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# New Insights into the Cell Biology of the Marginal Zone of the Spleen

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In the marginal zone of the spleen the bloodstream passes through an open system of reticular cells and fibers in which various myeloid and lymphoid cells are located. Macrophages in this region are well equipped to recognize pathogens and filter the blood by virtue of unique combinations of pattern recognition receptors. They interact with a specific set of B cells that can be found only in the marginal zone and that are able to react rapidly to bacterial antigens in particular. This combination of strategically located cells is an important factor in our defense against blood-borne pathogens. New data on the development of the marginal zone itself and the marginal zone B cells are reviewed and discussed in light of the function of the spleen in host defense.

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## I. Introduction

The marginal zone of the spleen is strategically positioned between the lymphoid compartment of the spleen, the white pulp, and the more innate, scavenging red pulp compartment. Most of the arterial blood that enters the spleen runs through the marginal zone where sets of cells, such as macrophages expressing unique combinations of pattern recognition receptors as well as marginal zone B cells that can be readily activated, are located to intercept and react to pathogens in the blood.

Although the important function of the spleen has been appreciated for a long time, the anatomical basis for its function has only gradually emerged. Less than 25 years ago Bishop and Lansing (1982) started an overview of


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normal and pathological anatomy of the human spleen stating that the spleen is "an organ of unique anatomic and functional importance, and yet the morphologic correlates of its various functions remain somewhat mysterious." Our own review, which appeared a decade later (Kraal, 1992), was probably the first that focused on the marginal zone only and described the constituents and function of this area as they were known at that time. It is striking how much data and new insights have been generated since then, and from how many different angles of interest the spleen and the marginal zone have been studied. Especially from the field of developmental immunology, but also from that of studies on pattern recognition receptors and B cell immunology, many exciting new data on the function of the spleen and the interactions between the various cells and molecules have been generated and have helped to solve at least some of the mysteries. Here we will describe these findings correlated with the function of the spleen, and the intriguing marginal zone in particular.

The spleen is composed of a branching splenic artery that eventually ends in venous sinuses. The arterial branches, called central arterioles, are surrounded by a layer of lymphoid tissue as a continuous sheath, the white pulp. It consists of T cell areas and B cell follicles, more or less resembling the organization found in lymph nodes, except for the apparent absence of high endothelial venules. Although smaller branches of the central arteriole run through the white pulp, most of the arterial blood (Schmidt *et al*., 1993) ends in a sinusoid system in the area surrounding the T and B cell zones, thereby forming an anatomical border between the white and red pulp, the marginal zone (Kraal, 1992). It is here that the blood opens up in sinusoid spaces formed by lining cells continuous with the endothelium of the arterioles. Especially in rodents, in which the spleen has been investigated most thoroughly, the marginal zone is a conspicuous area consisting of a network of sinus lining cells and reticular fibroblasts through which the blood freely percolates on its way to the red pulp. Macrophage populations and marginal zone B cells are firmly attached to this network, thus allowing a continuous scan of the blood for pathogens and debris. In humans the marginal zone can be divided into an inner and outer area with a small rim of T cell zone in between and surrounded by a more diffuse perifollicular zone where macrophages can be found that surround endings of blood vessels (Mebius and Kraal, 2005; Steiniger *et al*., 2001). The blood runs from the marginal zone through the red pulp cords into the venous sinuses, whereby the structure of the venous cords and sinuses, forming the spleen's red pulp, leads to a functional slow bloodstream, enabling the spleen to exert its function as a filter of the blood. The passage from the cords into the sinuses is an important step for the selection of effete red blood cells and their removal by the macrophages of the red pulp. Interestingly, the splenic vein is connected to the hepatic portal vein, so that all the blood leaving the spleen is then filtered by the portal system of the liver.

## II. Cells of the Marginal Zone

Within the reticular framework of the marginal zone, resident cell types can be found that form more or less permanent populations that are not present in other lymphoid organs (Fig. 1). These include the marginal zone macrophages, the marginal metallophilic macrophages, and the marginal zone B cells. In addition, many cell types present in the blood are located in the marginal zone as passing cells, including lymphocytes and granulocytes. In addition, a rather large number of dendritic cells can be found here. They are thought to reside temporarily in the marginal zone and to migrate into the white pulp after stimulation and antigen uptake. Also, lymphocytes may dwell here for longer periods during the process of transmigration into the white pulp. The characteristics of the resident cells will first be described, after which an attempt will be made to correlate the findings on the various cells in this area to the function of the marginal zone in innate and adaptive immunity.

### A. Macrophages

Armed with a unique set of pattern recognition receptors, the marginal zone macrophages occupy their strategic position in the marginal zone. In fact, it is impressive to observe the efficiency and speed by which these cells can clear the blood from an experimentally administrated antigen for which they have the appropriate receptors, such as FITC-Dextran. Similar to tissue macrophages, they display receptors involved in recognition and phagocytosis of opsonized particles, such as Fc and complement receptors, but their outstanding capacity for binding and uptake of nonopsonized particles is based upon the expression of surface receptors such as the C-type lectin SIGNR1 (Geijtenbeek *et al*., 2002a; Kang *et al*., 2003, 2004), and MARCO, a type I scavenger receptor (Elomaa *et al*., 1995; van der Laan *et al*., 1999) (Fig. 1A and B).

#### 1. Structure and Function of SIGNR1

That marginal zone macrophages are capable of recognizing polysaccharide antigens and play a role in T cell-independent antibody responses has been well established in earlier studies (Amlot and Hayes, 1985; Amlot *et al*., 1985; Humphrey and Grennan, 1981; Kraal *et al*., 1989b). With the discovery of DC-SIGN as a human dendritic cell-specific C-type lectin (van Kooyk and Geijtenbeek, 2003) and subsequent isolation of murine homologues, the SIGNR1 molecule was identified as an important C-type lectin present on

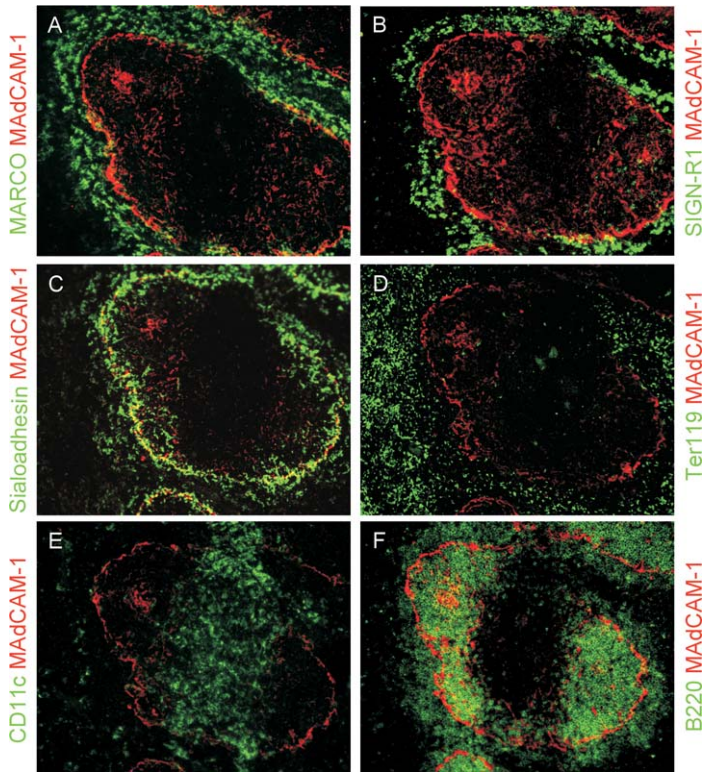


FIG. 1 The cellular constituents of the marginal zone. Serial sections of a mouse spleen have been stained with various monoclonal antibodies to reveal the position of the different cell populations in the marginal zone. In all sections the expression of MAdCAM-1 (red) on the cells of the marginal sinus is used to clearly indicate the demarcation between white pulp and marginal zone. In addition, MAdCAM-1 stains cells, probably follicular dendritic cells in the B cell follicles. In (A) and (B) the marginal zone macrophage population is clearly visible based on its expression of MARCO [(A) green] and SIGNR1 [(B) green]. In (C) the compact ring of sialoadhesin-positive marginal metallophilic macrophages can be seen. In (D) the staining for Ter119 (green) on erythrocyte membranes demonstrates the strict separation of freely passing blood in the marginal zone and the red pulp and the absence of erythrocytes in the white pulp, clearly illustrating the selective barrier that exists for leukocytes to enter the white pulp from the marginal zone. In (E) the localization of dendritic cells, based on the expression of CD11c, in the T cell zone of the white pulp, in a bridging channel [visible in the top of (E)], and in the marginal zone is seen. Finally, in (F) the presence of B cells, most of them belonging to the distinct population of marginal zone B cells, can be seen outside the MAdCAM-1-positive sinus. In addition, within the white pulp two B cell follicles (green), separated by the T cell zone, can be seen. Magnification: $\times 10$.

marginal zone macrophages and responsible for recognition of carbohydrate antigens (Geijtenbeek *et al.*, 2002a; Park *et al.*, 2001). The C-type lectin domain of mouse SIGNR1 has 74% similarity with that of human DC-SIGN, and the amino acid residues important for both $Ca^{2+}$ and ligand binding are conserved in the murine homologue (Geijtenbeek *et al.*, 2002b). The ligand-binding specificities of SIGNR1 are therefore quite similar to the ones described for DC-SIGN, based on the presence of the EPN sequence motif in the carbohydrate recognizing domain (CRD). The EPN motif determines their affinity, in particular to mannose-containing carbohydrates, but additional amino acids in the CRD will determine the specific structures that are recognized (Appelmelk *et al.*, 2003; Feinberg *et al.*, 2001; Galustian *et al.*, 2004), and many of the pathogens that are recognized by DC-SIGN can also interact with SIGNR1 (see Table I). Detailed analysis of the carbohydrate specificity of SIGNR1 showed interaction with mannose-, fucose-, and *N*-acetylglucosamine-terminating oligosaccharides, as demonstrated by their ability to react with high mannose *N*-glycans and proteins containing *N*-linked glycans such as invertase and soybean agglutinin. That SIGNR1 also reacts with fucose was inferred from binding studies with Lewis antigens (Galustian *et al.*, 2004).

The SIGNR1 molecule is sufficient for both binding and internalization of ligands, after which the complex is localized in lysosomes, as shown by

TABLE I
Overview of Pathogens and Molecules that Bind to SIGNR1[a]

| | Pathogen or molecule | References |
|---|---|---|
| | ICAM-2 | (Geijtenbeek *et al.*, 2002a) |
| Yeast | Zymosan | (Takahara *et al.*, 2004; Taylor *et al.*, 2004) |
| | *Candida albicans* | (Nagaoka *et al.*, 2005) |
| *Bacteria* | *Mycobacterium tuberculosis* (ManLAM) | (Koppel *et al.*, 2004) |
| | *Streptococcus pneumoniae* | (Lanoue *et al.*, 2004) |
| | *Escherichia coli* | (Nagaoka *et al.*, 2005) |
| | *Salmonella typhimurium* | (Nagaoka *et al.*, 2005) |
| Virus | Coronavirus (S protein) | (Marzi *et al.*, 2004) |
| | Marburg virus | (Marzi *et al.*, 2004) |
| | Ebola virus (glycoprotein) | (Marzi *et al.*, 2004) |
| | HIV (gp120) | (Geijtenbeek *et al.*, 2002a) |

[a]In parentheses is the ligand or subcellular part of the pathogen recognized by SIGNR1.

transfected cells (Geijtenbeek *et al.*, 2002a). The cytoplasmic region of the murine SIGNR1 lacks a dileucine motif that is responsible for the internalization of human DC-SIGN, but it contains a triacid cluster (DDD) in its cytoplasmic region (Koppel *et al.*, 2004; Park *et al.*, 2001). This motif functions as an internalization motif in DEC-205, an endocytic receptor on dendritic cells, where it is responsible for targeting to lysosomes for antigen processing (Mahnke *et al.*, 2000). It is therefore assumed that the lysosomal targeting of internalized ligand–SIGNR1 complexes is due to this triacidic cluster. Indications that the SIGNR1 receptor can also interact with other receptors begin to emerge. Cooperation with Dectin-1, the β-glucan receptor, leads to efficient uptake of zymosan and production of tumor necrosis factor (TNF)-α (Taylor *et al.*, 2004), although this is probably not as relevant for the situation in the marginal zone where dectin-1 is hardly expressed (Reid *et al.*, 2004). For marginal zone macrophages the observed interaction of SIGNR1 with the Toll-like receptor (TLR) 4 seems to be more important (Nagaoka *et al.*, 2005). In studies on the ligand specificity of SIGNR1 it was observed that lipopolysaccharide (LPS) from *Escherichia coli* bound to SIGNR1 via oligosaccharides in the nonreductive end of the LPS core region. In transfectant cells with both SIGNR1 and TLR4, LPS binding to SIGNR1 led to oligomerization of TLR4 and degradation of IκB-α and it was found that SIGNR1 associated with the TLR4–MD2 complex leading to cytokine production (Nagaoka *et al.*, 2005). It is obvious that this type of interaction greatly enhances the efficiency by which the marginal zone macrophages can eliminate pathogens.

In addition to interactions with apparent pathogens, SIGNR1 also interacts with murine ICAM-2, which is widely expressed on lymphocytes, and could therefore function as the leukocyte ligand mediating contact between SIGNR1$^+$ marginal zone macrophages and leukocytes in the marginal zone (Geijtenbeek *et al.*, 2002a). Close contacts between B cells and the marginal zone macrophages have been described, which suggests that the marginal zone macrophages may play a role in the migration and retention of B cells in the marginal zone.

In addition, as described in B cell-deficient mice and in mice where B cells were induced to disappear, B cells are essential for the early differentiation of the macrophage populations in the marginal zone but also for their maintenance during adult life (Crowley *et al.*, 1999; Karlsson *et al.*, 2003; Nolte *et al.*, 2004).

## 2. Role of MARCO on Marginal Zone Macrophages

As for SIGNR1, the expression of the Macrophage Associated Receptor with COllagenous structure (MARCO) is not restricted to marginal zone macrophages, but it is the combination of these receptors that makes

these cells special. Furthermore, the expression of MARCO seems to be constitutive on marginal zone macrophages, whereas it can be rapidly induced on many other macrophage populations, such as alveolar macrophages and macrophages in the liver (Arredouani *et al*., 2004; Elomaa *et al*., 1995; van der Laan *et al*., 1999). This enables the body to initiate a swift and functional upregulation of pattern recognition receptors under conditions of heavy pathogen load. For MARCO a broad range of pathogenic ligands has been described, whereby MARCO and SIGNR1 are often complementary in their specificity. Bacteria such as *Escherichia coli* and *Staphylococcus aureus* readily bind to MARCO, whereas yeast cells (zymosan) do not (Elomaa *et al*., 1995). Ficoll, which is avidly captured by the marginal zone macrophages, does not bind to MARCO, whereas acetylated low-density lipoprotein (LDL) does (Kraal *et al*., 2000). When MARCO is expressed in other cell types *in vitro* it induces changes in cell shape, and profound changes in cytoskeleton rearrangements, which may be related to the function of the molecule in the cellular changes needed to accommodate phagocytosis and engulfment of larger particles (Granucci *et al*., 2003; Pikkarainen *et al*., 1999).

MARCO belongs to the group of class A scavenger receptors (SR-A) that was originally defined as binding to LDLs and was studied extensively in relation to the development of artherosclerosis (Suzuki *et al*., 1997). Its structure resembles the SR-A1 molecule, consisting of a trimer of disulfide-bonded proteins with a collagenous structure. MARCO has short intracellular and transmembrane domains, as well as a large extracellular domain composed of a spacer domain, a long collagenous domain, and a C-terminal fifth domain, forming a scavenger receptor cysteine-rich domain (SRCR) (Elomaa *et al*., 1995).

SRCRs are ancient and highly conserved protein modules of ~100–110 amino acids, of either soluble or membrane-bound receptors expressed by hematopoietic and nonhematopoietic cells (Resnick *et al*., 1994; Sarrias *et al*., 2004). Based on the numbers of cysteine residues two groups are distinguished, with group A containing six cysteine residues, encoded by two exons, and group B usually containing eight cysteines and encoded by a single exon. Group A members, to which SR-A1 and MARCO belong, are multidomain mosaic proteins with single SRCR domains associated with other functional domains, such as enzymatic (protease) domains or collagenous regions. Group B members are composed of tandem repeats of SRCR domains, thought to be involved in oligomerization but never associated with protease domains. Representatives of either group are found in different animal species, from low invertebrates (sponges) to high vertebrates (mammals), and it is thought that based on the high degree of structural and phylogenetic conservation of SRCR domains, they have basic functions in innate immune defense (Lehrer, 2004; Sarrias *et al*., 2004).

Using human and mouse MARCO variants with deletions or single amino acid substitutions the bacteria-binding properties of MARCO were localized to the fifth SRCR domain, with an RXR motif as an essential element for high-affinity bacterial binding (Brannstrom *et al*., 2002). Interestingly, for SR-A1 the ligand-binding function has been localized to the collagenous domain, and so far not to the SRCR domain (Acton *et al*., 1993; Doi *et al*., 1993).

## B. Metallophilic Macrophages

In contrast to the position of the marginal zone macrophages that are located in a seemingly random way throughout the width of the marginal zone, the marginal metallophilic macrophages form a distinct line of cells at the border of the marginal zone at the white pulp side of the marginal sinus (Fig. 1A–C). As such their position closely resembles the rim of macrophages lying just underneath the subcapsular sinus of the lymph node (Nolte *et al*., 2000). Both sites form a transitional area, where cells and molecules can enter the white pulp or the lymph node parenchyma, suggesting an important role for the marginal metallophilic macrophage cells in selection and scavenging.

Originally, before the development of specific antibodies, the cells at this site had been characterized as macrophages by the presence of acid phosphatase, but differed from other macrophage populations by their high content of nonspecific esterase (NSE) (Eikelenboom, 1978). A role for this enzyme in cleaving fatty acids as well as for the detoxification of LPS has been described, as well as a direct association of NSE and removal of apoptotic T cells by macrophages in the thymus (Feng *et al*., 2002). This suggests that the marginal metallophilic macrophages are involved in scavenging pathogens and apoptotic cells based on the function of NSE in membrane destabilization. In line with this is the abundant expression of Siglec-1 (sialoadhesin) on these cells (Fig. 1C), a receptor associated with binding to oligosaccharide self ligands on many cells and extracellular matrix components (Crocker *et al*., 1995, 1997). The family of siglecs (sialic acid-binding Ig-like lectins) can be divided into two subsets, the highly related and large group of CD33-related siglecs, and a second group composed of sialoadhesin, CD22, and myelin-associated glycoprotein (MAG). Although the latter group is more distantly related, it is represented by well-conserved orthologues in all mammalian species examined so far (Crocker, 2005). Except for MAG, all siglecs are expressed on cells of the immune system and consist of an N-terminal V-set Ig domain that mediates sialic acid binding, and a number of C2-set Ig-like domains. Furthermore, several of them contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails, suggestive

of a role in immune regulation, but the precise role of the various siglecs, with the exception of CD22, is still not clear. Sialoadhesin, which in addition to its expression on marginal metallophilic macrophages is also found on macrophage subsets at inflammatory sites and in tumors, differs from other siglecs by the fact that it is constitutively active. Most others siglecs are thought to be masked through *cis* interactions with sialylated ligands on the same plasma membrane and have to be activated, e.g., by sialidases to become functional. That this is not the case for sialoadhesin could be due to the fact that with its 17 domains it stands out from the crowd of other molecules in the plasma membrane and is therefore less likely to interact in *cis* (Crocker *et al*., 1994; Munday *et al*., 1999).

The role of sialoadhesin in recognition of apoptotic cells was inferred from the expression of its ligands on many cells, in particular granulocytes, but remains circumstantial (Crocker *et al*., 1995). Based on the position of marginal metallophilic macrophages at the entrance site of the marginal zone and white pulp, a role for sialoadhesin in lymphocyte migration has been suggested. Activation of lymphocytes leads to changes in the glycosylation patterns of molecules such as CD43, CD45, and PSGL-1, all of which are ligands for sialoadhesin based on expression of clustered sialic acids. Their interaction may facilitate the retention of lymphocytes in the transit zone and subsequent entrance into the white pulp (Crocker *et al*., 1997). In fact, elimination of macrophages from the marginal zone with the use of toxic liposomes led to reduced entrance of lymphocytes into the white pulp, although it cannot be completely ruled out that this was related to general changes in the hemodynamics in the marginal zone (Kraal *et al*., 1989a). Also for blood-borne tumor cells with altered glycosylation, binding and removal through sialoadhesin have been suggested (Nath *et al*., 1999).

Although it was always assumed that the N-terminal V-set Ig domain of sialoadhesin was the sole ligand-binding site, it was recently found that sialoadhesin can also interact with ligands that bind to other parts of the molecule (Kumamoto *et al*., 2004). In a study on the migration of dermal macrophages into lymph nodes it was found that these cells interacted with the sialoadhesin expressed on the subcapsular macrophages in the node through M galactose C-type lectins (MGL1). This interaction was independent of sialic acid but involved *N*-glycans on the stalk of the sialoadhesin molecule (Kumamoto *et al*., 2004). In light of the aforementioned similarities between these subcapsular and the marginal metallophilic macrophages, similar interactions can be expected to occur in the marginal zone and it will be very interesting to see what cell types are involved.

Although sialoadhesin is not a phagocytic receptor, it may be involved in phagocytosis through interaction with other receptors. Uptake of pathogens for which recognition through sialic acid by sialoadhesin was crucial was reported for *Neisseria meningitides* (Jones *et al*., 2003), and for arterivirus in

the case of porcine alveolar macrophages (Delputte and Nauwynck, 2004). Together it is clear that both the strategic position of the marginal metallophilic macrophages and the expression of a receptor with some striking characteristics point to essential functions of these cells at the interface of the marginal zone and white pulp. However, full appreciation of their role needs further investigation.

## C. B Cells

Marginal zone B cells (MZ B cells) represent a distinct naive B cell lineage (Fig. 1F), different from mature follicular B cells and the B-1 cell lineage, predominantly nonrecirculating and specialized to respond rapidly to blood-borne pathogens (Gray *et al*., 1982; Martin *et al*., 2001; Oliver *et al*., 1997). Upon encounter with bacteria they respond swiftly by differentiation toward plasma cells that produce IgM as well as acquiring the capacity to function as antigen-presenting cells (Martin *et al*., 2001; Oliver *et al*., 1999). MZ B cells are particularly well equipped to deliver a response to T-independent antigens. With their rapid response to bacterial antigen and their strategic location in the marginal zone, where blood flows into open sinuses between the red and white pulp, they deliver a first line of defense against blood-borne pathogens and therefore help to fill the gap between the fast but nonspecific innate immune response and the adaptive, T cell-dependent antibody response that needs considerable time to reach its peak (Lopes-Carvalho and Kearney, 2004). In addition, they deliver immune complexes to follicular dendritic cells by migrating into the B cell follicles (Gray *et al*., 1984; Heinen *et al*., 1986).

### 1. Phenotype and Lineage Commitment of Marginal Zone B Cells

MZ B cells have a unique phenotype by expression of high levels of IgM, CD21, and CD1d, and low levels of IgD, CD23, and B220, whereas follicular B cells are characterized by high levels of IgD and CD23, but intermediate to low levels of IgM, CD1d, and CD21 (Martin and Kearney, 2002). In addition, CD9 has been identified as a marker to delineate MZ B cells from follicular B cells (Martin and Kearney, 2002).

To what extent are MZ B cells different from follicular B cells and how is this determined? Together with the B-1 B cells, MZ B cells are encompassed in the group of innate lymphocytes, of which the antigen receptor repertoire is germ-line encoded with limited diversity (Bendelac *et al*., 2001), and they are not part of a constantly migrating lymphocyte pool, but instead reside at certain anatomical sites. The B-1 B cells form an early lineage that already develops in neonatal life and shows a restricted repertoire of its V genes

biased to recognize T-independent antigens. B-1 B cells are mainly found in the peritoneal cavity and along mucosal surfaces (Bendelac *et al*., 2001). MZ B cells develop later in life and are also characterized by a restricted repertoire and a fixed position. It is now well established that in addition to negative selection of B cells with the B cell receptor (BCR) reacting strongly with self (Rolink and Melchers, 1996), B cells are also subjected to positive selection during their development based on interactions of their BCR, whereby the affinity of the receptor, accessory signaling molecules, and the nature of the antigen itself play a role (Lopes-Carvalho and Kearney, 2004). It is becoming clear that the signaling through the BCR is an important cell-fate decision step in the differentiation of MZ B cells (Pillai *et al*., 2005), whereby interaction with self-ligands plays an important role in their recruitment and selection (Dammers and Kroese, 2005; Wen *et al*., 2005). Positive selection, based on the very nature of the antigen, was very nicely demonstrated by Martin and Kearney, using mice transgenic for different Ig heavy chains. They showed clearly that the ability to become an MZ B cell, based on both phenotypic characteristics and localization, was dependent on the composition of the Ig molecule (Martin and Kearney, 2000). This means that the distinct subsets of B cells, B-1 cells, MZ B cells, and follicular B cells, not only occupy different domains of the immune system, but do so with different specificities, evolved to deal with antigens that are most likely encountered at their sites.

## 2. Precursor Relationships between MZ B Cells and Follicular B Cells

MZ B cells are quite long-living cells, compared to follicular B cells, as demonstrated in conditional knockout Rag-1 mice. Whereas in these mice, upon deletion of *Rag* follicular B cells gradually disappear, the numbers of MZ B cells are stable. This is suggestive of a self-renewing capacity, either through replication of MZ B cells themselves or from recirculating precursors (Hao and Rajewsky, 2001; Pillai *et al*., 2005).

Newly formed B cells, transitional type 1 (T1), emerge from the bone marrow and predominantly depend on BCR stimulation for their survival. These cells develop into T2 cells, which need additional survival signals such as BAFF (B cell-activating factor of the TNF family). These T2 cells will give rise to both follicular and marginal zone B cells, whereby intermediate precursor stages are discerned based on expression of various markers (Fig. 2). The existence of a separate $CD23^{+}$ $CD21/35^{high}$ precursor cell for MZ B cells was suggested (Loder *et al*., 1999). Cells with this phenotype are found only in spleen and not in bone marrow, blood, or lymph nodes, and are absent in mice that lack MZ B cells such as Aiolos null mice and $Notch2^{-/-}$ mice. Because of their absence in Aiolos null mice, they are

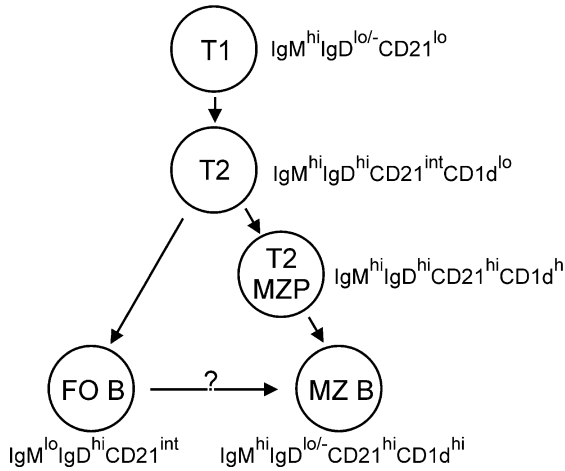


FIG. 2 Developmental pathways of marginal zone B cells and follicular B cells. Both B cell populations are derived from transitional type 2 (T2) B cells. The existence of a distinct marginal zone precursor B cell (T2 MZP) has been described but debated, as is the differentiation of marginal zone B cells from follicular B cells.

thought to be different from precursors for follicular B cells, since the development of the latter is normal in these mice (Cariappa *et al.*, 2001; Loder *et al.*, 1999; Saito *et al.*, 2003). Other investigators support the idea that these $CD23^{+}$ $CD21/35^{high}$ cells are transitional cells that develop from mature follicular B cells (Allman *et al.*, 2004; Srivastava *et al.*, 2005) (Fig. 2). This is based on observations using lymphopenic mice in which follicular B cells were transferred and gave rise to marginal zone B cells.

Irrespective of the precise precursor relationship, it is clear that MZ B cells use differentiation pathways that are distinct from follicular B cells. In models proposed to explain the two pathways, a major difference emerges: the difference in signaling strength of the BCR needed to develop the two cell types. MZ B cell development requires weak BCR signaling, whereas intermediate signaling is required for follicular B cells. Important data to support this came from studies in Aiolos null mice. As a zinc-finger protein of the Ikaros family, Aiolos is mainly expressed in B cells, and functions to repress target genes. When Aiolos is absent, the strength of BCR signaling increases as seen by enhanced B cell proliferation upon BCR crosslinking, leading to expanded numbers of follicular B cells, but MZ B cells do not develop (Wang *et al.*, 1998). This was related to the fact that Aiolos negatively influences Bruton's tyrosine kinase (Btk) activity (Cariappa *et al.*, 2001). Btk is a member of the Tec family of protein tyrosine kinases (PTKs) and plays a vital role in many cellular processes. Mutations in the Btk gene cause

the primary immunodeficiency disease X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice (Lindvall *et al.*, 2005). In Xid mice and Btk null mice, follicular B cells are strongly reduced in numbers, but MZ B cells are generated, although in smaller numbers (Kraal *et al.*, 1988a; Loder *et al.*, 1999). In the absence of both Aiolos and Btk, Cariappa *et al.* (2001) showed that MZ B cells do develop, indicating that Btk is a negative regulator of MZ B cell development, supportive of the idea that MZ B cells require low signal strength through their BCR to differentiate. In contrast, indications that strong BCR signaling, especially from low-dose self-antigens, is essential for MZ B cell maturation have recently been given using a monoclonal BCR mouse (Wen *et al.*, 2005). It is becoming clear that the decision steps for lineage commitment depend on many factors, partly BCR related, and are strongly influenced by environmental factors. Also signaling in B cells via the NF-κB pathway, and in p50 component in particular, is required for the proper development of MZ B cells (Cariappa *et al.*, 2000), but this pathway does not seem to interact directly with Btk activation or to influence the signaling strength of the BCR.

## 3. Notch Signaling and MZ B Cell Development

In addition to apparent differences in the threshold of BCR signaling, MZ B cells are critically dependent on Notch2 signaling for their survival and probably also for their maintenance. Notch signaling is important for many cell-fate decision steps during ontogenetic development, but also during adult life. Furthermore, it has been suggested that there is an inverse relationship of BCR and Notch signaling, whereby strong BCR signals could downregulate the Notch pathway by induction of inhibitors or downregulation of activating factors (Pillai *et al.*, 2004). Notch2 belongs to the group of four notch receptors described in mammals, which are composed of two noncovalently linked fragments in the cell membrane. Ligation induces the release and translocation into the nucleus of the intracellular domain of Notch (Notch IC), where it interacts with transcriptional repressors such as RBP-J (repressor recombination binding protein-J) to convert it into a transcriptional activator. Recent studies with mice targeted in several genes of the Notch pathways have revealed the role of this signaling route in MZ B cell development. It was first demonstrated that RBP-J was crucial for the proper development of MZ B cells (Tanigaki *et al.*, 2002), followed by the observations that Notch2 was specifically expressed in MZ B cells, and not in follicular B cells, coinciding with an absence of MZ B cells in Notch2$^{-/-}$ mice (Saito *et al.*, 2003). In addition, transfer of fetal liver cells from MINT$^{-/-}$ mice into RAG$^{-/-}$ mice led to the preferential development of MZ B cells (Kuroda *et al.*, 2003). MINT (Msx2-interacting nuclear target protein) negatively regulates activation through Notch by interfering with the interaction of Notch IC and RBP-J.

MINT is highly expressed in follicular B cells, consistent with suppression of Notch-regulated genes (Kuroda *et al.*, 2003).

## 4. Additional Transcription Factors

Recently a role for the helix-loop-helix protein E2A in MZ B cell development was described, balanced by its antagonist Id3 (Quong *et al.*, 2004). The E2A gene encodes for two proteins, E12 and E47, which are formed by alternative splicing, and these E proteins are involved in the early commitment and survival of lymphoid precursors into the B cell lineage (Lazorchak *et al.*, 2005). $E2A^{+/-}$ mice show an increase in levels of MZ B cells, whereas follicular B cell numbers decrease. In Id3 null mice more follicular B cells are generated (Quong *et al.*, 2004). A relationship with Notch signaling was proposed by the authors as it has been demonstrated that the Notch pathway negatively regulates E47 activity (Nie *et al.*, 2003). By controlling the levels of E47, Notch may regulate the cell-fate decision that leads to MZ B cells. Id3 levels, on the other hand, are controlled by Pyk-2 kinase activity, corresponding to the fact that in the absence of this kinase no MZ B cells can be formed (Guinamard *et al.*, 2000). A complex picture of signaling pathways that are necessary for the development of MZ B cells versus follicular B cells slowly starts to emerge, but the unraveling of the precise interactions of these routes awaits further studies (Fig. 3).

## 5. Extracellular Signals Involved in MZ B Cell Generation

In addition to these opposing interactions at the level of gene transcription, it is necessary to know more about the activating steps at the plasma membrane that lead to these intracellular signaling pathways. Signaling through Notch

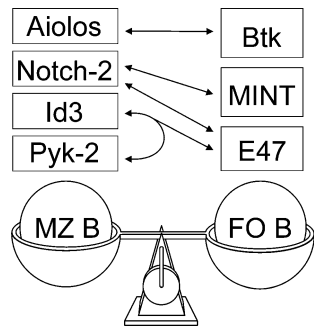


FIG. 3 Cell fate decisions between marginal zone B cells (MZ B) and follicular B cells (FO B). The points at which the decision for lineage commitment is made are dependent on the balance between various signaling cascades that either cooperate or counteract. See the text for details on these interactions.

as a receptor complex on the plasma membrane requires interactions with ligands on cells or extracellular matrix, different from cognate interaction with antigen and BCR. Delta-like-1 (Dl-1) is involved as a molecular ligand, since genetic ablation of Dl-1 also leads to significant loss of MZ B cells. Furthermore, Dl-1 was found to be expressed by dendritic cells in the spleen (Hozumi *et al*., 2004). Dendritic cells also produce growth and survival factors such as BAFF and APRIL (a proliferation–inducing ligand) (Balazs *et al*., 2002), and the signaling cascades through these factors may interfere with the above-mentioned transcription routes and lead to preferential induction or maintenance of MZ B cells.

Roles for other molecules on the surface of B cells related to BCR signaling such as CD19, CD21, and CD22 have been amply documented (Lopes-Carvalho and Kearney, 2004). The CD19 and CD21 molecules seem to act in concert and help to lower the threshold for BCR triggering. It is not clear what the ligand for CD19 is, but the molecule is important for the development of MZ B cells as shown in gene-targeted mice (Rickert *et al*., 1995), whereas the absence of the complement receptor CD21 leads to increased numbers of MZ B cells (Cariappa *et al*., 2001). Interestingly, the expression levels of CD21 on B cells seem to be related to the Notch pathway, since in Notch2$^{-/-}$ mice B cells exhibit reduced levels of CD21, while in E2$^{+/-}$ B cells the levels of CD21 are increased, again allowing a link between Notch2 signaling and the activity of E proteins (Quong *et al*., 2004; Saito *et al*., 2003).

CD22 is also a negative regulator of BCR signaling, based on the presence of an ITIM in its cytoplasmic tail and the reduced MZ B cell compartment in CD22–deficient mice (Samardzic *et al*., 2002). CD22 is a siglec molecule, with its N-terminal domain binding to glycans containing sialic acids. Recently it was found that CD22 is predominantly reacting *in cis* with sialic acids on other CD22 molecules, forming homomultimeric complexes, thereby masking its reactivity (Han *et al*., 2005). Interestingly, MZ B cells express an unmasked form of CD22, suggestive of an interaction with external stimuli (Danzer *et al*., 2003).

Dendritic cells have been mentioned as putative candidates with which the MZ B cells can interact during their development, but also the marginal zone macrophages are likely candidates to provide survival or differentiation signals. By *in vivo* infusion of an antibody against the MARCO scavenger receptor on marginal zone macrophages it was found that MZ B cells started to migrate out of the MZ and into the follicles (Karlsson *et al*., 2003). Recently we analyzed MARCO null mice and mice mutated in both MARCO and SR-A-I and found an impairment of the T-independent type 2 (TI-2) response in these mice, although no apparent differences in the presence of MZ B cells were found (Chen *et al*., 2005). The dependence of MZ B cell formation on NF-κB-p50 could point to interactions with microbial antigens encountered in the marginal zone and their signaling through

TLR via the NF-κB route. Together it is clear that the formation of MZ B cells is a complex process involving delicate balances in signaling pathways of both BCR and accessory molecules, through interactions with antigen, but also ligands produced by local cells in the marginal zone.

## 6. Retention Mechanisms for Marginal Zone B Cells

One of the most obvious outcomes of the complex differentiation schemes that lead to marginal zone B cells is the fact that they occupy a different niche and do not recirculate, as compared to follicular B cells. It is not clear whether the development of marginal zone B cells initially starts in the marginal zone or whether their retention is a result of this differentiation. At any rate, some of the molecular bases of why they remain localized are becoming more clear. An important breakthrough was derived from studies using the drug FTY720, which was found to have a major impact on lymphocyte egress from lymphoid organs (Cyster, 2005). Upon injection the drug becomes readily phosphorylated, thereby mimicking sphingosine-1-phosphate (S1P), and can engage four of the five known S1P receptors. S1P is a lysophospholipid, produced by many cells, including macrophages, and is found in high concentrations in blood compared to lymph. This concentration gradient from lymph to blood may be a driving force for lymphocytes to exit from lymph node upon expression of S1P receptors. Being G-protein coupled receptors they transduce signals upon binding to the lysophospholipids by association with G proteins, leading to a variety of downstream events, including survival signals as well as cell motility. The receptor $S1P_1$ is the most important S1P receptor associated with lymphocyte egress, as inferred from transfer studies with cells from $S1P_1$-deficient mice. $S1P_1$ on lymphocytes is downregulated and inactivated by the FTY720 drug, leading to the inability of cells to exit lymphoid organs (Matloubian *et al.*, 2004). Marginal zone B cells express high levels of $S1P_1$, which turned out to be important for the retention of the cells. Not only did treatment of mice with FTY720 lead to a rapid displacement of MZ B cells from marginal zones into the white pulp follicles, but in $S1P_1$-deficient mice marginal zones were absent (Cinamon *et al.*, 2004). Interestingly, normal numbers of B cells with the MZ B phenotype were found in their spleen, although the mice lacked a clearly localized population of MZ B cells, suggesting that the development of these cells may precede the lodging into their anatomical localization (Cinamon *et al.*, 2004).

In contrast to the active emigration of lymphocytes from lymph nodes upon $S1P_1$ engagement, MZ B cells use $S1P_1$ to retain their position. This apparent discrepancy may be explained by higher concentrations of S1P in the blood, to which the cells in the marginal zone are continuously exposed, leading to signaling cascades that favor retention over migration. Effects of

S1P on cytoskeleton rearrangements and adhesion have been described in other systems (Rosen and Goetzl, 2005), and a role for integrins and additional chemokines in MZ B cell retention has been given (Lu and Cyster, 2002). In this respect the selective expression of CD9 on MZ B cells deserves attention. CD9 belongs to the family of tetraspanin molecules that associates with signaling receptors on the plasma membrane of leukocytes, thereby forming so-called microdomains, which are thought to facilitate the signaling processes on leukocyte membranes (Wright *et al.*, 2004). CD9, in particular, is associated with integrin molecules (Shaw *et al.*, 1995), and its more or less restricted expression on marginal zone B cells, as well as on B-1 B cells and plasma cells, both of which are nonrecirculating cells, may imply a role in the adhesion and sessile nature of these cell types (Fig. 4).

In addition, $S1P_1$ may be involved in survival of the marginal zone B cells, because it is well established that the intracellular signaling cascades initiated by S1P can result in antiapoptotic survival signals (Rosen and Goetzl, 2005), and MZ B cells as a population are long–living cells (Hao and Rajewsky, 2001; Pillai *et al.*, 2004). This involvement of $S1P_1$ is not absolute, and other signaling pathways will also account for their survival, because MZ B cells are still present in $S1P_1$-deficient mice (Cinamon *et al.*, 2004). Activation with LPS or with cognate interaction overrides S1P-mediated retention and leads to immigration of the MZ B cells into the follicles of the spleen (Groeneveld *et al.*, 1985). This immigration is dependent on the chemokine CXCL13.

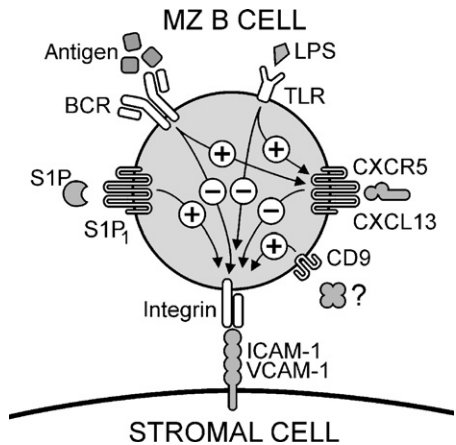


FIG. 4 Molecular interactions involved in the retention of marginal zone B cells. S1P is crucial for integrin activation and retention of MZ B cells through adhesion to stromal cells, and this signal is possibly strengthened through CD9. The signal can be negatively influenced by activation via the antigen receptor and via Toll-like receptors, leading to enhanced sensitivity of CXCR5 for CXCL13, whereupon the MZ B cell can migrate into the white pulp.

However, when this chemokine is absent, $S1P_1$ is no longer required to keep the B cells in the marginal zone, showing a hierarchy of responsiveness (Cinamon *et al*., 2004). Expression of $S1P_1$ on MZB cells was shown to be required to overcome the chemotactic response toward CXCL13, which is expressed in the B cell follicles. By triggering of $S1P_1$, B cells either fail to respond to CXCL13, or are induced to express adhesion molecules or other receptors required for retention in the marginal zone (Fig. 4). As such, expression of the integrins LFA-1 ($\alpha L\beta_2$) and $\alpha_4\beta_1$ has been implicated as adhesion molecules required for retention of MZB cells in this compartment (Lu and Cyster, 2002). Data from gene-targeted mice displaying defects in integrin and chemokine signaling support these findings. $DOCK2^{-/-}$ mice, which were shown to have a selective defect in chemokine-induced integrin activation (Nombela-Arrieta *et al*., 2004), have no MZ B cells. This could be attributed to the inability of lymphocytes to activate Rac, required for actin polymerization, which is in turn necessary for migration (Fukui *et al*., 2001). A strong reduction of MZ B cells was found in the absence of Lsc, which acts downstream of the chemokine receptors in polymerization of actin (Girkontaite *et al*., 2001). In all these mice, the inability to correctly signal through chemokine or lipid receptors can explain the defect in MZB cell localization.

However, there are still molecule(s) other than S1P involved in this lodging and retention, since in the absence of $S1P_1$ as well as CXCR5, the receptor for CXCL13, MZB cells are still retained within this compartment (Cinamon *et al*., 2004). Treatment with pertussis toxin leads to the disappearance of the B cells from the marginal zone. Since this toxin acts on $G\alpha$ protein signaling, additional chemokines could be involved to retain MZ B cells (Guinamard *et al*., 2000). However, pertussis toxin treatment will also lead to the disappearance of macrophages from the marginal zone, since marginal zone macrophages require the chemokines CCL21 and, to a lesser extent, CCL19 to localize to the marginal zone (Ato *et al*., 2004). Expression of the scavenger receptor MARCO by marginal zone macrophages was recently described as essential for localization of B cells to the marginal zone, because infusion of antibodies against MARCO led to migration of the B cells into the follicles, suggesting a disruption in the interaction of MZ B cells and macrophages (Karlsson *et al*., 2003) (Fig. 5).

## III. Marginal Zone as a Transit Area for Lymphocytes

Although extensive branching of the central arteriole can be found in the white pulp, it is generally assumed that there are no formal endings of the arteriolar bloodstream in the white pulp, and there are only scant indications

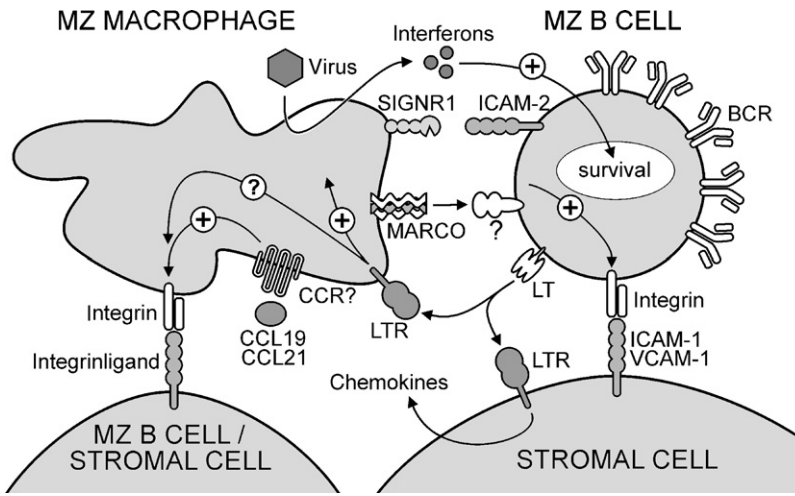


FIG. 5 The cellular interactions between cells in the marginal zone. Marginal zone macrophages (MZM) and MZ B cells influence each other through several pathways. Viral-induced production of interferons by MZM acts antiapoptotically and enhances the survival of MZ B cells. Lymphotoxin–lymphotoxin receptor interaction (LT-LTR) is thought to be necessary for the survival of MZM, possibly through positive effects on integrin-mediated retention. MARCO, acting through unknown mechanisms, influences the retention of MZ B cells. MZM and MZ B cells additionally interact via SIGNR1 and ICAM-2, whereas LT on B cells may be involved in triggering stromal cells to produce chemokines involved in the retention of MZ B cells and macrophages.

for specialized parts in blood vessels that allow cells to transverse from blood to white pulp, comparable to high endothelial venules in lymph nodes (Grayson *et al.*, 2003). This means that most, if not all, of the lymphoid and myeloid cells found in the white pulp enter and leave this region through the marginal zone. The fact that no red blood cells and almost no granulocytes can be found in the white pulp points to an active selection process, and suggests a role for chemokines (Fig. 1D). This was indeed demonstrated using pertussis toxin, which blocks chemokine receptor-associated G$\alpha$1 protein signaling and led to a blockade of the entry of lymphocytes into the white pulp (Cyster and Goodnow, 1995). The chemokines involved in the localization of the T- and B-lymphocytes into their respective white pulp compartments were similar to those in lymph node homing: CCR7–CCL19/CCL21 interactions are necessary for the localization of T cells in the white pulp T cell zone, and cooperation between CCR7 and CXCR5 and their ligands is required for B cell entry into the follicles (Muller *et al.*, 2003). Whether chemokine receptor triggering is also essential for the initial activation of lymphocytes in the marginal zone is not formally proven.

The selectivity and chemokine involvement point to mechanisms resembling the entry modes described for the entry of lymphocytes into lymph nodes through high endothelial venules, where the key adhesion molecules are well established (Kraal and Mebius, 1997). Initial rolling to ligands for L-selectin or integrins on high endothelial venules is followed by chemokine-triggered activation and firm adhesion of the cell onto the endothelial wall by interactions of integrins and integrin receptors. Important integrins that play a role in the adhesion and diapedesis are LFA-1(CD11a/CD18), $\alpha_4\beta_1$, and $\alpha_4\beta_7$. In the spleen, however, L-selectin was not found to be important, as inferred from experiments in which trypsin was used to remove this important rolling ligand from lymphocytes, nor did LFA-1 seem to play a major role based on studies with LFA-$1^{-/-}$ mice (Nolte *et al.*, 2002). In later studies, using blocking antibodies in transfer experiments, LFA-1 interaction did seem to be important, especially in combination with $\alpha_4\beta_1$ (Lo *et al.*, 2003). As counterreceptors, both ICAM-1 and VCAM-1 were required, but, because a complete blockade of the immigration could never be found using mixtures of antibodies against LFA-1 and $\alpha_4\beta_1$ and their counterreceptors, other ligands, such as fibronectin, may be involved (Lo *et al.*, 2003).

An obvious molecular candidate that is supposed to play an important role in the immigration process of cells from marginal zone into white pulp is MAdCAM-1 (see Fig. 1). Originally described as an integrin ligand on high endothelial venules in lymphoid organs at mucosal sites, such as mesenteric lymph nodes and Peyer's patches, it is expressed on sinus-lining cells of the marginal zone, right at the border of the white pulp (Kraal *et al.*, 1995). Several attempts to link this suggestive expression at such a strategic position to a functional role in cell migration using classic antibody interference approaches have so far not led to any results (Kraal *et al.*, 1995; Lo *et al.*, 2003; Nolte *et al.*, 2002). Nevertheless, indirect evidence for a role of MAdCAM-1 comes from two sources (Girkontaite *et al.*, 2004; Pabst *et al.*, 2000). First, targeted disruption of NKX2.3, a transcription factor important for tissue differentiation, led to the finding that this factor was essential for the expression of MAdCAM-1 in the spleen, and that this expression was not only necessary for the proper structure of the splenic marginal zone, but was also required for cell migration based on the diminished size of the spleen (Pabst *et al.*, 2000). However, the NKX gene also affected the position of marginal metallophilic macrophages, and may therefore have more profound effects than can be ascribed to the lack of MAdCAM-1 only. The second indication for a role of MAdCAM-1 comes from a study on the $S1P_3$ receptor (Girkontaite *et al.*, 2004). We previously showed that S1P is an important ligand in the retention of MZ B cells by interactions with its receptors $S1P_1$ and $S1P_3$ (Cinamon *et al.*, 2004).

In addition, it has been shown that S1P is an important factor in controlling the organization of endothelial cells through $S1P_3$. Using mice

deficient in $S1P_3$, a disruption of the normal organization of the marginal zone was found, and the effects on lymphocyte localization could be attributed to the initial effects of the lack of S1P signaling on the endothelial cells expressing MAdCAM-1 (Girkontaite *et al.*, 2004). Again, it cannot be ruled out that additional effects on the overall structure of the spleen are more important, and that the effects on MAdCAM-1 are secondary and not indicative of its precise function in the spleen.

In addition to lymphocytes, the marginal zone is also an important area for the entry and transit of dendritic cells (DC) (Fig. 1C). It is assumed that blood-borne DC reside in this zone for longer times and that activation upon pathogen encounter (Leisewitz *et al.*, 2004) or the uptake of apoptotic cells (Morelli *et al.*, 2003) will trigger them to actively migrate into the white pulp T cell zone to present processed antigens. The expression of the chemokine receptor CCR7 seems to be crucial for this migration process (Gunn *et al.*, 1999).

The cells that are responsible for the local production of chemokines in the marginal zone are either stromal cells or endothelial cells, and the induction of chemokine synthesis is probably dependent on lymphotoxin signaling. This is based upon the fact that in mice deficient for components of this signaling pathway, such as LTα, LTβ, and LTβ-receptor (LTβ-R) KO, an intact marginal zone is absent (Martin and Kearney, 2002). The expression of lymphotoxin $\alpha_1\beta_2$ on marginal zone B cells as well as on immigrating follicular B cells could be needed to induce LTβ-R expressing stromal and/or endothelial cells to upregulate the required chemokines. This means that the continuous interaction between stromal cells and resident cells is necessary for an optimal configuration and function of the marginal zone (Fig. 5).

## IV. Functions of the Spleen in Host Defense

The major factors in the marginal zone that are instrumental in the removal and destruction of pathogens or the initiation of adaptive immune responses against them are the macrophage subtypes, the marginal zone B cells, and the dendritic cells. New insights in the interactions between the various pattern recognition receptors on these cells involved in the uptake of particles have made it clear that phagocytosis inevitably leads to some sort of activation, and whether this activation has a more pro- or anti–inflammatory nature is dependent on whether the phagocytized material is apoptotic cells, bacteria, viruses, or parasites (Stuart and Ezekowitz, 2005). In some extreme cases, especially after parasitic infections, activation can lead to major changes in the overall organization of the marginal zone that impede the clearance of the infection (Engwerda *et al.*, 2002; Weiss, 1990). At any rate, it is clear that

these activation signals can stimulate other cells leading to an effective cooperation between the various cells in the marginal zone in their effort to control homeostasis.

A good example is the role of SIGNR1 on marginal zone macrophages in the control of pulmonary *Streptococcus* infection (Koppel *et al*., 2005). Mice deficient in this important receptor fail to clear pneumococcal infections (Koppel *et al*., 2005; Lanoue *et al*., 2004); it was suggested that this was mainly because capture and concentration of the bacteria in the spleen were insufficient to activate the marginal zone B cells to produce protective IgM antibodies (Koppel *et al*., 2005). A similar dependence on the marginal zone macrophages to control infections by adaptive immune responses has been described for viral infections (Oehen *et al*., 2002). Although uptake and clearance could be important factors to initially reduce the viral load, marginal zone macrophages can also contribute to viral protection as important producers of interferons (Eloranta and Alm, 1999). In addition, interferons give an anti-apototic signal to B cells (Fig. 5) and enhance BCR signaling (Braun *et al*., 2002).

## A. Marginal Zone and T-Independent Immune Responses

The spleen is regarded as the major lymphoid organ capable of mounting immune responses against multivalent TI-2 antigens. This is related to the efficient capturing of these antigens, many of them bacterial capsular polysaccharides, by the macrophages in the marginal zone and the role of MZ B cells in producing antibodies against them. Splenectomy results in increased susceptibility against encapsulated bacteria, and vaccination against these pathogens before the age of two is difficult, probably because the marginal zone is not yet well developed (Cowan *et al*., 1978; Likhite, 1976). The important role of marginal zone B cells became clear from experiments in various mice with gene deficiencies correlating a defect in MZ B cell development with a concomitant inability to handle gram-negative bacteria or their TI-2 antigens (Cariappa *et al*., 2000; Guinamard *et al*., 2000; Tanigaki *et al*., 2002). Their rapid maturation into plasmablasts upon antigen encounter is partly due to their BCR, which can respond more strongly, and probably also to the presence of TLRs on their surface that will help in the recognition of the bacterial antigens, leading to additional intracellular signaling and maturation of the cell. Also CD21, the complement receptor, which is highly expressed on MZ B cells, may aid in concentrating complement-coated polysaccharides (Guinamard *et al*., 2000; Martin *et al*., 2001). Although the MZ B cells are independent of T help for the production of IgM antibodies in the initial responses, they do interact and need dendritic cells for their stimulation. Dendritic cells in the marginal zone provide factors such as

BAFF and APRIL that will sustain the MZ B cell population and are involved in the generation of T-independent responses. This interaction is thought to replace the CD40 ligand–CD40 interaction between Th and B cells during B cell activation (Balazs *et al*., 2002; Litinskiy *et al*., 2002).

## B. Marginal Zone and T-Dependent Immune Responses

In addition to the presence of marginal zone B cells with their distinct features as outlined in the previous sections, especially in humans and rats, there is ample evidence for the existence of B memory cells in the marginal zone. These cells have been generated as a result of classical T-dependent responses, involving germinal center reactions, and showing somatic hypermutation and high affinity of their BCRs (Dunn-Walters *et al*., 1995; Liu *et al*., 1991; Shih *et al*., 2002; Tierens *et al*., 1999). They may react swiftly to antigen, and as such the marginal zone can form a reservoir of memory B cells.

MZ B cells also play a role in T-dependent responses as a result of their ability to function as antigen-presenting cells. Their basal levels of the costimulatory molecules CD80 and CD86 can be upregulated by stimulation via their antigen receptor, CD40 ligation, or LPS, and it has been shown using mice in which a transgenic anti-HEL BCR was introduced that MZ B cells can, in fact, readily prime naive CD4 T cells, whereas follicular B cells cannot (Attanavanich and Kearney, 2004).

A third level at which the marginal zone is important for the generation of T-dependent immune responses is activation of dendritic cells. It is assumed that the dendritic cells reside in the marginal zone as sentinels, which start to migrate into the white pulp upon appropriate activation. It is not known whether the dendritic cells are efficient enough to scavenge material from the blood on their own, or benefit from the concentrating effect of the large marginal zone macrophage population, or from the observed direct interactions with marginal zone B cells.

## C. Autoimmunity and Tolerance

In view of the selection of MZ B cells on the basis of recognition of self-antigens, there is a potential risk for the development of autoreactive B cell clones. But in general, MZ B cells do not produce natural antibody and they do not engage in T cell-dependent responses with high affinity maturation, whereas autoimmune, pathogenic antibodies are almost always highly somatically mutated (Lopes-Carvalho and Kearney, 2004). Nevertheless, in several autoimmune-prone mice models, indications that MZ B cells could be involved in the expansion of the autoimmune disease have been given.

In the autoimmune NZB mouse strain, enhanced numbers of MZ B cells are found, showing an activated phenotype (Wither *et al*., 2000), and in a lupus-prone model, early expansion of MZ B cells is found, which is responsible for the production of anti-DNA antibodies (Schuster *et al*., 2002). Also, studies in which autoreactive transgenic BCRs were introduced showed that negative selection of these transgenes occurred but that the marginal zone B cells, in particular, were autoreactive (Goodnow, 1996). Mice overexpressing BAFF, the factor critical for the survival and maturation of MZ B cells, spontaneously develop an autoimmune SLE-like syndrome with increased numbers of MZ B cells (Mackay *et al*., 1999). Although these and several other studies (Viau and Zouali, 2005) point to a role for MZ B cells in autoimmunity, a direct mechanistic link between MZ B cells and the initiation of autoimmunity is not clear.

Autoimmune disorders can be seen as the perturbance of tolerance to self, which is a highly regulated intrinsic characteristic of B and T cell development. In addition, immunologic tolerance can be induced against exogenous antigens as a result of an active immune response. The best studied examples are immune responses that are generated along mucosal surfaces against protein antigens, involving the generation of mucosal regulatory cells (Faria and Weiner, 2005; Samsom, 2004). The majority of these tolerogenic responses do not lead to any involvement of the spleen (Samsom *et al*., 2005; Unger *et al*., 2003), with the exception of immune responses that are generated in the eye (Camelo *et al*., 2005; Stein-Streilein and Streilein, 2002). Antigen introduced into the anterior chamber of the eye is carried to the marginal zone by a subset of macrophages, expressing the F4/80 antigen, and leads to the induction of CD8 T cells that can suppress the immune response against the antigen. This anterior chamber-associated immune deviation (ACAID) model is clearly dependent on the marginal zone of the spleen, whereby the F4/80 molecule plays a crucial role (Lin *et al*., 2005). Whether this involvement of the marginal zone is typical for eye-associated tolerance or whether the induction of CD8 suppressor cells is a general function of the marginal zone is an interesting question that needs further investigation.

## V. Embryonic Development of the Marginal Zone of the Spleen

### A. Development of the Spleen

During embryonic development of the mouse, the first evidence of splenic development is the condensation and proliferation of mesenchymal tissues in the dorsal part of the mesogastrium seen at dE12.5 (embryonic day 12.5 or

12.5 days postcoitus) (Green, 1967; Roberts *et al.*, 1994). In the following days the spleen increases in size because of expanding centers of myelopoiesis and erythropoiesis (Metcalf and Moore, 1971; Sasaki and Matsumura, 1988). The first macrophages that carry the F4/80 antigen, characteristic of the macrophages in the red pulp, can be detected from dE15, while a subset of Siglec-1 (sialoadhesin)-positive macrophages appears between dE18 and birth (Morris *et al.*, 1991, 1992). At this time there is no apparent organization of the macrophage populations. Only in the late phase of splenic development can lymphocytes be found, and the first mature $IgM^+$ B cells are present at dE17, although their precursors can be isolated from the spleen as early as dE13.5 (Godin *et al.*, 1999; Velardi and Cooper, 1984). In rodents the first mature T cells leave the thymus in large numbers only after birth, leading to the subsequent development of separate splenic compartments from that time on (Friedberg and Weissman, 1974; Metcalf and Moore, 1971; Rugh, 1968).

During the development of the spleen, as early as dE13.5, a population of $CD4^+CD3^-$ cells can be found in the splenic anlage. These lineage-restricted progenitors have been well characterized in early lymph nodes and have the potential to differentiate to antigen-presenting cells, NK cells, and follicular cells, but not to T or B cells (Mebius *et al.*, 1996, 1997). Importantly, they are crucial for the development of several lymphoid organs such as nasal-associated lymphoid tissue (NALT), Peyer's patches (PP), and lymph nodes (Eberl *et al.*, 2004; Finke *et al.*, 2002; Fukuyama *et al.*, 2002) by giving instructive signals to stromal cells in the organ anlagen that lead to further differentiation (Mebius *et al.*, 1997). This has been well documented using mice deficient in genes of the TNF or NF-κB superfamilies. Interestingly, so far none of these defects, which in some cases can lead to complete absence of any lymph node, affects the initial formation of the spleen, although developmental aberrations are found (see below). This means that the formation of the spleen is regulated by early developmental genes and differs from the development of other secondary lymphoid organs.

Again, with the help of genetically altered mice some of the molecular interactions that are intrinsic to the formation of the spleen have been identified. A mesodermal-derived cell layer, the splanchnic mesodermal plate (SMP), which is part of asymmetric left–right morphogenic development, can be viewed as an organizing center for the development of the spleen. When the SMP is not formed, as in mice lacking the dominant hemimelia (Dh) gene or the homeobox transcription factor Bapx1, no spleen is formed (Green, 1967; Hecksher-Sorensen *et al.*, 2004; Lettice *et al.*, 1999; Tribioli and Lufkin, 1999). In mice that are deficient in the homeobox gene Hox11 the anlage of the spleen begins normally at dE11.5, but is rapidly followed by resorption due to apoptosis (Dear *et al.*, 1995) or persists as an unorganized rudiment (Kanzler and Dear, 2001). In aggregation chimeras

generated between Hox11$^{-/-}$ and normal mice the defect could not be rescued, which indicates that this defect is intrinsic to this cell population and is not due to the inability of the splenic anlage to attract and retain lymphocyte precursors (Kanzler and Dear, 2001). Also, deficiency in the basic helix-loop-helix transcription factor Capsulin (Lu *et al*., 2000), and the Wilm's tumor gene product Wt1 (Herzer *et al*., 1999), results in the complete absence of the spleen. Some of these genes may act in concert, whereby Bapx1 and Dh control the development of the SMP. Wt1 and Hox11 are probably regulated independently of each other (Herzer *et al*., 1999). Future studies will be necessary to uncover the precise molecular interactions that are essential for the development of the spleen.

## B. Development of the Marginal Zone

In both rats and mice the marginal zone is still not distinguishable at day of birth and gradually develops in the course of the next 2 weeks (Takeya and Takahashi, 1992). Macrophages that will later enter into the marginal zone can be found dispersed throughout the entire spleen during the first neonatal days, while the actual formation of the marginal zone starts around day 5 in mice. From this day on these macrophages start to localize at the interface of the red and white pulp and establish a marginal zone, which is then also populated by marginal zone B cells (Kraal *et al*., 1988b; Morris *et al*., 1992; Takeya and Takahashi, 1992). Around day 10 the marginal zone attains its full mature appearance, and at this stage marginal zone bridging channels can also be recognized, which represent protrusions of the white pulp area across the marginal zone into the red pulp (Mitchell, 1973; Takeya and Takahashi, 1992).

## C. Molecules Involved in the Organization of the Marginal Zone

Although little is known about the molecular interactions that regulate the formation of the separate splenic compartments, development of genetically altered mice has revealed molecules and pathways that are important in this process. A large number of knockout mice have been generated in the past decade that appeared to have minor or major defects in splenic organization. In mice that lack a single member of the TNF or NF-κB superfamily, severe defects in the formation of lymphoid tissue, such as the absence of (certain) lymph nodes and/or Peyer's patches, are seen (Mebius, 2003). In all these mice a spleen is formed; however, anatomical abnormalities, with respect to

the formation of distinct B and T cell areas as well as the marginal zone, are observed in the spleens of these mice.

## 1. Lymphotoxin and Its Receptors

Lymphotoxins (LT) are members of a complex communication system between lymphocytes and surrounding stromal cells. With its close homology to TNF, it is encompassed in the TNF superfamily, and although TNF and LT often work together in signaling networks, sharing receptors, it has become clear that LT has quite a distinct role, especially in the development of the immune system. Two distinct structural forms of LT can be discerned, LTα and LTβ, which form trimeric molecules. As a membrane-bound form it can be found as a heterotrimer, either $LT\alpha_1\beta_2$ or $LT\alpha_2\beta_1$, and in in secreted form as a homotrimer, $LT\alpha_3$ (Ruddle, 1992; Ware, 2005). Homotrimeric forms of both TNF-α and LTα can bind and activate each of the two defined TNF receptors, TNF-RI (p55) and TNF-RII (p75), while the LT heterotrimers, but not $LT\alpha_3$, interact through the LTβ-R (Fu and Chaplin, 1999; Ware *et al*., 1995).

Genetic disruption of either the LTα gene or the LTβ gene results in complete disruption of the splenic microarchitecture: follicles, their follicular dendritic cells (FDC), and germinal centers are lacking, as well as normal constituents of the marginal zone, such as macrophages, B cells, and sinus lining cells (Alimzhanov *et al*., 1997; Banks *et al*., 1995; De Togni *et al*., 1994; Matsumoto *et al*., 1996). In LTα-deficient mice, T and B cells have no separate areas and are completely intermingled, while LTβ-deficient mice show B cells localized in a ring around a central T cell area (Koni *et al*., 1997). Compared to LTβ-deficient mice, LTβ-receptor-deficient mice showed an even more severe phenotype, suggesting the involvement of additional ligands. It was found that LIGHT, a closely related trimeric ligand, was responsible (Wang *et al*., 2002). The introduction of LIGHT as a transgene in LTα-deficient mice did lead to restoration of most of the defects in the spleen, except for the organization of the marginal zone, emphasizing the importance of LTα in the development of this region. The importance of LT for the architecture of the spleen was also evident from studies that used a soluble LTβ-R-Ig fusion protein to block normal LTαβ/LTβ-R interactions. Injection of this fusion protein as a decoy receptor during embryonic life severely interferes with the formation of the marginal zone and the T and B cell compartments of the white pulp, comparable to the phenotype of LTβ-deficient mice (Ettinger *et al*., 1996; Rennert *et al*., 1996). Interestingly, experiments in which adult animals with a functional marginal zone were treated with the LTβ-R-Ig fusion protein showed a disturbed marginal zone and loss of B cell follicles. This clearly indicates that signaling through LTβ-R is not only important for adequate development of normal

splenic organization, but also for its maintenance in adult life (Mackay *et al.*, 1997).

## 2. TNF-α and Its Receptors

Although LT signaling appears to be crucial for proper postnatal development and proper compartmentalization of the spleen, signaling through TNF-α and its receptors is also important in this process: in mice that are deficient in either TNF-α or TNF-RI the marginal zones are present, but they are clearly less well developed than in wild-type mice. Reduced numbers of marginal zone and metallophilic macrophages are found, while MAdCAM-1 expression is completely absent (Matsumoto *et al.*, 1996; Neumann *et al.*, 1996b; Pasparakis *et al.*, 2000). In addition these mice lack splenic follicles and FDCs, although T and B cells are segregated as seen in LTβ-deficient mice (Körner *et al.*, 1997; Le Hir *et al.*, 1996; Pasparakis *et al.*, 1996). In contrast, spleens of TNF-RII-deficient mice appear normal, indicating that this receptor is either not important in splenic formation or that its function can be taken over by TNF-RI (Matsumoto *et al.*, 1996; Neumann *et al.*, 1996a; Pasparakis *et al.*, 2000).

Soluble TNF-RI-Ig can reduce the expression of MAdCAM-1 (not other marginal zone markers) and disrupt the splenic T/B cell compartments, but only when this receptor decoy is administered during ontogeny (Rennert *et al.*, 1996, 1997). In contrast to the findings with LTβ-R, injections of soluble TNF-RI-Ig in adult mice did not lead to any effects, thus indicating that signaling through this receptor is important only for splenic development, but not for its maintenance (Mackay *et al.*, 1997). Importantly, bone marrow transplantation experiments have revealed that expression of TNF-RI and LTβ-R, required for proper development of the splenic microarchitecture, is necessary on radioresistant stromal cells, while LTα, LTβ, and TNF-α are derived from cells of hematopoietic origin, which are most likely B cells (Endres *et al.*, 1999; Matsumoto *et al.*, 1996, 1997; Mebius *et al.*, 1998; Tkachuk *et al.*, 1998). This could explain why the marginal zone in B cell-deficient mice is not developed, and when B cells are experimentally depleted in adult life, this leads to a disappearance of marginal zone macrophages and the expression of MAdCAM-1 (Nolte *et al.*, 2004). LTβ-R or TNF-R triggering leads downstream to the activation of NF-κB. This ubiquitously expressed transcription factor family is involved in numerous cellular responses and serves as a critical regulator of the inducible expression of many genes (de Winther *et al.*, 2005). In mammalian systems, this family is composed of subunits p50, p52, RelA (p65), c-Rel, and RelB, which can all form dimeric complexes, depending on the cell type and activation state (Baeuerle and Henkel, 1994; Ghosh *et al.*, 1998; Mercurio and Manning, 1999). These dimers exist in the cytoplasm in an inactive form, due to

interaction with inhibitory proteins termed IκBs. Upon activating signals, these IκBs are phosphorylated and consequently degraded, which results in nuclear translocation of the NF-κB dimeric complex and transcription of its target genes (de Winther *et al.*, 2005; Hayden and Ghosh, 2004).

Several phenotypes that affect the marginal zone by deficiencies in the NF-κB route exhibit defects that are similar to those seen with LTβ-R-deficient mice, being parts of the same activation and signaling route. This is the case for mice deficient in NIK, the natural mutant *aly/aly* mice (Shinkura *et al.*, 1999), which in addition to lacking lymph nodes and Peyer's patches, have no marginal zone (Karrer *et al.*, 1997; Koike *et al.*, 1996; Miyawaki *et al.*, 1994; Yamada *et al.*, 2000). Also, relB expression is absolutely required for the development of the splenic marginal zone (Weih *et al.*, 2001). RelB acts downstream of LTβ-R, and perhaps TNF-R, and is most likely expressed by endothelial and/or stromal cells present in the marginal zone (Weih and Caamano, 2003).

## VI. Concluding Remarks

In summarizing the multitude of data that have been generated on the function and development of the splenic marginal zone, the most obvious conclusion involves the complex interplay that takes place in this region between cell types from completely different lineages. Compared to our knowledge a decade ago, it is clear that a general picture of what the marginal zone was and how it functioned existed, but little was known about these intricate cellular interactions. It is clear that the marginal zone is a highly dynamic region in which the continuous interaction of the various resident and transmigrating cells is necessary to maintain its active state. In particular, the interaction between macrophages, MZ B cells, and dendritic cells is crucial for the integrity of this region. Through growth factors and hormones and cell–cell contacts, a picture of delicately balanced interactions is emerging, and any perturbance of this balance can have direct consequences for the function of the marginal zone.

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## Further Reading

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