STRUCTURE AND FUNCTION OF THE SPLEEN

Reina E. Mebius and Georg Kraal

Abstract | The spleen combines the innate and adaptive immune system in a uniquely organized way. The structure of the spleen enables it to remove older erythrocytes from the circulation and leads to the efficient removal of blood-borne microorganisms and cellular debris. This function, in combination with a highly organized lymphoid compartment, makes the spleen the most important organ for antibacterial and antifungal immune reactivity. A better understanding of the function of this complex organ has been gained from recent studies, as outlined in this Review article.

VENOUS SINUSOIDAL SYSTEM
Blood sinuses in which the blood is collected from the cords in the splenic red pulp and transported to the efferent vein of the spleen (the vena lienalis). The structure of the wall of the sinuses allows the removal of ageing erythrocytes from the circulation.

TRABECULA
A bar of connective tissue that protrudes from the capsule into the splenic tissue. Together, the capsule and the trabeculae form a supporting, three-dimensional framework that provides some rigidity to the spleen.

Department of Molecular Cell Biology and Immunology, Vrije Universiteit Medical Center, v.d. Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. Correspondence to R.E.M. e-mail: r.mebius@vumc.nl
doi:10.1038/nri1669

Located in the abdomen, directly beneath the diaphragm, and connected to the stomach, the spleen is the body's largest filter of the blood[1,2]. In essence, the spleen is organized as a 'tree' of branching arterial vessels, in which the smaller arterioles end in a VENOUS SINUSOIDAL SYSTEM. The organ is surrounded by a fibrous capsule of connective tissue, stemming from which are TRABECULAE that support the larger vasculature (FIG. 1). The smaller branches of the arterial supply are sheathed by lymphoid tissue (FIG. 1), which forms the white pulp of the spleen. In rodents, some of the smallest arterial branches terminate in the marginal sinus — the space between the white pulp and the surrounding marginal zone — whereas others traverse the marginal zone to form the venous system of the red pulp, which is named after the large, blood-filled sinuses. In humans, part of the bloodstream ends in the perifollicular zone (FIG. 1b). With its location in the circulatory system and with the unusual structure of its lymphoid compartments, the spleen is a unique lymphoid organ. This is also reflected in its embryological development, which differs from that of other lymphoid organs (BOX 1). The combination of highly adapted macrophages and specific anatomical features, of the marginal zone in particular, underlies the fact that the spleen is a crucial site of early exposure to encapsulated bacteria[1]. In this article, we discuss how splenic macrophages not only have a role in the recognition and uptake of pathogens by PATTERN-RECOGNITION RECEPTORS but also are involved in the structural organization of the organ. We highlight how splenic macrophages fight bacteria by competing for iron and discuss recent insights into the molecular interactions that lead to differentiation into (BOX 2), and migration of, follicular versus marginal-zone B cells. These and other recent developments have rekindled interest in the spleen and have led to better insight into the relationship between the structure and the function of this important organ. This overview is mainly based on studies in rodents, the spleens of which are slightly different on an anatomical level than those of humans. The anatomical differences between primate and rodent spleens are schematically illustrated in FIG. 1b.

The red pulp

In this section, we describe the efficient blood-filtering system of the spleen and its importance for iron recycling by splenic macrophages of the red pulp. In addition, we discuss that, in these macrophages, the processes that are involved in iron metabolism are also involved in the removal of bacteria from the blood.

Filtering the blood. The specialized structure of the venous system of the red pulp gives this area its unique capacity to filter the blood and remove old erythrocytes. Arterial blood arrives into cords in the red pulp, which consist of fibroblasts and reticular fibres and form an open blood system without an endothelial lining[3]. In these cords, many macrophages are found.

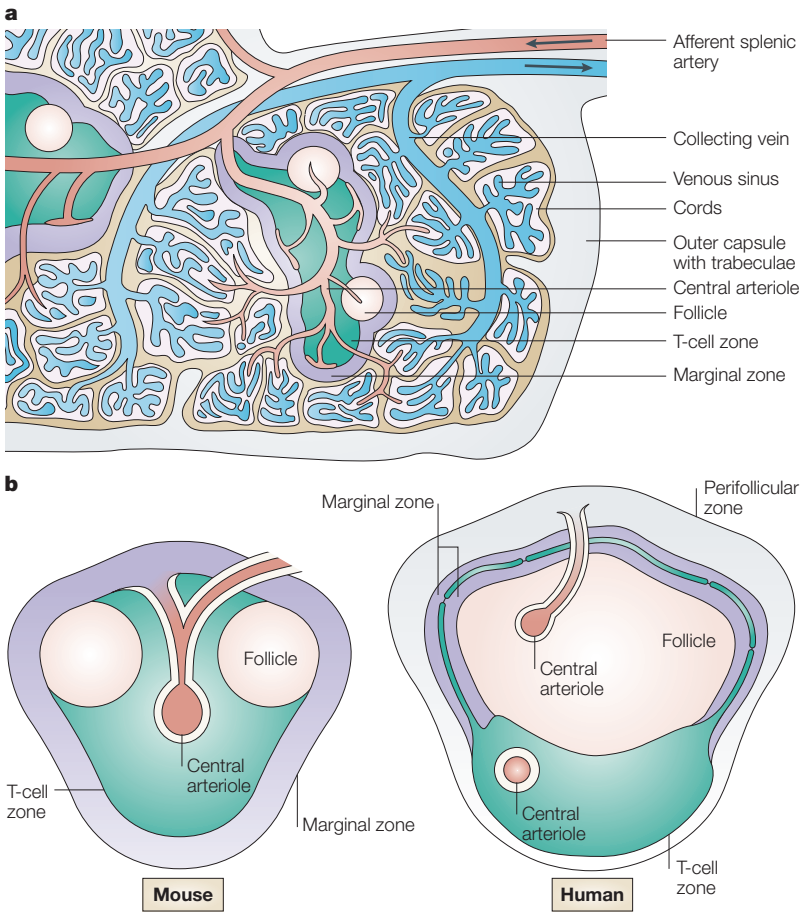

Figure 1 | **Structure of the spleen. a** | Schema of the spleen. The afferent splenic artery branches into central arterioles, which are sheathed by white-pulp areas; these white-pulp areas consist of the T-cell zone (also known as the periarteriolar lymphoid sheath, PALS), arterioles and B-cell follicles. The arterioles end in cords in the red pulp, from where the blood runs into venous sinuses (FIG. 2), which collect into the efferent splenic vein. The larger arteries and veins run together in connective-tissue trabeculae, which are continuous with the capsule that surrounds the spleen. **b** | Comparison of the structure of the white pulp in rodents and primates. The main differences are found in the structure of the marginal zone, which surrounds the white pulp. In contrast to mice, humans have an inner and an outer marginal zone, which is surrounded by a large perifollicular zone. In the perifollicular zone, some blood vessels terminate, and the endings of these capillaries are sheathed by macrophages. These macrophages express sialic-acid-binding immunoglobulin-like lectin 1 (SIGLEC1)[98,99].

PATTERN-RECOGNITION RECEPTOR
A receptor that recognizes unique structures that are present at the surface of microorganisms. Signalling through these receptors leads to the production of pro-inflammatory cytokines and chemokines and to the expression of co-stimulatory molecules by antigen-presenting cells. The expression of co-stimulatory molecules, together with the presentation of antigenic peptides, by antigen-presenting cells couples innate immune recognition of pathogens with the activation of adaptive immune responses.

From the cords, the blood passes into the venous sinuses of the red pulp, which collect into the efferent VENA LIENALIS. These sinuses are lined by endothelium that has an unusual discontinuous structure, with stress fibres extending underneath the basal plasma membrane, running parallel to the cellular axis[4]. The stress fibres connect the endothelial cells to components of the extracellular matrix and are composed of actin- and myosin-like filaments, indicating that there might be a sliding filament action by which the spaces between the endothelial cells are controlled (FIG. 2). The arrangement of the stress fibres, together with the parallel arrangement of the endothelial cells of the sinuses, forces the blood from the cords into the sinuses, through the slits that are formed by the stress fibres[5]. This passage becomes difficult for ageing erythrocytes, which have stiffening membranes[6], such that they stick in the cords and are phagocytosed by the red-pulp macrophages that are located in the cords. The contractility of the stress fibres might also aid in the retention of erythrocytes in the spleen (as has been observed in various mammals, such as dogs and horses), thereby forming a reservoir of erythrocytes and reducing stress on the heart by reducing the viscosity of the blood during rest[7].

Recycling iron. Erythrophagocytosis is important for the turnover of erythrocytes, and recycling of iron is an important task of splenic macrophages, in conjunction with those of the liver[8]. Erythrocytes are hydrolysed in the PHAGOLYSOSOME of macrophages, from which haem is released after the proteolytic degradation of haemoglobin. Haem is then further catabolized into biliverdin, carbon monoxide and ferrous iron (Fe^{2+}), after which the iron is either released from cells or stored[9]. Iron that is not used or released by a cell is stored as ferritin, which is a cytosolic protein. For the storage of larger amounts of iron in a cell, ferritin can aggregate into haemosiderin, which is an insoluble complex of partially degraded ferritin[8], deposits of which can easily be observed in red-pulp macrophages. Iron can be released from macrophages as ferritin or as low-molecular-weight species, and these rapidly bind plasma transferrin, which functions as a transporter protein.

In addition to such phagocytosis of erythrocytes, a considerable proportion of erythrocytes are also destroyed intravascularly throughout the body, as a result of continuing damage to their plasma membrane. This leads to the release of haemoglobin[8], which is bound rapidly by HAPTOGLOBIN. Receptor-mediated endocytosis of CD163, a haemoglobin-specific receptor at the cell surface of macrophages[10], leads to scavenging of haemoglobin from the circulation in the spleen. The release of iron from its storage in splenic macrophages is regulated by the requirements of the bone marrow, but the underlying mechanisms are not well understood. Iron uptake by most cells is mediated by a pH-dependent transporter for divalent metals — natural-resistance-associated macrophage protein 2 (NRAMP2) — which is found in transferrin-receptor-positive recycling endosomes, where it mediates the transport of ferritin iron across the endosomal membrane into the cytoplasm[11]. Interestingly, macrophages and monocytes express another NRAMP molecule, NRAMP1 (REF. 12). NRAMP1 was originally found to be involved in the resistance of inbred mice to certain intracellular pathogens, and this turned out to result from the ability of this molecule to transport iron across the phagosomal membrane. Although there is some debate on the direction of this transport, the result is that there is interference with the iron metabolism of the bacterium, thereby limiting its growth[13]. Interestingly, NRAMP1 seems to result from a basic iron-transport mechanism being adapted to fight pathogens in specific cells that are already engaged in iron metabolism through erythrophagocytosis, thereby linking two important functions of the splenic red pulp.

Box 1 | **Development of the spleen**

During embryogenesis, the initial event in the development of the spleen is the formation of the splanchnic mesodermal plate (SMP) at embryonic day 12 (E12), which is one of the processes in formation of the asymmetrical left–right axis. The SMP, which is derived from the mesoderm, can be seen as an organizing centre — that is, an anlage — for the formation of the spleen. When formation of the SMP is defective, as occurs in mice that are deficient in dominant hemimelia (DH) or the homeobox transcription factor bagpipe homeobox homologue 1 (BAPX1), then no spleen is formed[82,83]. Also, when the cells that form the SMP fail to proliferate, as occurs in mice that are deficient in the factor homeobox 11 (HOX11), then no further development of the spleen occurs[84]. In addition, both the basic helix–loop–helix transcription factor capsulin and Wilm's tumour 1 (WT1), which are already expressed in the splenic anlage at E12, are indispensable for the formation of the spleen[85,86].

The first cells that colonize the spleen are progenitors of the erythroid and myeloid lineages; at E14.5, after the progenitors have entered, the first haematopoietic stem cells lodge in the spleen (reviewed in REF. 87). On day E13.5, lymphoid-tissue-inducer cells, which are phenotypically identified as $CD4^+CD3^-CD45^+$ cells, are present in the spleen[88]. These cells provide the inductive signal for the development of the Peyer's patches and the nasopharynx-associated lymphoid tissue (NALT) and are thought to be crucial in the delivery of a similar signal for the development of the lymph nodes[89]. The formation of the lymph nodes and the Peyer's patches follows a scheme that is highly similar, in which signalling through the lymphotoxin-β receptor is crucial for further development[89]. However, the formation of the spleen depends on molecular interactions that are distinct from those involved in the generation of the lymph nodes and the Peyer's patches.

Iron is important for survival of both the host and the bacterium. Several pathogens compete for iron in serum and tissue by secreting SIDEROPHORES, which are molecules with a high affinity for iron, and these molecules are transported back into bacteria by specific receptors[14]. After macrophages encounter bacteria and signalling through TOLL-LIKE RECEPTORS has been initiated, macrophages can secrete molecules such as lipocalin-2, which complex with siderophores and, consequently, limit the growth of bacteria[15]. Lipocalin-2 is produced by several myeloid cells, but its production can easily be induced, particularly in red-pulp macrophages. These examples show that the red pulp not only is anatomically well suited for its blood-filtering function, by the combination of an open and sinusoidal venous system, but also contains macrophages that have special properties for fighting bacteria and facilitating iron metabolism.

Box 2 | **Generation of marginal-zone versus follicular B cells**

Recently, various publications have reported that whether marginal-zone B cells or follicular B cells are generated is a cell-fate decision of mature B cells that is controlled by signalling through Notch proteins and by the activity of E proteins[90,91]. In the absence of Notch-2 or RBP-J (Igκ joining-region recombination signal-binding protein 1; a DNA-binding molecule that is essential for Notch signalling), marginal-zone B cells do not develop, which indicates that Notch-2 signalling is required for the development of marginal-zone B cells[90,92]. Consistent with this is the observation that Notch-2 can be found in the marginal zone, presumably in mature B cells, which can still develop into either marginal-zone B cells or follicular B cells[93]. Conversely, high levels of MINT (MSH-homeobox-homologue 2-interacting nuclear target), which competes with RBP-J for binding to the intracellular domain of Notch, can be found in follicular B cells[93]. In the presence of high levels of MINT, Notch signalling is efficiently prevented, and follicular B cells develop preferentially. So, the concentration of MINT can determine the cell fate of mature B cells in the spleen.

In a similar manner, the levels of the helix–loop–helix transcription factor E2A and its antagonist ID3 (inhibitor of DNA binding 3) can determine the cell fate of mature B cells[91]. Lower levels of E2A result in preferential generation of marginal-zone B cells, whereas the absence of ID3 results in the generation of more follicular B cells. Because both Notch-2-dependent signalling and high levels of ID3 lead to the differentiation of mature B cells into marginal-zone B cells, Notch might control the levels of ID3. The level of Notch signalling is, in turn, determined by the amount of MINT that is expressed by mature B cells.

Others have proposed that the strength of signalling through the B-cell receptor (BCR) also contributes to cell-fate decisions in mature B cells[94], in which the zinc-finger transcription factor Aiolos and Bruton's tyrosine kinase (BTK) have opposite effects on the development of follicular versus marginal-zone B cells. In the absence of Aiolos, marginal-zone B cells cannot form, whereas in the absence of BTK, which is activated on BCR signalling, marginal-zone, but not follicular, B cells are present[95,96]. In the absence of Aiolos, increased BCR signalling is also observed, which has led to the hypothesis that Aiolos is a negative regulator of BTK[94]. In this model, mature B cells can only develop into follicular B cells after strong signalling through the BCR, whereas weak signalling through the BCR allows the formation of marginal-zone B cells.

Taken together, a complex picture of B-cell cell-fate decisions emerges from these findings, including the involvement of BCR signal strength, Notch-2 and E proteins. Further studies are required to fully elucidate the mechanism of this process[97].

VENA LIENALIS
The efferent vein of the spleen.

PHAGOLYSOSOME
An intracellular vesicle that results from the fusion of phagosomes, which enclose extracellular material that has been ingested, and lysosomes, which contain lytic enzymes.

HAPTOGLOBIN
A plasma protein that can bind free haemoglobin in the bloodstream.

SIDEROPHORE
A compound that is secreted by microorganisms that efficiently bind iron.

TOLL-LIKE RECEPTOR
A type of pattern-recognition receptor that is expressed by macrophages and dendritic cells. These receptors recognize unique structures that are present at the surface of microorganisms.

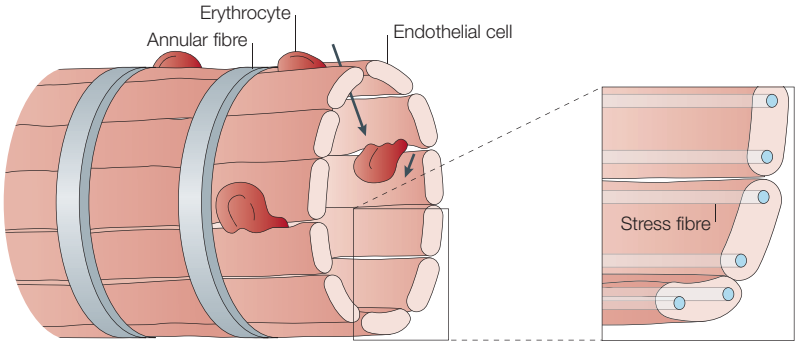

Figure 2 | **Venous sinuses in the red pulp of the spleen.** Schema of a venous sinus located in the cords of the red pulp. Blood from the cords collects in the sinuses (shown by arrows). The venous sinuses consist of a lining of endothelial cells that are positioned in parallel and connected by stress fibres to annular fibres, which are composed of extracellular-matrix components. The stress fibres run along the long axis of the endothelial cells and are most prominent where the endothelial cells are in contact. Contractility of the stress fibres allows the formation of slits between the endothelial cells, thereby regulating the passage of blood and blood cells from the red-pulp cords into the sinuses and back into the venous system. Because the red-pulp cords contain a large number of macrophages, ageing erythrocytes that are no longer able to pass through the slits are phagocytosed.

Producing antibodies. The red pulp is also known to be a site where PLASMABLASTS and PLASMA CELLS lodge. After antigen-specific differentiation in the follicles of the white pulp, plasmablasts migrate into the red pulp, initially just outside the marginal zone[16]. However, the exact anatomical position and cellular interactions that are involved in the retention of these cells are not clear (reviewed in REF. 17). The position of plasmablasts in the red pulp resembles the localization of plasmablasts in the medullary cords of lymph nodes, and this extrafollicular antibody production leads to rapid entry of antibody into the bloodstream. Evidence indicates that plasmablasts are attracted to the red pulp after upregulating their expression of the chemokine receptor CXC-chemokine receptor 4 (CXCR4); this receptor binds the chemokine CXC-chemokine ligand 12 (CXCL12), which is expressed in the red pulp[18]. This coincides with downregulation of expression of the chemokine receptors CXCR5 and CC-chemokine receptor 7 (CCR7), which bind the homeostatic chemokines that are present in the B-cell follicles and T-cell zone of the white pulp. Interestingly, it has been found that plasmablasts require CD11c^{hi} dendritic cells (DCs) to survive in the red pulp and to make the transition into plasma cells[19]. The presence of CD11c^{hi} DCs in the T-cell zone of the white pulp, as well as in the bridging channels that extend into the red pulp, might be of assistance in this transition. The bridging channels are where antibody-forming cells have been described to temporarily reside after antigenic challenge[20].

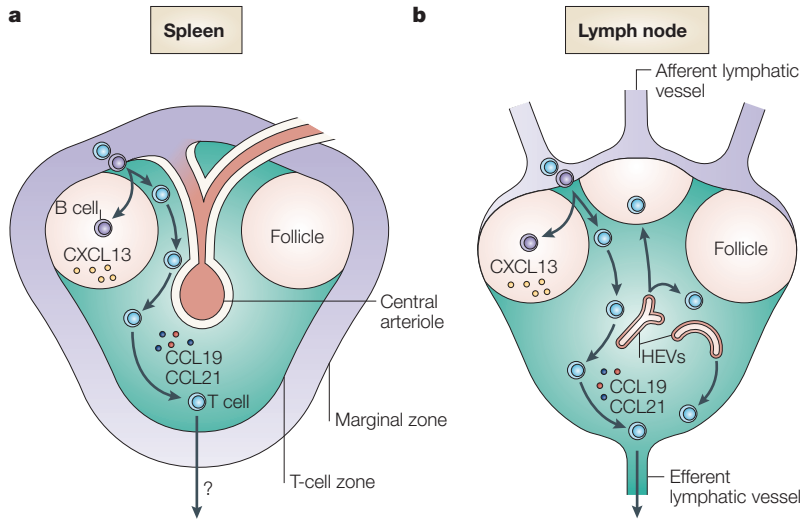

Figure 3 | **Comparison of lymphoid compartments and the migration pathways of lymphocytes into the splenic white pulp and the lymph nodes. a** | The spleen. Lymphocytes enter the white pulp of the spleen from the marginal zone, and entry is mediated by signalling through chemokine receptors. B cells are attracted to the B-cell follicles by CXC-chemokine ligand 13 (CXCL13), whereas T cells are directed to the T-cell zone by responding to CC-chemokine ligand 19 (CCL19) and CCL21. It is unclear how lymphocytes eventually leave the white pulp. **b** | The lymph nodes. Few lymphocytes enter a lymph node from the afferent lymphatic vessels. Most enter through specialized blood vessels that are known as high endothelial venules (HEVs) and then migrate to the B-cell follicles or the T-cell zone, which again is regulated by CXCL13, and CCL19 and CCL21, respectively. Lymphocytes exit lymph nodes in efferent lymphatic vessels, and they then re-enter the bloodstream from the lymph.

Organization of the lymphoid compartments

In this section, we describe the structure of the lymphoid region of the spleen — the white pulp — with an emphasis on new insights into how the cells of the immune system migrate and lodge in the various compartments of the white pulp.

White pulp. The white pulp is organized as lymphoid sheaths, with T- and B-cell compartments, around the branching arterial vessels, so it closely resembles the structure of a lymph node. The correct organization and maintenance of the white pulp is controlled by specific chemokines that attract T and B cells to their respective domains, thereby establishing specific zones within the white pulp (FIG. 3). In the T-cell zone (also known as the periarteriolar lymphoid sheath, PALS), T cells interact with DCs and passing B cells, whereas in the B-cell follicles (also known as the B-cell zones), clonal expansion of activated B cells can take place, which leads to isotype switching and somatic hypermutation. CXCL13 is required for B cells to migrate to the B-cell follicles[21], whereas CC-chemokine ligand 19 (CCL19) and CCL21 are involved in attracting T cells and DCs to the T-cell zones of the white pulp[22,23] (FIG. 3). Expression of these chemokines is controlled by lymphotoxin-$\alpha_1\beta_2$ (LT-$\alpha_1\beta_2$) and tumour-necrosis factor (TNF)[24]. When signalling through the LT-β receptor (LT-βR) or TNF receptor 1 (TNFR1) is lacking, levels of the homeostatic chemokines CXCL13, CCL19 and CCL21 are reduced in the spleen, which results in disorganization of white-pulp regions[24] (TABLE 1). Both LT-βR and TNFR1 are expressed by radiation-resistant stromal cells, whereas their ligands are expressed by haematopoietic cells, most probably B cells, as occurs in RADIATION CHIMERAS[25–27]. After engagement of these receptors, nuclear factor-κB becomes activated, resulting in induction of expression of the various chemokines.

Table 1 | **Summary of mutant mice with defective splenic organization or lack of specific cellular subsets**

Deficiency*	Marginal-zone macrophages	Marginal-zone metallophilic macrophages	Marginal-zone B cells	MADCAM1+ sinus-lining cells	Segregation of B- and T-cell zones	Follicular dendritic cells	Germinal centres	References
LT-α	–	–	–	–	–	–	–	25,100–105
LT-β	–	–	–	–	+	–	↓↓	102,103, 105,106
LT-β and TNF	–	–	–	–	↓↓	–	–	105
LT-α, LT-β and TNF	–	–	–	–	–	–	–	107
LT-βR	–	–	–	–	–	–	↓↓	108
LT-β in B cells	↓↓	↓↓	↓↓↓	↓↓	↓↓	↓↓↓	↓↓↓	30
LT-β in T cells	+	+	+	+	+	+	+	30
LT-β in B and T cells	↓↓↓	↓↓↓	–	↓↓	↓↓	–	–	30
LIGHT and LT-β	–	–	↓↓	–	↓↓	–	–	109
TNF	↓↓	↓↓	+	–	↓	–	–	110–112
TNFR1	↓↓	↓↓	+	–	↓	–	–	110,113
TNFR2	+	+	+	+	+	+	+	114
NIK	–	–	–	–	–	–	–	115–118
NF-κB p50	+	+	–	+	+	+	+	119,120
NF-κB p52	+	–	ND	–	↓↓	–	–	119,121,122
REL-B	–	–	–	–	–	–	–	119,123
REL	ND	+	↓↓	+	↓↓	ND	–	119
BCL-3	–	↓↓	ND	↓↓	+	↓↓	–	119,124
PYK2	+	+	–	ND	+	+	+	53
$S1P_1$‡	ND	ND	+ (not in MZ)	+	+	ND	ND	48
$S1P_3$	ND	+§	↓↓	+§	ND	ND	ND	49
Aiolos¶	ND	ND	–	ND	ND	ND	ND	94
NKX2.3	–	–	–	–	–	ND	ND	125

*Indicates mice (or cells, in a conditional gene-knockout model) that are deficient in specific cell-surface molecules or transcription factors that lead mainly to defects in organization of the spleen or to a lack of specific cellular subsets in the spleen. ‡Chimeras of fetal liver cells from mutant mice were transferred into wild-type recipients. §Distributed over a larger area. ¶A representative example of the many deficiencies that affect differentiation into marginal-zone B cells (BOX 2), but because their effects on splenic organization have not been reported, they are not included in this table. +, present; –, absent; ↓, slightly reduced; ↓↓, reduced; ↓↓↓, strongly reduced; BCL-3, B-cell lymphoma 3; LT, lymphotoxin; LT-βR, LT-β receptor; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; MZ, marginal zone; ND, not determined, NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; NKX2.3, NK2-transcription-factor related, locus 3; PYK2, protein tyrosine kinase 2; $S1P_1$, sphingosine 1-phosphate receptor 1; $S1P_3$, sphingosine 1-phosphate receptor 3; TNF, tumour-necrosis factor; TNFR, TNF receptor.

CXCL13, which is crucial for attracting B cells to the B-cell follicles, is produced by $CD35^+$ FOLLICULAR DENDRITIC CELLS (FDCs) and stromal cells that are adjacent to these FDCs[21]. B cells express the receptor CXCR5, which mediates their migration towards the B-cell follicles. In addition, signalling through CXCR5 induces the expression of LT-$\alpha_1\beta_2$ at the surface of B cells, which in turn induces the differentiation of FDCs and their expression of CXCL13, thereby mediating a positive-feedback loop.

There is a similar mechanism for regulating the integrity of the T-cell zone, in which the chemokines CCL19 and CCL21 (which are mainly produced by stromal cells in the T-cell zone) are crucial for the attraction and retention of T cells[28] (FIG. 3). In addition, DCs in the T-cell zone produce CCL19, although to a lesser extent than do stromal cells[28]. During development of the T-cell-zone stromal compartment, LT-$\alpha_1\beta_2$-expressing B cells are required for sufficient expression of CCL21 by stromal cells. This crucial interaction of B cells and stromal cells occurs in early neonatal life[29]. Conversely, it has been shown that, after immunization of mice with a B-cell-specific LT-β deficiency, the levels of CCL21 are only marginally lower than in wild-type mice, indicating that the instructive role of LT-$\alpha_1\beta_2$-expressing B cells is less strict than the previous study described[30]. In these experiments, however, chemokine expression was always measured after immunization. This could indicate that immunization induces expression of LT-$\alpha_1\beta_2$ by cells other than B cells (which could account for an increase in chemokine levels) or that other TNF-family members can induce CCL21 expression by stromal cells after immunization.

PLASMABLAST
An activated B cell at an intermediate stage of differentiation. Plasmablasts leave germinal centres and migrate through the lymph and the blood to distant sites, such as the skin and the lamina propria of the intestines, to become antibody-producing plasma cells.

PLASMA CELL
A terminally differentiated cell of the B-cell lineage that secretes large amounts of antibodies.

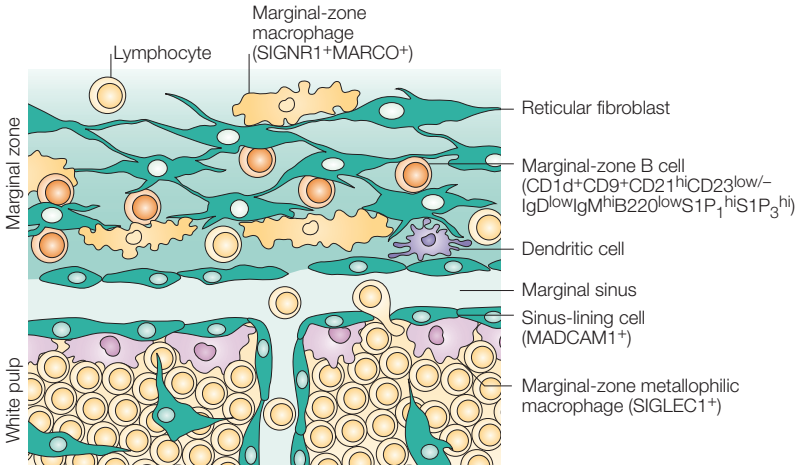

Figure 4 | **Anatomical localization of the cell types that comprise the marginal zone.** A framework of reticular fibroblasts forms the basis of the marginal zone and is continuous with the reticular fibroblasts in the red pulp and the sinus-lining cells of the marginal sinus. In this framework, the most distinctive cell type is the marginal-zone macrophage, which expresses both MARCO (macrophage receptor with collagenous structure) and SIGNR1 (a mouse homologue of DC-SIGN). Another important cell type in the marginal zone is the marginal-zone B cell. In addition to these two resident cells, many lymphocytes and dendritic cells, as well as granulocytes, can also be found in transit, because part of the bloodstream flows through the marginal zone into the red pulp (FIG. 1). Lymphocytes and dendritic cells can enter the white pulp from the marginal sinus by passing through a layer of sinus-lining cells that form a barrier between the marginal zone and the white pulp. These sinus-lining cells express mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1). Directly beneath the sinus-lining cells is a ring of sialic-acid-binding immunoglobulin-like lectin 1 (SIGLEC1)$^+$ macrophages, which are known as marginal-zone metallophilic macrophages. $S1P_1$, sphingosine 1-phosphate receptor 1; $S1P_3$, sphingosine 1-phosphate receptor 3.

Marginal zone. The continuous migration of haematopoietic cells from the blood into the lymphoid organs and back to the blood is an efficient way for these cells to search for pathogens and antigens. In the spleen, the marginal zone is an important transit area for cells that are leaving the bloodstream and entering the white pulp. This is an active process, which involves signalling through G-protein-coupled receptors[31], and it is probably similar to the multistep transmigration process that has been described for leukocytes transversing inflamed endothelium[32]. The exact molecular interactions by which lymphocytes enter the white pulp are still under debate.

In addition to being a transit area, the marginal zone contains a large number of resident cells that not only have unique properties but also seem to depend on each other for their localization, thereby establishing and maintaining the integrity of the marginal zone (FIG. 4). Two subsets of specific macrophages can be found there. The first subset — MARGINAL-ZONE MACROPHAGES — forms an outer ring of macrophages, and these cells are characterized by expression of the C-type lectin SIGNR1 (which is a mouse homologue of DC-SIGN)[33–35] and the type I scavenger receptor MARCO (macrophage receptor with collagenous structure)[36] (FIG. 4). The second subset — MARGINAL-ZONE METALLOPHILIC MACROPHAGES — is located closer to the white pulp and forms an inner ring of macrophages, and these cells are characterized by expression of the adhesion molecule SIGLEC1 (sialic-acid-binding immunoglobulin-like lectin 1; also known as sialoadhesin)[37]. Located between these two macrophage subsets are the marginal-zone B cells and a subset of DCs[38,39] (FIG. 4).

An important role in the organization and integrity of the marginal zone has been attributed to B cells. Studies in which B cells were absent from the time of birth or were induced to disappear led to disappearance of the two macrophage subsets[40,41]. In lymph-node development and organization, $CD4^+CD3^-CD45^+$ inducer cells (known as LYMPHOID-TISSUE INDUCER CELLS) lead to upregulation of expression of chemokines and adhesion molecules by stromal cells, through LT-βR signalling[42,43], and a similar role can be envisaged for B cells in organization of the marginal zone. B cells that express LT-$\alpha_1\beta_2$ can bind LT-βR that is expressed by endothelial and/or stromal cells present in the marginal zone, and this leads to induction of expression of a range of chemokines that could, in turn, influence the lodging and retention of the various cellular subsets in this region. This induction could occur when mature B cells either lodge in the marginal zone as marginal-zone B cells or transit through the marginal zone on their way to the B-cell follicles. This correlates with the findings that marginal-zone macrophages depend on CCL19 and CCL21 for their localization: in the absence of these chemokines, marginal-zone macrophages are no longer found in their specific domain next to the endothelial lining of the marginal sinus, whereas in the absence of CXCL13, a mannose-receptor-binding subset of DCs is absent from the marginal zone[38,44]. Although no chemokine has so far been identified to be crucial for the attraction and/or retention of the marginal-zone metallophilic macrophages in the marginal zone, one can envisage that chemokines are important in these processes, given the crucial role of B cells in the maintenance of these cells[40]. Conversely, it has been shown that MARCO expressed by marginal-zone macrophages is essential for the retention of marginal-zone B cells[45], indicating that these cells can influence each other.

So, although marginal-zone B cells can be induced to translocate to the white pulp, they are regarded as a unique and relatively sessile population of the marginal zone, with features that clearly distinguish them from follicular B cells. Recently, insights have been gained into the mechanisms that are involved in their lodging and retention in the marginal zone. Studies have revealed that there is a complex interplay between lipid and chemokine receptors. Marginal-zone B cells express both $S1P_1$ and $S1P_3$ — receptors for the lysophospholipid sphingosine 1-phosphate (S1P) — which have been shown to be crucial for the exit of T cells from the thymus and lymph nodes[46,47]. $S1P_1$ and $S1P_3$ are expressed at higher levels by marginal-zone B cells than by follicular B cells[48]. Accordingly, marginal-zone B cells, and not follicular B cells, respond vigorously to S1P *in vitro*[48]. This *in vitro* response can mainly be attributed to the expression of $S1P_3$ by marginal-zone B cells, because marginal-zone B cells from $S1P_3$-deficient mice fail to migrate towards S1P[48,49]. However, in the absence

RADIATION CHIMERA
An animal that contains cell populations of different genotypes as a result of the transfer of haematopoietic stem cells from fetal liver or bone marrow to a recipient in which haematopoietic-cell populations (and other actively dividing cell populations) have been fully or partially destroyed by lethal or sub-lethal ionizing radiation.

FOLLICULAR DENDRITIC CELL (FDC). A stromal cell that is crucial for the development of germinal centres in B-cell follicles. The interaction between FDCs and B cells is thought to be essential for isotype switching and somatic hypermutation.

MARGINAL-ZONE MACROPHAGE
A large macrophage in the splenic marginal zone that is characterized by the expression of a unique set of pattern-recognition receptors.

of $S1P_3$ expression, marginal-zone B cells can still localize to the marginal zone, showing that migration towards S1P does not have a role in the initial localization of marginal-zone B cells in the marginal zone.

For B cells to localize to the marginal zone, signalling through $S1P_1$ is required, because $S1P_1$-deficient B cells fail to localize to the marginal zone and migrate to the B-cell follicles[48] (TABLE 1). However, in the absence of CXCL13, which attracts B cells to the B-cell follicles, the ability of $S1P_1$-deficient B cells to lodge in the marginal zone is restored. These experiments indicate that S1P-mediated signalling prevails over the attraction cues from CXCL13 (REF. 48). Such an aspect of S1P-mediated signalling has been described for T cells, which fail to deliver a chemotactic response towards a chemokine in the presence of S1P at concentrations that are present in the blood and the lymph[50,51]. For B cells in the marginal zone, this could be similar, because after stimulation with lipopolysaccharide (LPS) or after antigen encounter, marginal-zone B cells downregulate expression of $S1P_1$ and $S1P_3$ and can subsequently relocalize to the B-cell follicles[48]. In the absence of $S1P_1$ expression, these B cells can either respond to CXCL13 and migrate to the B-cell follicles or downregulate the expression of adhesion molecules that are used for retention of marginal-zone B cells in the marginal zone. The adhesion molecules that are thought to be involved in the retention of marginal-zone B cells are the integrins lymphocyte function-associated antigen 1 (LFA1; $\alpha_L\beta_2$-integrin) and $\alpha_4\beta_1$–integrin, which interact with intercellular adhesion molecule 1 (ICAM1) and vascular cell-adhesion molecule 1 (VCAM1), respectively[52]. In support of this is the observation that downregulation of expression of both the receptors $S1P_1$ and $S1P_3$ and the integrins LFA1 and $\alpha_4\beta_1$-integrin occurs after stimulation of marginal-zone B cells with LPS[48,52]. However, marginal-zone B cells from $S1P_1$-deficient mice can still bind strongly to ICAM1 and VCAM1 *in vitro*, excluding a direct effect of $S1P_1$ signalling on the expression of these integrins. Support for the involvement of yet other chemokine receptors or lipid receptors comes from the observation that, in the absence of both CXCR5 and $S1P_1$, marginal-zone B cells remain in the marginal zone, whereas treatment with pertussis toxin, which blocks signalling through chemokine receptors, causes marginal-zone B cells to leave the marginal zone[48,53]. In this model, ligation of chemokine receptors or lipid receptors at the surface of marginal-zone B cells could lead to upregulation of integrin expression. Taken together, these findings show that the lodging of marginal-zone B cells involves a complex and unique range of molecular interactions, emphasizing the special function of these cells in the marginal zone.

For correct development of the anatomy of the marginal zone, the expression of $S1P_3$ by non-haematopoietic cells is required. In the absence of $S1P_3$, endothelial cells expressing mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1) no longer form the continuous, single-celled layer that usually lines the marginal sinus. Instead, in $S1P_3$-deficient mice, $MADCAM1^+$ cells are spread over a wider region, which surrounds the white pulp. Furthermore, there are more marginal-zone B cells in the absence of $S1P_3$, and these cells form a broader, less well organized ring of B cells around the white pulp (TABLE 1). As a result of this altered structure of the marginal zone, an effective response against T-cell-independent antigens seems to be lacking in $S1P_3$-deficient mice[49]. These studies show that the marginal zone and/or its endothelial lining form a physical barrier, because in $S1P_3$-deficient mice, marginal-zone B cells could migrate to the B-cell follicles more rapidly after stimulation with LPS[49].

Innate versus adaptive immunity in the spleen

The way that the spleen is structured such that most of the blood flow passes through the marginal zone and directly along the white pulp leads to efficient monitoring of the blood by the immune system. In this section, we explain how the various cells function in their specific locations and how this is associated with the structure of the lymphoid compartments in which they reside.

In the spleen, both innate and adaptive immune responses can be efficiently mounted, making it an important organ for immune homeostasis. Whereas the white pulp is restricted to being involved in adaptive immunity, the marginal zone is involved in both innate and adaptive immunity, through its specific macrophage populations and marginal-zone B cells.

The innate immune response. For the efficient trapping of blood-borne pathogens and antigens, resident marginal-zone cells express specific receptors, some of which are unique to this region (FIG. 4). In addition to pattern-recognition receptors (such as Toll-like receptors), which are expressed by most tissue macrophages for the clearance of pathogens[54], marginal-zone macrophages express the C-type lectin SIGNR1 and the type I scavenger receptor MARCO. SIGNR1 efficiently binds polysaccharide antigens, such as mannosylated lipoarabinomannan, which is present at the surface of *Mycobacterium tuberculosis*[34,55]. This binding leads to internalization of *M. tuberculosis* and, subsequently, to its targeting to lysosomes for degradation[35]. Furthermore, SIGNR1 has been shown to be crucial for the uptake and clearance of *Streptococcus pneumoniae*[33,56]. SIGNR1 might also be involved in the uptake of viruses, because various viruses have been shown to bind SIGNR1 (REFS 35,57). This correlates with earlier findings showing that marginal-zone macrophages are crucial for the clearance of viruses[58].The other important receptor present at the surface of marginal-zone macrophages, MARCO, can recognize many pathogens, including *Escherichia coli* and *Staphylococcus aureus*[36], and there is a striking complementary recognition of SIGNR1 and MARCO by these pathogens.

Marginal-zone metallophilic macrophages express SIGLEC1, which can bind not only sialic-acid-containing molecules expressed at the surface of cells

MARGINAL-ZONE METALLOPHILIC MACROPHAGE
A macrophage that is located at the border of the white pulp and the marginal zone of the spleen. The precise function of these cells at this site is not known.

LYMPHOID-TISSUE INDUCER CELL
A cell that is present in developing lymph nodes, Peyer's patches and nasopharynx-associated lymphoid tissue (NALT). Lymphoid-tissue inducer cells are required for the development of these lymphoid organs. The inductive capacity of these cells for the generation of Peyer's patches and NALT has been shown by adoptive transfer, and it is generally assumed that they have a similar function in the formation of lymph nodes.

of the immune system but also sialic-acid residues at the cell surface of pathogens. SIGLEC1 belongs to the growing family of SIGLEC molecules, which are characterized on the basis of recognition of sialic acid, as well as through structural similarities[59]. SIGLECs are expressed at the surface of cells of the haematopoietic system, including monocytes and DCs, and are thought to have a role in cellular interactions. With the exception of SIGLEC1, the ligand-binding motif of SIGLEC molecules, such as CD22 and CD33, is masked by *cis* interactions with sialic-acid residues at the surface of the same cell, and this motif is unmasked only on activation of the cell. The expression of high levels of the constitutively unmasked SIGLEC, SIGLEC1, by marginal-zone metallophilic macrophages can be viewed as a mechanism to concentrate blood-borne pathogens in this area, leading to their subsequent clearance through phagocytosis, as shown for SIGLEC-mediated capture and uptake of LPS derived from *Neisseria meningitidis*[60].

What happens after the uptake of pathogens in the marginal zone? It has been shown that marginal-zone metallophilic macrophages are the main producers of interferon-α and interferon-β after a viral challenge, whereas marginal-zone macrophages produce these cytokines to a lesser extent[61]. Marginal-zone macrophages are reported to lack the expression of MHC class II molecules, and it has been proposed that the subsequent activation of marginal-zone B cells occurs through shedding of pathogen-degradation products that are opsonized by complement[62].

Marginal-zone B cells are another important population of the marginal zone. Marginal-zone B cells are specialized to detect blood-borne pathogens, after which they either respond swiftly by differentiating into IgM-producing plasma cells or gain the capacity to function as antigen-presenting cells (APCs)[63]. Marginal-zone B cells can be distinguished phenotypically from splenic follicular B cells[39]: marginal-zone B cells express high levels of IgM, CD1d, CD9, CD21 and CD22, and low levels of IgD, CD23 and B220; and follicular B cells express high levels of IgD, lower levels of IgM, CD21 and CD22 than marginal-zone B cells, and no detectable CD1d or CD9.

After activation in the marginal zone, some resident marginal-zone cells, such as marginal-zone B cells and subpopulations of DCs, might migrate into the white pulp. After uptake of soluble antigen, marginal-zone B cells become potent APCs within the white pulp, and they activate naive $CD4^+$ T cells[64]. After stimulation with LPS, marginal-zone B cells downregulate $S1P_1$ and $S1P_3$, which allows them to respond to CXCL13 produced by FDCs and to migrate to the B-cell follicles[48]. Similarly, one can envisage that the mannose-receptor-binding marginal-zone DCs, which have been shown to express CXCR5, are also maintained in the marginal zone through expression of $S1P_1$ and/or $S1P_3$ and that immune activation causes downregulation of the expression of these receptors[38]. This would subsequently allow the migration of these DCs into the B-cell follicles, as has been described[38].

After activation, blood-borne DCs that are temporarily present in the marginal zone are induced to migrate into the white pulp. This process is crucial for the initiation of adaptive immune responses, as shown after infection with *Leishmania donovani*[65], which leads to downregulation of CCR7 expression by DCs. This subsequently prevents the migration of these APCs into the splenic white pulp, which leads to spread of the parasite. In these studies, transfer of activated bone-marrow-derived DCs, which expressed high levels of CCR7, resulted in a strong reduction in the proliferation of parasites. This indicates that the migration of DCs into the white pulp enhances the protection of the host, although in this example it can not be excluded that the increase in DC numbers (through transfer) itself accounts for this effect[65].

The crucial role of the spleen in protection against blood-borne pathogens was shown in studies of splenectomized patients and mice, which cannot mount appropriate responses to several bacterial products. Splenectomy in humans therefore leads to the lifelong requirement for prophylactic intake of antibiotics[66].

The adaptive immune response. The entry of APCs to the white pulp, in particular to the T-cell zone, is an important step in the initiation of the adaptive immune response. The overall organization and immunophysiology of the white pulp is highly similar to lymph-node structure and function[67]. One important difference is the manner in which lymphocytes enter the different lymphoid organs. For the lymph nodes, most lymphocytes enter through HIGH ENDOTHELIAL VENULES (HEVs) and afferent lymphatic vessels. For the spleen, all cells enter the white pulp through the marginal zone (FIG. 4). In the marginal zone, cells of the innate immune system, as well as marginal-zone B cells, are strategically located to effectively eliminate blood-borne pathogens. In the initial response to intact bacteria, blood DCs have been shown to be responsible for the capture of bacteria in the blood and for their subsequent transport to the spleen. On entry to the spleen, these DCs mediate the initial differentiation and survival of B cells to become antibody-producing plasmablasts, which occurs in the bridging channels[68]. On entry of activated APCs to the white pulp, T cells become activated, and this results in upregulation of CXCR5 expression and downregulation of CCR7 expression by these T cells, which allows them to migrate to the edge of the B-cell follicles[69]. Similarly, in the B-cell follicles, the binding of antigen to the antigen receptor induces the upregulation of CCR7 expression by B cells. This leads to their migration to the edge of the B-cell follicles, where they receive help from the activated T cells[70]. (The migration of lymphocytes within the lymph nodes is regulated in a similar manner[71].) After contact with activated T cells, B cells switch their isotype within the B-cell follicles, after which they either migrate to the red pulp and the marginal zone or remain in germinal centres in the spleen[72].

HIGH ENDOTHELIAL VENULE (HEV). A specialized venule that occurs in secondary lymphoid organs, except the spleen. HEVs allow continuous transmigration of lymphocytes as a consequence of the constitutive expression of adhesion molecules and chemokines at their luminal surface.

In a further comparison between the lymph nodes and the splenic white pulp, the presence of a tubular network has been reported for both lymphoid organs[73,74]. This tubular network, which is known as the conduit system, allows rapid transport of small molecules (such as chemokines and peptides) through the lymphoid compartments of the lymph nodes and the spleen. A key difference between the spleen and the lymph nodes is that molecules that enter the conduit of the white pulp are present in the blood, whereas molecules that enter the lymph nodes are carried by the lymph[73–76]. Also, some of the molecules that can enter the conduit of the splenic white pulp cannot enter the lymph-node conduit and vice versa, indicating that the requirements for entry to the conduit of the different lymphoid organs differ slightly[73,74]. It was shown that the conduit of the lymph nodes is formed by a core of collagen fibres (composed of type I collagen), surrounded by a microfibrillar zone, in which the structure recognized by the ERTR7 antibody is localized. This, in turn, is surrounded by a basement membrane and then by reticular fibroblasts[77]. In the spleen, the conduit was shown to colocalize with the antigen recognized by ERTR7 (REF. 74). The reticular fibroblasts that surround the conduit system of the white pulp are characterized by expression of the stromal marker glycoprotein 38 (gp38), as well by expression of VCAM1 (R.E.M. and G.K., unpublished observations). Similarly, in lymph nodes, it was shown that reticular fibroblasts express gp38 and that cultured gp38$^+$VCAM1$^+$ stromal cells produce extracellular-matrix molecules that contain the ERTR7 antigen[77,78]. This process was shown to depend on the activation of stromal cells by lymphocytes, in a TNFR- and LT-βR-dependent manner[78]. So, one can envisage that a close interaction between haematopoietic cells and reticular fibroblasts affects the conduit system in the lymphoid organs. Indeed, it was shown that, during an immune response, the extracellular matrix, and therefore presumably also the conduit, of lymph nodes was completely remodelled[78].

What can be transported through these narrow tubes of the conduit system? In lymph nodes, the conduit allows rapid transport of chemokines that are brought in by the afferent lymph to the luminal side of HEVs[73,75,76]. This can lead to rapid changes in the repertoire of chemokines that are displayed at the surface of HEVs when inflammation occurs in the draining area of the lymph node, leading to the entry of cells that usually would not enter the lymph node through HEVs, such as granulocytes and monocytes. Similarly, in the spleen, chemokines are associated with the conduit system. In the absence of HEVs, chemokines presumably direct migration, as well as retention, of cells in the white pulp[74]. Furthermore, the reticular fibroblasts that surround the conduit system are probably also themselves producing chemokines that can subsequently be transported through the conduit. Indeed, the response of reticular fibroblasts to signalling through TNFRs and LT-βR provides a mechanism for induction of expression of the homeostatic chemokines (that is, CCL19, CCL21 and CXCL13)[78,79]. In addition, one can envisage that blood-borne, low-molecular-weight antigens might enter the splenic white pulp through the conduit, where they would be taken up by DCs that are in proximity to the conduit. This uptake would occur in the absence of an activation signal, thereby possibly contributing to the induction of immune tolerance to such antigens.

How do lymphocytes exit from these organs? Lymphocytes can exit lymph nodes through the efferent lymphatic vessels (FIG. 3), which requires that they downregulate expression of $S1P_1$. Exclusion of lymphocytes from the white pulp is also regulated and is highly functional. For example, CD8$^+$ effector T cells downregulate expression of CCR7, which results in their exclusion from the splenic white pulp, allowing these cells to enter the blood and thereby the peripheral tissues. Downregulation of CCR7 expression allows an effective antiviral response to be mounted, because preventing this change in CCR7 expression results in reduced viral clearance[80].

The precise molecular mechanisms by which lymphocytes leave the white pulp and the exact anatomical route that they use are unknown. It is assumed that lymphocytes leave the white pulp through the marginal zone and then re-enter the bloodstream. This might occur through bridging channels that have been described as passageways and can be regarded as protrusions of white-pulp areas through the marginal zone into the red pulp[81].

Concluding remarks

The spleen is a fascinating organ that accommodates the efficient phagocytosis of erythrocytes and recycling of iron, the capture and destruction of pathogens and the induction of adaptive immune responses. These discrete functions are uniquely combined in one organ through its compartmentalization into different regions with adaptations that are not observed for other lymphoid organs. Although the overall picture of the spleen and the function of its regions have become clear as a result of many studies, several cellular interactions and molecular mechanisms that underlie the important processes in the spleen need further analysis. For example, the conduit system has been described to transport molecules in the splenic white pulp; however, in the red pulp, there is an extensive extracellular-matrix network that allows the rapid transport of small molecules. The precise composition of this network and its function need further clarification. Also, the requirements by which the different cell types are retained in, and released from, the marginal zone have not been completely determined. The pathways that lymphocytes use to exit from the white pulp are unclear, as are the processes that underlie the dependence of marginal-zone B cells and marginal-zone macrophages on each other and the implications of this dependence for immune responses. Further insight into these processes and into how the innate and adaptive immune systems cooperate will allow us to optimize our fight against pathogens and design more efficient vaccines.

1. Kraal, G. Cells in the marginal zone of the spleen. *Int. Rev. Cytol.* **132**, 31–73 (1992).
2. Steiniger, B. & Barth, P. Microanatomy and function of the spleen. *Adv. Anat. Embryol. Cell Biol.* **151**, III–IX; 1–101 (2000).
3. Groom, A. C., Schmidt, E. E. & MacDonald, I. C. Microcirculatory pathways and blood flow in spleen: new insights from washout kinetics, corrosion casts, and quantitative intravital videomicroscopy. *Scanning Microsc.* **5**, 159–174 (1991).
4. Drenckhahn, D. & Wagner, J. Stress fibers in the splenic sinus endothelium *in situ*: molecular structure, relationship to the extracellular matrix, and contractility. *J. Cell Biol.* **102**, 1738–1747 (1986).
5. MacDonald, I. C., Ragan, D. M., Schmidt, E. E. & Groom, A. C. Kinetics of red blood cell passage through interendothelial slits into venous sinuses in rat spleen, analyzed by *in vivo* microscopy. *Microvasc. Res.* **33**, 118–134 (1987).
6. Bratosin, D. *et al*. Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review. *Biochimie* **80**, 173–195 (1998).
7. Stewart, I. B. & McKenzie, D. C. The human spleen during physiological stress. *Sports Med.* **32**, 361–369 (2002).
8. Knutson, M. & Wessling-Resnick, M. Iron metabolism in the reticuloendothelial system. *Crit. Rev. Biochem. Mol. Biol.* **38**, 61–88 (2003).
9. Maines, M. D. The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.* **37**, 517–554 (1997).
10. Kristiansen, M. *et al*. Identification of the haemoglobin scavenger receptor. *Nature* **409**, 198–201 (2001).
This paper shows that CD163, which is expressed at the cell surface of macrophages, is a scavenger receptor for haemoglobin and haptoglobin-bound haemoglobin. This provides insight into a molecular mechanism of iron recycling by macrophages.
11. Gruenheid, S. *et al*. The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. *J. Exp. Med.* **189**, 831–841 (1999).
12. Gruenheid, S. & Gros, P. Genetic susceptibility to intracellular infections: Nramp1, macrophage function and divalent cations transport. *Curr. Opin. Microbiol.* **3**, 43–48 (2000).
13. Hackam, D. J. *et al*. Host resistance to intracellular infection: mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification. *J. Exp. Med.* **188**, 351–364 (1998).
14. Ratledge, C. & Dover, L. G. Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* **54**, 881–941 (2000).
15. Flo, T. H. *et al*. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* **432**, 917–921 (2004).
The authors of this paper describe a new mechanism of the control of bacterial growth by macrophages, the increased production of lipocalin-2 after bacterial encounter. This molecule sequesters iron and thereby limits the growth of bacteria.
16. Sze, D. M., Toellner, K. M., Garcia de Vinuesa, C., Taylor, D. R. & MacLennan, I. C. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *J. Exp. Med.* **192**, 813–821 (2000).
17. MacLennan, I. C. *et al*. Extrafollicular antibody responses. *Immunol. Rev.* **194**, 8–18 (2003).
18. Hargreaves, D. C. *et al*. A coordinated change in chemokine responsiveness guides plasma cell movements. *J. Exp. Med.* **194**, 45–56 (2001).
19. Garcia De Vinuesa, C. *et al*. Dendritic cells associated with plasmablast survival. *Eur. J. Immunol.* **29**, 3712–3721 (1999).
20. Leenen, P. J. M. *et al*. Heterogeneity of mouse spleen dendritic cells: *in vivo* phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J. Immunol.* **160**, 2166–2173 (1998).
21. Ansel, K. M. *et al*. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* **406**, 309–314 (2000).
22. Gunn, M. D. *et al*. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J. Exp. Med.* **189**, 451–460 (1999).
23. Förster, R. *et al*. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* **99**, 23–33 (1999).
24. Ngo, V. N. *et al*. Lymphotoxin α/β and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J. Exp. Med.* **189**, 403–412 (1999).
25. Matsumoto, M. *et al*. Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science* **271**, 1289–1291 (1996).
26. Mebius, R. E., Van Tuijl, S., Weissman, I. L. & Randall, T. D. Transfer of primitive stem/progenitor bone marrow cells from LT-$\alpha^{-/-}$ donors to wild-type hosts: implications for the generation of architectural events in lymphoid B cell domains. *J. Immunol.* **161**, 3836–3843 (1998).
27. Endres, R. *et al*. Mature follicular dendritic cell networks depend on expression of lymphotoxin β receptor by radioresistant stromal cells and of lymphotoxin β and tumor necrosis factor by B cells. *J. Exp. Med.* **189**, 159–168 (1999).
28. Luther, S. A., Tang, H. L., Hyman, P. L., Farr, A. G. & Cyster, J. G. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the *plt/plt* mouse. *Proc. Natl Acad. Sci. USA* **97**, 12694–12699 (2000).
29. Ngo, V. N., Cornall, R. J. & Cyster, J. G. Splenic T zone development is B cell dependent. *J. Exp. Med.* **194**, 1649–1660 (2001).
30. Tumanov, A. *et al*. Distinct role of surface lymphotoxin expressed by B cells in the organization of secondary lymphoid tissues. *Immunity* **17**, 239–250 (2002).
31. Cyster, J. G. & Goodnow, C. C. Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. *J. Exp. Med.* **182**, 581–586 (1995).
In this paper, the authors show that entry of lymphocytes to the white pulp depends on signalling through G-protein-coupled receptors.
32. Johnston, B. & Butcher, E. C. Chemokines in rapid leukocyte adhesion triggering and migration. *Semin. Immunol.* **14**, 83–92 (2002).
33. Kang, Y. S. *et al*. The C-type lectin SIGN-R1 mediates uptake of the capsular polysaccharide of *Streptococcus pneumoniae* in the marginal zone of mouse spleen. *Proc. Natl Acad. Sci. USA* **101**, 215–220 (2004).
34. Kang, Y. S. *et al*. SIGN-R1, a novel C-type lectin expressed by marginal zone macrophages in spleen, mediates uptake of the polysaccharide dextran. *Int. Immunol.* **15**, 177–186 (2003).
35. Geijtenbeek, T. B. *et al*. Marginal zone macrophages express a murine homologue of DC-SIGN that captures blood-borne antigens *in vivo*. *Blood* **100**, 2908–2916 (2002).
This paper shows that expression of the mouse homologue of DC-SIGN, SIGNR1, is restricted in the spleen to marginal-zone macrophages. It functions there as a pattern-recognition receptor for blood-borne antigens.
36. Elomaa, O. *et al*. Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. *Cell* **80**, 603–609 (1995).
37. Munday, J., Floyd, H. & Crocker, P. R. Sialic acid binding receptors (siglecs) expressed by macrophages. *J. Leukoc. Biol.* **66**, 705–711 (1999).
38. Yu, P. *et al*. B cells control the migration of a subset of dendritic cells into B cell follicles via CXC chemokine ligand 13 in a lymphotoxin-dependent fashion. *J. Immunol.* **168**, 5117–5123 (2002).
39. Martin, F. & Kearney, J. F. Marginal-zone B cells. *Nature Rev. Immunol.* **2**, 323–335 (2002).
40. Nolte, M. A. *et al*. B cells are crucial for both development and maintenance of the splenic marginal zone. *J. Immunol.* **172**, 3620–3627 (2004).
41. Crowley, M. T., Reilly, C. R. & Lo, D. Influence of lymphocytes on the presence and organization of dendritic cell subsets in the spleen. *J. Immunol.* **163**, 4894–4900 (1999).
42. Cupedo, T. *et al*. Initiation of cellular organization in lymph nodes is regulated by non-B cell-derived signals and is not dependent on CXC chemokine ligand 13. *J. Immunol.* **173**, 4889–4896 (2004).
43. Cupedo, T. *et al*. Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. *J. Immunol.* **173**, 2968–2975 (2004).
44. Ato, M., Nakano, H., Kakiuchi, T. & Kaye, P. M. Localization of marginal zone macrophages is regulated by C-C chemokine ligands 21/19. *J. Immunol.* **173**, 4815–4820 (2004).
45. Karlsson, M. C. *et al*. Macrophages control the retention and trafficking of B lymphocytes in the splenic marginal zone. *J. Exp. Med.* **198**, 333–340 (2003).
46. Matloubian, M. *et al*. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **427**, 355–360 (2004).
47. Allende, M. L., Dreier, J. L., Mandala, S. & Proia, R. L. Expression of the sphingosine 1-phosphate receptor, $S1P_1$, on T-cells controls thymic emigration. *J. Biol. Chem.* **279**, 15396–15401 (2004).
48. Cinamon, G. *et al*. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nature Immunol.* **5**, 713–720 (2004).
This paper shows that $S1P_1$ is required for retention of marginal-zone B cells in the marginal zone, 'overwriting' the effects of the chemoattractant CXCL13, which is produced in B-cell follicles. This finding adds another level of complexity to the regulation of the integrity of the marginal zone.
49. Girkontaite, I. *et al*. The sphingosine-1-phosphate (S1P) lysophospholipid receptor $S1P_3$ regulates MAdCAM-1^+ endothelial cells in splenic marginal sinus organization. *J. Exp. Med.* **200**, 1491–1501 (2004).
50. Graeler, M., Shankar, G. & Goetzl, E. J. Suppression of T cell chemotaxis by sphingosine 1-phosphate. *J. Immunol.* **169**, 4084–4087 (2002).
51. Goetzl, E. J., Wang, W., McGiffert, C., Huang, M. C. & Graler, M. H. Sphingosine 1-phosphate and its G protein-coupled receptors constitute a multifunctional immunoregulatory system. *J. Cell. Biochem.* **92**, 1104–1114 (2004).
52. Lu, T. T. & Cyster, J. G. Integrin-mediated long-term B cell retention in the splenic marginal zone. *Science* **297**, 409–412 (2002).
53. Guinamard, R., Okigaki, M., Schlessinger, J. & Ravetch, J. V. Absence of marginal zone B cells in Pyk-2 deficient mice defines their role in the humoral response. *Nature Immunol.* **1**, 31–36 (2000).
54. Gordon, S. Pattern recognition receptors: doubling up for the innate immune response. *Cell* **111**, 927–930 (2002).
55. Koppel, E. A. *et al*. Identification of the mycobacterial carbohydrate structure that binds the C-type lectins DC-SIGN, L-SIGN and SIGNR1. *Immunobiology* **209**, 117–127 (2004).
56. Lanoue, A. *et al*. SIGN-R1 contributes to protection against lethal pneumococcal infection in mice. *J. Exp. Med.* **200**, 1383–1393 (2004).
57. Marzi, A. *et al*. DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus. *J. Virol.* **78**, 12090–12095 (2004).
58. Oehen, S. *et al*. Marginal zone macrophages and immune responses against viruses. *J. Immunol.* **169**, 1453–1458 (2002).
59. Crocker, P. R. & Varki, A. Siglecs, sialic acids and innate immunity. *Trends Immunol.* **22**, 337–342 (2001).
60. Jones, C., Virji, M. & Crocker, P. R. Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Mol. Microbiol.* **49**, 1213–1225 (2003).
61. Eloranta, M. L. & Alm, G. V. Splenic marginal metallophilic macrophages and marginal zone macrophages are the major interferon-α/β producers in mice upon intravenous challenge with herpes simplex virus. *Scand. J. Immunol.* **49**, 391–394 (1999).
62. Van Rooijen, N. Antigen processing and presentation *in vivo*: the microenvironment as a crucial factor. *Immunol. Today* **11**, 436–439 (1990).
63. Lopes-Carvalho, T. & Kearney, J. F. Development and selection of marginal zone B cells. *Immunol. Rev.* **197**, 192–205 (2004).
64. Attanavanich, K. & Kearney, J. F. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *J. Immunol.* **172**, 803–811 (2004).
65. Ato, M., Stager, S., Engwerda, C. R. & Kaye, P. M. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nature Immunol.* **3**, 1185–1191 (2002).
66. Amlot, P. L. & Hayes, A. E. Impaired human antibody response to the thymus-independent antigen, DNP–Ficoll, after splenectomy. Implications for post-splenectomy infections. *Lancet* **1**, 1008–1011 (1985).
67. Nolte, M. A., Hoen, E. N., van Stijn, A., Kraal, G. & Mebius, R. E. Isolation of the intact white pulp. Quantitative and qualitative analysis of the cellular composition of the splenic compartments. *Eur. J. Immunol.* **30**, 626–634 (2000).
This paper shows, by isolation of intact white pulp, that the cellular composition and organization of the lymphoid compartment of the spleen is highly similar to that of lymph nodes.
68. Balazs, M., Martin, F., Zhou, T. & Kearney, J. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity* **17**, 341–352 (2002).
69. Ansel, K. M., McHeyzer-Williams, L. J., Ngo, V. N., McHeyzer-Williams, M. G. & Cyster, J. G. *In vivo*-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J. Exp. Med.* **190**, 1123–1134 (1999).

70. Reif, K. *et al*. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* **416**, 94–99 (2002).
71. Garside, P. *et al*. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science* **281**, 96–99 (1998).
72. Pape, K. A. *et al*. Visualization of the genesis and fate of isotype-switched B cells during a primary immune response. *J. Exp. Med.* **197**, 1677–1687 (2003).
73. Gretz, J. E., Norbury, C. C., Anderson, A. O., Proudfoot, A. E. I. & Shaw, S. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J. Exp. Med.* **192**, 1425–1440 (2000).
The authors show that there is a fine tubular network in the lymph nodes that allows rapid transport of small molecules. Others have shown that this conduit system is important for the transport of chemokines and that it is also present in the spleen.
74. Nolte, M. A. *et al*. A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. *J. Exp. Med.* **198**, 505–512 (2003).
75. Palframan, R. T. *et al*. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J. Exp. Med.* **194**, 1361–1373 (2001).
76. Baekkevold, E. S. *et al*. The CCR7 ligand ELC (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J. Exp. Med.* **193**, 1105–1112 (2001).
77. Sixt, M. *et al*. The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity* **22**, 19–29 (2005).
78. Katakai, T., Hara, T., Sugai, M., Gonda, H. & Shimizu, A. Lymph node fibroblastic reticular cells construct the stromal reticulum via contact with lymphocytes. *J. Exp. Med.* **200**, 783–795 (2004).
79. Dejardin, E. *et al*. The lymphotoxin-β receptor induces different patterns of gene expression via two NF-κB pathways. *Immunity* **17**, 525–535 (2002).
80. Unsoeld, H., Voehringer, D., Krautwald, S. & Pircher, H. Constitutive expression of CCR7 directs effector CD8 T cells into the splenic white pulp and impairs functional activity. *J. Immunol.* **173**, 3013–3019 (2004).
81. Mitchell, J. Lymphocyte circulation in the spleen. Marginal zone bridging channels and their possible role in cell traffic. *Immunology* **24**, 93–107 (1973).
82. Green, M. C. A defect of the splanchnic mesoderm caused by the mutant gene dominant hemimelia in the mouse. *Dev. Biol.* **15**, 62–89 (1967).
83. Hecksher-Sorensen, J. *et al*. The splanchnic mesodermal plate directs spleen and pancreatic laterality, and is regulated by Bapx1/Nkx3.2. *Development* **131**, 4665–4675 (2004).
84. Roberts, C. W., Shutter, J. R. & Korsmeyer, S. J. Hox11 controls the genesis of the spleen. *Nature* **368**, 747–749 (1994).
85. Lu, J. *et al*. The basic helix–loop–helix transcription factor capsulin controls spleen organogenesis. *Proc. Natl Acad. Sci. USA* **97**, 9525–9530 (2000).
86. Herzer, U., Crocoll, A., Barton, D., Howells, N. & Englert, C. The Wilms tumor suppressor gene *WT1* is required for development of the spleen. *Curr. Biol.* **9**, 837–840 (1999).
87. Seifert, M. F. & Marks, S. C. J. The regulation of hemopoiesis in the spleen. *Experientia* **41**, 192–199 (1985).
88. Mebius, R., Rennert, P. D. & Weissman, I. L. Developing lymph nodes collect $CD4^+CD3^-LT\beta^+$ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity* **7**, 493–504 (1997).
89. Mebius, R. E. Organogenesis of lymphoid tissues. *Nature Rev. Immunol.* **3**, 292–303 (2003).
90. Saito, T. *et al*. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* **18**, 675–685 (2003).
91. Quong, M. W. *et al*. Receptor editing and marginal zone B cell development are regulated by the helix–loop–helix protein, E2A. *J. Exp. Med.* **199**, 1101–1112 (2004).
92. Tanigaki, K. *et al*. Notch–RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nature Immunol.* **3**, 443–450 (2002).
93. Kuroda, K. *et al*. Regulation of marginal zone B cell development by MINT, a suppressor of Notch/RBP-J signaling pathway. *Immunity* **18**, 301–312 (2003).
94. Cariappa, A. *et al*. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by Aiolos, Btk, and CD21. *Immunity* **14**, 603–615 (2001).
95. Loder, F. *et al*. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J. Exp. Med.* **190**, 75–89 (1999).
References 90–95 provide evidence that the differentiation of B cells into marginal-zone B cells or follicular B cells is a cell-fate decision that is regulated by many factors.
96. Makowska, A., Faizunnessa, N. N., Anderson, P., Midtvedt, T. & Cardell, S. $CD1^{high}$ B cells: a population of mixed origin. *Eur. J. Immunol.* **29**, 3285–3294 (1999).
97. Pillai, S., Cariappa, A. & Moran, S. T. Marginal zone B cells. *Annu. Rev. Immunol.* **23**, 161–196 (2005).
98. Steiniger, B., Ruttinger, L. & Barth, P. J. The three-dimensional structure of human splenic white pulp compartments. *J. Histochem. Cytochem.* **51**, 655–664 (2003).
99. Steiniger, B., Barth, P. & Hellinger, A. The perifollicular and marginal zones of the human splenic white pulp: do fibroblasts guide lymphocyte immigration? *Am. J. Pathol.* **159**, 501–512 (2001).
100. De Togni, P. *et al*. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* **264**, 703–707 (1994).
101. Banks, T. A. *et al*. Lymphotoxin-α-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* **155**, 1685–1693 (1995).
102. Koni, P. A. *et al*. Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. *Immunity* **6**, 491–500 (1997).
103. Alexopoulou, L., Pasparakis, M. & Kollias, G. Complementation of lymphotoxin α knockout mice with tumor necrosis factor-expressing transgenes rectifies defective splenic structure and function. *J. Exp. Med.* **188**, 745–754 (1998).
104. Pasparakis, M., Kousteni, S., Peschon, J. & Kollias, G. Tumor necrosis factor and the p55TNF receptor are required for optimal development of the marginal sinus and for migration of follicular dendritic cell precursors into splenic follicles. *Cell. Immunol.* **201**, 33–41 (2000).
105. Kuprash, D. V. *et al*. TNF and lymphotoxin β cooperate in the maintenance of secondary lymphoid tissue microarchitecture but not in the development of lymph nodes. *J. Immunol.* **163**, 6575–6580 (1999).
106. Alimzhanov, M. *et al*. Abnormal development of secondary lymphoid tissues in lymphotoxin β-deficient mice. *Proc. Natl Acad. Sci. USA* **94**, 9302–9307 (1997).
107. Kuprash, D. V. *et al*. Redundancy in tumor necrosis factor (TNF) and lymphotoxin (LT) signaling *in vivo*: mice with inactivation of the entire TNF/LT locus versus single-knockout mice. *Mol. Cell. Biol.* **22**, 8626–8634 (2002).
108. Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M. H. & Pfeffer, K. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* **9**, 59–70 (1998).
109. Scheu, S. *et al*. Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin β in mesenteric lymph node genesis. *J. Exp. Med.* **195**, 1613–1624 (2002).
110. Pasparakis, M. *et al*. Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor. *Proc. Natl Acad. Sci. USA* **94**, 6319–6323 (1997).
111. Pasparakis, M., Alexopoulou, L., Episkopou, V. & Kollias, G. Immune and inflammatory responses in TNFα-deficient mice: a critical requirement for TNFα in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J. Exp. Med.* **184**, 1397–1411 (1996).
112. Körner, H. *et al*. Distinct role for lymphotoxin-α and tumor necrosis factor in organogenesis and spatial organization of lymphoid tissue. *Eur. J. Immunol.* **27**, 2600–2609 (1997).
113. Pfeffer, K. *et al*. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* **73**, 457–467 (1993).
114. Erickson, S. L. *et al*. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* **372**, 560–563 (1994).
115. Koike, R. *et al*. The splenic marginal zone is absent in alymphoplastic *aly* mutant mice. *Eur. J. Immunol.* **26**, 669–675 (1996).
116. Matsumoto, M. *et al*. Involvement of distinct cellular compartments in the abnormal lymphoid organogenesis in lymphotoxin-α-deficient mice and alymphoplasia (*aly*) mice defined by the chimeric analysis. *J. Immunol.* **163**, 1584–1591 (1999).
117. Shinkura, R. *et al*. Alymphoplasia is caused by a point mutation in the mouse gene encoding NF-κB-inducing kinase. *Nature Genet.* **22**, 74–77 (1999).
118. Yamada, T. *et al*. Abnormal immune function of hemopoietic cells from alymphoplasia (*aly*) mice, a natural strain with mutant NF-κB-inducing kinase. *J. Immunol.* **165**, 804–812 (2000).
119. Weih, F. & Caamano, J. Regulation of secondary lymphoid organ development by the nuclear factor-κB signal transduction pathway. *Immunol. Rev.* **195**, 91–105 (2003).
120. Cariappa, A., Liou, H. C., Horwitz, B. H. & Pillai, S. Nuclear factor κB is required for the development of marginal zone B lymphocytes. *J. Exp. Med.* **192**, 1175–1182 (2000).
121. Franzoso, G. *et al*. Mice deficient in nuclear factor (NF)-κB/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J. Exp. Med.* **187**, 147–159 (1998).
122. Poljak, L., Carlson, L., Cunningham, K., Kosco-Vilbois, M. H. & Siebenlist, U. Distinct activities of p52/NF-κB required for proper secondary lymphoid organ microarchitecture: functions enhanced by Bcl-3. *J. Immunol.* **163**, 6581–6588 (1999).
123. Weih, D., Yilmaz, Z. & Weih, F. Essential role of RelB in germinal center and marginal zone formation and proper expression of homing chemokines. *J. Immunol.* **167**, 1909–1919 (2001).
124. Franzoso, G. *et al*. Critical roles for the Bcl-3 oncoprotein in T cell-mediated immunity, splenic microarchitecture, and germinal center reactions. *Immunity* **6**, 479–490 (1997).
125. Pabst, O., Förster, R., Lipp, M., Engel, H. & Arnold, H. H. NKX2.3 is required for MAdCAM-1 expression and homing of lymphocytes in spleen and mucosa-associated lymphoid tissue. *EMBO J.* **19**, 2015–2023 (2000).

Acknowledgements

We thank all of our collaborators, particularly B. Steiniger, for helpful discussions.

Competing interests statement

The authors declare no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to:

Entrez Gene:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene

CCL19 | CCL21 | CCR7 | CXCL13 | LT-βR | MARCO | NRAMP1 | $S1P_1$ | $S1P_3$ | SIGLEC1 | SIGNR1 | TNFR1

Access to this interactive links box is free online.