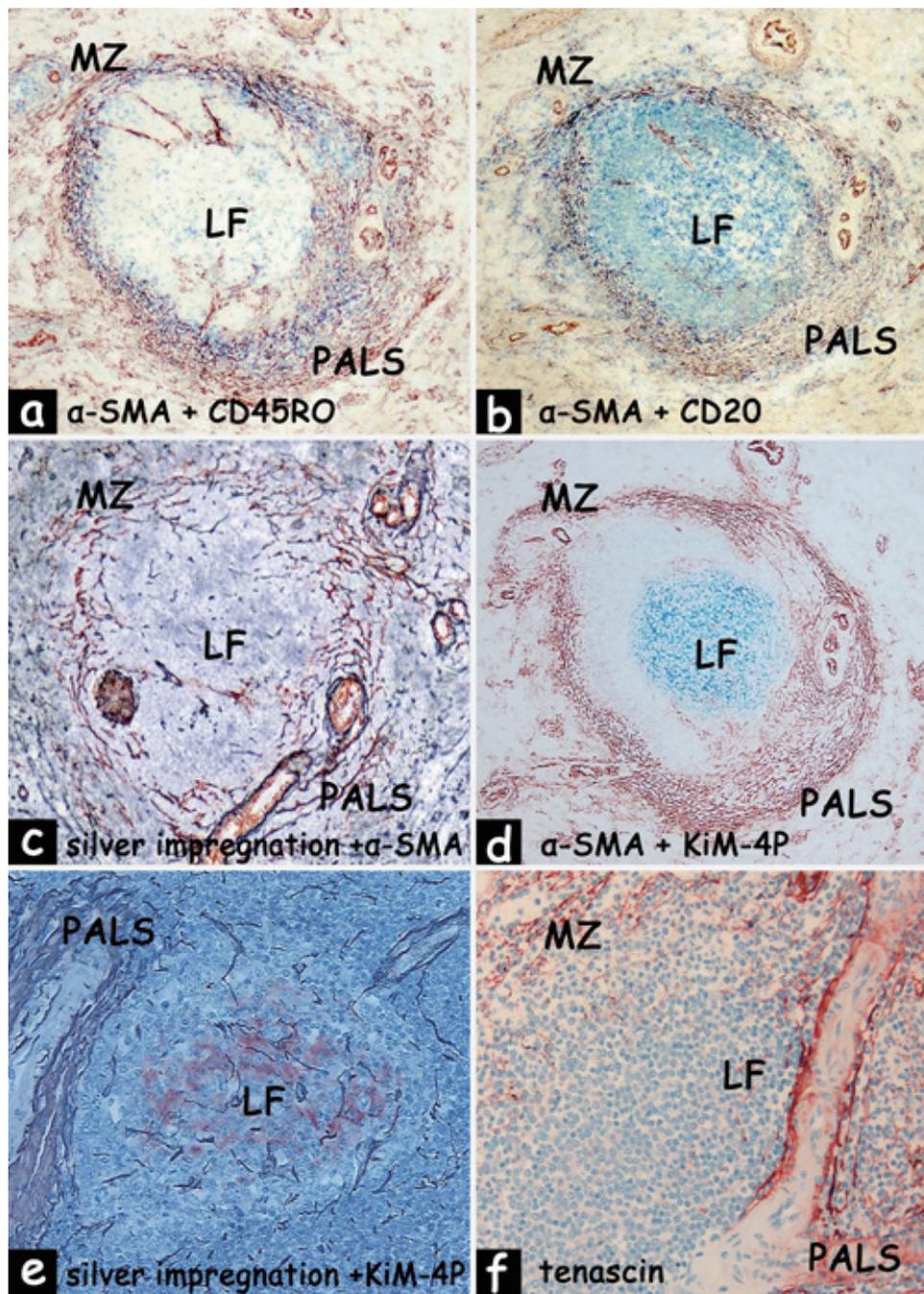
Immunostaining of podoplanin revealed the lymphatic vessels accompanying the central arteries of the PALS. Reticulum cells in the WP and MZ were podoplanin-negative.

### Electron microscopy

The number and spatial orientation of the reticulin fibers were different from those on light microscopy. The framework of the WP was formed by reticulum cells and reticulin fibers. In the PALS, the cytoplasm of the reticulum cells ensheathed reticulin fibers (Fig. 3a, b), although some reticulin fibers were not enclosed. Reticulum cells had bundles of microfilaments with dense bodies. In the LF, reticulin fibers were distributed sparsely. FDCs elongated their slender cytoplasmic processes among the lymphocytes and covered the reticulin fibers. In the MZ, reticulum cells and reticulin fibers formed the mesh structure (Fig. 4a, b). The reticulum cells encased the fine reticulin fibers. The cytoplasm of the reticulum cells contained bundles of microfilaments with dense bodies and connected with other reticulum cells. An intermediate junction was observed between their cytoplasm.

### Expression and localization of MAdCAM-1

Expression of MAdCAM-1 was observed in the PALS, and at the border of the LF and MZ (Fig. 5a). Double immunostaining of MAdCAM-1 and Ki-M4P revealed no expression of MAdCAM-1 at the mesh of FDCs in the LF (Fig. 5b). Confocal laser scanning microscopy demonstrated MAdCAM-1 expression on the  $\alpha$ -SMA-positive reticular framework, which was localized at the surface of  $\alpha$ -SMA-positive reticulum cells (Fig. 6). On immunoelectron microscopy,

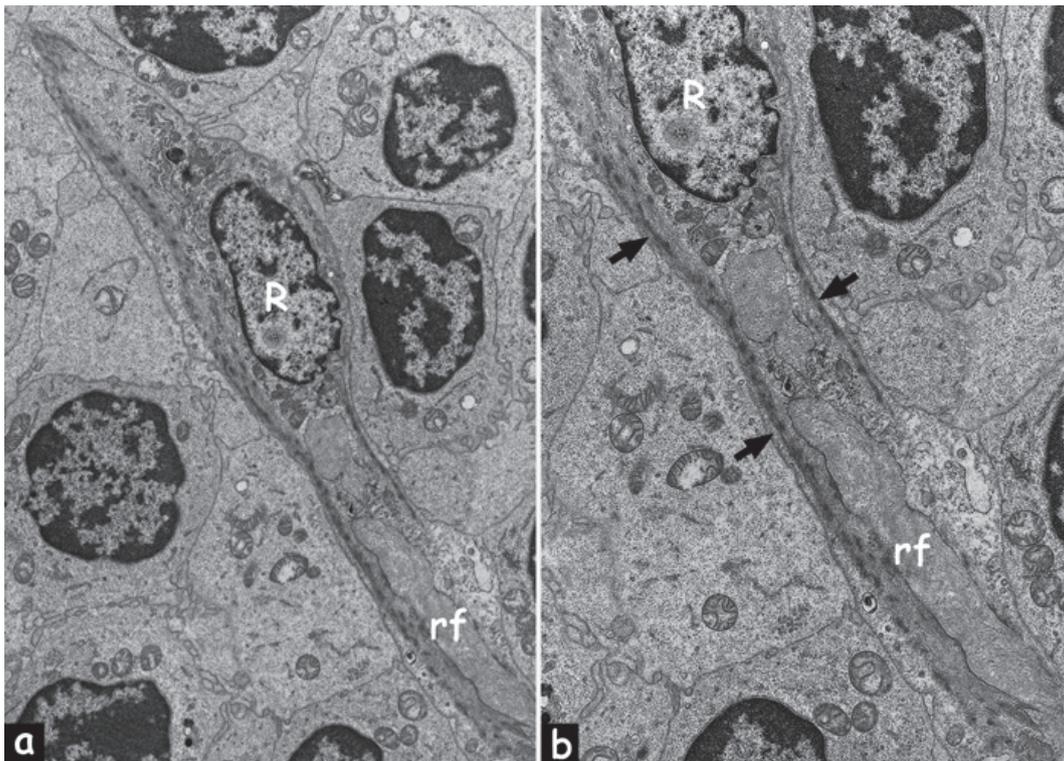


**Fig. 2.** Immunohistochemistry  
*a* Double immunostaining of  $\alpha$ -SMA (brown) and CD45RO (blue).  $\alpha$ -SMA-positive reticulum cells form the reticular framework of the PALS. x 50.  
*b* Double immunostaining of  $\alpha$ -SMA (brown) and CD20 (blue).  $\alpha$ -SMA-positive reticulum cells are not found in the LF. x 50.  
*c* Silver impregnation and immunostaining of  $\alpha$ -SMA.  $\alpha$ -SMA-positive reticulum cells ensheath the reticulin fibers. x 100.  
*d* Double immunostaining of  $\alpha$ -SMA (brown) and KiM-4P (blue). A mesh of FDCs was found in the germinal center. x 50.  
*e* Silver impregnation and immunostaining of Ki-M4P. The reticulin fibers were involved in the mesh of the FDCs and encased by the cytoplasm of the FDCs. x 100.  
*f* Immunostaining of tenascin. Tenascin was found in reticulin fibers of the PALS and MZ. x 200.

immuno-gold labeling for MAcCAM-1 was predominantly on the plasma membrane of reticulum cells (Fig. 7).

## DISCUSSION

In the lymphatic tissues, reticulum cells and reticulin fibers form the basic reticular framework that supports the



**Fig. 3.** Ultrastructure of the reticulum cell in the PALS  
**a** The cytoplasm of the reticulum cell (R) ensheathes the reticulin fibers (rf). x 3000.  
**b** The reticulum cell (R) has bundles of microfilaments with dense bodies (arrows). x 5000.

tissue structure. Recent studies reported that the reticular framework is heterogeneous in T and B lymphocyte areas of the WP and MZ.<sup>7,13,14</sup> Yoshida *et al.* confirmed the antigenic heterogeneity of the reticular framework of the PALS and LF in the mouse spleen.<sup>14</sup> In the present study, the reticular framework of the PALS was formed by  $\alpha$ -SMA-positive reticulum cells. Immunoreactivity to  $\alpha$ -SMA and ultrastructural findings demonstrated myofibroblastic differentiation of the reticulum cells in the PALS. In the LF, reticulum cells were  $\alpha$ -SMA-negative and the mesh of FDCs involved the reticular framework of the germinal center. FDCs may play a role in the formation of the reticular framework of the germinal center of the LF.

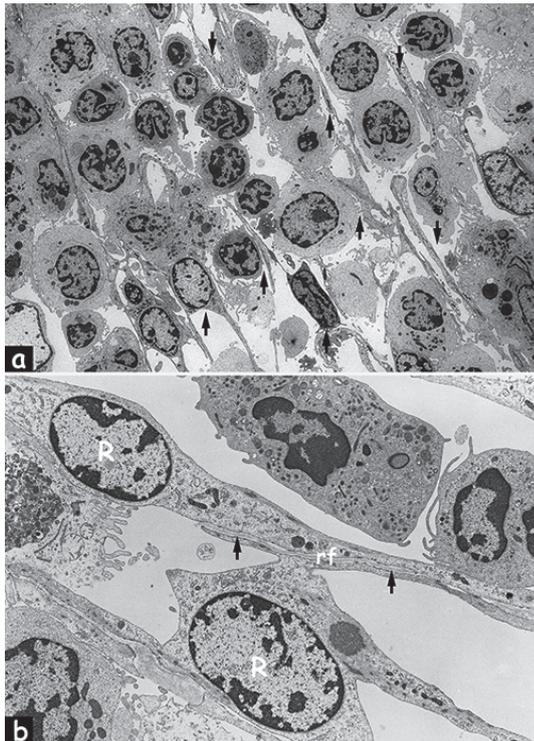
The MZ is a discrete compartment and functions as an immunological filter where blood-borne antigens are trapped.<sup>20,21</sup> The major cell population of the MZ is medium-sized B lymphocytes.<sup>5</sup> Their immunophenotype is CD23-, CD27+, KiB3-, IgD-, and enzyme histochemistry revealed them to have alkaline phosphatase activity.<sup>3-6</sup> The immunophenotype and enzyme phenotype distinguish MZ B lymphocytes from the B lymphocytes in the mantle zone of the LF.<sup>3-6</sup> The reticulum cells in the MZ were  $\alpha$ -SMA-positive and their immunohistochemical pattern was different from that in the LF.

The distribution of extracellular matrix protein in reticulin fibers was also heterogeneous in the PALS, LF and MZ. The reticulin fibers of the WP and MZ were immunostained for fibronectin, laminin and type IV collagen. However, tenascin was found in reticulin fibers of the PALS and MZ.

In previous reports, immunohistochemical analysis revealed the reticular localization of tenascin in the PALS and around the follicle.<sup>15,17</sup>

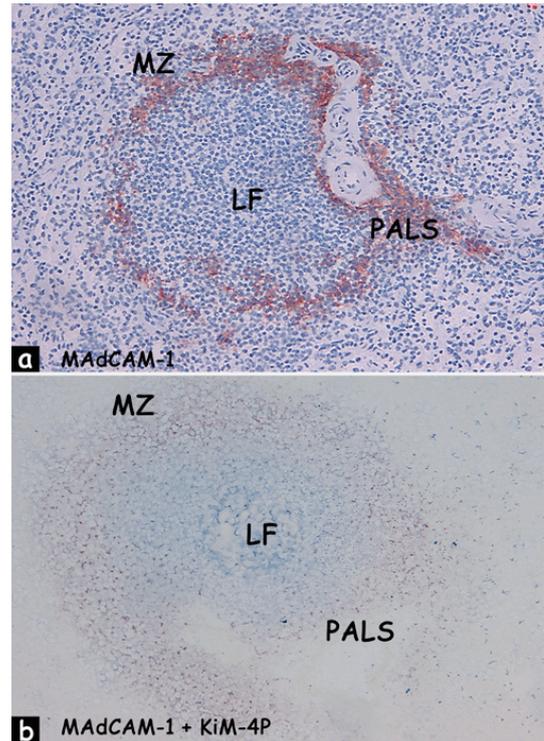
The T and B lymphocyte areas are involved in the reticular framework, and a specific microenvironment is prepared for the lymphocytes in each area. Dijkstra *et al.* demonstrated that during the regeneration process of heterotopic autotransplanted splenic implants, T and B lymphocytes exhibit a specific homing pattern in the developing WP and the newly formed reticulum determines the distribution of the homing lymphocytes.<sup>22</sup> The antigenic heterogeneity of the reticular framework is also observed in the human fetal spleen, which induces the organization of the PALS, LF and MZ.<sup>16</sup> In intrasplenic lymphocytic migration, the reticular framework of the WP and MZ may function in the segregation of the lymphoid subclass and the homing mechanism.<sup>7,14,16</sup>

In the human spleen, the blood cells flow in an open circulation.<sup>11,12,23</sup> T and B lymphocytes flow out from the arterial terminal and migrate in the reticular framework. Lymphocyte homing is regulated by differential expression of cell surface homing receptors and their selective interactions with tissue-selective vascular addressins at sites of lymphocyte recruitment from the blood.<sup>24</sup> A lymphocyte adhesion molecule, sialoadhesin was first described on the metallophilic macrophages in the mouse and rat spleen.<sup>25</sup> MAdCAM-1 is selectively expressed on the endothelium of mucosal venules, and regulates direct lymphocyte traffic into Peyer's patches and the intestinal lamina propria.<sup>26</sup> MAdCAM-1 expression in the spleen was reported in recent



**Fig. 4.** Ultrastructure of the reticular framework in the MZ  
**a** The reticular framework shows the mesh structure (arrows). x 1500.  
**b** The reticulum cells (R) encase the fine reticulin fibers (rf) (arrows). x 6000.

studies.<sup>17,18,27,28</sup> Kraal *et al.* noted the expression of MAdCAM-1 on marginal sinus lining cells in the marginal zone of the mouse spleen and its ultrastructural localization on the side of the cell facing the sinus.<sup>27</sup> Tanaka *et al.* examined mouse spleens and observed the expression of MAdCAM-1 not on marginal sinus lining cells, but at the reticular mesh exclusively in the perifollicular region, which was confined to the plasma membranes of the fiber-forming reticular cells and processes of the perifollicular reticular mesh.<sup>17</sup> In the human spleen, the expression of MAdCAM-1 has been previously reported.<sup>18,28</sup> The present study revealed that MAdCAM-1 was expressed on the  $\alpha$ -SMA-positive reticular framework, consistent with the findings of Steiniger *et al.*<sup>18,28</sup> They also demonstrated that MAdCAM-1 was more highly expressed on  $\alpha$ -SMA-positive stromal cells of the superficial PALS. Immunoelectron microscopy in the present study demonstrated the ultrastructural localization of MAdCAM-1 on the plasma membrane of reticulum cells. Interaction of MAdCAM-1 expressed on the plasma membrane with cell surface homing receptor on lymphocytes may regulate lymphocytic homing and segregation in the PALS, LF and MZ. The reason why MAdCAM-1 is expressed in the human spleen is not clear. In ontogeny, the spleen originates from the gut wall. MAdCAM-1-positive stromal cells were found to play an important role in spleen tissue organogenesis.<sup>29</sup> MZ B cells, which are the most frequent cell type contacting MAdCAM-1+ stromal cells, recirculate between the spleen and

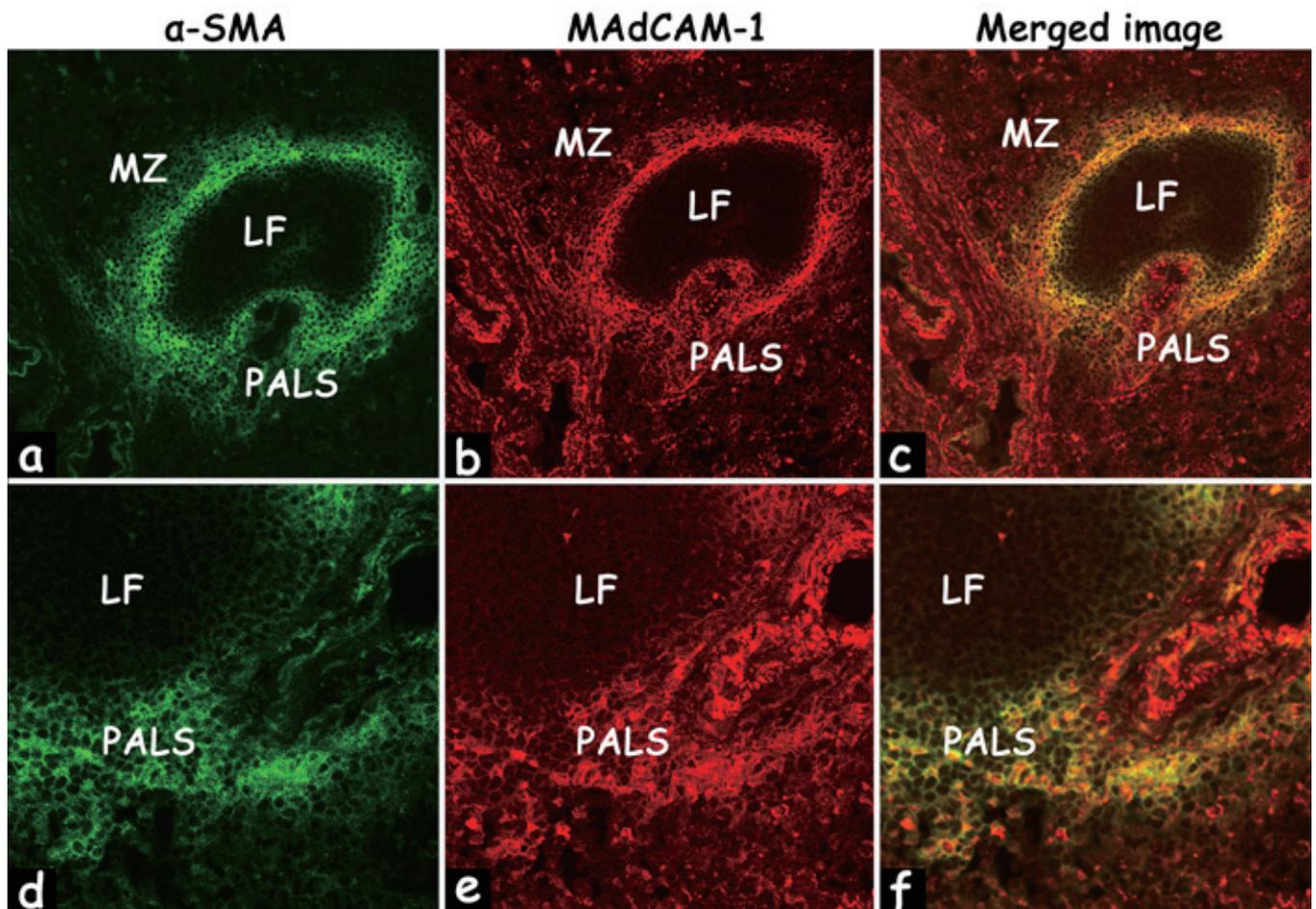


**Fig. 5.** Expression and localization of MAdCAM-1  
**a** Immunostaining of MAdCAM-1. Expression of MAdCAM-1 is observed in the PALS, and at the border of the LF and MZ. x 100.  
**b** Double immunostaining of MAdCAM-1 (brown) and Ki-M4P (blue). Expression of MAdCAM-1 is not found at the mesh of FDCs in the LF. x 100.

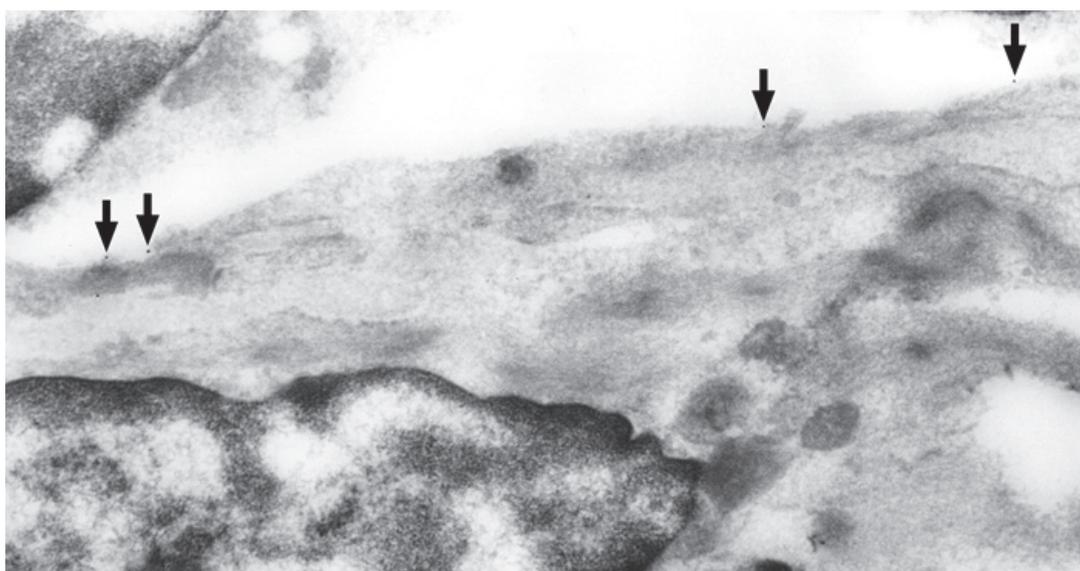
gut-associated tissues, and may depend on MAdCAM-1+ stromal cells for their survival and activity regulation.<sup>28</sup> It was also suggested that the specialized stromal marginal reticular cells form a barrier inside the MZ, which guarantees the maintenance of gradients necessary for positioning of migratory B and T cells in the WP.<sup>30</sup>

In addition to cell adhesion molecules, the  $\alpha$ -SMA-positive reticular framework expresses the chemokines CCL21, CXCL12 and CXCL13 in the human spleen.<sup>28,31</sup> In the ontogeny of the human fetal spleen,  $\alpha$ -SMA-positive stromal cells were demonstrated to express CCL21 and CXCL13, and induce the colonization of B and T lymphocytes into the WP.<sup>32</sup>

In the spleen, lymphatic vessels lie around the central artery of the WP and run out of the spleen through the trabeculae, which functions as a route of splenic exit for recirculating lymphocytes.<sup>33</sup> In the mouse spleen, podoplanin was expressed not only in lymphatic vessels, but also in stromal cells of the WP and podoplanin-positive stromal cells formed fine meshworks.<sup>34</sup> This suggests that the reticular framework of the WP acts as an extravascular lymphatic pathway and plays a role in the lymphocytic traffic in the spleen. In the present study, reticulum cells of the WP and MZ were podoplanin-negative. Podoplanin was not expressed in stromal cells of human spleen paraffin sections, nor in cryosections in a previous study.<sup>28</sup> The phenotype of the stromal



**Fig. 6.** Confocal laser scanning microscopy  
*a, d*  $\alpha$ -SMA. *b, e* MAdCAM-1. *c, f* Merged image. MAdCAM-1 is expressed on the  $\alpha$ -SMA-positive reticular framework and localized to the surface of  $\alpha$ -SMA-positive reticulum cells. *a, b, c*  $\times 50$ . *d, e, f*  $\times 200$ .



**Fig. 7.** Immunoelectron microscopy of MAdCAM-1  
 Immuno-gold labeling of MAdCAM-1 is observed on the plasma membrane of a reticulum cell (arrows).  
 $\times 25000$ .

cells of the WP may be species-specific.

The present study revealed that MAdCAM-1 is selectively expressed on the  $\alpha$ -SMA-positive reticular framework. The reticular framework may function in lymphocyte homing and segregation into the PALS, LF and MZ. In further investigations, the expression of other lymphocyte adhesion molecule and lymphocyte integrins, including  $\alpha 4\beta 7$ , which is the exclusive integrin receptor for MAdCAM-1, must be examined.

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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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