Sinusoid-lining cells are novel myeloid-endothelial innate cells that form splenic niches for marginal zone B cell activation and plasma cell survival

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To Irene Puga,

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THESIS ABSTRACT

Sinusoid vessels promote the slow percolation of venous blood through the red pulp of the spleen, thereby favoring antigen capture by phagocytes and lymphocytes of the local immune system. Strategically positioned around sinusoids and historically known as reticulo-endothelial cells, sinusoid-lining cells (SLCs) have an enigmatic biology and thus can be viewed as an orphan component of our immune system. We found here that SLCs were a human-specific population of endothelial-like cells that expressed typical endothelial molecules such as von Willenbrand factor, CD31 (PECAM-1), CD54 (ICAM-1), CD102 (ICAM-2), CD105 (endoglin) and CD141 (thrombomodulin). However, unlike endothelial cells, SLCs also expressed the stromal molecules vimentin and smooth muscle actin along with several myeloid molecules such as CD14, CD36, CD163, MR, DEC-205 and TLR4. Accordingly, SLCs showed a prominent macrophage-like gene signature that included microbial sensors, scavanger receptors, immune mediators, and regulators of phagocytosis and antigen presentation. Besides phagocytosing particulate antigens through an actin-dependent mechanism, SLCs released BAFF, APRIL, IL-6 and CXCL10, which enhanced the recruitment, activation and survival of marginal zone (MZ) B cells, a splenic lymphocyte subset specialized in innate-like antibody responses to blood-borne antigens. Thus, SLCs are endothelial-myeloid cells that serve as sentinels endowed with phagocytic and antibody-enhancing functions.

RESUMEN DE LA TESIS

Los sinusoides del bazo humano promueven la lenta percolación de la sangre, favoreciendo la captura de antígeno por los fagocitos y linfocitos del sistema inmune local. Estratégicamente posicionadas delimitando los vasos, e históricamente conocidas como células retículo-endoteliales, las células que delinean los sinusoides (SLCs), tienen una biología enigmática y podrían ser vistas como un componente desconocido del sistema inmunológico. En esta tesis hemos observado que las SLCs poseían un fenotipo simil-endotelial, eran específicas de humano y expresaban moléculas típicamente asociadas al linaje endotelial como el factor de von Willenbrand, y las moléculas CD31, CD54, CD102, CD105 y CD141. Sin embargo, a diferencia de las células endoteliales, las SLCs también expresaban moléculas típicamente asociadas al linaje estromal como la vimentina y la actina de músculo liso, junto con varias moléculas del linaje mieloide como CD14, CD36, CD163, MR, DEC-205 y TLR4. A si mismo, las SLCs evidenciaron una huella genética típicamente macrofágica que incluía sensores microbianos, receptores de tipo "scavenger", mediadores de la respuesta inmunitaria y reguladores de la fagocitosis y la presentación antigénica. Además de fagocitar antígenos a través de un mecanismo actina-dependiente, las SLCs secretaban BAFF, APRIL, IL-6 y CXCL10, induciendo el reclutamiento, la activación y la supervivencia de células B de la zona marginal, un subtipo celular especializado en la respuesta de anticuerpos frente a antígenos provenientes de la sangre. Por lo tanto, las SLCs son células endotelio-mieloides que funcionan como centinelas dotados de funciones fagocíticas y que ayudan en la producción de anticuerpos.

PREFACE

Blood-borne infections by *Streptococcus pneumoniae*, *Haemophilus influenzae, Neisseria meningitides*, *Escherichia coli* and *Staphylococcus aureus* account for about 2.5 million deaths among neonates and infants every year. These encapsulated bacteria take advantage of capsular polysaccharides (CPS) to evade protective responses by the innate immune system, including opsonization and phagocytosis. CPS surround the cell wall of encapsulated bacteria and consist of repetitive carbohydrate units that bind some germline-encoded pattern-recognition receptors (PRRs) expressed by various effector cells of the innate immune system[1]. CPS also bind to somatically recombined antibodies expressed by MZ B cells, a unique subset of innate-like lymphocytes strategically positioned at the interface between the splenic immune system and the general circulation[2]. While conventional follicular B cells mount protective antibody responses to proteins through a slow T cell-dependent (TD) pathway, MZ B cells generate protective antibodies to carbohydrates and lipids through a swift T cell-independent (TI) pathway.

These pathways generate antibody-mediated protection against encapsulated bacteria in response to distinct vaccine formulations that show age-dependent efficacy. Vaccines containing multiple unconjugated (or native) CPS generate protective antibodies in adults, but not in infants and children, probably because the MZ is not fully developed until 5 years of age[3]. Unconjugated vaccines are also poorly immunogenic in patients with congenital, functional or post-operatory asplenia. In both these patients and children, antibody-mediated protection is achieved by using vaccines containing protein-conjugated

CPS[4], which bypass the TI pathway by preferentially stimulating follicular B cells through the TD pathway. However, also conjugated vaccines present major limitations. Firstly, conjugated vaccines have higher costs of preparation, which limits their widespread use in developing countries. Secondly, conjugated vaccines are poorly effective in patients with primary or acquired immunodeficiencies that compromise the number and/or function of $CD4^+$ T cells. Thirdly, conjugated vaccines contain CPS from only three serotypes of encapsulated bacteria, whereas unconjugated vaccines contain CPS from up to 23 serotypes and thus provide broader protection[3,4].

Because of these limitations and the emergence of bacterial strains unresponsive to conjugated CPS, there is an urgent need to develop novel, more efficacious and less expensive vaccine strategies against encapsulated bacteria, including antibiotic-resistant bacteria. However, this task is hampered by our poor knowledge of the mechanisms underlying antibody production by MZ B cells in humans[5]. Growing evidence shows that TI antibody responses require the activation of MZ B cells and their plasma cell progeny by helper signals derived from cells of the innate immune system, including dendritic cells (DCs), monocytes, macrophages and granulocytes. The goal of the present studies was to elucidate the MZ B cell-helper function of SLCs, a mysterious endothelial-like cell type that surrounds the MZ and forms an extensive vascular network in the red pulp of the spleen. By gaining new insights into the biology and function of these and other cells of the splenic innate immune system, it may be possible to identify novel molecules and strategies to enhance the production of protective CPS-specific antibodies by MZ B cells.

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PART I

INTRODUCTION AND AIMS

Chapter I

Introduction

1. The immune system and the antibody response

The immune system develops to provide protection against virtually any challenge arising from the external environment. The immune response is comprised of a series of processes that function as sequential defensive walls against intruding microbes. Nonspecific physical and chemical barriers, such as mucus, acid secretions and epithelial surfaces, provide a first layer of protection. When these nonspecific barriers are breached, a number of progressively more specific innate and adaptive immune responses are activated to stop the invasion. Innate immune responses involve PRRs that rapidly mount a first line of protective effector functions by detecting a broad array of highly conserved molecules expressed by intruding microbes[6]. Although swift and generally efficient, innate immune responses are poorly specific and thus require help from slower but highly specific adaptive immune responses[7]. These responses involve a vast repertoire of somatically recombined and highly diversified antigen receptors that mount immunological reactions capable to generate full protection and immunological memory[7].

Though historically viewed as two separate and functionally distinct compartments, the innate and adaptive immune systems continually cooperate to generate protection. This cooperation involves an extensive crosstalk between multiple subsets of immune effector cells generally referred to as leukocytes. In general, granulocytes, monocytes, macrophages, DCs, mast cells, natural killer (NK) cells and innate lymphoid cells (ILCs) of the innate immune system express invariant PRRs that generate early but nonspecific protection, whereas

T and B lymphocytes of the adaptive immune system express highly diversified antigen receptors that mediate slow but specific protection[8]. A third category of immune effector cells includes lymphocytes that blur the conventional boundaries between the innate and adaptive immune systems. These “in-betweeners” include γδ T cells, NKT cells, B-1 cells and MZ B cells, which are characterized by the expression of somatically recombined but semi-invariant antigen receptors[9]. Remarkably, extrafollicular B-1 and MZ B cells mediate poorly specific but extremely rapid antibody responses that bridge the temporal gap required for the production of specific antibodies by follicular B cells.

In general, B cells are chiefly characterized by the expression of a somatically recombined transmembrane receptor termed B cell receptor (BCR). Engagement of the BCR by a specific antigen triggers the differentiation of B cells into plasma cells that secrete soluble forms of BCR molecules known as antibodies or immunoglobulins (Igs). By targeting native antigenic determinants (or epitopes) associated with intruding microbes, antibodies induce opsonization, neutralization and cell-mediated cytotoxic processes that generate immune protection[10]. Antigen-activated B cells further differentiate into memory B cells, which rapidly mount anamnestic responses after recognizing previously encountered antigens.

Mature B cells develop in the bone marrow from early B cell progenitors through an antigen-independent gene diversification process known as Ig V(D)J gene recombination. This process generates transitional B cells that expressing a primary repertoire of functionally competent BCR molecules of the IgM and IgD type.

After emerging from the bone marrow, transitional B cells colonize the spleen and lymph nodes and differentiate into either follicular or MZ B cells. In the presence of antigen, these mature conventional B cells (also known as B-2 cells in mice) undergo a second wave of Ig gene diversification processes known as Ig V(D)J gene somatic hypermutation (SHM) and Ig heavy (IgH) chain class switch recombination (CSR). While SHM generates antibodies with higher affinity for antigen, CSR replaces the primary repertoire of IgM and IgD antibodies with a secondary repertoire of IgG, IgA and IgE antibodies that deploy novel effector functions[11].

Eventually, antigen-activated B cells become long-lived plasma cells that home to the bone marrow, where they release large amounts of high-affinity antibodies in the circulation. Alternatively, antigen-activated B cells become memory B cells, which enter the circulation and patrol secondary lymphoid organs. In the presence of secondary exposure to a previously encountered antigen, memory B cells mount explosive responses that rapidly generate antibody-secreting plasma cells with high affinity for antigen[12]. Though essential for antibody-mediated protection, Ig V(D)J gene recombination and SHM have the potential to generate pathogenic B cell clones capable to recognize self-antigens. To avoid this possibility, immature and mature B cells transit through several checkpoints that promote tolerance toward molecules expressed by the host[13].

a. BCR: the hallmark of a B cell

In mature B cells, the BCR is composed of a transmembrane variable Ig receptor complex that mediates antigen binding and a transmembrane invariable CD79 receptor complex that mediates signal transduction[14]. The Ig receptor complex includes a protein tetramer composed of two identical IgH chains and two identical Ig light (IgL) chains that are held together by disulphide bonds. The IgL chains are present in Igκ and Igλ flavors with mutually exclusive expression. Both IgH and IgL chains have a constant (C) region that determines the effector function of an Ig molecule and a variable (V) region that account for epitope recognition. The signaling complex includes an invariant heterodimer composed of Igα (CD79a) and Igβ (CD79b) chains. The cytoplasmic domain of each CD79 molecule contains immune receptor tyrosine-based activation motifs (ITAMs) that are essential for the initiation of signal transduction and the ensuing activation of a variety of cellular processes, including B cell proliferation, differentiation or apoptosis[15]. In early B cell progenitors called pre-B cells, the BCR lacks conventional IgL chains and instead expresses surrogate IgL chains composed of invariant VpreB and λ5 proteins. In immature B cells, this pre-BCR is converted into a canonical BCR following replacement of surrogate IgL chains with conventional Igκ or Igλ chains (Figure 1.1)[16].

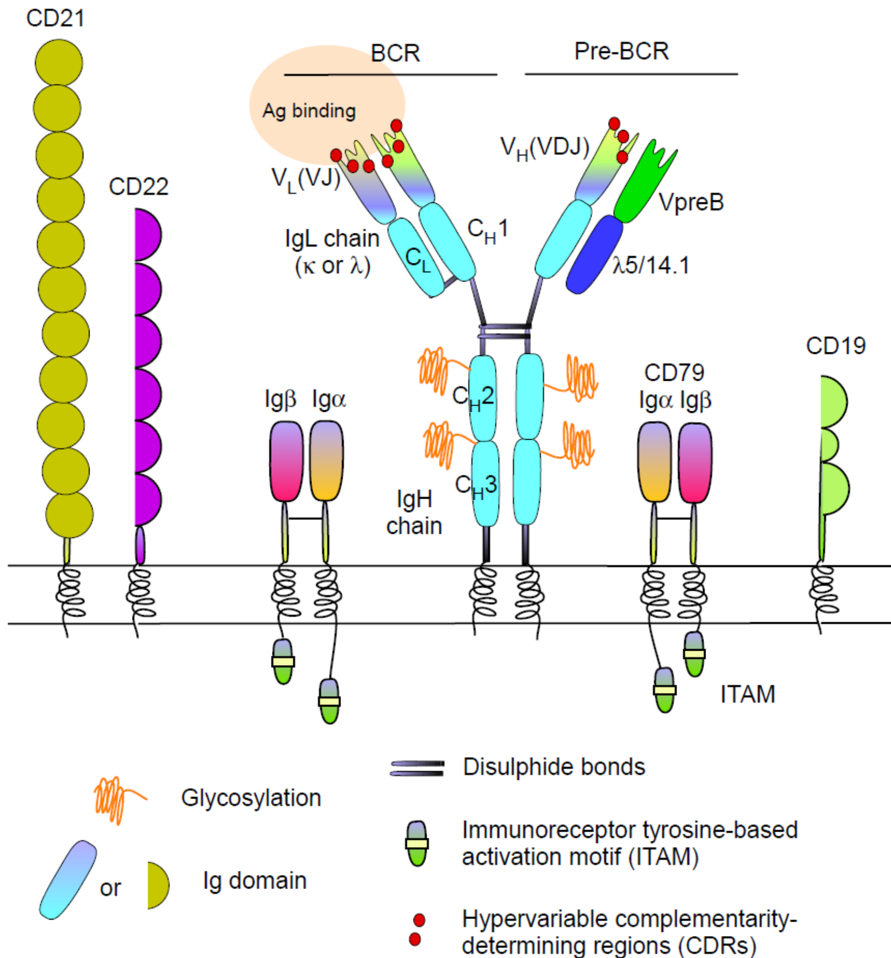


Figure 1.1 Composition and function of the BCR complex. In pre-B cells, the pre-BCR complex includes two V_HDJ_H-Cμ IgH chains and two surrogate VpreB-λ5 IgL chains. In immature B cells, two V_LJ_L-C_L IgL chains replace the surrogate VpreB-λ5 IgL chains to form the BCR complex. In the IgM component of this complex, IgH is composed of V_H and C_H1, C_H2 and C_H3 domains, whereas IgL includes V_L and C_L domains. The C_L domain is covalently joined to the C_H1 domain by a disulphide bond. The BCR complex further includes invariant Igα and Igβ signaling subunits, also termed CD79. Additional co-receptors, including CD19, CD21 and CD22, finely tune the signaling activity of the BCR complex.

b. Antibody structure and subclasses

Secreted antibodies are spliced Ig variants that lack the transmembrane domain of the BCR and instead express a secretory tail. Digestion of these antibodies with the enzyme papain yields two cleavage products known as Fc and Fab fragments. The Fc fragment includes the C_H region and mediates effector functions by binding to Fc receptors (FcRs) on innate immune cells, whereas the Fab fragment includes V_H and V_L regions and thus mediates antigen binding. In humans there are five antibody classes or isotypes named IgM, IgD, IgG, IgA and IgE[17]. Each class is associated with specific FcR-dependent effector functions that are mainly determined by the structure and glycosylation of the Fc region[18]. In contrast, Fab region-dependent functions such as neutralization and opsonization of toxins, microbes and infected or neoplastic cells are common to all antibody classes.

IgM. This antibody isotype is expressed as a transmembrane receptor by immature, transitional and mature naïve B cells[19]. IgM is also released by plasma cells as a soluble pentameric complex held together by a joining chain. IgM activates complement and usually recognizes multiple phylogenetically conserved microbial structures. This is antibodies, which are present prior to immunization or infection[17]. In general, IgM antibodies have a low affinity but high avidity for antigen[20].

IgD. This antibody isotype is expressed as a transmembrane receptor by transitional and mature naïve B cells together with IgM by means of alternative splicing. Although highly conserved during evolution,

the function of IgD remains obscure[21]. In humans, some plasma cells located in the upper respiratory tract secrete soluble monomeric IgD after undergoing a non-canonical form of CSR. Soluble IgD binds to various subsets of innate effector cells, including basophils, and probably functions as an ancestral immune surveillance system at the interface between immunity and inflammation[22].

IgG. This antibody isotype is expressed as a transmembrane receptor by memory B cells. IgG is also released by plasma cells as a monomer and represents the most abundant antibody in the serum[23]. IgG activates complement and mediates phagocytosis and antibody-dependent cellular toxicity (ADCC) by binding to activating FcγRs on phagocytes and NK cells, respectively[24]. Based on structural and functional differences in the Cγ chain, IgG can be distinguished into four IgG1, IgG2, IgG3 and IgG4 subclasses numbered on the basis of their abundance in the serum[25,26]. IgG subclasses differ with respect to number of disulfide bonds, glycosylation patterns and length of the hinge region connecting the Fab and Fc segments of the molecule. These structural variations translate into differences in the ability of each IgG subclass to bind specific types of activating or inhibitory FcγRs[24].

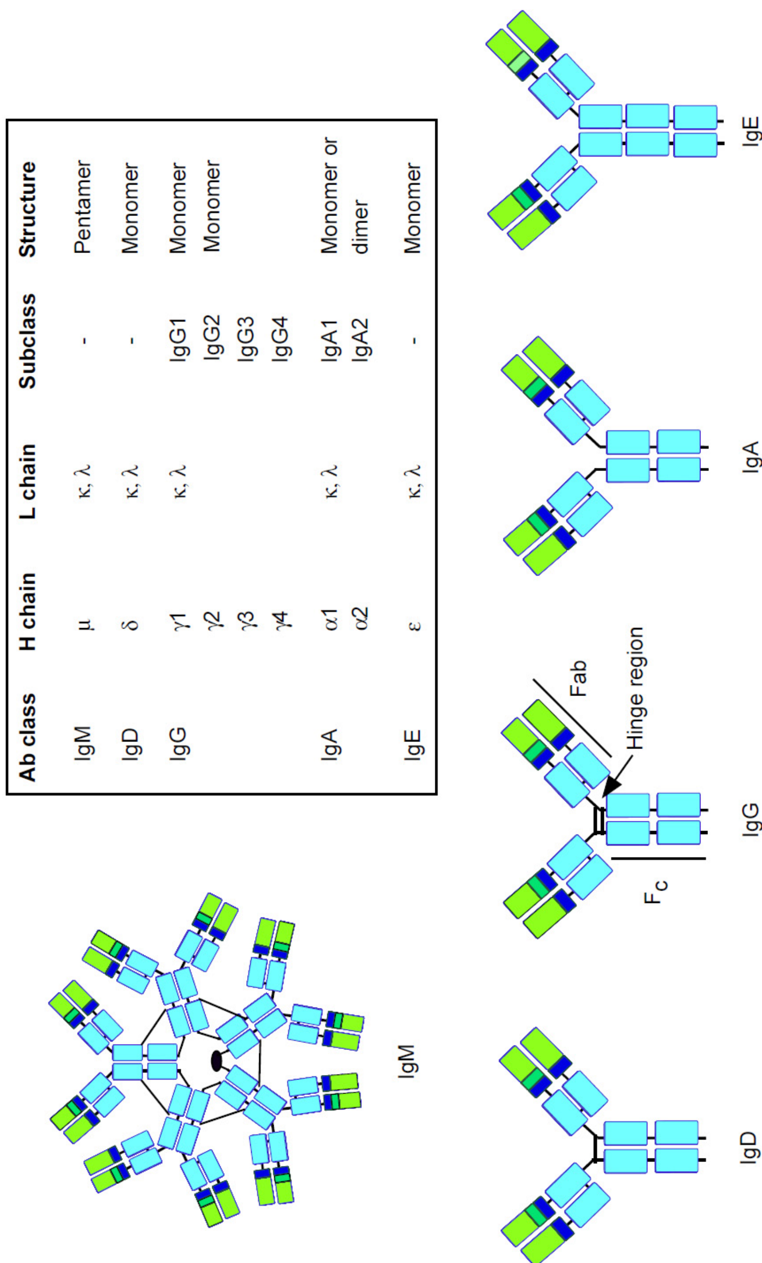
IgA. This antibody isotype is expressed as a transmembrane receptor by memory B cells. IgA is also released by plasma cells and plays a crucial role in mucosal immune surveillance[27]. IgA includes IgA1 and IgA2 subclasses[23]. IgA1 monomers are found in the circulation, whereas IgA1 and IgA2 dimers stabilized by a J chain are found in mucosal secretions. The J chain in IgA dimers interacts with the polymeric Ig receptor (pIgR) expressed on the basolateral surface of

mucosal epithelial cells. This interaction leads to transcytosis of IgA across epithelial cells to form secretory IgA (SIgA). The main function of SIgA is to neutralize toxins and pathogens in mucosal fluids without causing inflammation due to the inability of SIgA to activate complement[28]. In addition, SIgA blocks the entrance of commensal bacteria into the intestinal mucosa by anchoring them to the mucus layer[29]. Of note, SIgA cooperates with many other signals from epithelial cells and DCs to enhance intestinal tolerance against food antigens and commensal bacteria[30].

IgE. This antibody isotype is released by some plasma cells and mediates immunity to parasites[23]. IgE binds a high-affinity FcεRI receptor expressed by mast cells, eosinophils and basophils. The expression of FcεRI is up-regulated upon binding to IgE, creating a positive feedback that enhances FcεRI signal[31]. IgE also triggers the release of powerful pro-inflammatory mediators, including proteases, cytokines, histamine, leukotrienes and platelet activating factor (Figure 1.2)[32].

c. Pre- immune antibodies

Mice raised under germ-free conditions and fed with an antigen-free diet retain the capacity to form antibodies. These pre-immune or natural antibodies are produced by innate-like B cells such as B-1 and MZ cells in the absence of external antigenic stimulation through poorly understood mechanisms and constitute the first line of defense



Ab class	H chain	L chain	Subclass	Structure
IgM	μ	κ, λ	-	Pentamer
IgD	δ	κ, λ	-	Monomer
IgG	$\gamma1$	κ, λ	IgG1	Monomer
	$\gamma2$		IgG2	Monomer
	$\gamma3$		IgG3	
	$\gamma4$		IgG4	
IgA	$\alpha1$	κ, λ	IgA1	Monomer or
	$\alpha2$		IgA2	dimer
IgE	ε	κ, λ	-	Monomer

Figure 1.2 Structure of immunoglobulins. Human antibodies include five antibody classes or isotypes named IgM, IgD, IgG, IgA and IgE. Each class is associated with specific effector functions that are mainly determined by the C_H region.

against blood-borne microorganisms and recognize multiple phylogenetically conserved structures known as pathogen associated molecular patterns (PAMPs)[33]. Natural antibodies are mosly of the IgM class, bind antigens with low affinity but high avidity and neutralize or destroy them by activating the complement cascade[34,35]. Although predominantly belonging to the IgM class, natural antibodies also include IgA, IgG and even IgE[33,36].

Natural IgM provides protection by lysing microbes through the activation of the complement cascade. In addition, some IgM-induced complement fragments, including C3b and C3d, engage the CD21 co-receptor on B cells, thereby lowering the threshold for BCR activation. IgM-induced complement proteins also target DCs to enhance antigen presentation[37]. Natural IgG binds to microbes opsonized by ficolin or mannose-binding lectin (MBL), two soluble PRRs of the innate immune system. The resulting immunocomplex provides protection through a complement-independent mechanism that involves binding of natural IgG to FcγRI on phagocytes, including macrophages. Natural IgM and IgG further enhance immunity by shuttling antigens to the splenic MZ to induce swift antibody production via a TI pathway.

Natural IgM and IgG antibodies also recognize self-antigens and may cooperate with complement to clearance of apoptotic cells in a process that minimizes the onset of uncontrolled inflammation and autoimmunity[20]. Other functions of natural antibodies may include clearance of senescent erythrocytes and platelets[38].

In mice, natural antibodies mostly originate from innate-like MZ B cells and B-1 cells. Strategically positioned between the circulation and the immune system, splenic MZ B cells have a pre-activated phenotype that permits them to mount swift antibody responses to blood borne antigens, including bacteria. This innate-like lymphocytes bridge the early innate immune response and the slower adaptive antibody response[39]. The origin, phenotype and activation of MZ B cells will be discussed in subsequent chapters. B-1 cells are mainly located in serosal cavities and emerge from progenitors distinct from those giving rise to conventional B-2 cells, including follicular and MZ B cells[40]. A hallmark of B-1 cells is the expression of CD5, a scavenger receptor that negatively regulates signals from the BCR[41]. Due to the tonic inhibition provided by CD5, B-1 cells require strong signals from the BCR to become activated. Remarkably, B-1 cells spontaneously release low-affinity IgM antibodies that react against common microbial structures present on microbes, including encapsulated encapsulated bacteria. Natural IgM antibodies from B-1 cells are poorly diversified and thus are enriched in reactivity against some self-antigens[42].

Recently, a subset of $IgD^{+}IgM^{+}CD5^{+}$ B cells has been identified in humans and proposed to be equivalent to mouse B-1 cells. However, the frequency and function of these human B-1 cells is highly debated[43–45], which suggests that other B cells in humans provide natural antibody-mediated protection similar to that exerted by mouse B-1 cells. In humans, it seems likely that $IgD^{lo}IgM^{+}CD27^{+}$ MZ B cells are the main source of natural antibodies and antibodies to encapsulated bacteria[39]. Compared to their murine counterpart, human

MZ B cells show a largely overlapping phenotype but also display several differences and indeed are often referred to as IgM memory B cells. This is probably a misnomer, given the existence in humans of an additional subset of truly memory $IgD^{-}IgM^{+}CD27^{+}$ B cells[46]. Unlike mouse MZ B cells, humans MZ B cells enter the general circulation and contain Ig V(D)J gene somatic mutations, which are acquired both during fetal life and after birth[39,47,48].

d. Geography of the antibody response

Mature B cells mount antigen-specific antibody responses in lymph nodes, spleen and mucosa-associated lymphoid tissues[49]. These secondary lymphoid organs have distinct architectures, but all provide a specialized microenvironment that favors the interaction of B cells of B cells with antigen and other immune cells, including DCs, macrophages and T cells[50]. Lymph nodes collect antigen and antigen-presenting cells through afferent lymphatic vessels that originate from non-lymphoid organs, including the skin, whereas mucosal lymphoid organs acquire antigen directly from the mucosal lumen across epithelial cells. Finally, the spleen receives antigens from the general circulation.

i. Lymph nodes

Lymph nodes are encapsulated bean-shaped structures that act as filters for antigens trafficking along the lymphatic vasculature[50]. The

subcapsular sinus receives lymphatic fluifs from afferent vessels and surround three separate compartments known as cortex, paracortex and medulla. The cortex contains densely packed B cells arranged in follicles that also contain follicular dendritic cells (FDCs) and macrophages. The paracortex has a more inner location and provides the site for the interaction of antigen-presenting DCs with T cells. Resident stromal cells regulate this interaction by recruiting T cells and DCs through chemotactic factors[51]. Antigen-activated T cells further interact with B cells to provide helper signals that lead to the formation of the germinal center (GC), a specialized microenvironment that fosters B cell expansion, differentiation and selection through the induction of SHM and CSR[52]. The medulla consists of medullary cords where plasma cells actively secrete antibodies. Around the cords, medullary sinuses join efferent lymphatic vessels that collect lymphatic fluid from the node[53].

ii. The spleen

1. Spleen structure. The spleen is a lymphoid organ that filters antigen circulating in the blood[54]. A fibrous capsule surrounds the splenic parenchyma, which is organized in two regions termed white pulp and red pulp (Figure 1.3)[55].

In humans, the white pulp includes B cell follicles completely surrounded by the MZ[56]. These follicles are adjacent to the periarteriolar lymphoid sheath (PALS), a T cell-containing area that surrounds the central arteriole[54,57,58] and contains $CD4^+$ T cells but few

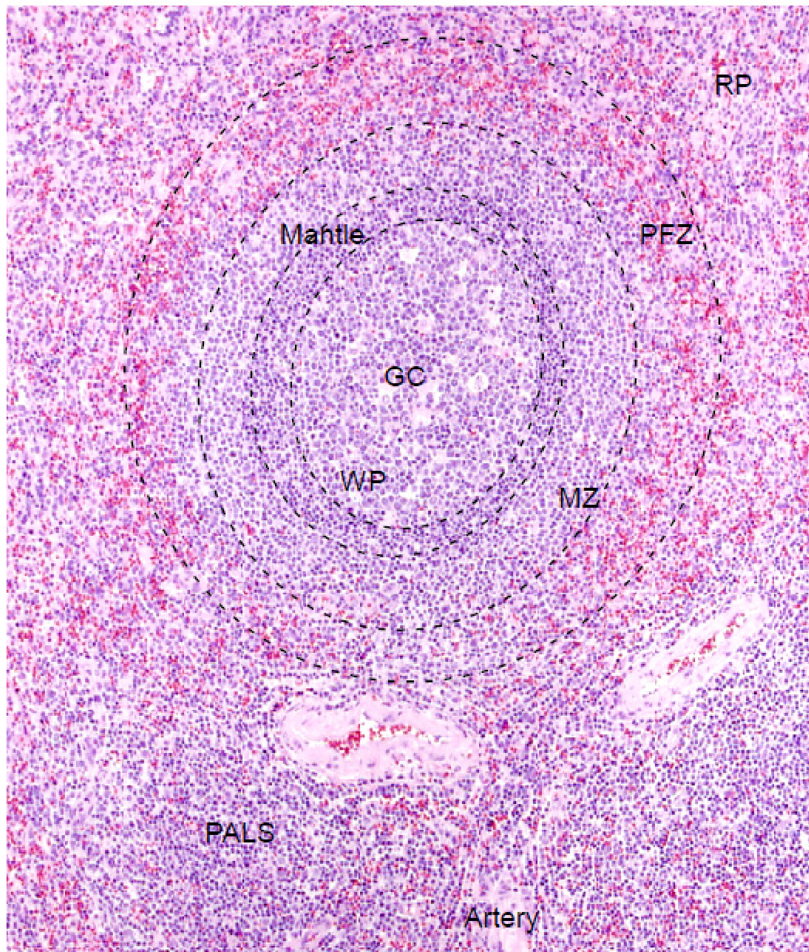


Figure 1.3 Structure of the human spleen. Hematoxilin and eosin (H&E) staining of a section of the splenic white pulp (WP) A secondary B cell follicle is proximal to a periarteriolar lymphoid sheath (PALS) containing T cells. The secondary lymphoid follicle includes the GC, the mantle zone and the MZ. The mantle area is formed by naïve B cells that surround a GC containing macrophages distinguishable by their large cytoplasm. The perifollicular zone (PFZ) and red pulp (RP) contain sinusoidal vessels that convey splenic blood into the venous system. Original magnification: 100x (courtesy of L. Comerma).

or no DCs[59]. The human MZ is bathed by arterial blood originating from capillaries and penicillary branches of the central arteriole[57]. The low flow rate of the blood passing through the MZ enable circulating antigen to interact with $IgD^{low}IgM^{+}CD27^{+}$ MZ B cells that are poised to initiate rapid antibody responses[39]. Indeed, human MZ B cells are in a pre-activated state that includes low expression of IgD and high expression of CD21 and CD35 complement receptors and CD1 MHC-I-like molecules such as CD1c[5,60]. In addition, human MZ B cells lack CD5, CD9, CD10 and CD23[5,60]. Besides canonical MZ B cells, the outer portion of the human MZ contains some $IgD^{+}IgM^{+}CD27^{-}$ B cells similar to follicular B cells and some canonical class-switched memory $IgD^{-}IgM^{-}CD27^{+}$ B cells[61].

In mice, the MZ receive blood-borne antigens from the marginal sinus, a fenestrated vessel that communicates with the central arteriole and separates the MZ from follicles[59]. This marginal sinus includes specialized metallophilic macrophages that express the lectin receptor SIGLEC-1 (also known as MOMA-1). Additional MZ macrophages express the C-type lectin receptor SIGNR1 and the scavenger receptor MARCO[62]. These and other PRRs enable resident macrophages and non-resident $CD11c^{low}$ DCs to capture blood-borne antigens, which are subsequently presented to MZ B cells[63].

Due to the lack of the marginal sinus, the human MZ lacks metallophilic macrophages, raising questions as to how MZ B cells capture antigen[64]. This process could involve DCs expressing the C-type lectin receptor DEC-205 (CD205) and some pericapillary macrophages expressing the sialoadhesin CD169[65]. As recently shown by our group, outer MZ B cells may also interact with a subset of

neutrophils termed B helper neutrophils (N_{BH}) cells[66]. These granulocytes appear to colonize the spleen in response to postnatal mucosal colonization by microbes and deliver powerful helper signals to MZ B cells through mediators that include B cell-activating factor of the TNF family (BAFF), a proliferation-inducing ligand (APRIL), and interleukin (IL)-21[39,66]. N_{BH} cells probably originate from the reprogramming of circulating neutrophils by cues released in the local microenvironment, including granulocyte monocyte-colony stimulating factor (GM-CSF) produced by $ROR\gamma t^{+}$ ILCs[67]. In addition to regulating the generation and/or survival of N_{BH} cells, these splenic ILCs establish a crosstalk with MAdCAM-1^{+} stromal cells through the release of tumor-necrosis factor (TNF) and lymphotoxin (LT). Besides activating stromal cells, this crosstalk enhances the survival of ILCs and their ability to stimulate MZ B cells via BAFF, CD40 ligand (CD40L) and the NOTCH2 ligand Delta-like 1 (DLL1)[67].

In both mice and humans, the red pulp harbors a vascular network that drains the blood into the splenic vein[55]. In humans, a unique feature of the red pulp relates to the presence of an extensive sinusoidal meshwork comprised of discontinuous vessels interconnected with blood-filled spaces known as cords of Billroth[55]. Unlike conventional blood vessels, splenic sinuses are not lined by a continuous monolayer of endothelial cells, but instead include a discontinuous layer of poorly understood and somewhat ambiguous cells known as reticulo-endothelial cells[68], splenic sinusoidal endothelial cells[69], splenic sinusoidal lining cells[70], endothelial cells of the sinuses[54,71] or littoral cells[72]. In the present study, we refer to these cells as SLCs. Although traditionally referred to as sinusoid endothelial

cells, SLCs have very unusual morphological and ultrastructural features[68]. In particular, SLCs have a fusiform shape and express myeloid molecules such as CD8α and mannose receptor (MR) as well as stromal molecules such as vimentin and thrombomodulin (CD141) in addition to canonical endothelial molecules such as the coagulation proteins von Willebrand factor (vWF) and Factor VIII[69–74]. Remarkably, SLCs are also present in the PFZ, a poorly understood peri-MZ area interconnected with both the red pulp and cords of Billroth[59,66,75].

2. Splenic vasculature. The spleen operates as a filter intercalated in the general bloodstream. In humans, four to six branches of the splenic artery enter the spleen and further divide into smaller vessels that form small arterioles surrounded by PALS. These arterioles are embedded in a fibrous collagenous tissue and their endothelial lining ends abruptly in the PFZ to generate a specialized vascular structure termed sheathed capillaries surrounded by sheaths of periarteriolar $CD169^+$ macrophages[59]. Additional arterioles terminate deep in the red pulp, but there is no consensus over their ability to form sheathed capillaries[73,76]. The path followed by the blood after its exit from arterioles remains unclear, but recent studies indicate that the capillaries emanating from arterioles are not directly connected to sinusoids, which suggests a model of completely open circulation in the human spleen[76,77].

After reaching the red pulp, blood first flows into the cords of Billroth and then enters the sinusoidal system after crossing the discontinuous endothelium of sinusoids. At this stage, SLCs may function as a biological filter that clears blood-borne antigens, including damaged

hematopoietic cells (Figure 1.4). Finally, sinusoids drain into the splenic vein, which is connected to the portal venous system.

3. Fetal development. The human spleen can be first identified in the 5th week of gestation as a collection of primitive reticular cells located in the dorsal mesogastrium[57]. After the 14th week, developing blood vessels become surrounded by erythrocytes and macrophages with phagocytic activity[78]. This developmental phase is called preliminary stage[79], which is associated with signs of active erythropoiesis[55,80]. The transformation stage begins in the 15-17 week and leads to the development of the red pulp, which involves formation of discontinuous venous sinusoids and additional recruitment of hematopietic cells[79]. The lymphoid colonization stage begins after 18 weeks with the formation of B cell clusters and accumulation of T cell precursors[79]. Follicles arise from B cell clusters located in the vicinity of T cell regions at a later gestational stage[78].

After birth and up to two years of age, the spleen lacks an anatomical or fully functional MZ, because neonates and infants fail to respond to TI antigens such as native CPS[81]. Remarkably, some MZ B cells are present in the circulation of neonates and progressively increase along with canonical memory B cells in the following years[5], which indicates that the anatomical MZ may not be required for the development of MZ B cells. At any rate, the signals required for the post-natal development of a fully functional MZ remain unknown, but may involve signals from the gut microbiota. Indeed, children with congenital defects in signaling proteins involved in microbial signaling through a category of PRRs known as Toll-like receptors (TLRs) have a profound defect of MZ B cells.

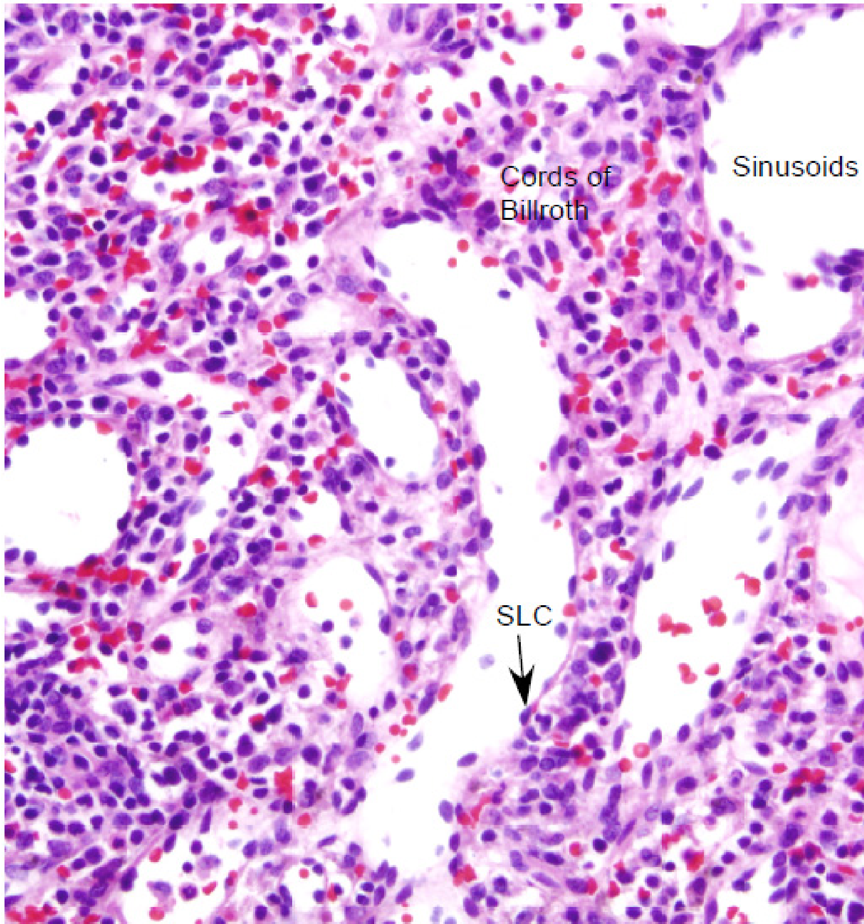


Figure 1.4 Sinusoids in the human red pulp. H&E staining of a human splenic tissue section showing the structure of sinusoids in the red pulp and PFZ. Sinusoids are lined by SLCs and communicate with blood-filled spaces tha lack endothelial lining and are known as cords of Billroth. 200X magnification (courtesy of L. Comerma).

4. Defensive function of the spleen. Owing to its specific microanatomy, the spleen fosters massive interactions between the circulation and the immune system[55]. As a result of these interactions, the spleen promotes the clearance of aged erythrocytes and platelets

from the circulation, recycles iron and serves as a reservoir for blood cells. In addition, the spleen captures and destroys blood-borne bacterial, viral and fungal pathogens by initiating both innate and adaptive immune responses[54]. Each of these functions takes place in specific and highly dynamic splenic compartments.

2. B cell diversification mechanisms

a. Development of B cells

B cells arise from multipotent hematopoietic stem cells (HSCs) that appear at 7 weeks of fetal life in the liver. By the 20th week, HSCs can also be found in the bone marrow, which after birth becomes the only developing site for B cells[82]. The B cell production rate of the bone marrow is maintained from mid-gestation throughout the eighth decade of life, although the diversity of developing B cells progressively decreases with age[83,84]. B cell development initiates from a common lymphoid progenitor (CLP), proceeds through several intermediate stages, requires a specialized niche, and involves changes in the expression of transcription factors, immunoglobulin gene products and multiple cell-surface molecules[85].

B cell development from the CLP follows three major stages (Figure 1.5)[85]. Pro-B cells express the B cell lineage-specific transcription factor paired box protein 5 (PAX5) and sequentially undergo DJ_H and V_HDJ_H recombination through a process that requires recombination-activating gene (RAG) endonucleases[86,87]. Pre-B cells down-regulate RAG expression and synthesize Igμ along with surrogate IgL chain to form a surface pre-BCR associated with the CD79 signaling complex[13,82]. Signals emanating from this pre-BCR regulate the expansion of pre-B cells and their further differentiation into immature B cells[13]. Finally, immature B cells re-express RAG proteins and undergo V_LJ_L recombination to assemble a mature and functional

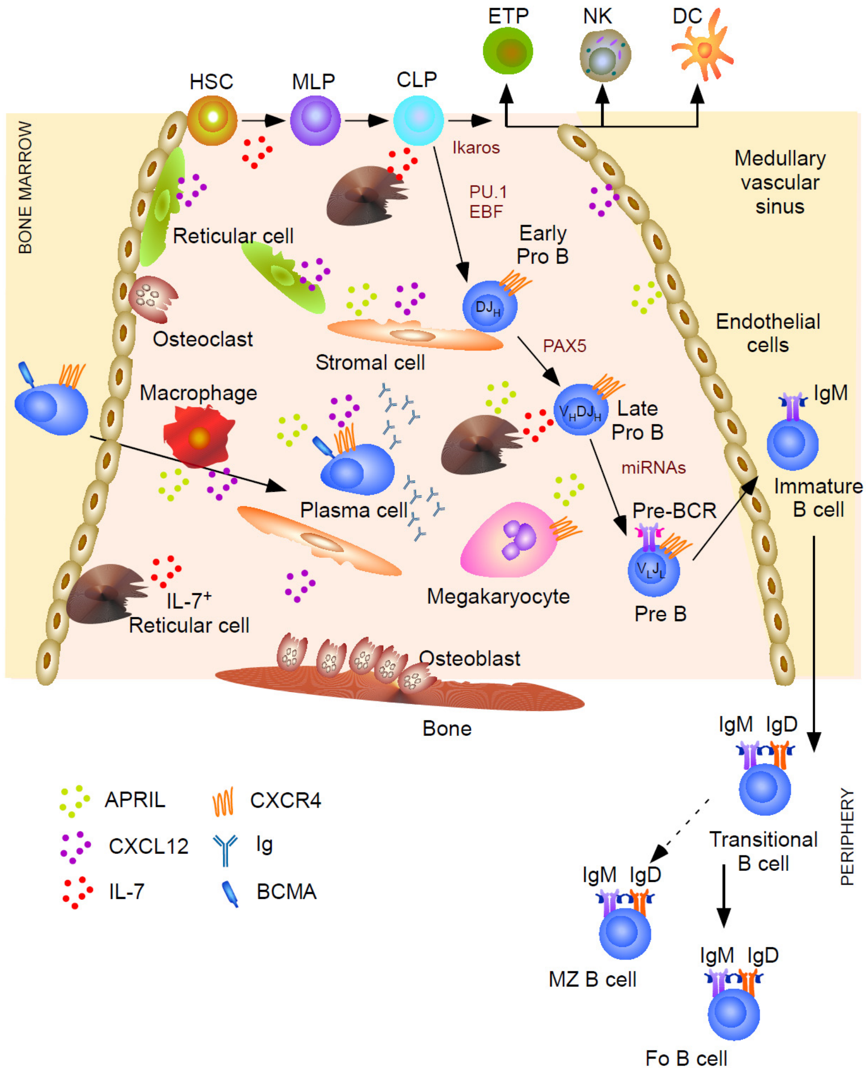


Figure 1.5 Development of B cells. The precursor haematopoeitic stem cells (HSC) gives rise to common lymphoid precursor (CLP) through myeloid-lymphoid progenitor (MLP). Early thimic progenitor (ETP), NK, DCs and Pro-B cells emerge from CLP cells and include early pro-B and late pro-B cells that undergo DJ_H and V-DJ_H gene rearrangements, respectively. These DNA recombination events require RAG1 and RAG2 endonucleases that are expressed as a result of changes in the expression of transcription factors including PAX5, PU.1 and EBF. Late pro-B cells differentiate to large pre-B cells that express a surface pre-BCR molecule composed of a V_H-

Cμ IgH chain and surrogate IgL chain formed by the Vpre-B and λ5 proteins. Large pre-B cells with in-frame V_HDJ_H rearrangements undergo positive selection and further differentiate to small pre-B cells, which down-regulate surface pre-BCR expression, and undergo V_L-J_L recombination via RAG proteins. Subsequent assembly of two IgH and two IgL chains leads to the formation of a surface BCR in immature B cells. Then, immature B cells differentiate to transitional B cells that express both surface IgM and IgD through alternative splicing of a long V_HDJ_H-Cμ-Cδ mRNA. Transitional B cells exit the bone marrow and further differentiate to either mature naïve B cells or mature MZ B cells in secondary lymphoid organs (Adapted from Cerutti et al. The immune sytem and Nagasawa 2006[85]).

BCR of the IgM type[13,82]. Strong signals from the BCR generated by self-proteins cause clonal deletion of immature B cells, thereby providing a critical central checkpoint. After becoming independent from stromal factors, immature B cells leave the bone marrow via the blood stream as <u>transitional B cells</u>[88]. In humans, transitional B cells express a $CD19^{+}CD24^{hi}CD38^{hi}IgM^{+}IgD^{+}$ phenotype and may be further sub-divided in at least two discrete stages known as T1 and T2[86,88].

In mice, the survival of transitional B cells depends on signals from the BCR as well as signals from the TNF family member BAFF[89–91]. According to the signal-strength model, the differentiation of transitional cells into either $IgM^{+}IgD^{lo}CD27^{+}$ MZ B cells or follicular $IgM^{+}IgD^{+}CD27^{-}$ B cells is dictated by the reactivity of the BCR to self-antigens through a pathway that involves Bruton's tyrosine kinase (BTK)[9,92,93]. Additional signals from the NOTCH2 receptor are also important[94]. In mice, robust BCR signals via BTK block the

differentiation of transitional B cells into MZ B cells by inhibiting MZ B cell inductive signals from NOTCH2[92,95,96]. Accordingly, B cell-conditional deletion of NOTCH2 results in the loss of MZ B cells but not follicular B cells[94]. Although there are several NOTCH ligands, the development of MZ B cells is specifically dependent on DLL-1[97]. This NOTCH2 ligand may be specifically expressed by radio-resistant endothelial cells present in the splenic red pulp and MZ[98]. Recent studies have confirmed that the development of MZ B cells requires NOTCH2 also in humans[99,100].

Accordingly, patients with Alagille syndrome have a NOTCH2 haploinsufficiency and show a marked reduction of circulating MZ B cells but not class-switched memory B cells[100]. Additional studies indicate that TLR receptors can also influence the development and/or maintenance of MZ B cells[101,102].

1. Origin of MZ B cells. The origin of $IgM^{+}IgD^{lo}CD27^{+}$ MZ B cells in humans is still debated. Indeed, MZ B cells share many properties with canonical class-switched $IgM^{-}IgD^{-}CD27^{+}$ memory B cells, including presence of mutated Ig V_H genes, expression of CD27, prominent responsiveness to TLR ligands, and striking propensity to rapidly secrete large amounts of antibodies upon stimulation[103]. Furthermore, some MZ B cells show signs of active proliferation and clonal expansion[104] and contain V(D)J gene mutation patterns similar to those of GC B cells[105]. Thus, it has been proposed that human MZ B cells may represent a subset of IgM-expressing memory B cells that exit the GC after undergoing SHM but not CSR through a canonical TD pathway. However, there is also abundant evidence that argues against a GC origin of MZ B cells. Indeed, MZ B cells have an Ig

V(D)J gene mutation frequency about two-fold lower than that of memory B cells[103]. Furthermore, compared to GC B cells, MZ B cells show a more diverse V(D)J gene repertoire due to a more limited antigen-driven clonal expansion[5,106]. When present, clonally expanded MZ B cells are not clonally related to GC B cells and show fewer cell divisions as compared to canonical memory B cells[46,104]. Moreover, MZ B cells but not memory B cells remain present and functional in immunodeficient patients with a form of hyper-IgM syndrome that disrupts the GC reaction due to the lack of CD40 signaling[107,108]. Similarly, immunodeficient patients with defective TLR signaling have a selective loss of MZ but not memory B cells[101]. Additional evidence indicates that MZ B cells mostly respond to TI antigens and derive from transitional precursors exposed to TLR ligands[39,102]. However, in agreement with studies performed in mice, some MZ B cells can also enter the GC in response to TD antigens and later emerge from the follicle as memory-like B cells..

i. V(D)J recombination

The IgH and IgL chains of antibodies include antigen-binding V regions encoded by recombined V_HDJ_H and V_LJ_L exons, respectively. Developing B cells assemble these exons from individual variable (V), diversity (D) and joining (J) gene segments, which are organized in multiple families in the IgH and IgL loci. An enzymatic complex including RAG1 and RAG2 endonucleases promotes V(D)J recombination by introducing double-stranded DNA breaks in specific recombination signal sequences (RSSs) flanking each V, D and

J exon to generate a wide diversity of V_HDJ_H and V_LJ_L combinations[109]. Pro-B cells initially recombine a DJ_H segment that subsequently recombines with a V_H segment to form an in-frame V_HDJ_H gene[110]. Later on, immature B cells begin the rearrangement of V_L and J_L segments to form an in-frame V_LJ_L gene (Figure 1.6)[111].

Antibody recognition diversity is further enhanced by developing B cells through nucleotide deletions in the 32 end of V_H segments, non-germline-encoded nucleotide additions, germ line-encoded palindrome nucleotide additions, transcription of D regions in any of three potential open reading frames as well as fusion and inversion of D regions[112–114]. Most of these diversification mechanisms are centered in hypervariable specific segments of V_HDJ_H exons called complementarity-determining regions (CDRs), which play a major role in antigen binding[23]. Overall, RAG-mediated V(D)J recombination is further regulated by epigenetic changes and formation of extensive chromosome loops that allow equal access of RAG proteins to widely dispersed gene elements[115].

b. B cell activation

Mature naïve B cells initiate antibody production and generate memory by undergoing several differentiation steps that vary in relationship to the nature of the antigen, which can be either a protein or a lipid or a carbohydrate.

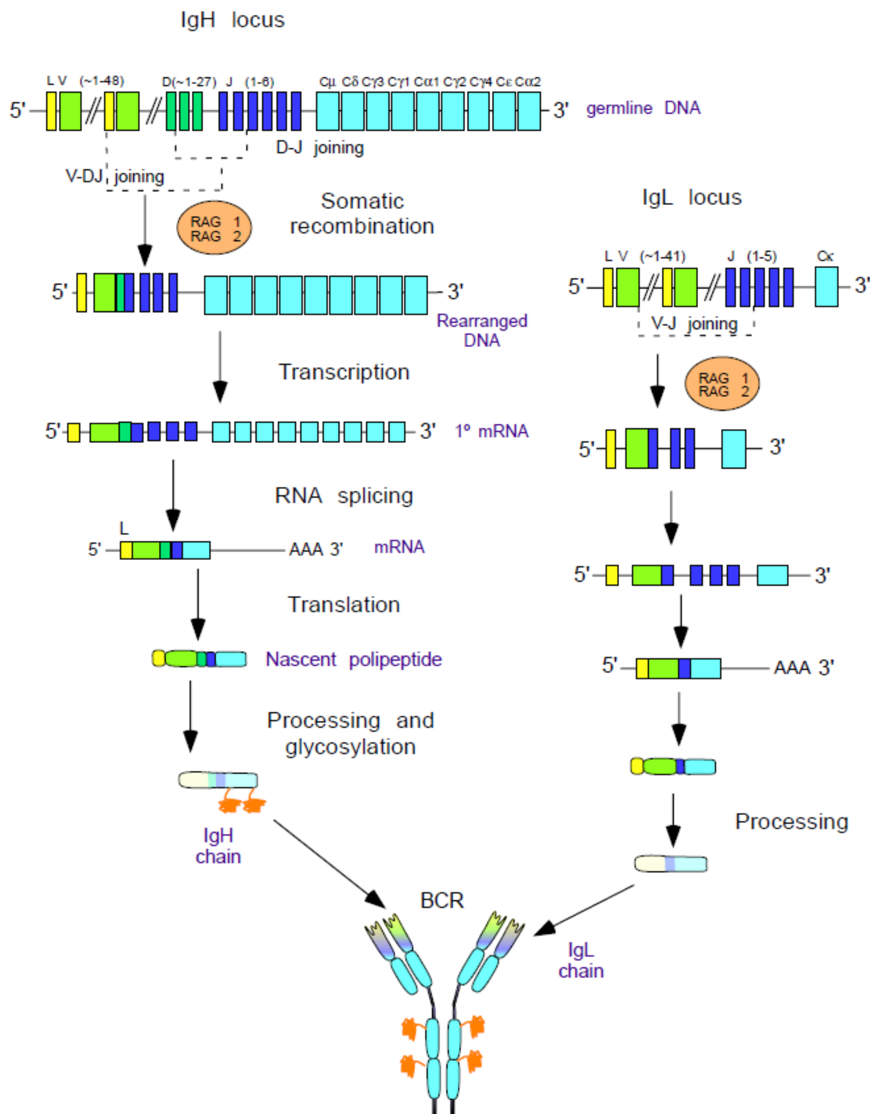


Figure 1.6 V(D)J recombination and BCR expression. The Ig loci include multiple V, D, and J (IgH locus) and V and J (IgL loci termed Igκ and Igλ) gene segments that undergo germ-line rearrangement during B-cell development in the bone marrow through a process involving RAG endonucleases. In the IgL loci, a V_L gene segment rearranges with a J_L gene segment to generate a V_LJ_L exon. In the IgH locus, recombination initiates with D and J segments to form a D_HJ_H segment followed by V

recombination to assemble a V_HDJ_H complete gene. Transcription of the rearranged genes, subsequent splicing, polyadenylation and translation generates mature IgL and IgH proteins. In each Ig locus, V gene transcription initiates at the level of a leader (L) sequence positioned upstream of each V segment. Ultimately, two identical IgH and two identical IgL proteins are assembled to generate the BCR. (Adapted from Cerutti et al. The immune sytem.)

i. TD pathway

Protein antigens usually trigger antibody production through a TD pathway involving the activation of follicular B cells by T follicular helper (T_{FH}) cells. Subcapsular macrophages or paracortcal DCs capture particulate antigen and then expose it to follicular naïve B cells. Alternatively, follicular B cells directly capture soluble antigen through the BCR. After internalizing antigen through the BCR, follicular B cells process antigen in the context of major histocompatibility complex-II (MHC-II) molecules and up-regulate T cell co-stimulatory molecules to acquire antigen-presenting activity. This antigen-specific B cell migrates to the boundary between the follicle and the T cell zone, also known as T-B border, to present antigen to T_{FH} cells through a process known as cognate interaction. T_{FH} cells are $CD4^+$ T cells with professional B cell-helper activity and emerge from a cognate interaction between naïve $CD4^+$ T cells and antigen-presenting DCs. By expressing CD40L and cytokines such as IL-21 and IL-4, T_{FH} cells drive the activation, expansion and differentiation of follicular B cells which initiate the GC reaction[116].

ii. GC reaction

The GC forms approximately two weeks after immunization[117] and represents a microenvironment specialized in the generation of class-switched B cells with high affinity for antigen. Chemokine gradients mostly generated by follicular stromal cells known as FDCs determine the specific positioning of both B and T cells in different areas of the GC, including the light and dark zones. Early T_{FH} cells and activated B cells expressing elevated levels of the chemokine receptor CXCR5 move into the follicle in response to CXCL13, a CXCR5 ligand produced by FDCs[118,119]. In the follicle, B cells enter a Bcl-6-regulated differentiation program[120–122] that involves massive proliferation, SHM, CSR and antigen-driven selection[123]. This Bcl-6 program is largely orchestrated by signals from T_{FH} cells, including the TNF family member CD40L and the cytokine IL-21, and leads to the formation of a GC surrounded by a follicular mantle[124]. This mantle area contains naïve B cells pushed aside by antigen-reactive B cells as they undergo clonal expansion[125,126].

In the GC, the proliferation of antigen-specific B cells is coupled with SHM and CSR, two Ig gene diversifying processes that require the enzyme AID[127]. SHM introduces point mutations in the V(D)J regions, thereby providing a correlate for the selection of GC B cells expressing BCRs with higher affinity for antigen[128,129]. These high-affinity B cells survive and further differentiate into memory or plasma cells, whereas low-affinity and autoreactive B cells brought about by the mutational process die by apoptosis[130]. Finally, CSR replaces IgM

with IgG, IgA or IgE to modulate the antibody effector functions without changing its antigen specificity.

In the GC, the dark zone contains large dividing centroblasts that undergo SHM and CSR in response to T_{FH} cells (Figure 1.7). Centroblasts further differentiate into smaller non-dividing centrocytes that move to the light zone of the GC. These centrocytes use their newly mutated BCRs to recognize native antigen trapped on the surface of FDCs[131,132]. Antigen-selected centrocytes expressing high-affinity BCRs receive additional survival and differentiation signals by establishing a cognate interaction with T_{FH} cells[122,133]. Of note, centrocytes expressing suboptimal BCRs traffic back to the dark zone to undergo a further round of SHM. This interzonal movement is regulated by changes in the expression of CXCR5 and CXCR4 chemokine receptors, which draw GC B cells toward the dark and light zones, respectively, in response to CXCL13 and CXCL12 from FDCs[134].

Unlike follicular B cells, centroblasts and centrocytes lack the intracellular anti-apoptotic factor Bcl-2 and instead express other Bcl-2 family members with pro-apoptotic activity[135,136]. This feature renders GC B cells highly susceptible to apoptosis, which allows their rapid elimination in the absence of high-affinity engagement of BCR by antigen[137]. Other pro apoptotic factors involved in the selection of GC B cells include FasL interaction, is expressed by T_{FH} cells. Of note, GC B cells up-regulate the expression of Fas after engagement of CD40 by CD40L on T_{FH} cells[138,139]. This death-inducing signal is overridden by strong “rescue” signals generated by a high-affinity BCR.

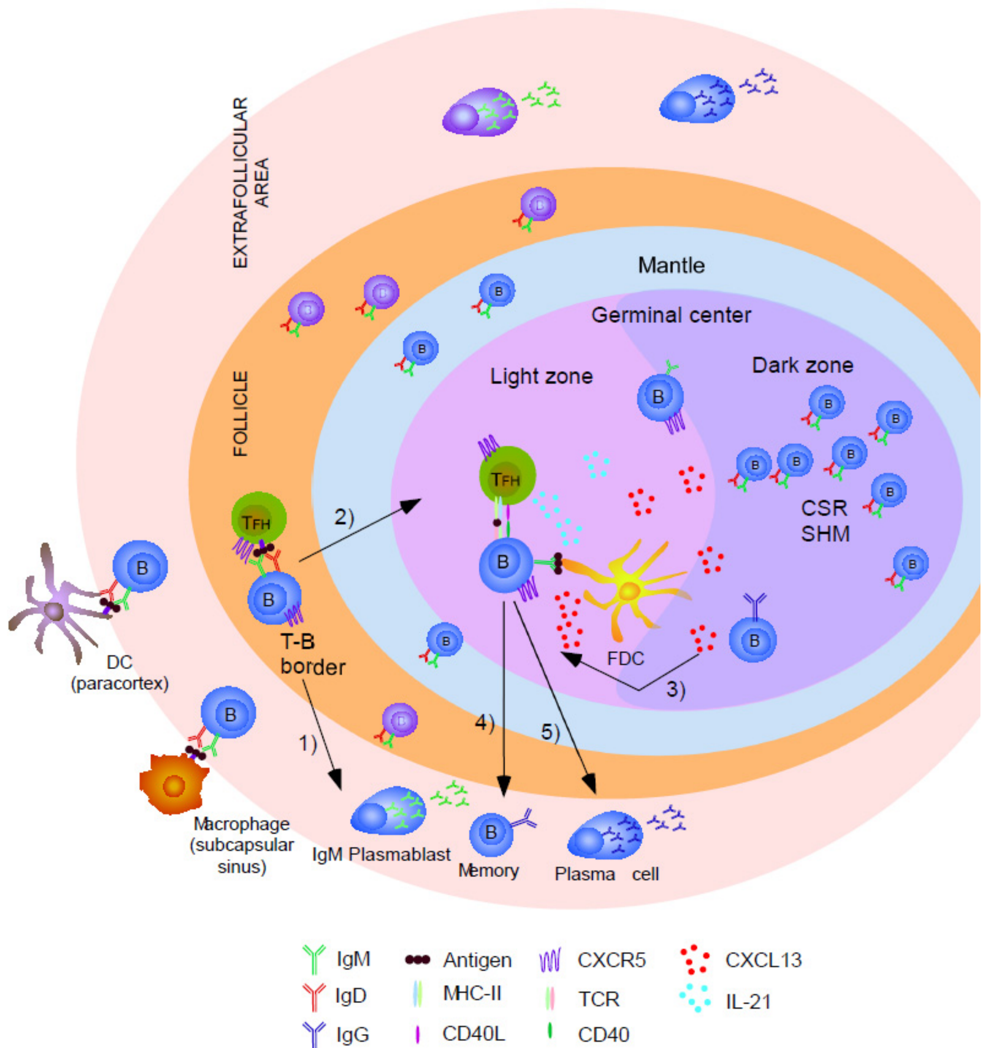


Figure 1.7 Germinal center reaction. Naïve B cells capture native antigen from subcapsular sinus macrophages and paracortical DCs through the BCR (both IgM and IgD molecules) and subsequently establish a cognate interaction with T_{FH} cells located at the boundary between the follicle and the extrafollicular area. After activation by T_{FH} cells via CD40L and cytokines such as IL-21, B cells enter either an extrafollicular pathway to become **1)** short-lived IgM-secreting plasmablasts or **2)** a follicular pathway to become germinal center centroblasts. In the dark zone of the germinal

center, centroblasts undergo extensive proliferation, express AID, and induce SHM and CSR from IgM to IgG, IgA, or IgE (the figure only shows IgG). **3)** After exiting the cell cycle, centroblasts differentiate into centrocytes that interact with FDCs located in the light zone of the germinal center. FDCs expose immune complexes containing native antigen to the BCR and centrocytes with low affinity for antigen die by apoptosis, whereas centrocytes with high affinity for antigen differentiate to **4)** long-lived memory B cells or **5)** plasma cells expressing high-affinity and class-switched antibodies. Memory B cells recirculate, whereas plasma cells migrate to the bone marrow (Adapted from Cerutti et al. The immune system and Cerutti et. al. EMBO Reports 2012[140]).

Centrocytes expressing high-affinity BCRs eventually differentiate into either long-lived memory B cells or long-lived plasma cells expressing IgG, IgA or IgE[52,117], whereas centrocytes expressing low-affinity BCRs undergo apoptosis to become phagocytosed by resident macrophages[117]. The nature of the signals required by centrocytes to enter a memory or plasma cell differentiation pathway remains unclear, but may be established by different subsets of T_{FH} cells expressing distinct sets of co-stimulatory molecules and cytokines. Finally, although predominantly activating follicular B cells, T_{FH} cells can also initiate a GC reaction after establishing a cognate interaction with MZ B cells responding to a TD antigen. Conversely, MZ B cells responding to TI antigens have some features of GC B cells responding to TD antigens, including SHM[141,142]. However, it must be noted that MZ B cells have a mutational loads and patterns of mutation largely different from those present in GC and memory B cells.

ii. CSR and SHM

Antibody class switching is a unique and irreversible process of intrachromosomal DNA recombination. In response to antigen stimulation and costimulatory signals, special G-rich tandem switch (S) regions located upstream of each IgH are exscinded to have a different somatic gene composition[143,144]. This event replaces the constant region of the IgM molecule with the encoding region of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgE[145].

The recombination is initiated by the B cell specific enzyme AID, which deaminates deoxycytosine (dC) into deoxyuridine (dU) in a single stranded-DNA in both the donor and acceptor S regions[127]. This deamination starts a process followed by the action of the uracil DNA glycosylase (UNG) which exscinds the region inside the two tagged S regions[146]. Finally the resulting a-basic sites are nicked by a apurinic/apyrimidinic endonuclease (APE) and other mismatch-repair proteins convert the single-strand DNA breaks to double-strand breaks (DSB) leaving them for appropriate end-joining recombination[147]. Fusion of double-stranded DNA breaks via the non-homologous end-joining pathway induces a looping-out deletion of the intervening DNA with subsequent replacement of $C\mu$ with a downstream C_H gene. The resulting juxtaposition of the recombined VDJ gene with a $C\gamma$, $C\alpha$ or $C\varepsilon$ gene permits B cells to produce an Ig with novel functions but identical specificity for antigen(Figure 1.8)[145].

CSR in B cells requires two signals. The first is normally delivered by cytokines, which target specific C_H genes for transcription; the second

is delivered in the case of TD antigens by interaction of CD40 on B cells with its ligand CD40L on activated T cells. The T_H2-type cytokines, IL-4 and IL-13 activate Cγ4 and with IL-21 are also potent inducers of Cε, while TGF-β predominantly induces Cα1 and Cα2 genes[148–151]. IL-4, IL-10 with IL-21 mostly target Cγ1, Cγ2 and Cγ3 genes[152,153]. CSR is severely impaired in patients and mice deficient in CD40L or CD40, although low levels of IgG and variable levels of IgA are still detected in serum[154,155]. BAFF and APRIL are two TNF family members that have been shown to activate CSR in human B cells and hence may contribute to residual CSR in CD40L and CD40 deficiency[142,156].

In SHM, AID introduces single-point mutations into the binding pocket of the variable region of IgH and IgL chains, to increase the affinity of the antibodies[157]. Affinity maturation by SHM does not depend on isotype switching and may occur also in B cells producing IgM[158,159]. Equally to CSR, AID starts the process by converting dC to dU, followed by error-prone DNA polymerases that complete the hypermutation process[129]. However, CSR and SHM do not appear to occur simultaneously in a cell. It is still unclear which AID interactions determine whether a cell will undergo CSR or SHM instead[145]. This highly mutagenic process has to be tightly controlled to avoid the generation of self-reactive antibodies, but also mutations have to be restricted to specific regions to induce affinity maturation[160].

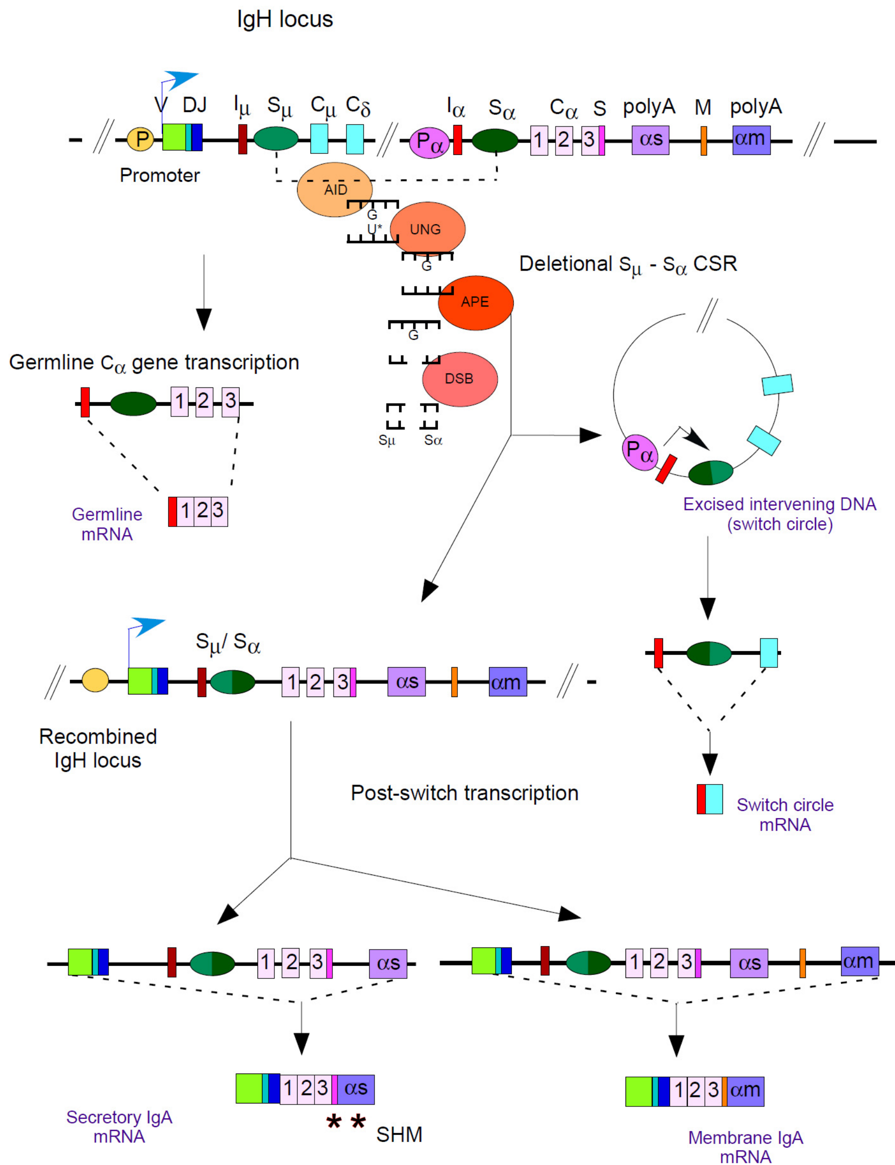


Figure 1.8 CSR mechanism. The IgH locus contains a rearranged V_HDJ_H exon encoding the antigen-binding domain of an immunoglobulin. Following rearrangement of the IgL locus, B cells produce intact IgM and IgD through a transcriptional process driven by a promoter (P) upstream of V_HDJ_H. Production of downstream IgG, IgA or IgE with identical antigen specificity but different effector function occurs through CSR. CSR and SHM processes are initiated by AID, which deaminates dC into dU coupled

to the transcription elongation process. The diagram shows the mechanism of IgA CSR, but a similar mechanism underlyes IgG and IgE CSR. Appropriate stimuli induce germline transcription of the Cα gene from the promoter (Pα) of an intronic α (Iα) exon (black arrow) through an intronic switch α (Sα) region located between Iα and Cα exons. In addition to yielding a sterile Iα-Cα mRNA, germline transcription renders the Cα gene substrate for AID, an essential component of the CSR machinery. AID expression occurs following activation of B cells by helper signals from T_{FH} cells (in the TD pathway) or innate immune cells (in the TI pathway). By generating and repairing DNA breaks at Sμ and Sα, the CSR machinery rearranges the IgH locus, thereby yielding a reciprocal deletional DNA recombination product known as Sα-Sμ switch circle. This episomal DNA transcribes a chimeric Iα-Cμ mRNA under the influence of signals that activate Pα. Post-switch transcription of the IgH locus generates mRNAs for both secreted and membrane IgA proteins. Cα1-3, exons encoding the Cα chain of IgA; S, 3' portion of Cα3 encoding the tailpiece of secreted IgA; M, exon encoding the transmembrane and cytoplasmic portions of membrane-bound IgA; αs, polyadenylation site for secreted IgA mRNA; αm, polyadenylation site for membrane-bound IgA mRNA (Adapted from Cerutti et al. NRI 2008 and from Cerutti et al. The immune system).

iv. TI pathway

Follicular B cells mount high-affinity antibody responses to protein antigens through a TD pathway that requires 5 to 7 days, which is too much of a delay to control blood-borne pathogens. To compensate for this limitation, extrafollicular MZ B cells and B-1 cells mount low-affinity antibody responses to carbohydrate and lipid antigens through

a TI pathway that only requires 1-3 days. In this alternative pathway, the production of IgM as well as class-switched IgG and IgA antibodies is orchestrated by CD40L-related factors released by DCs, macrophages and granulocytes of the innate immune system, including BAFF and APRIL[66,142]. By engaging a receptor called transmembrane activator and calium-modulating cyclophilin ligand interactor (TACI), these cytokines activate CD40-independent pathways that cooperate with both BCR and TLRs, thereby bridging the temporal gap required for the induction of high-affinity antibodies by follicular B cells.

In mice, both MZ B cells and B-1 cells express largely unmutated V(D)J genes that encode polyreactive antibodies capable to recognize multiple antigens with low affinity[161]. Classically, TI antigens are classified into type-1 (TI-1), and type-2 (TI-2) antigens. TI-1 antigens activate B cells in a polyclonal manner through TLRs or stimulate antigen-specific B cells by co-engaging BCRs and PRRs, including TLRs[162,163],[164],[165]. TI-2 antigens activate B cells through extensive BCR cross-linking and include carbohydrates with repetitive structure such as CPS from encapsulated bacteria[14,15]. These antigens are often opsonized by complement and therefore induce extensive activation of CD21 and CD35 complement receptors, which considerably lowers the activation threshold of the BCR complex in B cells. Irrespective of their recognition mechanisms, TI-1 and TI-2 antigens rapidly induce the differentiation of B-1 and MZ B cells into short-lived plasma cells that secrete massive amounts of low-affinity antibodies. In mice, TI antigens are generally poor inducers of immunological memory, but in humans this aspect remains controversial[166]. In addition, humans immunized with TI-2 antigens can induce the production of high-

affinity antibodies encoded by mutated V(D)J genes. This response may largely derive from the activation of splenic MZ B cells.

3. TI antibody responses in the spleen

a. Antigen capture by myeloid cells

A key component of antibody responses relates to the mechanisms by which B cells interact with antigen. In TD antibody responses, follicular B cells capture antigen from either subcapsular macrophages or paracortical DCs from lymph nodes. In TI antibody responses, MZ B cells capture antigen from metallophilic macrophages positioned along the marginal sinus and resident MZ macrophages[64,66,167]. Antigen-transporting DCs and inflammatory monocytes derived from the general circulation may also play a role[65]. In humans, the splenic MZ mostly lacks professional macrophage subsets, raising the possibility that other cells of the innate immune system mediate the initial capture of TI antigens and their presentation to MZ B cells (Figure 1.9)[39,167]. In both mice and humans, myeloid cells not only make available TI antigens as BCR and PRR (mainly TLR) ligands to B cells, but also release cytokines that cooperate with antigen to stimulate MZ B cells.

1. Macrophages. In mice, the marginal sinus surrounding the splenic white pulp conveys blood-borne antigens into the MZ, which is occupied by metallophilic macrophages and resident MZ macrophages. These macrophages capture antigen through scavenger

receptors, TLRs and C-type lectin receptors (CLRs)[168]. These PRRs may convey TI antigens to a non-degradative intracellular compartment that subsequently recycles antigen onto the cell surface, thereby permitting the activation of MZ B cells via antigen-responsive BCR and TLR pathways[39].

In humans, the MZ has neither marginal sinus nor specific populations of macrophages, but some macrophages form pericapillary sheaths in the PFZ[169]. Similar to subcapsular sinus macrophages from lymph nodes, these pericapillary macrophages express CD169, a PRR that recognizes syalic acid associated with CPS from certain strains of encapsulated bacteria[169]. Similar to mice, humans have additional macrophages in the red pulp of the spleen. These nonspecific macrophages express the scavenger receptor CD68 and the hemoglobin receptor CD163[39,64,167]. In addition to clearing structurally altered red blood cells, platelets and leukocytes from the circulation, red pulp macrophages may capture antigen and then expose it to MZ B cells[39]. Indeed, while mouse MZ B cells are sessile in nature, human MZ B cells appear to be highly mobile and may therefore interact with red pulp macrophages after entering the open circulation of the PFZ. In addition to exposing native antigen to B cells, both specific and nonspecific splenic macrophages release BAFF, APRIL, IL-6, IL-10, IFN and CXCL10 in response to antigenic stimulation. These cytokines may help TI antibody responses by enhancing the activation of antigen-reactive MZ B cells and by promoting the differentiation and survival of antibody-secreting plasma cells[39].

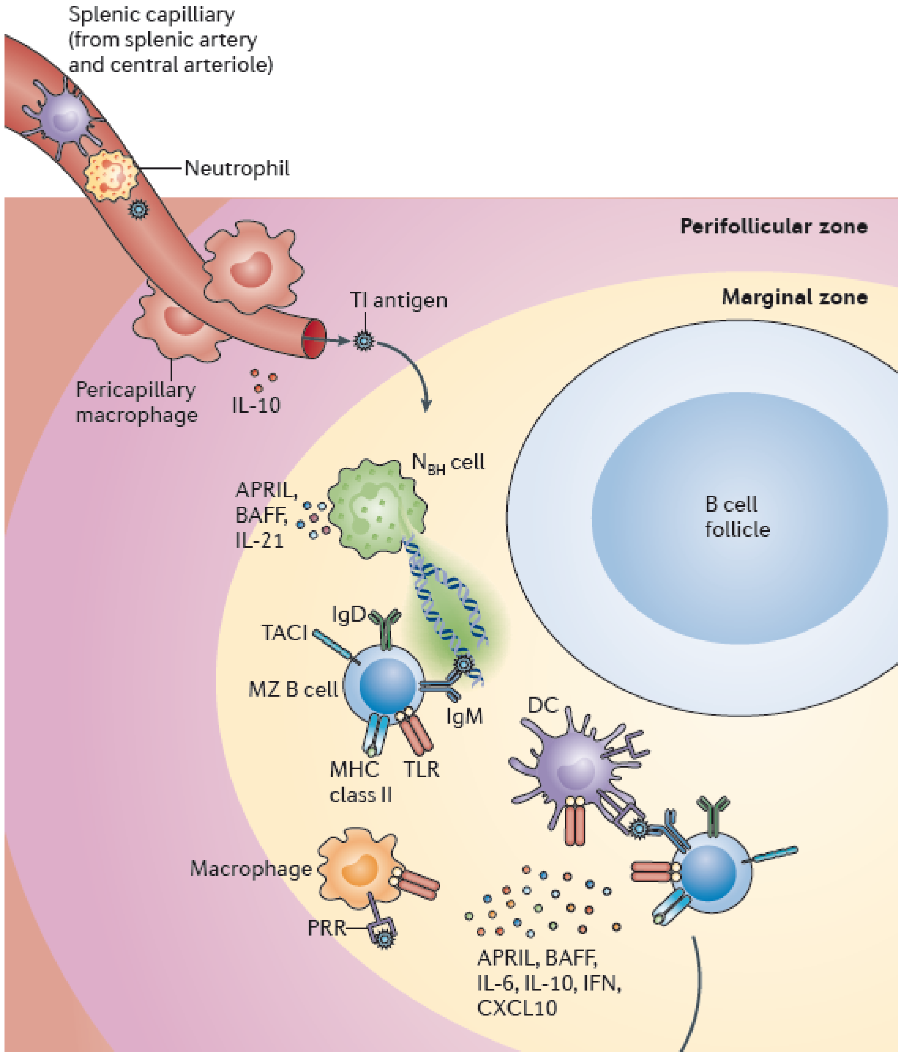


Figure 1.9 Antigen capture and presentation in the MZ. TI antigens are thought to enter the MZ through the perifollicular zone. Once in the MZ, they may be captured by neutrophil extracellular trap (NET)-like structures emanating from N_{BH} cells. Antigen capture may also involve reticular cells, macrophages, SLC and DCs. In addition to making TI antigens available to the BCR and TLRs on MZ B cells, antigen-capturing cells release BAFF and APRIL, which engage transmembrane activator and CAML interactor (TACI) on MZ B cells. N_{BH} cells also release IL-21, thereby inducing class-

switch recombination, somatic hypermutation and antibody production in MZ B cells. The generation of plasmablasts secreting IgM or class-switched IgG and IgA involves the production of IL-6, IL-10, IL-21 and CXCL10 by antigen-capturing cells. Arrows indicate the putative path followed by antigens through the spleen. IFN, interferon; PRR, pattern-recognition receptor. (Adapted from Cerutti et al. NRI 2013)

2. DCs. In mice, circulating DCs expressing low levels of CD11c and possibly corresponding to plasmacytoid DCs migrate to the MZ after capturing blood-borne bacteria. Similar to macrophages, these DCs release BAFF and APRIL to stimulate TI antibody production in MZ B cells. In humans, DCs expressing CD11c and the endocytic receptor DEC-205 (also known as CD205) inhabiting MZ and PFZ areas may exert a similar MZ B cell-helper function[39,65,170].

3. Neutrophils. As recently shown by our group, the PFZ of the human spleen contains a subset of neutrophils that have powerful MZ B cell-helper function. These neutrophils have a phenotype and gene expression profile distinct from those of circulating neutrophils. Indeed, splenic neutrophils express more immunostimulating molecules such as human leukocyte antigen (HLA)-II, CD86, IL-1β, IL-6, IL-8, IL-12 and TNF, which suggests their local activation by splenic signals. These signals may include microbial products that possibly originate from mucosal surfaces. Splenic neutrophils are also characterized by an elevated expression of immunoregulatory molecules such as progranulin, secretory leukocyte protease inhibitor (SLPI), arginase, indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS), which may account for the T cell-suppressive activity of splenic neutrophils, at least in vitro. Echoing

previously published studies performed with activated circulating neutrophils[66,171], splenic neutrophils also express elevated levels of BAFF and APRIL, which stimulate antibody production in MZ B cells in a TI manner[66]. Of note, splenic neutrophils colonize the spleen soon after birth, possibly in response to gut colonization by commensal bacteria[66]. Splenic neutorphils receive additional signals from a distinct population of splenic ILCs that release granulocyte-macrophage colony-stimulating factor (GM-CSF)[67]. This cytokine is likely to play a major role in the determination of some of the specific features of splenic neutrophils, including their activated phenotype and enhanced B cell-helper function[67].

b. Activation of MZ B cells

In both mice and humans, MZ B cells express a broad array of PRRs that permit them to recognize TI antigens in cooperation with the BCRs. In mice, these BCRs are encoded by mostly unmutated V(D)J genes and usually recognize multiple TI antigens with low-affinity[5,39]. In some cases, the recognition profile of these polyreactive BCRs is broadly similar to that of TLRs and indeed some TI antigens such as lipopolysaccharide (LPS) or peptidoglycan have been shown to co-engage BCR and TLR molecules on MZ B cells to initiate swift antigen-specific antibody production without any help from T cells[39]. Due to its rapidity, this low-affinity antibody response bridges the temporal gap required for the induction of high-affinity antibody production by conventional follicular B cells expressing monoreactive BCRs. In humans, MZ B cells express BCRs encoded by moderately

mutated V(D)J genes and appear to recognize TI antigens such as CPS with a relatively low affinity. As discussed earlier, these moderately mutated BCR appear to emerge from an "extrafollicular" form of SHM, which may involve the activation of TLRs.

In both mice and humans, MZ B cells are in a pre-activated state and indeed express elevated levels of PRRs, including the complement receptors CD21 and CD35 and various microbial sensors of the TLR family. This PRR expression profile is reminiscent to that present in DCs, macrophages and neutrophils and would permit MZ B cells to cross over the conventional boundaries between the innate and adaptive immune systems. When exposed to native antigen on macrophages, DCs or neutrophils, MZ B cells may co-activate NF-κB-dependent signaling pathways emanating from both BCR and TLR molecules and thereafter undergo massive activation, expansion and differentiation. In addition to inducing the production of large amounts of IgM [172], BCR and TLR ligands from TI antigens cooperate with BAFF, APRIL and other cytokines from macrophages, DCs or neutrophils to undergo CSR from IgM to IgG and IgA[66,173]. Our group has recently shown that this response is further enhanced by BAFF and DLL1 from splenic ILCs[67]. These MZ B cell-helper signals from ILC are particularly powerful when ILCs receive activation and survival signals from IL-1β and IL-23 produced by DCs and macrophages and from IL-7 produce by a subset of MAdCAM-1-expressing stromal cells residing in the human splenic MZT. The resulting CD40-independent activation of extrafollicular MZ B cells may account for the persistence of CPS-reactive antibodies in immunodeficient patients affected with type-1 or type-3 hyper-IgM

syndromes, who lack CD40-dependent follicular B cell response due to the lack of functional CD40L on T_{FH} cells and CD40 on B cells, respectively[46,108].

c. Splenic plasma cell response

After interacting with macrophages, DCs or neutrophils, MZ B cells rapidly differentiate to short-lived plasmablasts that produce large amounts of IgM as well as class-switched IgG and IgA. These antibodies recognize commensal antigens from mucosal surfaces in addition to blood-borne pathogens[66], which indicates that MZ B cells contribute to both pre-immune (or natural) and post-immune (or post-infection) humoral immune responses. In mice, plasmablasts emerging from activated MZ B cells migrate to the red pulp in response to CXCL12 expressed by local stromal cells and macrophages through a process that requires the down-regulation of surface receptors involved in MZ retention, including integrins, S1P receptors as well as CXCR5 and CCR7 chemokine receptors[174]. Once in the red pulp, plasmablasts receive survival signals from DCs and possibly macrophages, which indeed secrete BAFF and APRIL in response to various TLR ligands and cytokines such as IFN-α[156,175]. BAFF and APRIL support plasmablast survival by engaging B cell maturation antigen (BCMA) and possibly TACI receptors[66,74,166,176,177]. Remarkably, plasmablasts receive additional maturation and survival signals from IL-6, IFN-α, and CXCL10. This CXCR3 ligand stimulates activated B cells to release IL-6, which in turn enhances CXCL10 production by CD163-expressing macrophages[178,179].

Altogether, these findings indicate that plasmablasts emerging from antigen-activated MZ B cells may require a multi-component survival niche to deliver antibodies in the red pulp of the spleen. The composition of this splenic niche is poorly understood, but may involve nurse-like cells similar to those that support plasma cell survival in the bone marrow. However, additional nurse-like cells may be unique to the spleen.

1. DCs. In mice, TI antigens induce exponential growth of MZ B cells and massive plasma cell differentiation in extrafollicular foci that are located at the junction between T and B zones and contain clusters of conventional myeloid DCs[180]. In addition to CD11c, these DCs express DEC-205 and vigorously support the differentiation of plasmablasts into plasma cells independently of $CD4^+$ T cells[181].

2. Stromal cells. In both mice and humans, the spleen contains multiple subsets of stromal cells, including MAdCAM-1-expressing stromal cells positioned along the marginal sinus, marginal reticular cells (MRCs) located in the MZ, fibroblastic reticular cells (FRCs) inhabiting the red pulp, and FDCs positioned in the follicle. Besides playing a fundamental scaffolding function, some of these splenic stromal cells regulate immune responses by producing collagen-rich reticular fibres and chemokines that guide the trafficking of antigens and lymphocytes via dynamic conduit networks[182]. Only a few data are available on the role of stromal cells in plasma cell survival. In humans, poorly defined stromal cells located in the red pulp promote the recruitment of plasma cells via CXCL12 and enhance their survival through a mechanism that requires IL-6 but not BAFF and APRIL[183]. Furthermore, MAdCAM-1-expressing MRCs located in the MZ may

support plasma cells emerging from TI antibody responses by enhancing the survival and activation of splenic ILCs through both contact-dependent and contact-independent signals, including IL-7. These splenic ILCs release GM-CSF and IL-8 to co-opt neutrophils, which stimulate plasma cells through a mechanism that may involve BAFF and APRIL[67].

3. SLCs. In humans, the PFZ and red pulp of the spleen contain an enigmatic and somewhat protean population of endothelial cells that has been qualified in different ways, including littoral cells[69,70,73]. We generally refer to these cells as to SLCs. Originally described as a component of the splenic reticulo-endothelial system, SLCs have been suggested to have phagocytic activity by some studies[70], whereas others have confined this function to macrophages located in the splenic cords[68,184]. In general, the function of SLCs remains unknown, partly as a result of the technical difficulties associated with the isolation and propagation of these cells. In addition to canonical endothelial molecules such as coagulation factors, SLCs express DC molecules such as CD8α and, as recently shown by our group[74], MR, a CLR family member. Additional recent studies show that SLCs uniquely express signal regulatory protein (SIRP)-α and formin homology domain 1 (FHOD1)[72]. These proteins are also expressed by splenic angiomas, a benign neoplasia that possibly originates from SLCs[72]. Of note, SIRP-α is a transmembrane protein that binds CD47 and participates in the regulation of leukocyte migration and phagocytosis, whereas FHOD1 regulates actin filaments and stress fibers in smooth muscle cells. These functions highlight the protean nature of SLCs.

In a recent work, we demonstrated that SLCs support the survival and activation of malignant B-1/MZ-like B cells from patients with chronic lymphocytic leukemia (CLL) through a mechanism involving BAFF and APRIL[74]. These findings imply that SLCs may also contribute to the formation of splenic niches for the activation and survival of non-malignant B cells. In agreement with this hypothesis, the present study was designed to define the nature and function of SCLs, including their ability to support MZ B cell activation and plasma cell survival in the PFZ and red pulp of the human spleen.

Chapter II

Aims

The overall goal of this project was to characterize the geography, species specificity, nature and function of SLCs, an enigmatic population of endothelial cells that demarcate sinusoidal vessels in the spleen. We hypothesized that SLCs constitute a unique and human-specific subset of endothelial cells. We also hypothesized that SLCs enhance antibody production by forming splenic niches that promote the recruitment, activation and survival of MZ B cells and plasma cells.

AIM 1: To determine the topography, species specificity, phenotype, gene expression profile and innate immune properties of splenic SLCs.

AIM 2: To elucidate whether splenic SLCs deliver helper signals to enhance survival, activation, CSR and antibody production in MZ B cells.

AIM 3: To evaluate whether splenic SLCs deliver chemotactive and survival signals to antibody-secreting plasma cells.

PART II

MATERIALS AND METHODS

Chapter III

Materials and Methods

Human samples. Spleens from organ donors and individuals undergoing post-traumatic splenectomy without clinical signs of infection or inflammation and normal histology were used for functional assays and immunofluorescence analysis (IFA). Cells were also obtained from tonsils, colon and liver with normal histology. Formalin-fixed and paraffin-embedded tissue sections from healthy subjects and fetal demises were obtained from local tissue repositories from Hospital del Mar and Hospital Sant Joan de Deu. The Institutional Review Board of Institut Municipal d'Investigació Mèdica, Hospital Sant Joan de Deu and Hospital clinic de Barcelona approved the use of blood, lymphoid organs and tissues.

Animals. Spleens were collected from healthy rhesus macaques, wild type C57BL6/6 mice (thanks to J. Yelamos), and wild type rat F344 (thanks to M. Riera). All mice were housed in specific pathogen–free conditions. Male and female mice were used at 8–12 weeks of age. All tissue extractions from mice and rats were in accordance with approved protocols from the Institutional Animal Care at PRBB.

Cell isolation. Human splenocytes or intestinal, tonsillar and hepatic cell suspensions were obtained by mechanical tissue disruption with a scalpel, followed by 1 mg/ml collagenase type IV (Invitrogen) and 50 ng/ml DNAse I (NewEnglandBiolabs) with 1 ml of human serum digestion treatment in Hank's Balanced Salt Solution (HBSS; Sigma) for 45 min at 37 ºC and washed with cold HBSS to stop digestion. For the isolation of human SLCs, splenic tissues were enzymatically digested as above and followed by separation on a Ficoll-Hypaque gradient. Cells were isolated from the mononuclear ring and endothelial cells were cultured in EGM-bulletkit (Lonza) selective

media in p75 flasks for 3 to 7 d or directly FACS sorted for RNA purification. Non-adherent cells were washed out with PBS after 24-48 h culture. SLCs were sorted by flow cytometry as $CD45^-$ $CD31^+CD14^+CD8\alpha^+$ in Influx or Aria II cell sorters (BD Biosciences) with a 100 μm nozzle in PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 5% human serum. Splenic monocytes/macrophages were sorted as $CD45^+CD14^+$ from fresh splenocytes. $CD19^+IgD^{lo}CD27^+$ MZ B cells, $CD19^+IgD^{hi}CD27^-$ follicular B cells, $CD19^+IgD^-CD27^+CD38^-$ memory B cells and $CD19^+IgD^-CD27^{hi}CD38^{hi}$ plasmablasts/plasma cells were FACS sorted with an Aria II cell sorter (BD Biosciences) with a 70 μm nozzle in PBS after separation from the mononuclear ring using Ficoll Histopaque-1077 (Sigma).

Cultures and Reagents. SLCs and human umbilical vein endothelial cells were cultured in 12 wells plates or in 96-well flat bottom plates for B cell co-cultures, precoated with 0.1% gelatin (Sigma). SLCs and endothelial cells were detached using Accumax solution (Millipore) for 10 min at 37 ºC and washed for functional analysis. Half of supernatant was changed every 2-3 d in culture, except in B cell co-culture experiments where initial media was not changed. Half of EGM media was replaced with RPMI (Lonza) supplemented with 10% FBS, 5 mM Glutamine and 10 mM penicillin-streptomycin in B cell co-cultures. SLC cells were seeded 48 h before B cell addition, to reach confluence. Conditioned media was obtained by culturing SLCs for 24 h in EGM with or without 1 μg/ml of LPS. SLC to B cell ratio was 1 to 10 or 1 to 20 depending on the experiment. SLCs and B cells were co-cultured for different time points, depending on the particular

experiment. B cells were cultured alone or with conditioned media in round bottom 96 well plates. In blocking experiments, reagents were used at the following concentrations: BAFF-R-Ig, TACI-Ig and control Ig (R&D) were used at 25 ug/ml; neutralizing mAb to IL-6, TGF-β and isotype-matched control mAb (InVivogen) were used at 10 μg/ml.

Flow cytometry. Freshly isolated cells were incubated with total human IgG (Fc blocking reagent Miltenyi Biotec) at 4 °C with various combinations of antibodies (**Table 1**). 4'-6-diamidine-2'-phenylindole (DAPI) (Boehringer Mannheim) or 7-amino-actinomycin (7-AAD) (BD Pharmingen) were used to exclude dead cells from the analysis. B cell survival was assessed by DAPI staining or with Annexin-V Apoptosis Detection Kit II (BD Pharmingen). All gates and quadrants were drawn to give ≤ 1% total positive cells in the sample stained with isotype control antibodies. Cells were acquired using a FACS Fortessa (BD Biosciences) and analyzed using the FlowJo 7.6.5 software (Tree Star).

Immunohistochemistry (IHC). Formalin-fixed paraffin embedded tissue sections with a thickness of 3-4 μm were stained with Bond Max Immunohistochemical Stainer (Leica Biosystems) to stain CD8α, MR, vWF, CD34, CD68, vimentin, smooth muscle α-actinin (SMA), CD31, CD141 and CD45, with Kit Bond Polymer Refine Detection (Leica Biosystems) to stain CD8α and MR, and with Kit Bond Polymer Refine Red Detection (Leica Biosystems) to stain CD138, IgD, IgA and IgM. Sections were counterstained with haematoxylin.

Fluorescence microscopy and confocal microscopy. OCT-frozen tissues, paraffin blocks and cells were fixed and washed as reported [156,175] and stained with various combinations of antibodies (**Table 1**). Nuclear DNA was stained with DAPI. *S. aureus* inactivated-bacteria were stained with tracing marker carboxyfluorescein succinimidyl ester (CFSE). Coverslips were applied with FluorSave reagent (Calbiochem). Confocal images were generated with a Leica TCS SP5 up-right confocal microscope (Leica) by acquiring at least 12 different z-planes (Carl Zeiss Microimaging) with optimal z spacing (~ 0.03 μm). Fluorescence microscopy imaging was performed using Leica TCS SP5 up-right confocal microscope (Leica) with an open pinhole 3AU or were acquired with a BX-61 Olympus microscope. Fiji-ImageJ software was used to analyze and merge channels.

ELISA. Immunoglobulins to various antigens were detected in microplates coated overnight at 4 ºC in carbonate-bicarbonate buffer. Total IgM, IgG and IgA as well as BAFF, APRIL were detected as reported[156,175]. IL-6 (PeproTech) and IP-10 (BD Biosciences) were measured using a commercially available kit. TGF-β was measured by using a cytometric bead array (CBA) kit (BD Biosciences) using FACScalibur flow cytometer (BD) and analyzed using FlowCytomix Pro software. Values were expressed as mean ± standard deviation of the mean (s.d.m.).

B cell proliferation assay. 2×10^5 MZ B cells were seeded in 96-well plates in the presence of SLCs for 5 d and pulsed with 1 μCi [^{3}H]-thymidine the last 18 h. Harvested cells were moved to another plate without detaching SLCs and washed with a harvester to remove excess [^{3}H]-thymidine. Filter discs were collected and embedded with

scintillation fluid to assess [^{3}H]-thymidine. The proliferative response was determined with a Tri Carb 2800TR beta counter (Perkin Elmer).

Phagocytosis. Phycoerythrin-labeled polystyrene microspheres (Polysciences) were coated with total serum proteins for 30 min at 37 in KREBS buffer containing: 5.5 mM glucose, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$ HBSS (Gibco). To inhibit phagocytosis, control cells were pre-treated with 2 mM Cytochalasyn D (Sigma) for 30 min. Cells were treated with coated-PE-particles for 30 min at 37 ºC or at 4 ºC (unspecific surface-binding control). Quantification of internalized particles was performed by using a BD FACS Fortessa or FACS LSRII (Becton Dickinson). Data was analyzed using FlowJo 7.6.5 software.

RT-PCR, Southerns and QRT-PCR. RNA was extracted using Qiagen kit according to the manufacturer's instructions. DNA was reverse transcribed from total RNA using Superscript III kit (Invitrogen). After treatment of RNA with DNase I (Invitrogen), random hexamers (Invitrogen) were used for first-strand cDNA synthesis. PCRs were made semiquantitative by varying the number of amplification cycles and performing dilutional analysis so that there was a linear relationship between the amount of cDNA used and the intensity of the PCR product. $I_{\gamma}1$-$C_{\gamma}1$ (603 bp), $I_{\gamma}2$-$C_{\gamma}2$ (597 bp), $I_{\gamma}3$-$C_{\gamma}3$ (670 bp), $I_{\gamma}4$-$C_{\gamma}4$ (411 bp), $I_{\alpha}1$-$C_{\alpha}1$ (1,194 bp), $I_{\alpha}2$-$C_{\alpha}2$ (1,181 bp), $C_{\gamma}4$ (~415 bp), $I_{\gamma}1/2$-C_{μ} (557 bp), $I_{\gamma}3$-C_{μ} (608 bp) $I_{\gamma}4$-C_{μ} (358 bp) $I_{\alpha}1/2$-C_{μ} (666 bp) and I_{ϵ}-C_{μ} were PCR-amplified for 25 cycles with specific primer pairs (**Table 2**). Germline and switch circle transcripts were amplified by RT-PCR, hybridized with appropriate radiolabeled probes, and subjected to Southern blotting. PCR

conditions were: denaturation 1 min at 94 °C, annealing 1 min at 60 °C and extension 1 min at 72 °C. QRT-PCR was performed using SYBR green (Promega) with specific primer pairs (**Table 2**) in 96-well plates with a LightCycler 480 real-time PCR system (Roche Diagnostics). Gene expression was normalized to that of the gene encoding β-actin or CD20 in SLC- B cell co-cultures.

Southern blots. PCR products were fractionated onto agarose gels, transferred overnight onto nylon membranes and hybridized with specific radiolabeled probes (**Table 2**). SCs were hybridized with a probe that recognized the recombined S_{μ} region. Hybridization products appeared smeary on gel electrophoresis because CSR did not target a consensus DNA sequence, but it randomly occurred within a 1−10 kb S region. In each B cell, CSR yielded a single copy S_{μ}-S_X SC with a size that ranged from 500−4,000 bp. Circle transcripts were hybridized with a probe that encompassed nucleotides 1−250 of the first C_{μ} exon. Two $I_{\gamma}1/2$-C_{μ} and $I_{\alpha}1/2$-C_{μ} bands represented alternatively spliced forms, as reported[156].

Chemotaxis. 2 x 10^5 B cells were cultured for 18 to 24 h in RPMI (extend) with or without FBS (for starvation). Chemotaxis was performed with Cytoselect 96-well migration chamber kit (Cell Biolabs). Cells were seeded in the upper chamber and SLC-conditioning media or chemoattractants were set in the bottom and incubated for 4 h at 37 ºC. Migratory cells were transferred to a black non-adsorbent plate (Nunc) and labelled with CyQuant GR Dye (Cell Biolabs). Fluorescence was measured at 480/520 nm with a fluorescence plate reader (Migration index). Concentrations used for the chemokines: 600 pg/ml IP-10; 100 ng/ml CXCL13.

Microarray Hybridization. 100 ng of total RNA was labeled using LowInputQuick Amp Labeling kit (Agilent 5190-2305) following manufacturer instructions. mRNA was reverse transcribed in the presence of T7-oligo-dT primer to produce cDNA. cDNA was then in vitro transcribed with T7 RNA polymerase in the presence of Cy3-CTP to produce labeled cRNA. 600 ng of labeled cRNA was hybridized to the Agilent Human SurePrint G3 gene expression 8x60K microarray according to the manufacturer's protocol. The arrays were washed, and scanned on an Agilent G2565CA microarray scanner at 100% PMT and 3 mm resolution. Intensity data was extracted using the Feature Extraction software (Agilent). 4 samples of SLC, 5 of MCs and 6 samples of HUVECS were analyzed. 4 representative samples of each group were picked to construct the heatmaps.

Statistical Analysis. Statistical significance was assessed with the one-tailed unpaired Student's t-test with GraphPad Prism 5 software. Whole genome array statistical analyses were performed with the Bioconductor project (http://www.bioconductor.org/) in the R statistical environment (http://cran.r-project.org/)[185].

Preprocessing and differential expression analysis. Raw data was taken from the Feature Extraction output files and was corrected for background noise using the normexp method[186]. To assure comparability across samples we used quantile normalization [Bolstad, B. (2001). Probe Level Quantile Normalization of High Density Oligonucleotide Array Data. Unpublished manuscript http://bmbolstad.com/stuff/qnorm.pdf]. Differential expression analysis was carried out on non control probes with an empirical

Bayes approach on linear models (limma)[187]. Results were corrected for multiple testing according to the False Discovery Rate (FDR) method[188].

Venn diagrams, heatmaps, gene ontology gene selection. Selections of probe: "S vs H": probes with absolute fold change above 2 and an adjusted-Pvalue below 0.01. "S vs M": probes with absolute fold change above 2 and a p-value below 0.05. Additionally only probes which are present and reliable according to our criteria (Normalized signal intensity value > 250, non flagged by Feature Extraction, non flagged by saturation, non control, and foreground signal greater 2 fold the background signal) in at least 4 samples across the dataset were selected. Finally gene-level data was obtain by collapsing probe-level data by the median intensity. Values were expressed as mean ± standard error of the mean (s.e.m.). Statistical significance was assessed by using one-tailed unpaired Student's *t*-test.

TABLE 1. Antibodies

Antigen	Label	Isotype	Clone (if monoclonal)	Manufacturer	Use
AID		Goat IgG	-	Santa Cruz	IF
SMA		Mouse IgG1	HHF35	Ventana	IHC
APRIL		Rabbit IgG	ED2	Prosci	IF
APRIL	PE	Mouse IgG2a	T3-6	Biolegend	FC
BAFF		Mouse IgG1	1D6	eBioscience	IF
BAFF	PE	Mouse IgG1	1D6	eBioscience	FC
BAFF-R	PE	Mouse IgG2a	8A7	eBioscience	FC
BCMA	PE	Goat IgG		RnD	FC
CD8α		Mouse IgG2b	4B11	Leica	IHC/IF
CD8α		m IgG1	C8/144B	DAKO	IHC/IF
CD8α	FITC	Mouse IgG1	RFT-8	Southern Biotech	FC
CD10	PE	Mouse IgG1	HI10a	Biolegend	FC
CD11b	PE	Mouse-IgG2a	D12	BD Biosciences	FC
CD11c		m IgG2a	5D11	Leica	IF
CD11c	PE	Mouse IgG1	B-ly6	BD Biosciences	FC/IF
CD14	APC-Cy7	Mouse IgG1	HCD14	Biolegend	FC
CD16	PE	Mouse IgG1	3G8	BD Biosciences	FC
CD19	PE-Cy7	Mouse IgG1	HIB19	Biolegend	FC
CD20	FITC	Mouse IgG1	L27	BD Biosciences	FC
CD24	PE	Mouse IgG2a	ML5	BD Biosciences	FC
CD27	PE	Mouse IgG1	M-T271	Ancell	FC
CD31	Alexa 647	Mouse IgG2a	M89D3	BD	FC
CD31		Mouse IgG1	JC70	Ventana	IHC
CD33	PE	Mouse IgG1	WM53	Biolegend	FC
CD34		Mouse IgG1	QBEnd/10	Ventana	IHC
CD34	PE	Mouse IgG1	581	BD	FC
CD36	PE	Mouse IgG2a	5-271	Biolegend	FC
CD38	APC-Cy7	Mouse IgG1	HIT2	BD Biosciences	FC
CD38	PE	Mouse IgG1	HIT2	BD Biosciences	FC
CD40	PE	Mouse IgG1	5C3	Biolegend	FC
CD45	Pe-Cy7	Mouse IgG1	HI30	BD	FC
CD45		Mouse IgG1	2B11 & PD7/26	Ventana	IHC
CD54	PE	Mouse IgG1	HCD54	Biolegend	FC
CD68		Mouse IgG1	KP1	Dako	IF
CD89	PE	Mouse IgG1	A59	Biolegend	FC
CD102	PE	Mouse IgG1	CBR-IC2/2	Biolegend	FC
CD103	PE	Mouse IgG1	B-Ly7	Biolegend	FC

CD105	PE	Mouse IgG1		Immunotools	FC
CD134	PE	Mouse IgG1	ACT35	Biolegend	FC
CD138		Mouse IgG1	MI15	DAKO	IHC
CD141	PE	Mouse IgG1	M80	Biolegend	FC
CD141		Mouse IgG1	1009	DAKO	IHC
CD141		Rabbit IgG	EPR4051	epitomics	IHC
CD144	PE	Mouse IgG2a	BV9	Biolegend	FC
CD163	PE	Mouse IgG1	GHI/61	Biolegend	FC
CD172(SIRPα)	PE	Mouse IgG2a		RnD	FC
CD205	PE	Mouse IgG1	HD30	Biolegend	FC
CD206		mouse IgG1	19.2	BD pharmingen	IHC/IF
CD206		mouse IgG1	15-2	biolegend	IHC/IF
CD206		rabbit IgG	H300	santa cruz	IHC/IF
CD206*		goat IgG	C-20	santa cruz	IHC/IF
CD206	AF488	Mouse IgG1	15-2	Biolegend	FC
CD207(langerin)	PE	Mouse IgG1	10E2	Biolegend	FC
IgA		Rabbit IgG		DAKO	IHC
IgD		Rabbit IgG		Dako	IHC
IgD	FITC	Goat IgG F(ab')2		Southern Biotech	FC
IgD	Biotin	Goat IgG F(ab')2		Southern Biotech	IF
IgM		Rabbit IgG		Dako	IHC
HLA-DR	FITC	Mouse IgG2b	G46-6	BD	FC
Neutrophil Elastase		Rabbit IgG		Abcam	IF
TACI	PE	Mouse IgG2b	11H3	eBioscience	FC
TLR4	PE	Mouse IgG2b	HTA125	Biolegend	FC
VEGFR-1	PE	Mouse IgG1			FC
Vimentin		Mouse IgG2a	Vim 3B4	Ventana	IHC
Vimentin	PE	Mouse IgG1	RV202	BD	FC
vWF*		Rabbit IgG		Millipore	IF
vWF		Rabbit IgG		Dako	IF

* Antibodies used for mouse and rat stainings.

TABLE 2. Primers and probes

Target gene		Primer sequence
ACTB	S	GGATGCAGAAGGAGATCACT
	AS	CGATCCACACGGAGTACTTG
AICDA	S	AGAGGCGTGACAGTGCTACA
	AS	TGTAGCGGAGGAAGAGCAAT
CXCL1	S	GCGCCCAAACCGAAGTCATA
	AS	TCAGGAACAGCCACCAGTGA
CXCL2	S	AGAAAGCTTGTCTCAACCCCG
	AS	TCCTTCAGGAACAGCCACCA
CXCL3	S	GCGCCCAAACCGAAGTCATA
	AS	TCAGTTGGTGCTCCCCTTGT
CXCL5	S	TGCTGTGTTGAGAGAGCTGC
	AS	TTGGAGCACTGTGGGCCTAT
CXCL6	S	TGCGTTGCACTTGTTTACGC
	AS	CCCGTTCTTCAGGGAGGCTA
CXCL8	S	CCAAACCTTTCCACCC
	AS	ACTTCTCCACAACCCT
CCR1	S	TCACGGACAAAGTCCCTTGGAACC
	AS	GCCCCAAAGGCCCTCTCGTT
CCR2	S	CAGGTGACAGAGACTCTTGGGA
	AS	GGCAATCCTACAGCCAAGAGCT
CCR5	S	CATCCGTTCCCCTACAAGAA
	AS	GGCAGGGCTCCGATGTATAA
CCR6	S	GCAGCGGTAGCAGGAAAGTA
	AS	GGAGTCATCAGATTGTGGGG
CCR7	S	CTTCTCGGACATCAGCTGGC
	AS	GGACGCTTTTGTTGGGCATG
CCR8	S	ACCTCCAGAGAGGCTGCTGCT
	AS	AATCCATCAAGGCAGCGGGAC
CX3CR1	S	ACAGGGTGGCTGACTGGCAGA
	AS	CCCAAAGACCACGATGTCCCCA
CXCR1	S	TACTGTTGGACACACCTGGC
	AS	GGCATGCCAGTGAAATTTAG
CXCR2	S	GCCACACGCACACTGACCCA
	AS	ACAGGCAGGGCCAGGAGCAA
CXCR3	S	ACGAGAGTGACTCGTGCTGTAC
	AS	GCAGAAAGAGGAGGCTGTAGAG
CXCR4	S	AAGTGACGCCGAGGGCCTGA
	AS	CCCCTGAGCCCATTTCCTCGGT
CXCR5	S	TGAAGTTCCGCAGTGACCTGTC
	AS	GAGGTGGCATTCTCTGACTCAG
CXCR6	S	CTCTGCCCTTCTGGGCCTAT

	AS	GATGCCCAGTAGGCTCTTGC
CXCR7	S	CCAAGACCACAGGCTATGACAC
	AS	TGGTTGTGCTGCACGAGACTGA
PAX5	S	TTGCTCATCAAGGTGTCAGG
	AS	CTGATCTCCCAGGCAAACAT
TLR3	S	TGACTGAACTCCATCTCATGTCC
	AS	CCATTATGAGACAGATCTAATGTG
TLR4	S	CCCTGCGTGGAGGTAT
	AS	GCACCTGCAGTTCTGGGAAA
TLR8	S	TTCCTGTGAGTTATGCGCCG
	AS	TATTTGCCCACCGTTTGGGG
TLR9	S	ACAACAACATCCACAGCCAAGTGTC
	AS	AAGGCCAGGTAATTGTCACGGAG
$I\alpha 1/2$	S	CAGCAGCCCTCTTGGCAGGCAGCCAG
$C\alpha 1$	AS	GGGTGGCGGTTAGCGGGGTCTTGG
$C\alpha 2$	AS	TGTTGGCGGTTAGTGGGGTCTTCA
$I\gamma 1/2$	S	GGGCTTCCAAGCCAACAGGGCAGGACA
$C\gamma 1$	AS	ATTCTCCTCATCTGCGCCAGGA
$C\gamma 2$	AS	GTGGGCACTCGACACAACATTTGCG
$I\gamma 3$	S	AGGTGGGCAGCCTTCAGGCACCGAT
$C\gamma 3$	AS	TTGTGTCACCAAGTGGGGTTTTGAGC
$I\gamma 4$	S	TTGTCCAGGCCGGCAGCATCACCAGA
$C\gamma 4$	AS	ATGGGCATGGGGGACCATATTTGGA
$I\varepsilon$	S	GACGGGCCACACCATCC
$I\mu$	S	GTGATTAAGGAGAAACACTT TGAT
$C\mu$	AS	AGACGAGGGGGAAAAGGGTT
Cα probe	S	ACCTGAGGAGACGGTGACC
Cα probe	AS	CACTGTGTGGCCGGCAGGGT
Cε probe	S	CCTCCACACAGAGCCCATCC
Cε probe	AS	GAGACGGTCAGCAAGCTGATGGTG
Cγ probe	S	TGGTCAAGGACTACTTCCCCGAACCG
Cγ probe	AS	GAAGTTGCTGGAGGGCACGGT
Sμ probe	S	GGATGAACTGGAGGACATGGCAC
Sμ probe	AS	CTCAGCTCCCAGCACATGCAG

PART III

RESULTS

Chapter IV

Results

SLCs are unique myeloid-endothelial cells of the splenic innate immune system that form activation and survival niches for MZ B cells and plasma cells

1. SLCs are distinct from endothelial cells

SLCs, also known as littoral cells, are an unusual subset of endothelial cells that line the venous sinusoids of the red pulp of the spleen[68,184]. Earlier studies indicate that SLCs express typical endothelial features along with the lymphoid/myeloid molecule CD8α[71,74,189,190]. Despite these findings, the phenotype of SLCs remains largely unknown. In particular, it is unclear whether myeloid traits are truly specific to SLCs or rather reflect the presence of myeloid cells physically contiguous to SLCs. To address this issue, high-resolution images were obtained from human splenic tissue sections co-stained for SLC, endothelial and myeloid molecules and analyzed by three-color confocal microscopy.

We found strong expression of canonical endothelial molecules such as the coagulation protein vWF and the adhesion molecule CD31 (or PECAM-1) in both sinusoids from the red pulp and PFZ and arterioles from the white pulp (**Figure 4.1 A, B**). Unlike arterioles, sinusoids expressed MR, an endocytic receptor of the CLR family usually detected in some myeloid DCs, monocytes and macrophages (**Figure 4.1 A, B**). Sinusoids also expressed CD8α (**Figure 4.1 C**), a lymphoid molecule expressed by some DCs, at least in mice. Together with vWF and CD31, both MR and CD8α followed sinusoidal staining patterns in both PFZ and red pulp. An intense non-sinusoidal staining pattern characterized CD8α expression

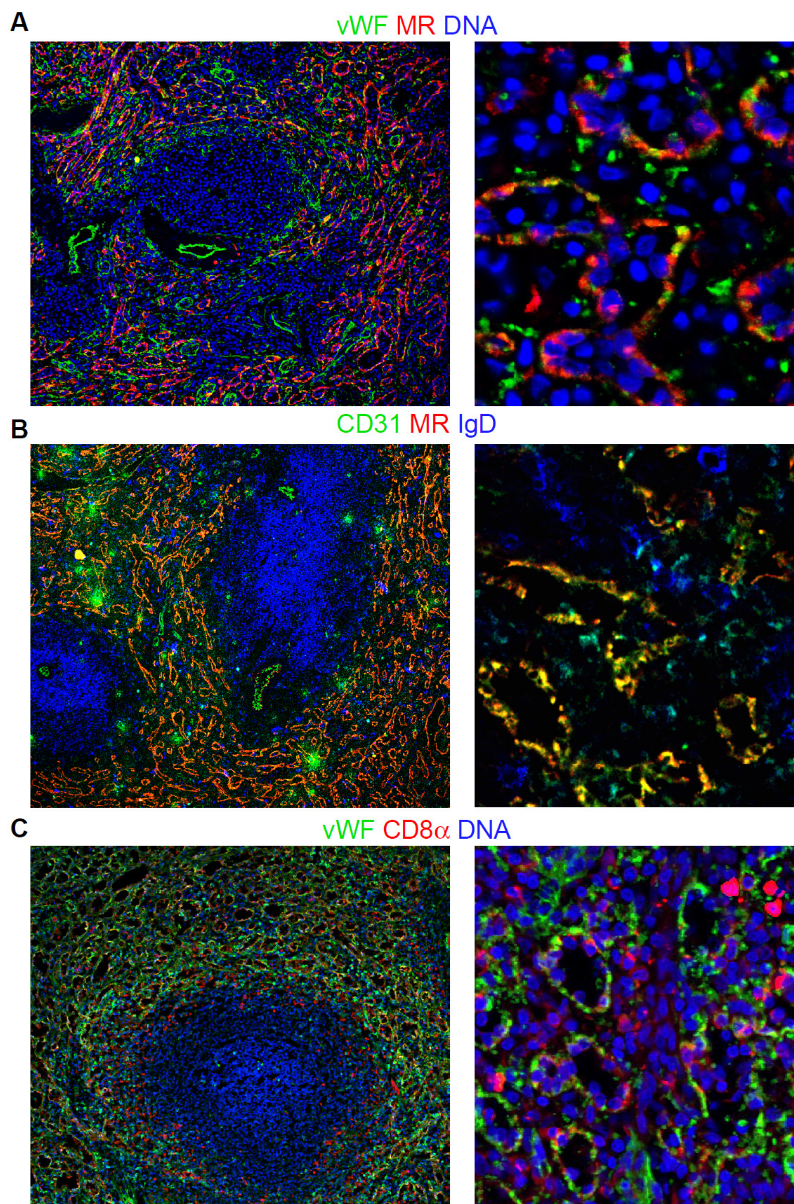


Figure 4.1 SLCs occupy the red pulp of the spleen and show a phenotype distinct from that of ECs. (A-C) Confocal microscopy of human spleen tissue sections co-stained for MR or CD8α (red), CD31 or vWF (green), and IgD or DNA (blue). Original magnification, 200x (left panels) or 630x (right panels). Data are from one of three experiments with similar results.

by round cells scattered in the red pulp, which possibly corresponded to $CD8^+$ T cells, NK cells or monocytes.

Of note, SLCs showed weak or no expression of the scavenger receptor CD68 and the integrin CD11c, two myeloid molecules that instead were abundant in PFZ and red pulp macrophages as well as follicular, MZ and PFZ DCs, respectively (**Figure 4.2 A, B**). Unlike SLCs, macrophages lacked MR and were often contiguous to SLCs, pointing to a possible functional cooperation between SLCs and macrophages in the sinusoidal meshwork. Finally, unlike SLCs but similar to macrophages, DCs did not express MR (**Figure 4.2 A, B**). Thus, SLCs express a hybrid $vWF^+CD31^+CD8\alpha^+MR^+$ phenotype distinct from the $vWF^+CD31^+CD8\alpha^-MR^-$ phenotype of vascular endothelial cells from canonical blood vessels. In addition, SLCs are phenotypically distinct from perisinusoidal macrophages and DCs.

2. SLCs express hybrid myeloid-endothelial features

The limited knowledge of the phenotypic features of SLCs has severely hampered their purification and functional evaluation. To further characterize the phenotype of SLCs, we performed detailed morphological and phenotypic studies on SLCs purified on the basis of their unique co-expression of CD31 and CD8α. Bright field microscopy and confocal microscopy demonstrated that SLCs had elongated-spindle-like morphology similar to that of canonical endothelial cells and expressed CD31 and vWF (**Figure 4.2 C, D**).

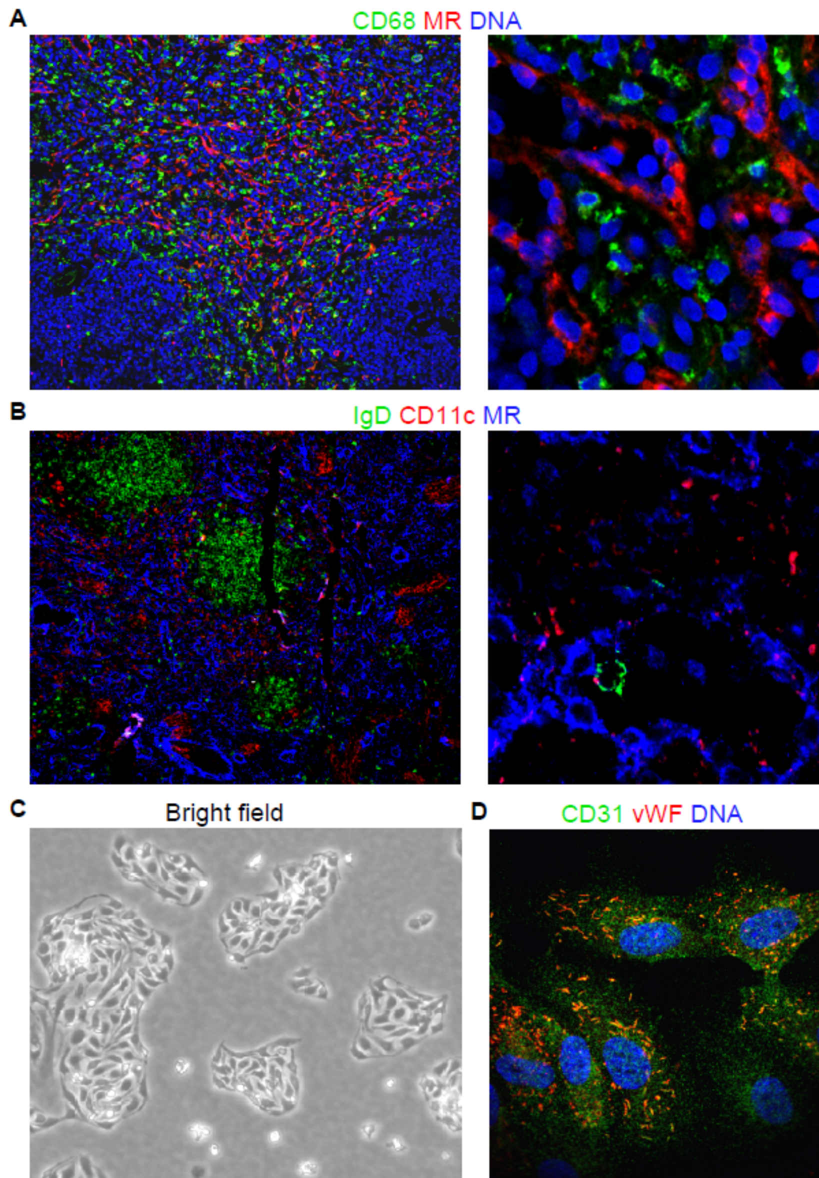


Figure 4.2 SLCs are associated with but distinct from macrophages. (A,B) Confocal microscopy of human spleen tissue sections stained for CD68 or IgD (green), MR or CD11c (red), and MR of DNA (blue). **(C)** Bright field images of in vitro propagated SLCs. **(D)** Confocal microscopy of *in vitro* propagated SLCs stained for CD31 (green), vWF (red) and DNA (blue). Original magnification: 200x, (A; B, left panels; C), 630x (A; B, right panels; D). Data are from one of three experiments with similar results.

Multiparametric flow cytometric and IHC analysis showed that, similar to endothelial cells, SLCs lacked the leukocyte-associated phosphatase CD45, but expressed the adhesion molecules CD54 (or ICAM-1), CD102 (or ICAM-2), the subunit of the TGF-β receptor CD105 (or endoglin), the thrombin-interacting protein CD141 (or thrombomodulin or BDCA-3), and the vascular endothelial growth factor receptor (or VEGFR)-1 (**Figure 4.3 A, B, C**).

Despite having several morphological and phenotypical similarities with endothelial cells, SLCs also showed striking differences. Unlike endothelial cells, SLCs lacked the adhesion molecules CD34 and CD144 (or VE-cadherin) and instead expressed multiple molecules usually associated with myeloid cells, including the LPS co-receptor CD14, the integrin CD18, the sialic acid-binding Ig-type lectin CD33 (or SIGLEC-3), the IgA receptor CD89 (or FcαRI), the CLR family members CD205 (or DEC-205) and CD206 (or MR), and the receptor for granulocyte colony-stimulating factor (G-CSF R) (**Figure 4.3 B)**.

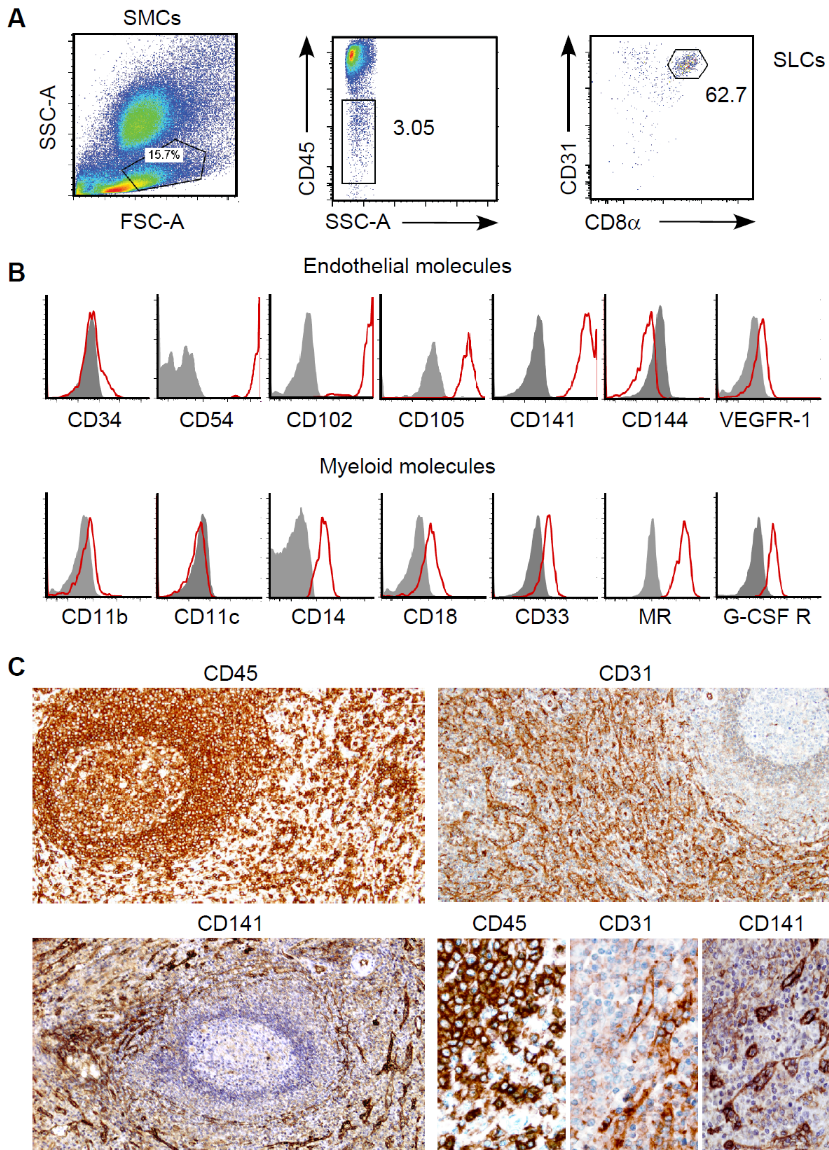


Figure 4.3 SLCs express a hybrid endothelial-myeloid phenotype. (A) Flow cytometric analysis of CD45, CD31 and CD8α on splenic mononuclear cells (SMCs). Gating strategy to identify SLCs is shown. **(B)** Flow cytometric analysis of CD11b, CD11c, CD14, CD18, CD33, CD34, CD54, CD102, CD105, CD141, CD144, VEGFR-1, MR and G-CSF R on SLCs. Solid profiles, isotype control. **(C)** IHC of hematoxilin-stained adult splenic tissue sections stained for CD45, CD31 and CD141 (brown). Original

magnification 100x (main images) and 400x (insets, bottom right). Data are representative of two-five experiments with similar results.

However, unlike typical macrophages and DCs, SLCs did not express the integrins CD11b and CD11c (**Figure 4.3 B**). Collectively, these findings demonstrate that SLCs express canonical endothelial molecules along with many molecules usually associated with myeloid cells of the innate immune system.

3. SLCs express stromal features

Splenic sinusoids are absent during the initial stages of fetal development[78]. In later stages, the spleen shows primordial sinusoids expressing CD34, an endothelial molecule that is typically undetectable in SLCs from adult spleens. We further characterized the phenotypic relationship between fetal and adult SLCs through IHC. Unlike adult spleens, fetal spleens lacked typical sinusoidal vessels lined by $CD8\alpha^+MR^+$ SLCs (**Figure 4.4**). Instead, fetal spleens showed abundant vessels demarcated by vWF^+CD34^+ endothelial-like cells.

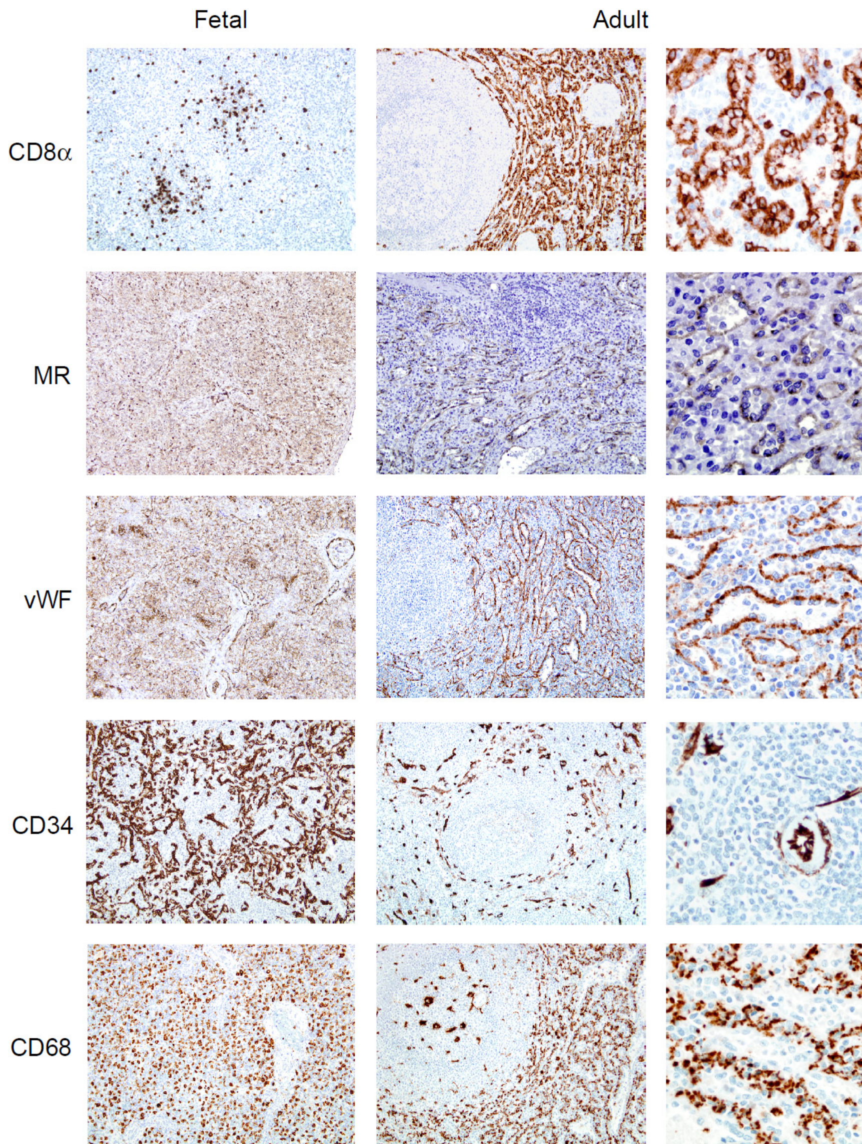


Figure 4.4 SLCs require postnatal signals to form a mature sinusoidal meshwork. IHC of hematoxilin-stained fetal (left panels) and adult (mid and right panels) splenic tissue sections stained for CD8α, MR, vWF, CD34 and CD68 (brown). Original magnification 100x (left and middle panels) and 400x (right panels). Data are from one of at least two different experiments.

As expected, both fetal and adult spleens contained nonspecific $CD68^+$ macrophages as well as $CD20^+$ B cells and $CD3^+$ T cells (**Figure 4.5 A**). While T cells appeared to form PALS-like areas, B cells did not generate organized follicles and were instead organized in small lymphoid aggregates. To further characterize fetal $CD34^+vWF^+$ endothelial like-cells, we evaluated their expression of the cytoskeletal proteins vimentin and smooth muscle α-actinin (SMA), which are highly expressed by endothelial-stromal progenitor cells[191]. Similar to these cells, SLCs from adult spleens expressed the stromal molecules vimentin and SMA (**Figure 4.5 B**). In agreement with our recently published findings, vimentin and SMA were also detected in marginal reticular cells (MRCs), a stromal cell subset from the splenic MZ that also expresses CD141 but not CD31[67] (**Figure 4.5 B and Figure 4.3 C**). In fetal spleens, vimentin expression was broad but did not characterize specific structures, whereas SMA expression was mostly confined to blood vessels (**Figure 4.5 B**). Collectively, these data demonstrate that SLCs have immature features during fetal life and may form a mature sinusoidal meshwork after birth. They also indicate that mature $CD34^-$ SLCs express stromal molecules along with endothelial and myeloid molecules and might derive from early $CD34^+$ fetal precursors.

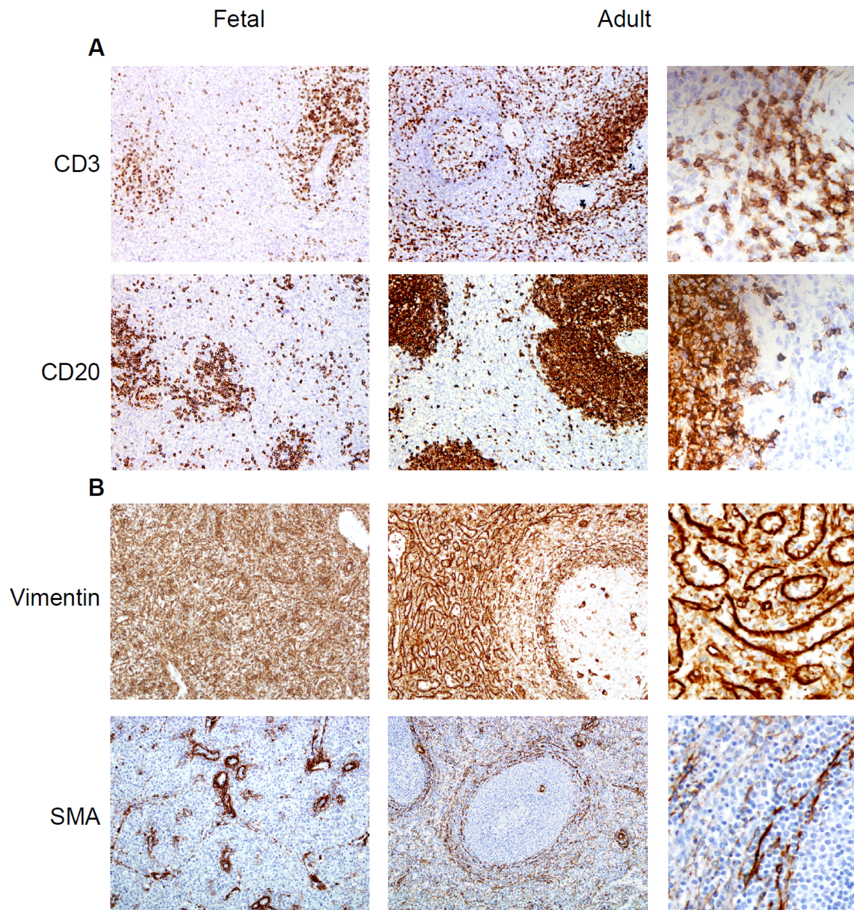


Figure 4.5 SLCs have stromal features similar to those of fetal perivascular cells and adult MRCs. (A-B) IHC of hematoxilin-treated fetal (left) and adult (middle and right) splenic tissue sections stained for (A) CD3, CD20, (B) vimentin or SMA. Original magnification 100x (left and middle panels) and 400x (right panels). Data are from one of at least two different experiments.

4. SLCs with endothelial-myeloid features are human-specific

Having shown that SLCs have unique endothelial-myeloid-stromal features, we wondered whether their presence relates to the unique nature of the splenic circulatory system in humans. Indeed, the splenic circulation is open in both human and non-human primates, but not in rodents, which have a closed or mixed circulation[192]. IFA evidenced MR^+ sinusoidal vessels that surrounded follicles containing IgD^+ B cells in spleens from both humans and rhesus macaques, but not in spleens from mice or rats (**Figure 4.6 A,B**). Instead of MR^+vWF^+ SLCs (please, see earlier vWF data), mice and rats showed conventional vWF^+MR^- endothelial cells in the red pulp. This splenic compartment also contained scattered isolated vWF^-MR^+ cells, possibly macrophages (**Figure 4.6 A,B**). Overall, these data indicate that mice and rats lack SLCs with endothelial-myeloid features.

5. SLCs inhabit the spleen but not other lymphoid organs

Endothelial cells express distinct morphological, phenotypic and functional features in blood vessels from different lymphoid organs. For example, high endothelial venules are post-capillary venous vessels specialized in lymphocyte recruitment to systemic and mucosal lymphoid organs and contain cuboidal endothelial cells that express a typical $MAdCAM\text{-}1^{lo}PNA^{hi}LT\text{-}\beta R^+$ phenotype, at least in mice[193–195]. Thus, we wondered whether SLCs were unique to the spleen or also inhabited other lymphoid organs. IFA identified perifollicular $CD31^+MR^+$ SLCs in spleens but not tonsils and intestine, which instead contained conventional $CD31^+MR^-$ endothelial cells.

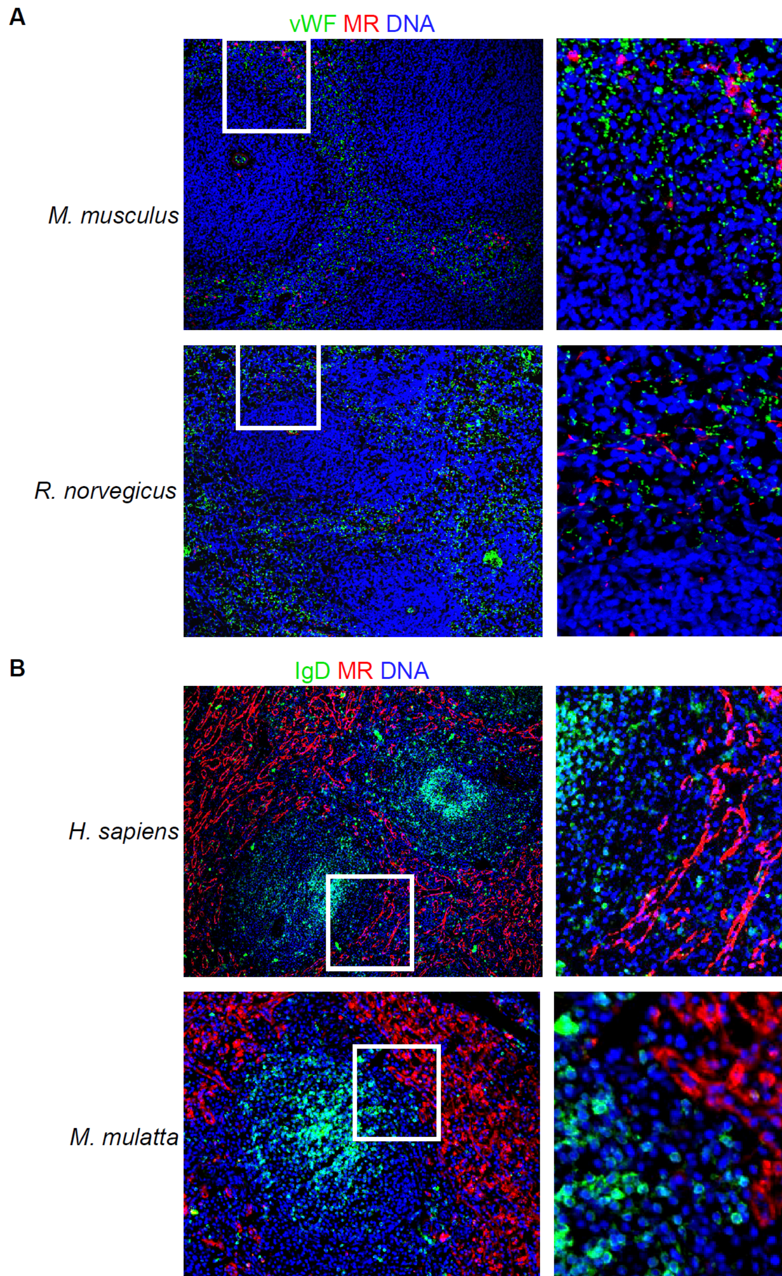


Figure 4.6 SLCs inhabit the spleen of humans and rhesus macaques, but not rodents. (A,B) Confocal microscopy of splenic tissue sections from mice (*Mus musculus*), rats (*Rattus norvegicus*), humans (*Homo sapiens*) and rhesus macaques (*Macaca mulatta*) stained for **(A)** vWF or **(B)** IgD (green), MR

(red), and DNA (blue). Original magnification, 200x (left panels) or 400x (insets). Data are from one of three experiments with similar results.

Accordingly, flow cytometry demonstrated the presence of $CD45^{-}$ $CD31^{+}$ SLCs expressing the myeloid molecules CD8α, CD14 and MR in the spleen but not tonsil or intestine (**Figure 4.7 A, B, C**). Whereas splenic $CD45^{-}CD31^{+}$ SLCs expressed CD54 and CD102 but lacked the endothelial lineage molecule CD34, tonsillar and intestinal $CD45^{-}$ $CD31^{+}$ endothelial cells expressed CD54 and CD102 along with CD34 (**Figure 4.7 A, B, C**). We then analyzed endothelial cells from the liver, an organ that contains abundant sinusoidal vessels engaged in the filtration of the venous blood collected by the portal system. In agreement with these properties, the liver showed SLC-like $CD31^{+}CD45^{-}$ endothelial cells that expressed CD14 and CD54 (**Figure 4.8**). However, compared to splenic SLCs, liver endothelial cells lacked CD8α and expressed lower amounts of CD31 and CD54. Overall, these data indicate that SLCs expressing a hybrid endothelial-myeloid phenotype are likely confined to the spleen.

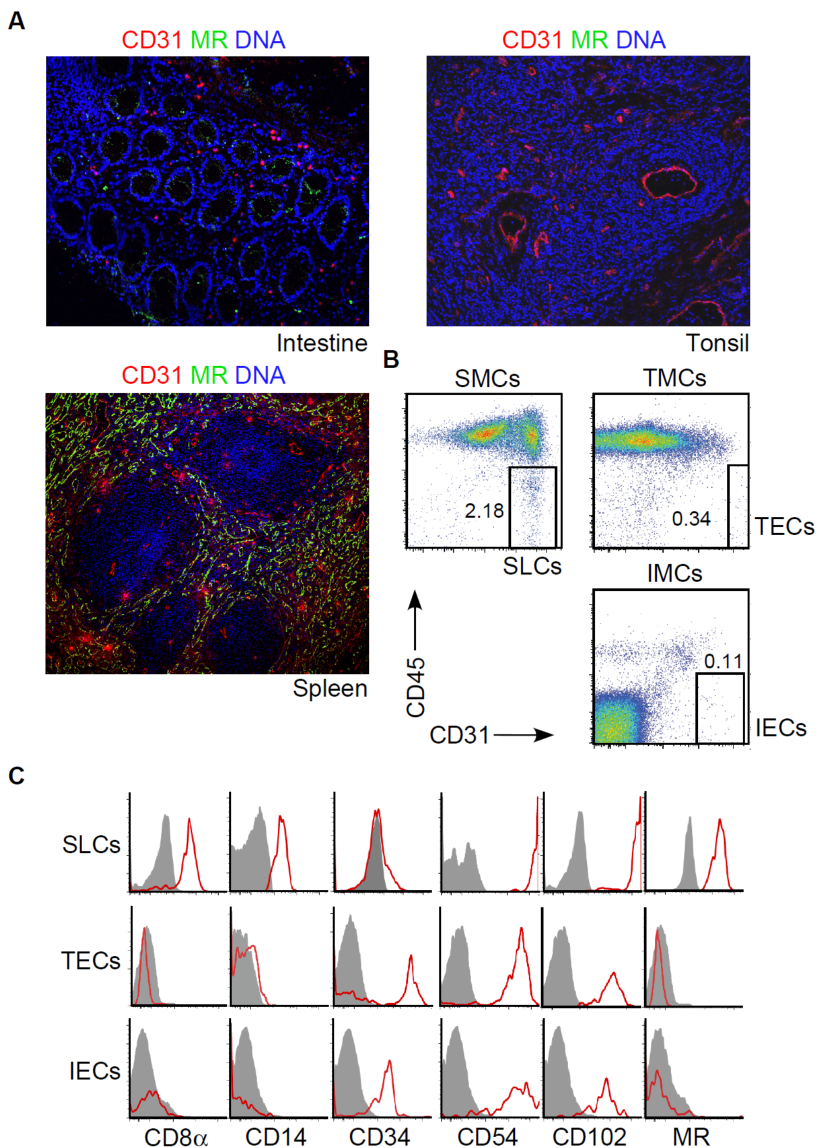


Figure 4.7 SLCs specifically inhabit the spleen. (A) IFA of splenic, tonsillar and intestinal tissue sections stained for MR (green), CD31 (red), or DNA (blue). Original magnification, 100x. **(B)** Flow cytometric analysis of CD45 and CD31 on splenic mononuclear cells (SMCs), tonsil mononuclear cells (TMCs) and intestinal mononuclear cells (IMCs). Gating strategy to identify SLCs, tonsil endothelial cells (TECs), and intestinal endothelial cells (IECs) is shown. **(C)** Flow cytometric analysis of CD8α, CD14, CD34,

CD54, CD102 and MR on SLCs, TECs, and IECs. Solid profiles, isotype control. Data are from at least three experiments with similar results.

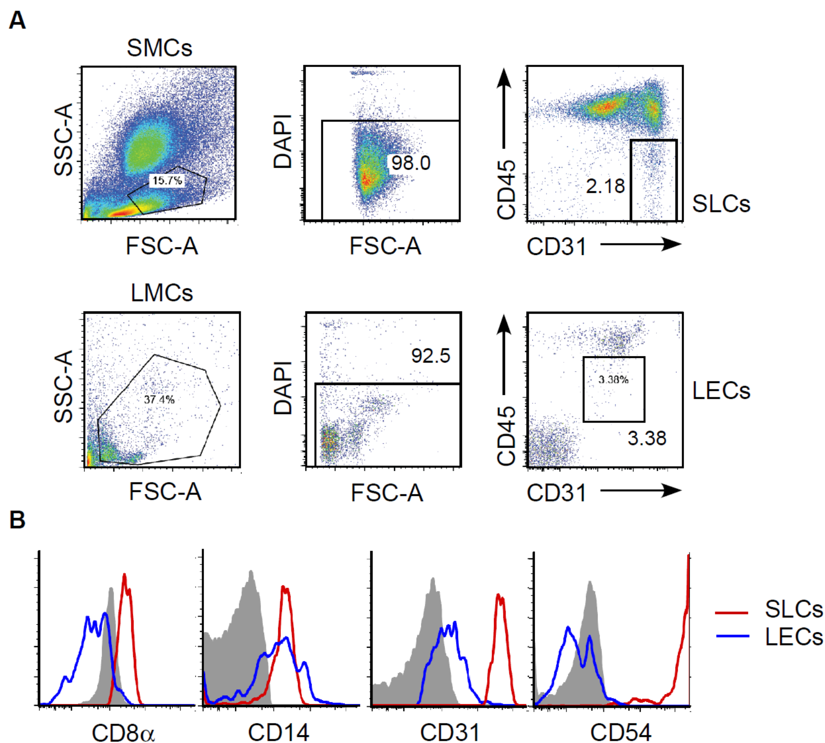


Figure 4.8 SLCs are phenotypically distinct from liver endothelial cells. **(A)** $CD45^{-}CD31^{+}$ splenic SLCs and $CD45^{-}CD31^{+}$ liver endothelial cells (LECs) electronically gated from splenic mononuclear cells (SMCs) and liver mononuclear cells (LMCs), respectively. **(B)** Flow cytometric analysis of CD8α, CD14, CD31 and CD54 on electronically gated $CD45^{-}CD31^{+}$ SLCs (red) and $CD45^{-}CD31^{+}$ LECs (blue). Solid profiles, isotype control. Data are from one experiment.

6. SLCs have a dominant myeloid gene expression signature

To further define the hybrid myeloid-endothelial phenotype of SLCs, we compared the gene expression profile of FACSorted splenic $CD45^{-}CD14^{+}$ SLCs with that of FACSorted splenic $CD45^{+}CD14^{+}$ myeloid cells, which mostly include macrophages and monocytes. Additional comparisons were established with $CD45^{-}CD14^{-}$ endothelial cells from the umbilical vein (**Figure 4.9 A**). The rationale for including umbilical vein endothelial cells in this analysis was two-fold. Firstly, we encountered technical difficulties in the isolation of sufficient amounts of endothelial cells from lymphoid organs. Secondly, umbilical vein endothelial cells have paradigmatic and well-characterized endothelial phenotype and function. Microarray analysis was followed by the generation of a heat map that included genes at least two-fold differentially expressed in SLCs compared to myeloid or endothelial cells. Venn diagrams were generated to further visualize and quantify differences.

We found that SLCs had a gene expression signature similar to that of myeloid cells, but different from that of endothelial cells (**Figure 4.9 B**). Accordingly, 1768 genes were enriched in SLCs compared to endothelial cells, whereas only 85 genes were enriched in SLCs compared to myeloid cells (**Figure 4.10 A**). Conversely, 2458 genes were less expressed in SLCs compared to endothelial cells, whereas only 275 genes were less expressed in SLCs compared to myeloid cells. Finally, we identified 108 and 64 genes in

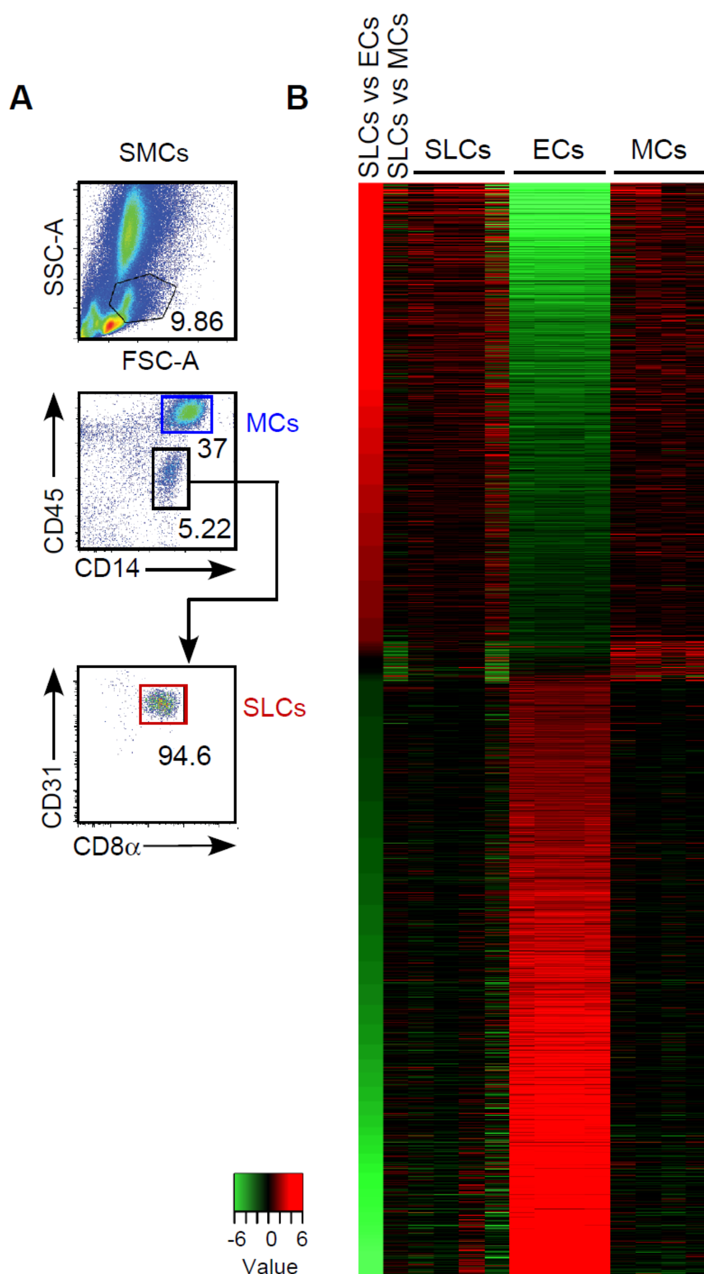


Figure 4.9 SLCs express a transcriptome similar to that of macrophages, but distinct from that of endothelial cells. (A) Flow cytometric analysis depicting sorting strategy to isolate splenic SLCs and myeloid cells (MCs) on the basis of physical parameters (FSC and SSC) and CD45, CD14, CD8α and CD31 expression. **(B)** Supervised analysis of differentially expressed genes (≥ 2 fold change) in splenic SLCs, splenic MCs

and umbilical vein endothelial cells (ECs). The first two columns represent the log_2 ratio of SLC vs EC and SLC vs MC group samples. From left to right, the x-axis of the heat map depicts SLC samples from 4 spleens, umbilical vein EC samples from 4 cultures, and MC samples from 4 spleens. The y-axis depicts genes with higher fold increase (top half of the heat map) and genes with higher fold decrease (bottom half of the heat map) in a comparative analysis of SLC vs EC samples (P value < 0,01). In color bar, red, black and green represent fold changes >2, = 0 and <2, respectively.

SLCs that were more and less expressed, respectively, compared to both endothelial and myeloid cells. Combined, these 172 genes were considered as part of an SLC-specific gene signature. To further elucidate the degree of relatedness of SLCs to myeloid and endothelial cells, we performed a Pearson's correlation coefficient analysis. In two identical cell populations, the gene expression correlation ratio (R) corresponds to 1. We found R values of 0.95 and 0.27 when the gene expression profile of SLCs was compared to that of myeloid and endothelial cells, respectively (**Figure 4.10 B**). These data indicate that SLCs express a transcriptome that closely relates to that of myeloid cells and profoundly differs from that of endothelial cells.

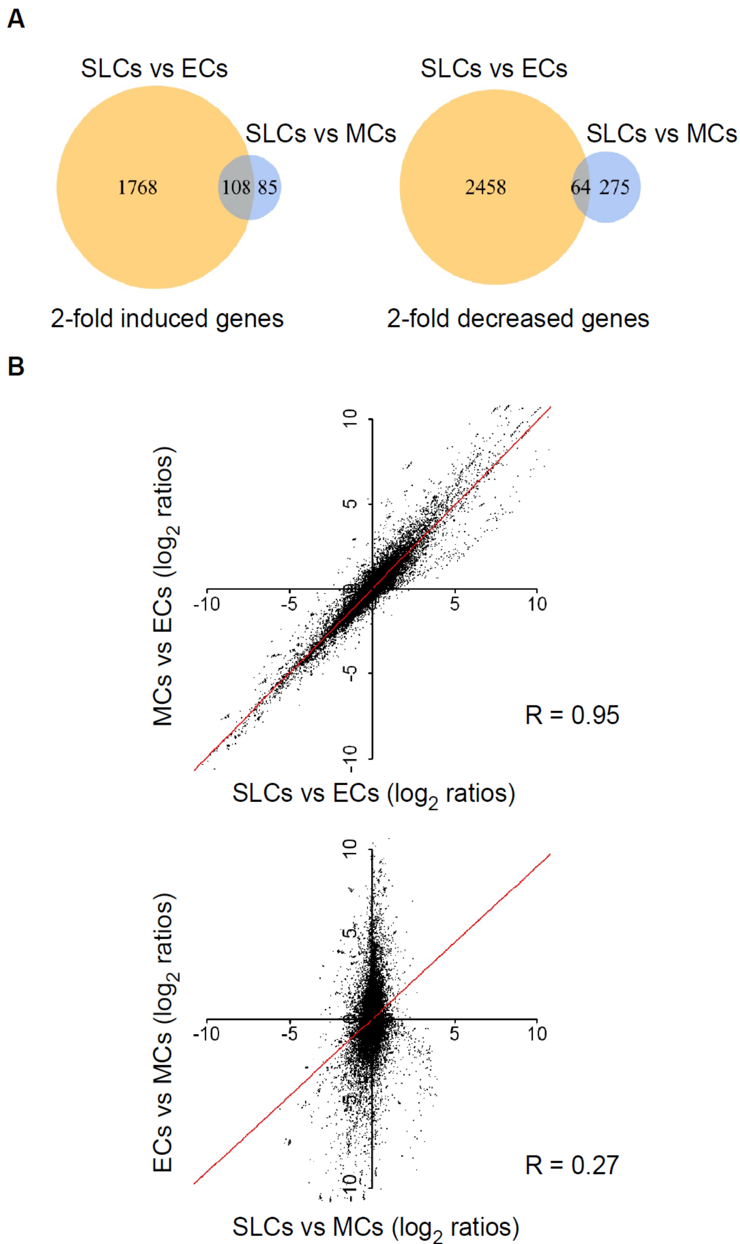


Figure 4.10 SLCs express a prominent myeloid gene expression signature. (A) Venn diagrams showing genes differentially expressed in SLCs, ECs and MCs. *P* value < 0,01. **(B)** Pearson's correlations of genes expressed by SLCs, ECs and MCs. R values indicate gene expression correlation ratios.

7. SLCs are highly enriched in genes involved in innate immune responses

To start elucidating the function of SLCs, we performed gene ontology analysis of genes showing at least two-fold increased expression in SCLs compared to myeloid or endothelial cells. We found that SLCs were enriched in the expression of genes involved in innate immune responses, cytokine production, hemopoiesis, response to wounding, inflammation, myeloid cell differentiation, endocytosis, phagocytosis and cell chemotaxis (**Figure 4.11 A**). Similar to macrophages, SLCs highly expressed genes that encoded proteins involved in microbial recognition (e.g., CLEC4A, TLR1, TLR2, CD14, TREX1, NAIP, AIM2, PYCARD), microbial receptor signaling (e.g., MyD88, IRAK3, IRAK4), signaling from IFN (e.g., STAT2, IRF1, IRF3, IRF7), antimicrobial responses (e.g., Mx2, OAS1, OAS2, SP100), phagocytosis (FcγRIIA, FcεRI), and antigen presentation (e.g., HLA molecules, CD74, TAP2) **(Figure 4.11 B).** Collectively, these data indicate that SLCs express a gene expression profile that may reflect functional properties similar to those of myeloid effector cells of the innate immune system.

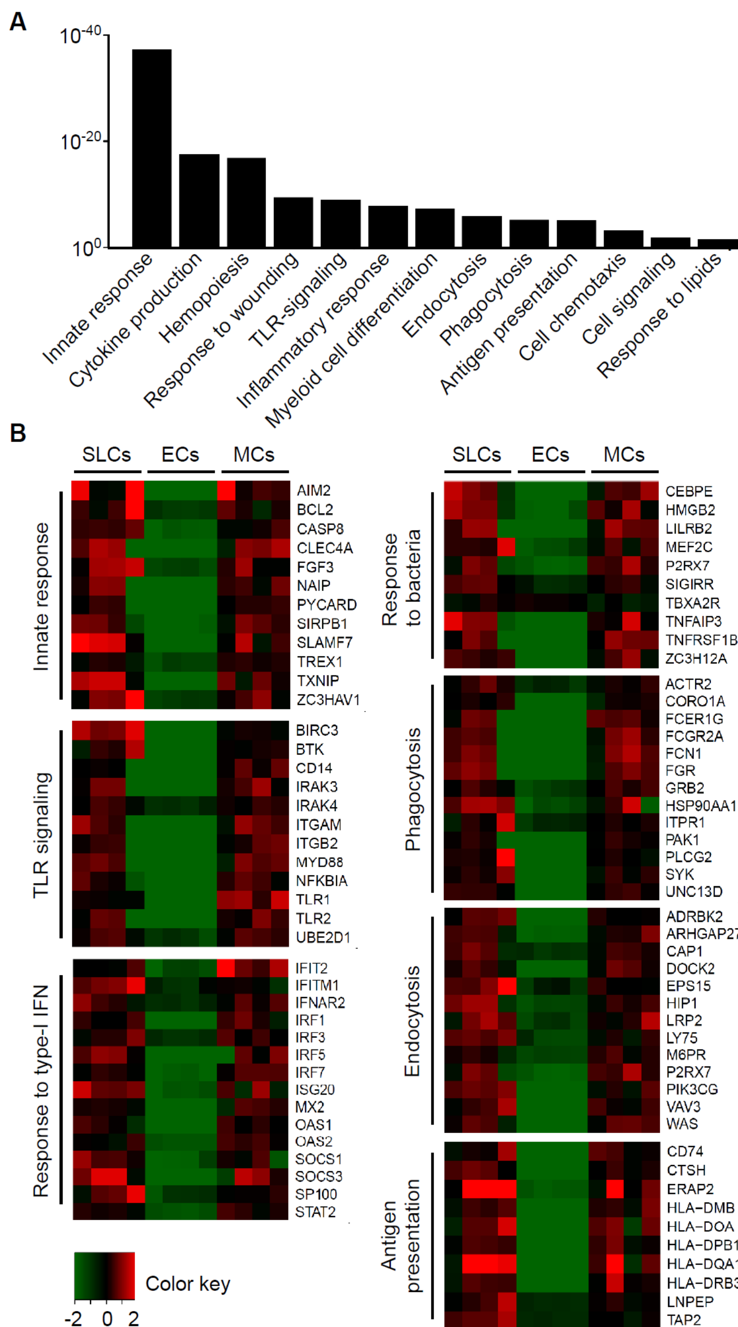


Figure 4.11 SLCs are enriched in myeloid gene products involved in microbial recognition and innate immune responses. (A) Bar plot representation of functional categories emerging from gene ontology analysis of genes showing at least two-fold enrichment in splenic SLCs compared to splenic MCs and umbilical vein ECs (*P* value <0.01). **(B)** Functional

categories of innate immune response genes showing at least two-fold enrichment in splenic SLCs compared to splenic MCs and umbilical vein ECs (*P* value <0.01). Functional categories and genes are listed on the left and right sides of the heat maps, respectively.

8. SLCs express multiple microbial sensors and mediate phagocytosis

Given that SLCs were highly enriched in gene products involved in microbial sensing, signaling and antigen presentation, we compared the expression of PRRs by SLCs, macrophages and endothelial cells by QRT-PCR and flow cytometry. We found that splenic SLCs expressed more TLR4, TLR8 and TLR9 than umbilical vein endothelial cells (**Figure 4.12A**). Compared to endothelial cells, SCLs also expressed more abundant antigen-presenting molecules such as HLA-DR, LPS receptors such as CD14 and TLR4, scavenger receptors such as CD36 and CD163, Fc receptors such as CD16 (FcγRIII) and CD89 (FcαRI), and carbohydrate receptors of the C-type lectin receptor family such as DEC-205 (CD205), MR and langerin (CD207) (**Figure 4.12 B**). The expression of these PRRs was largely overlapping in splenic SLCs and macrophages. Compared to endothelial cells, SLCs and macrophages expressed more SIRP-α (or CD172a), a signal regulatory protein previously reported to be unique to SLCs[72]. These results demonstrate that SLCs are equipped with a vast machinery of microbial sensors similar to those expressed by macrophages.

Given that SLCs were enriched in genes encoding scavanger receptors, Fc receptors and proteins involved in cytoskeleton remodeling, we determined whether SLCs undergo phagocytosis as macrophages do. Flow cytometry showed that at least 30% of SLCs specifically internalized fluorochrome-labeled beads through a temperature-sensitive and actin-dependent process that was inhibited by cytocalasin D, an inhibitor of actin polimerization (**Figure 4.13 A**). Confocal microscopy showed that SLCs also phagocytosed fluorochrome-labelled bacteria, including the pathogen *Staphylococcus aureus* (**Figure 4.13 B**). Co-staining of the surface adhesion molecule CD31 permitted to ascertain that while some bacteria were simply attached to the plasma membrane, many others were engulfed in the cytoplasm of SLCs. This engulfment occurred through a specific process that was inhibited by low temperature.

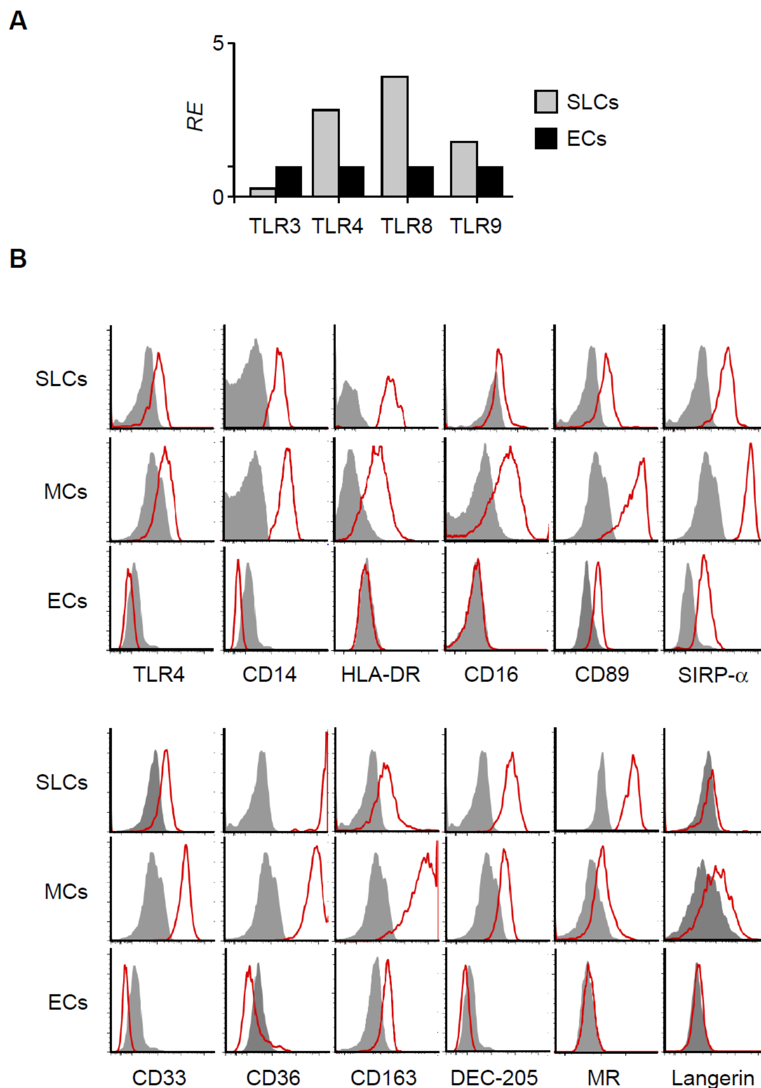


Figure 4.12 SLCs express multiple microbial sensors of the innate immune system. (A) QRT-PCRs of transcripts for TLR3, TLR4, TLR8 and TLR9 in splenic SLCs and umbilical vein ECs. RE, relative expression compared to ECs. One of three experiments yielding similar results. **(B)** Flow cytometric analysis of TLR4, CD14, HLA-DR, CD16, CD89, SIRP-α, CD33, CD36, CD163, DEC-205, MR and Langerin on splenic SLCs, splenic MCs and umbilical vein ECs. Solid profiles, isotype control. Data are representative of at least five experiments with similar results.

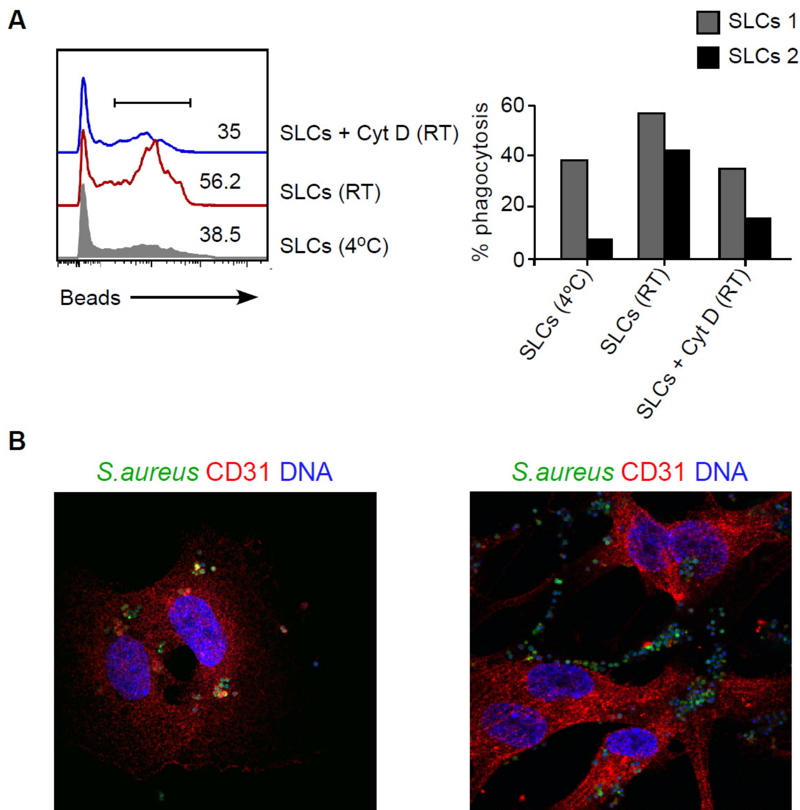


Figure 4.13 SLCs display phagocytic activity. (A) Flow cytometric analysis of phagocytosed fluorochrome-labelled beads by SLCs with or without cytocalasin D (Cyt D) at room temperature (RT). Staining at 4ºC corresponds to nonspecific binding of labeled beads to the surface of SLCs. **(B)** Confocal microscopy of SLCs cultured with heat-inactivated and fluorochrome-labelled bacteria (green) and stained for CD31 (red) and DNA (blue). Original magnification, 630x. Data are representative of two different experiments.

9. SLCs express BAFF and APRIL

Having shown that SLCs have macrophage-like features and knowing that macrophages produce powerful innate B cell-stimulating factors, we wondered whether these factors were also expressed by SLCs. This expression could enable SLC to deliver helper signaling to MZ B cells during TI antibody responses. Among innate B cell-stimulating factors, BAFF and APRIL play a dominant role in the activation of MZ B cells and initiation of TI antibody production[63,156].

Confocal microscopy showed typical $CD8\alpha^{+}MR^{+}CD31^{+}$ SLCs in the PFZ, which was adjacent to the MZ containing IgD^{lo} MZ B cells. (**Figure 4.14 A, B**). In addition, $CD8\alpha^{+}MR^{+}CD31^{+}$ SLCs from the red pulp were proximal to some IgD^{hi} B cells resembling those positioned in splenic follicles (**Figure 4.14 A, B**). These IgD^{hi} follicular B cells may be en route to reach the general circulation through splenic sinusoids.

IHC evidenced intense and widespread BAFF and APRIL expression in splenic sinusoids from the PFZ and red pulp (**Figure 4.15 A**). BAFF was also expressed in the germinal center of some secondary follicles, whereas APRIL was abundant in the MZ. In agreement with these data, confocal microscopy demonstrated that purified $CD31^{+}vWF^{+}$ SLCs contained BAFF and APRIL (**Figure 4.15 B**). While APRIL showed a predominant perinuclear localization, which possibly included Weibel-Palade bodies, BAFF showed a more diffuse expression pattern.

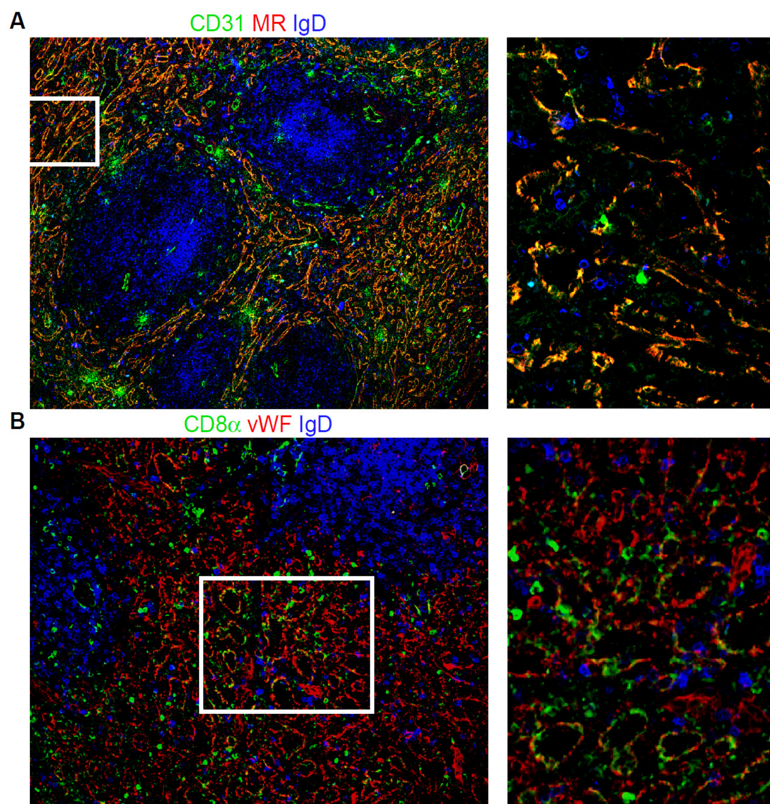


Figure 4.14 SLCs are proximal to MZ B cells. (A-B) Confocal microscopy of human spleen tissue sections stained for CD31 or CD8α (green), MR or vWF (red), and IgD (blue). Original magnification, 100x (upper left panel), 200x (lower left panel), or 400x (right panels). Data are representative of at least three different experiments.

Previously published studies indicate that myeloid cells release soluble BAFF and APRIL after cleaving precursor molecules localized on the plasma membrane and in the Golgi compartment, respectively[74,196,197]. Flow cytometry showed membrane-bound BAFF but not APRIL in splenic SLCs and conventional endothelial cells, but not splenic macrophages (**Figure 4.16 A**).

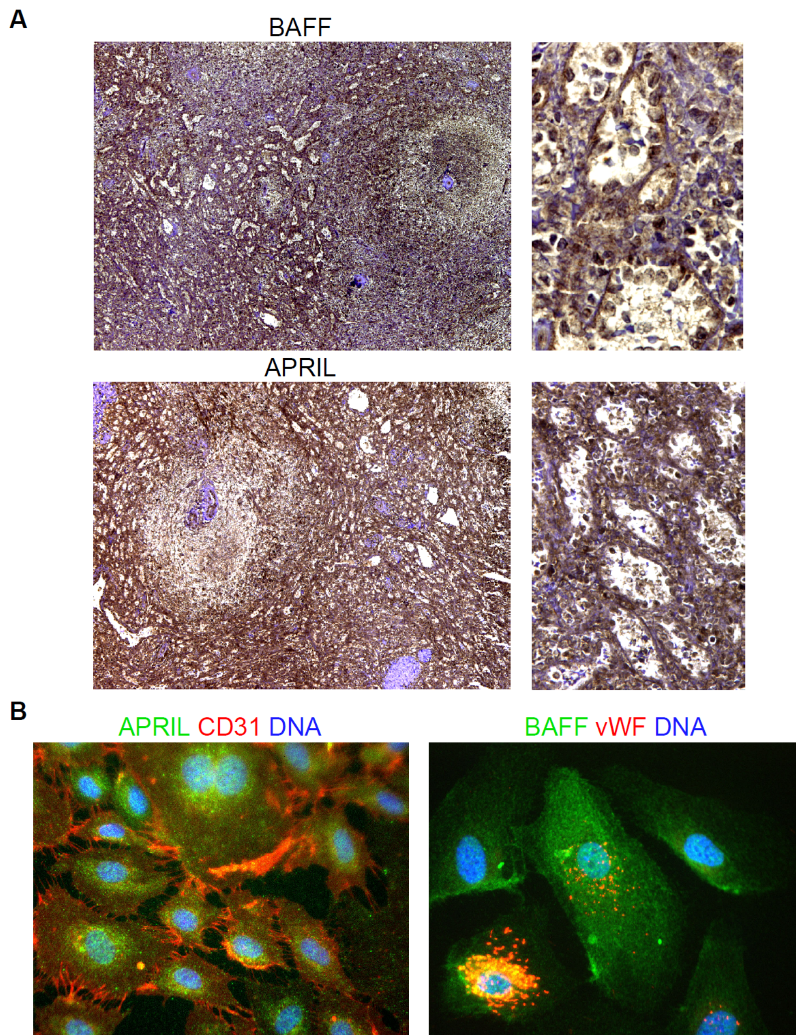


Figure 4.15 SLCs express BAFF and APRIL. (A) IHC of hematoxilin-treated adult splenic tissue sections stained for BAFF and APRIL (brown). Original magnification 100x (left panels) and 630x (right panels). **(B)** Confocal microscopy of SLCs stained for BAFF or APRIL (green), CD31 or vWF (red) and DNA (blue). Data are representative of at least three different experiments.

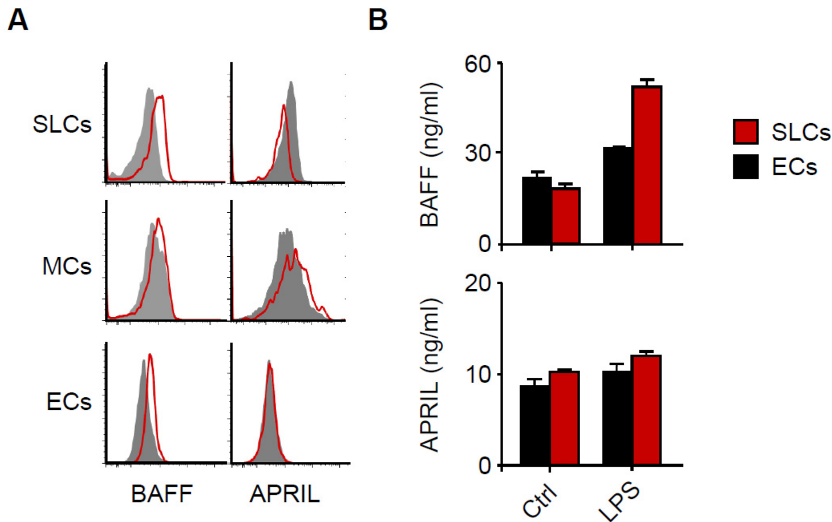


Figure 4.16 SLCs release BAFF and APRIL. (A) Flow cytometric analysis of membrane-bound BAFF and APRIL in splenic SLCs, splenic myeloid cells (MCs) and umbilical vein endothelial cells (ECs). Solid profiles, isotype control. Data are representative of at least three different experiments. **(B)** ELISA of soluble BAFF and APRIL from SLCs and umbilical vein ECs left untreated (Ctrl) or incubated with LPS for 48 h. Three different experiments are summarized in the graph.

A distinguishing trait of endothelial cells relates to the presence of perinuclear Weibel-Palade bodies containing pre-stored cytokines that can be rapidly released upon stimulation by various signals, including cytokines, TNF family members and TLR ligands[198]. Given the presence of some LPS in perifollicular areas of the spleen and having shown that SLCs express the LPS receptors TLR4 and CD14, we wondered whether LPS could stimulate BAFF and APRIL release by SLCs. ELISA assays showed that SLCs constitutively secreted BAFF and APRIL and further increased BAFF but not APRIL secretion in response to LPS (**Figure 4.16 B**). Collectively, these results indicate that SLCs are proximal to MZ B cells and some follicular B cells and

release powerful B cell-stimulating factors, including BAFF and APRIL.

10. SLCs enhance the survival of MZ B cells

BAFF and APRIL stimulate B cells and plasma cells by engaging BAFF-R, TACI and BCMA, three receptors characterized by distinct binding, expression and functional patterns[199]. BAFF-R is expressed by mature B cells but not plasma cells and delivers survival signals in response to BAFF[200]. TACI is expressed by mature B cells, including B-1 and MZ B cells, and delivers CSR and antibody-inducing signals in response to BAFF or APRIL[66,74,156,175,176,201,202]. Finally, BCMA is predominantly expressed by plasma cells and mostly delivers survival signals[66,74,176,177,181,203].

Considering the possible responsiveness of MZ B cells to BAFF and APRIL from SLCs, we compared the expression of BAFF-R, BCMA and TACI on multiple B cell subsets from human spleens, including $IgD^{lo}CD27^{+}$ MZ B cells, $IgD^{hi}CD27^{-}$ follicular B cells, $IgD^{-}CD27^{+}$ memory B cells, and $IgD^{-}CD27^{hi}CD38^{hi}$ plasmablasts/plasma cells (**Figure 4.17**). Flow cytometry showed that BAFF-R was highly expressed by all splenic B cell subsets, except plasmablasts/plasma cells. In contrast, TACI was predominantly detected on MZ B cells and memory B cells, whereas BCMA was only expressed by plasmablasts/plasma cells. These findings indicate that MZ B cells as well as other splenic B cell subsets have the potential to respond to BAFF and APRIL from SLCs.

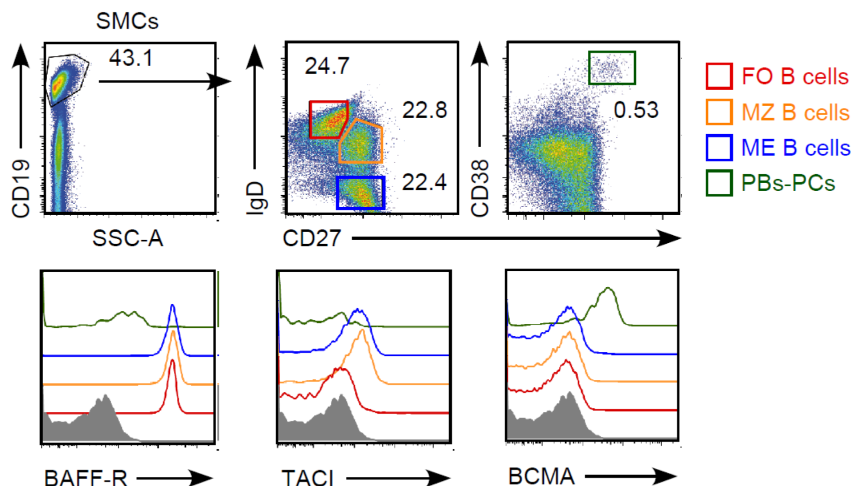


Figure 4.17 MZ B cells express TACI and BAFF-R but not BCMA. Flow cytometric analysis of BAFF-R, TACI and BCMA on $CD19^{+}IgD^{lo}CD27^{+}$ MZ B cells, $CD19^{+}IgD^{hi}CD27^{-}$ follicular (FO) B cells, $CD19^{+}IgD^{-}CD27^{+}$ memory (ME) B cells and $CD19^{+}IgD^{-}CD27^{hi}CD38^{hi}$ plasmablasts/plasma cells (PB/PC) electronically gated from splenic mononuclear cells (SMCs). Data from one of three independent experiments yielding similar results.

Next, we determined whether SLCs enhanced the survival of MZ B cells. Flow cytometry showed that the proportion of viable Annexin-$V^{-}DAPI^{-}$ MZ B cells strongly increased after exposure to SLCs for 5 days (**Figure 4.18 A**). This effect was not associated with a massive proliferation of MZ B cells, because thymidine incorporation assays demonstrated only a marginal increase of DNA synthesis in MZ B cells exposed to resting or LPS-activated SLCs (**Figure 4.18 B**).

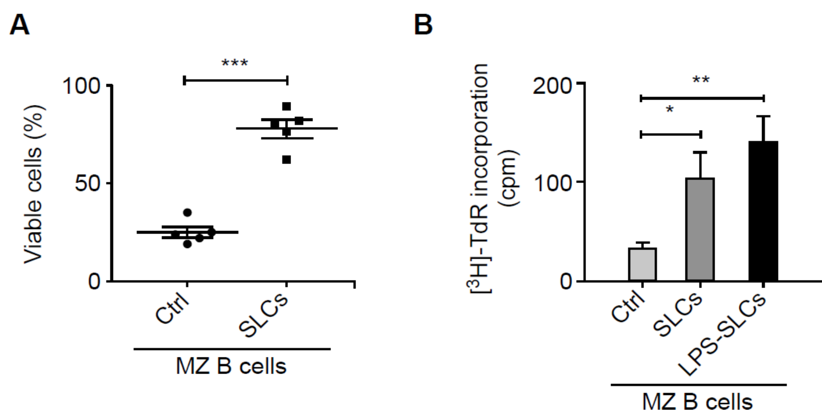


Figure 4.18 SLCs provide survival signals to MZ B cells. (A) Flow cytometric analysis of viable Annexin-V⁻DAPI⁻ MZ B cells from a 5-d co-culture of MZ B cells with or without SLCs. Data summarize 5 experiments. *** $P<0.0005$ (one-tailed unpaired Student's *t*-test). **(B)** ^{3}H-thymidine incorporation assays from co-cultures of MZ B cells with or without resting or LPS-stimulated SLCs. Data summarize two experiments. ** $P< 0.005$; * $P < 0.05$ (one-tailed unpaired Student's *t*-test).

These experiments indicate that SLCs stimulate the survival of MZ B cells, possibly through a mechanism involving BAFF and APRIL. Blocking experiments with TACI-Ig and BAFF-R-Ig decoy receptors were technically challenging due to paradoxical stimulating effects resulting from the engagement of FcγRs on SLCs by the Fc portion of decoy receptors.

11. SLCs induce CSR in MZ B cells

In addition to promoting B cell survival, BAFF and APRIL can induce CSR from IgM to IgG and IgA through a CD40-independent (i.e., TI) pathway[66,156]. For this reason, we verified whether SLCs could induce

CSR in MZ B cells. Initial experiments determined whether splenic MZ and PFZ areas contained AID, a short-lived DNA-editing enzyme that indicates ongoing CSR[204]. Tissue IFA demonstrated the presence of scattered IgD^+ B cells expressing AID in both MZ and PFZ areas that also contained neutrophils (**Figure 4.19 A**), an elastase-expressing granulocyte subset that inhabits splenic perifollicular areas and exerts MZ B cell-helper functions[66]. This finding suggests that some MZ B cells actively undergo CSR in response to local splenic signals, which might involve SLCs in addition to other cells of the local innate immune system.

Additional experiments determined whether SLCs could induce CSR in MZ B cells. QRT-PCR showed that SLCs increased the expression of transcripts encoding AID in MZ B cells after a 4-day co-culture (**Figure 4.19 B**). Subsequent RT-PCR assays verified whether SLCs could induce molecular byproducts of CSR in MZ B cells. When co-cultured with SLCs for 4 days, MZ B cells augmented the expression of germline Iγ1-Cγ1, Iγ3-Cγ3 and Iα2-Cα2 transcripts as well as the expression of switch circle Iγ1/2-Cμ, Iγ3-Cμ and Iα1/2-Cμ transcripts (**Figure 4.20**). These data indicate that, in principle, SLCs could induce CSR from IgM to IgG1, IgG3 and IgA2 in MZ B cells.

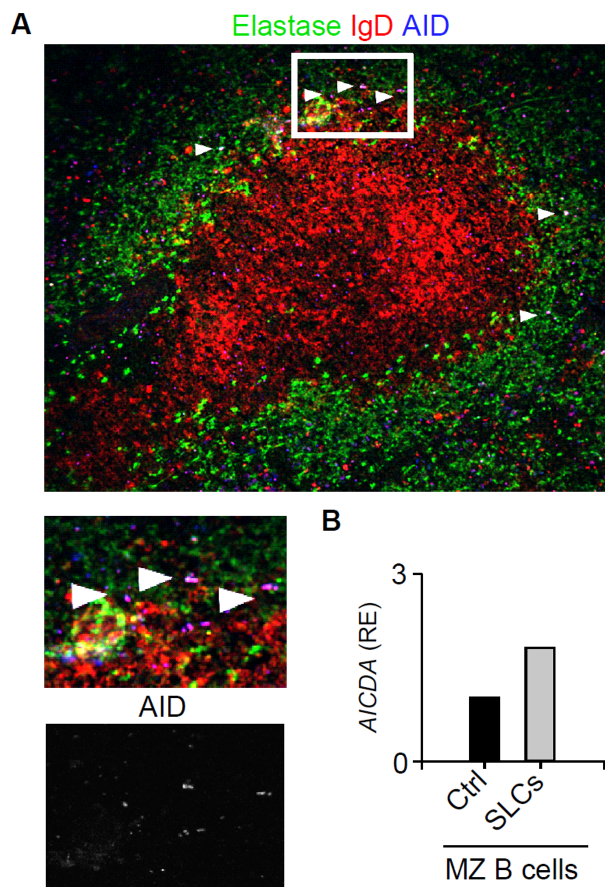


Figure 4.19 SLCs induce AID expression in MZ B cells. (A) IFA of human splenic tissue sections stained for neutrophil elastase (green), IgD (red) and AID (blue). Co-localization is shown in purple. AID single staining is shown in white. Original magnification, 100x. Bottom insets, 4x zoom. (**B)** QRT-PCR of transcripts encoding AID from MZ B cells co-cultured with or without SLCs for 4 d. Data are representative of two independent experiments.

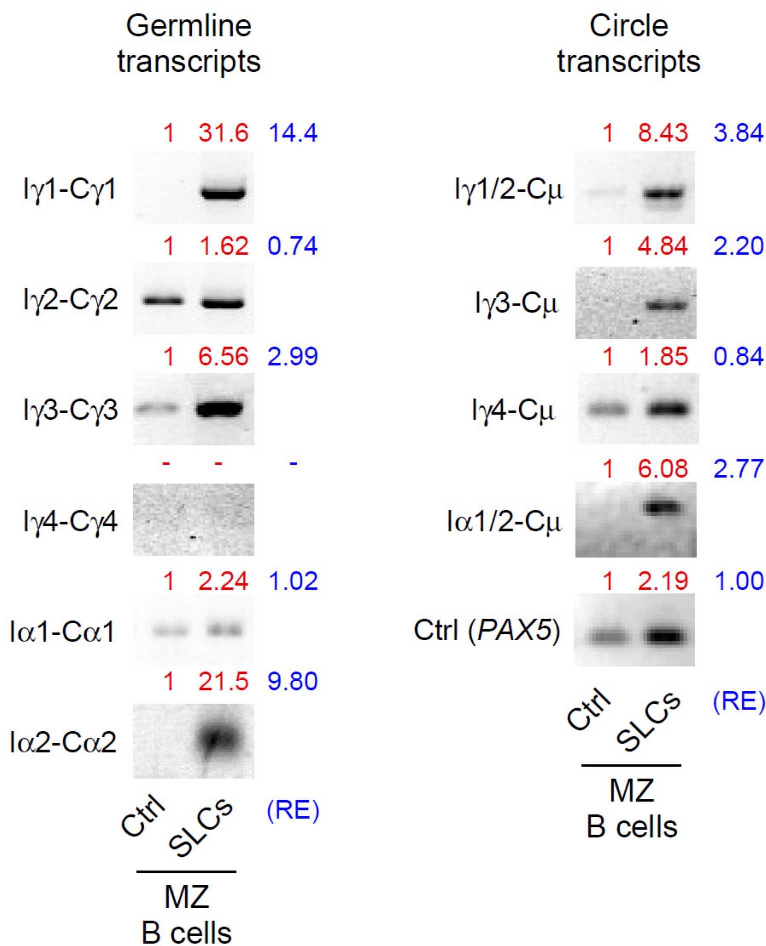


Figure 4.20 SLCs induce CSR from IgM to IgG and IgA in MZ B cells. RT-PCRs of germline Iγ1-Cγ1, Iγ2-Cγ2, Iγ3-Cγ3, Iγ4-Cγ4, Iα1-Cα1 and Iα2-Cα2 transcripts and switch circle Iγ1/2-Cμ, Iγ3-Cμ, Iγ4-Cμ and Iα1/2-Cμ transcripts from MZ B cells cultured for 4 d with or without SLCs. Transcripts encoding the B cell-specific transcription factor PAX5 were used as loading control. Red numbers indicate expression compared to MZ B cells alone in each transcript. Blue numbers indicate relative expression (RE) normalized to control (Ctrl) PAX5. Data represent one of three independent experiments yielding similar results.

12. SLCs induce plasma cell differentiation

MZ B cells engaged in TI antibody responses migrate to splenic extrafollicular areas, where they differentiate to short-lived plasmablasts and, in some cases, long-lived plasma cells[39]. Given that plasma cell differentiation involves signals from BAFF and APRIL, we determined whether SLCs could stimulate the differentiation of MZ B cells into plasmablasts/plasma cells. Flow cytometry showed that SLCs induced $CD27^{hi}CD38^{hi}$ plasmablasts after a 5-day co-culture with MZ B cells (**Figure 4.21 A**). This induction did not increase when SLCs were pre-stimulated with LPS to enhance BAFF and APRIL production. Consistent with their ability to trigger CSR along with plasma cell differentiation, SLCs also stimulated MZ B cells to secrete IgM, IgG and IgA, albeit at different level (**Figure 4.21 B**). ELISA assays demonstrated that conditioned medium from SLCs was sufficient to induce IgA and some IgM, but not IgG production. These findings indicate that SLCs stimulate the generation of antibody-secreting plasma cells through isotype-specific contact-dependent and contact-independent mechanisms.

Plasma cell differentiation further involves cytokines such as IL-6[178,183]. QRT-PCR assays showed that, in the presence of LPS, SLCs up-regulated the expression of transcripts encoding IL-6 (**Figure 4.22 A**). ELISA assays demonstrated that SLCs secreted IL-6 in amounts comparable to those secreted by conventional endothelial cells (**Figure 4.22 B**). However, after stimulation with LPS for two days, SLCs secreted more IL-6 than endothelial cells (**Figure 4.22 B**).

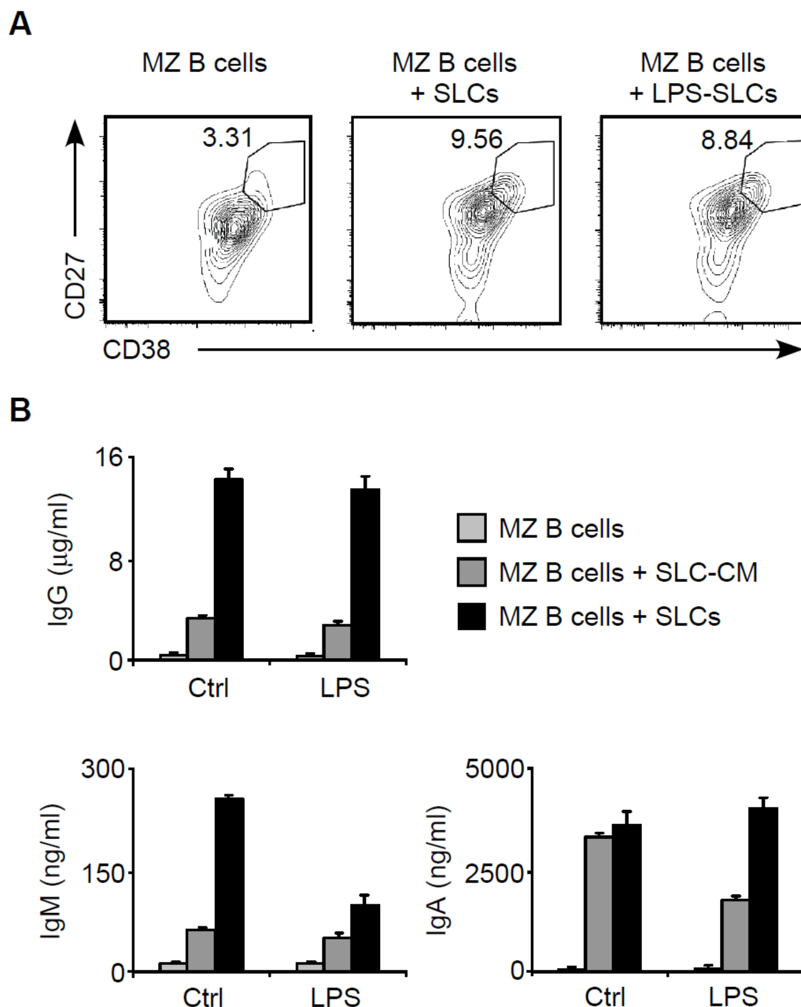


Figure 4.21 SLCs induce plasma cell differentiation and antibody secretion in MZ B cells. (A) Flow cytometry analysis of CD27 and CD38 on MZ B cells co-cultured for 5 days with or without SLCs left untreated or pre-stimulated with LPS. **(B)** ELISA of IgG, IgM and IgA secreted by MZ B cells co-cultured for 5 days with or without SLCs or SLC-derived conditioned medium (SLC-CM). Data are representative of two independent experiments.

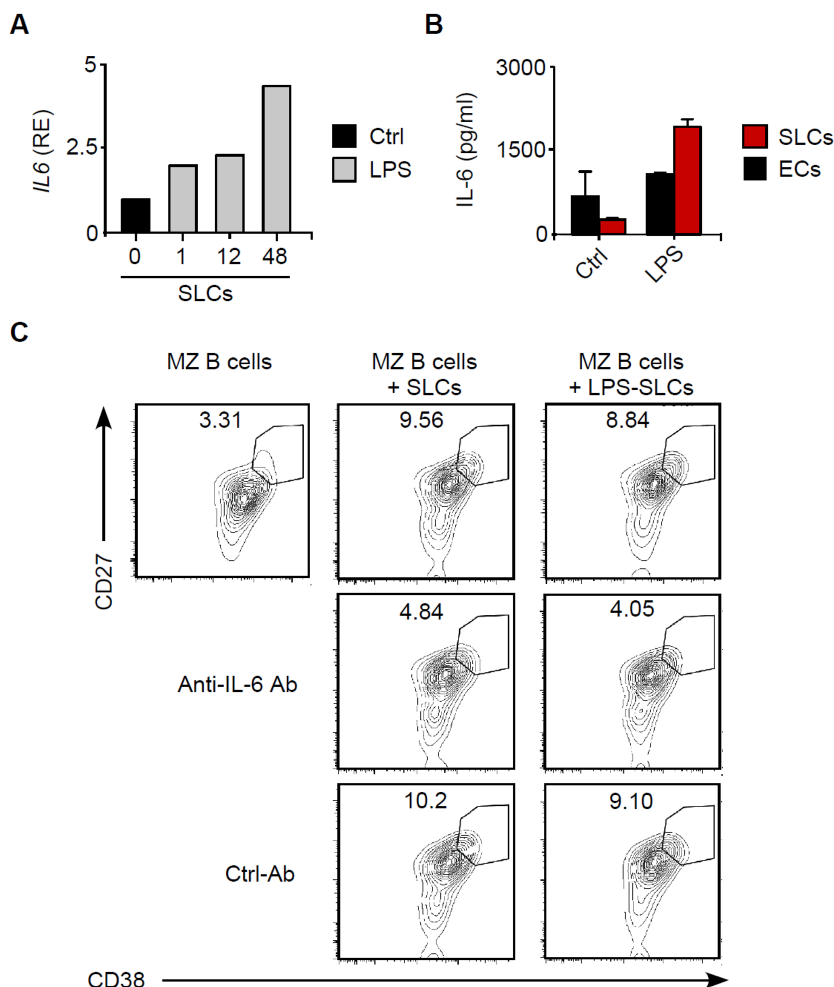


Figure 4.22 SLCs induce plasma cell differentiation by stimulating MZ B cells through IL-6. (A) QRT-PCR of transcripts encoding IL-6 from SLCs cultured with or without LPS. Transcripts for IL-6 were normalized to transcripts encoding β-actin. Data are representative of two experiments yielding similar results. **(B)** ELISA of IL-6 secreted by SLCs or umbilical vein endothelial cells (ECs) cultured with or without LPS. Summary of three experiments. **(C)** Flow cytometry analysis of CD27 and CD38 on MZ B cells cultured for 5 d with or without resting or LPS-stimulated SLCs in the presence or absence of a blocking anti-IL-6 antibody (Ab) or a control (Ctrl) isotype-matched Ab. One of two independent experiments yielding similar results.

Having shown that SLCs produce BAFF, APRIL and IL-6, we attempted to establish the relative contribution of these factors to the induction of plasma cell differentiation by SLCs. Flow cytometry showed that a blocking antibody to IL-6 reduced the formation of $CD27^{hi}CD38^{hi}$ plasmablasts form MZ B cells exposed to SLCs alone or pre-stimulated with LPS (**Figure 4.22 C**). Decoy TACI-Ig or BAFF-R-Ig receptors capable to block APRIL and/or BAFF had no effect or even increased plasma cell differentiation (data not shown), but this paradoxical effect could be due to engagement of activating FcγRs on SLCs by the Fc portion of TACI-Ig and BAFF-R-Ig. Thus, further experiments are needed to clarify the effect of BAFF and APRIL on SLC-induced antibody secretion. Overall, these results suggest that SLCs induce plasma cell differentiation by stimulating MZ B cells through a mechanism involving the release of IL-6.

13. SLCs release chemotactic factors for MZ B cells

Given their ability to induce CSR and plasma cell-inducing signals to MZ B cells, SLCs may have the capability to recruit MZ B cells to PFZ and red pulp area of the spleen through the release of specific chemokines. This possibility would be consistent with the notion that, unlike mouse MZ B cells, human MZ B cells are not sessile and thus can enter the splenic sinusoidal system to gain access to the general circulation. Earlier studies show that macrophages use the chemokine CXCL10 (or IP-10) to enhance TI antibody responses[179]. Consistent with these studies, microarray analysis showed that SLCs expressed more transcripts for CXCL10 than endothelial cells did. Accordingly, ELISA assays demonstrated that SLCs secreted CXCL10 and further

increased this secretion in response to LPS (**Figure 4.23 A**). Consistent with the striking myeloid nature of SLCs, conventional endothelial cells did not show any CXCL10 secretion.

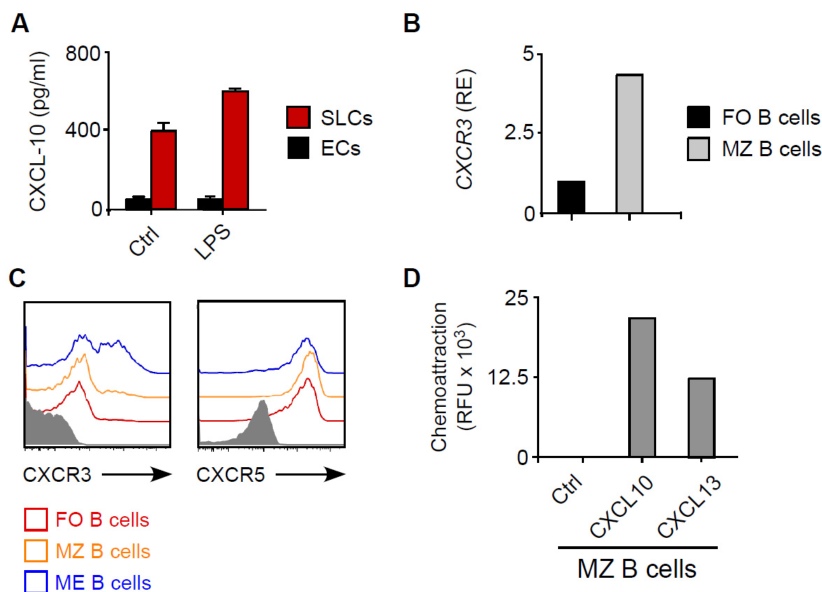


Figure 4.23 MZ B cells express CXCR3 and migrate in response to CXCL10. (A) ELISA of CXCL10 secreted by SLCs or umbilical vein endothelial cells (ECs) cultured with or without LPS. Summary of three experiments. **(B)** QRT-PCR of transcripts encoding CXCR3 on splenic follicular (FO) B cells and MZ B cells. Data are representative of two experiments yielding similar results. **(C)** Flow cytometric analysis of CXCR3 and CXCR5 on FO B cells, MZ B cells and ME B cells. Solid profile, isotype-matched control. **(D)** Chemotactic response of MZ B cells to CXCL10 or CXCL13. One of two independent experiments yelding similar results.

In mice, MZ B cells receive retention signals from S1P1 and S1P3 receptors, which recognize sphingosine-1 phosphate (S1P) present in the blood of the marginal sinus[174,205]. Ligation-induced down-regulation of S1P1 and S1P3 receptors causes MZ B cells to move to

the follicle in response to CXCR5 signals generated by CXCL13, a chemokine expressed by FDCs. This follicular shuttling of MZ B cells occurs on a constant basis to promote the transportation of blood-borne antigens to the follicle. MZ B cell trafficking to the follicle further increases in response to stimulation by TD antigens.

In the presence of TI antigens, activated MZ B cells down-regulate S1P1 and S1P3 receptors and move to the extrafollicular area, possibly in response to CXCR4 signals generated by CXCL12 from macrophages, DCs and stromal cells. The chemokine CXCL10, which binds CXCR3, may play an additional role. Thus, we set up to verify the expression of CXCR3 on MZ B cells and, for comparison, other splenic B cell subsets[39].

QRT-PCR showed that MZ B cells contained more abundant transcripts for CXCR3 than follicular B cells did **(Figure 4.23 B**). Flow cytometry demonstrated that all the major splenic B cell subsets, including follicular, MZ and memory B cells, expressed CXCR3 along with CXCR5. However, we also detected higher expression of CXCR3 on a fraction of MZ B cells and on a larger subset of memory B cells **(Figure 4.23 C**). Thus, CXCL10 from SLCs might guide the migration of MZ B cells as well as other subsets of splenic B cells to extrafollicular areas. To start testing this possibility, we exposed MZ B cells to a chemotactic gradient formed by CXCL10 or control CXCL13. We found that MZ B cells migrated in response to CXCL10 as well as control CXCL13 (**Figure 4.23 D**).

As suggested by our earlier findings, MZ B cells may undergo plasma cell differentiation following receiving activation signals from SLCs, including IL-6. The resulting plasma cells might take advantage of

similar signals to optimize their survival within SLC-containing niches. In agreement with this notion, IHC showed that the red pulp and PFZ of the spleen contained $MR^{+}CD8\alpha^{+}$ SLCs in close proximity of plasma cells accumulating cytopolasmic IgM or IgA and expressing CD138 (**Figure 4.24 A, B**). This latter is also known as syndecan-1 and undergoes strong up-regulation during plasma cell differentiation[206].

To formally elucidate the nurse-like function of SLCs, we exposed splenic plasmablasts/plasma cells to SLC-derived conditioned medium or SLCs. Flow cytometry showed that splenic plasmablasts/plasma cells up-regulated their survival in response to SLC-derived conditioned medium and that this up-regulation further increased in response to SLCs (**Figure 4.24 C**). Thus, SLCs provide survival signals not only to MZ B cells, but also to plasmablasts/plasma cells through both contact-dependent and contact-independent mechanisms. Ongoing blocking experiments are determining the involvement of IL-6 and CXCL10 in the pro-survival activity of SLCs on plasmablasts/plasma cells.

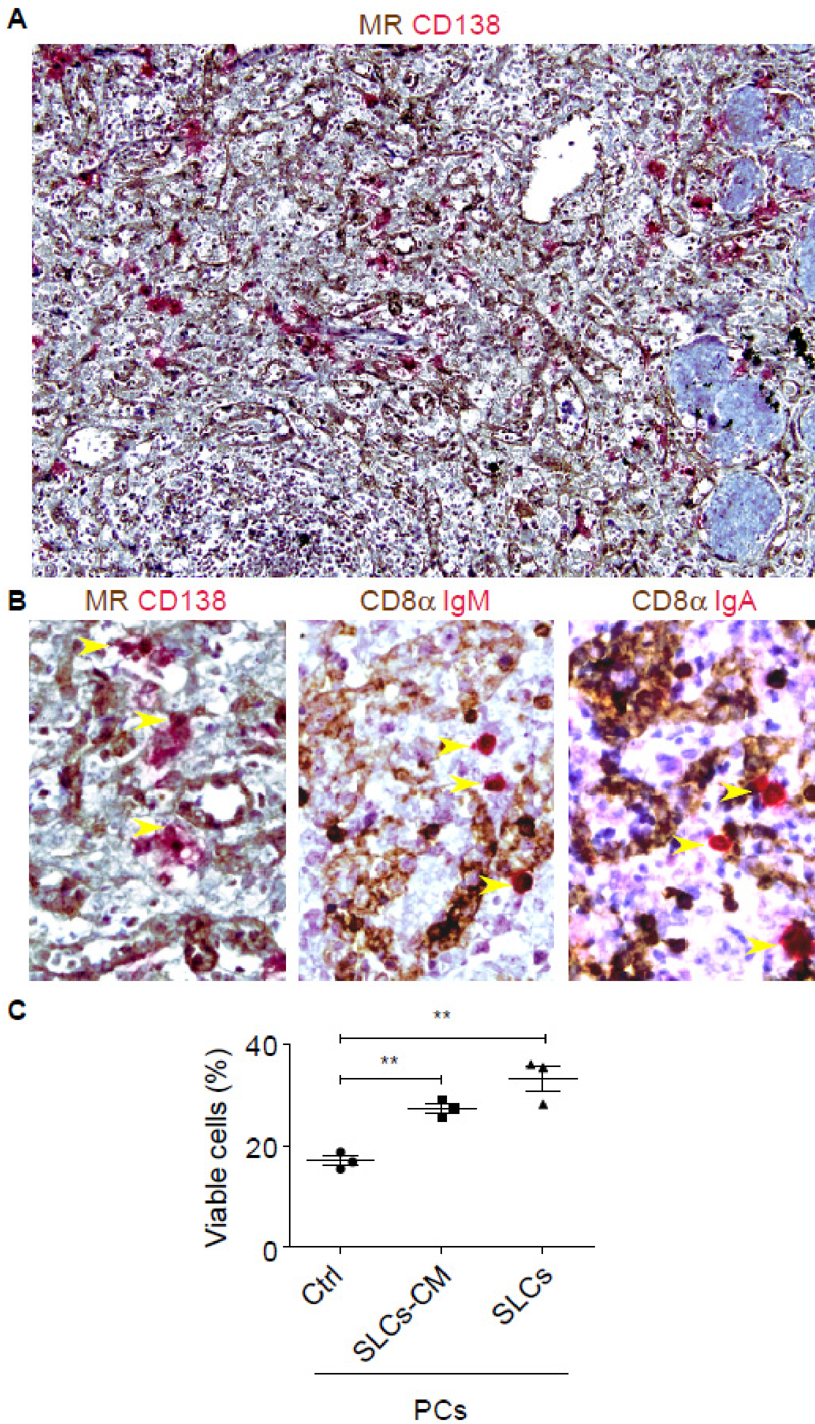


Figure 4.24 SLCs provide survival signals to splenic plasma cells. (A-B) IHC of hematoxylin-stained adult splenic tissue sections stained for MR

or CD8α (brown) and CD138, IgM or IgA (red). Original magnification, 100x **(A)** and 630x **(B)**. One of two experiments yielding similar results. **(C)** Flow cytometric analysis of viable Annexin-V$^-$DAPI$^-$ splenic plasma cells (PCs) after a 24-h co-culture with or without SLC-derived CM (SLC-CM) or SLCs. Data summarize four independent experiments. ** $P < 0.005$ (one-tailed unpaired Student's *t*-test).

PART IV

DISCUSSION AND CONCLUSIONS

Chapter V

Discussion

We have shown that SLCs expressing the leukocyte molecule CD8α specifically demarcated sinusoidal vessels in the splenic red pulp of humans and primates but not mice or rats. Similar to vascular endothelial cells, SLCs exhibited both endothelial topography and morphology and expressed typical endothelial molecules such as vWF, CD105 and CD141. Nonetheless, SLCs lacked the canonical endothelial lineage molecules CD34 and CD144, and instead strongly expressed multiple myeloid molecules, including CD14, CD36, CD163, MR, DEC-205 and TLR4.

Despite lacking the pan-leukocyte molecule CD45, SLCs showed a prominent macrophage-like gene signature. Accordingly, SLCs were enriched in transcripts encoding microbial sensors, scavanger receptors, immune mediators, and regulators of phagocytosis and antigen presentation. In addition to engulfing particulate antigens through an actin-dependent mechanism, SLCs released BAFF, APRIL, IL-6 and CXCL10 to enhance the recruitment, activation and survival of both MZ B cells and plasma cells.

We conclude that SLCs constitute a major population of hybrid endothelial-myeloid cells of the splenic innate immune system. Besides functioning as perisinusoidal sentinels with phagocytic properties, SLCs may enhance splenic antibody responses by forming niches for the activation and survival of MZ B cells and plasma cells.

SLC: an old but still enigmatic cell type

The spleen has co-developed with red blood cells as a condensation of the lymphomyeloid complex in the intestine of primitive

vertebrates[207,208]. As a result of this co-evolution, the organization of blood vessels has remained a major determinant of the structure and function of the spleen. Unlike the white pulp, the red pulp of the spleen contains sinusoidal vessels that serve as filter for the clearance of circulating antigens, antigen-transporting erythrocytes and senescent or perturbed hematopoietic cells, including erythrocytes[68]. Typical sinusoidal vessels demarcated by discontinuous walls first arose in mammals and typically contribute to an open vascular system that connects venous lakes termed cords of Billroth with the splenic vein. By permitting the slow percolation of blood through low-resistance areas, splenic sinusoids facilitate the interaction between blood-borne antigens and phagocytic cells strategically positioned around the sinusoid and known as SLCs[70].

The nature of SLCs has long remained mysterious. Owing to their perisinusoidal topography, possible phagocytic activity and intimate proximity to macrophages, SLCs have been traditionally referred to as reticulo-endothelial cells[209]. The long-held perception regarding the endothelial origin of SLCs was first questioned by studies showing that SLCs were not surrounded by reticulin fiber sheaths and lacked junctional side processes typical of endothelial cells[210,211]. Additional studies showed that SLCs have some ultrastructural features reminiscent of phagocytes and stromal cells and show a unique phenotype that entails expression of the leukocyte molecule CD8α, the actin nucleator FHOD1, and the signalling inhibitor SIRPα along with the expression of myeloid molecules, including the microbial sensor MR[70,72].

Despite these advances, the precise phenotype, gene expression profile and function of SLCs remain uncertain. This is mainly due to the following major limitations. Firstly, mice have an asinusal spleen that lacks sinusoidal vessels and thus no mouse model is available to study the biology and function of SLCs. Secondly, it has not been possible so far to isolate and propagate SLCs from human spleens due to elevated mortality and incomplete knowledge of their phenotype and growth requirements. The present study is the first to overcome these limitations and shows that SLCs are a large human-specific population of endothelial-myeloid cells of the splenic innate immune system.

SLCs express endothelial and stromal features

We found that SLCs had a unique phenotype that included the expression of both endothelial and myeloid lineage molecules. Among endothelial molecules, SLCs typically expressed elevated levels of the adhesion molecules CD31 (PECAM-1), CD54 (ICAM-1), CD102 (ICAM-2), CD105 (endoglin) and CD141 (thrombomodulin) along with the coagulation factor vWF and the endothelial growth factor receptor VEGF-R1. This endothelial phenotype was associated with perisinusoidal topography, endothelial-like elongated morphology and endothelial-like expression of intracellular Weibel-Palade bodies, which are perinuclear organelles for the storage of cytokines, chemokines and coagulation factors, including vWF[198,212].

Despite showing these typical endothelial features, SLCs lacked the canonical endothelial lineage molecule CD34 and the adhesion molecule CD144 (VE-cadherin), which were instead expressed by endothelial cells from other lymphoid organs, including tonsils and

intestine. Furthermore, SLCs expressed multiple myeloid molecules and showed a macrophage-like gene expression profile, which suggests that SLCs may derive from a unique endothelial-myeloid precursor.

At this stage, the nature of the SLC precursor remains unknown, but may include a pluripotent cell type capable to also generate vascular endothelial cells and macrophages. This SLC precursor may already be present during fetal life, as fetal spleens contained macrophage-like cells expressing the scavanger receptor CD68 along with endothelial-like cells expressing CD34 and vWF. Consistent with this possibility, some evidence points to the initial formation of SLCs within stromal foci, also known as stromal lacunae, which contain $CD34^+$ sinusoid-forming vessels that develop during late fetal life[78]. Our data indicate that, unlike SLCs, endothelial cells from fetal spleens lacked CD8α and did not form a mature sinusoidal network like the one typically present in the red pulp of adult spleens.

Given the dynamic nature of the splenic environment and its continuous exposure to self and microbial antigens, including commensal antigens, there is the possibility that splenic SLC precursors persist during adult life. In general, postnatal neovascularization is thought to result exclusively from the proliferation, migration and remodeling of fully differentiated endothelial cells occupying pre-existing blood vessels[213]. However, there is also some evidence that points to the presence of a circulating multipotent cell with endothelial-myeloid-stromal differentiation potential. Besides expressing the endothelial-stem cell molecule CD34, the pan-leukocyte molecule CD45, the monocyte-macrophage molecule CD14 along with type-I collagen, this circulating progenitor

cell shows mixed monocytic, endothelial and mesenchymal molecular traits and differentiates to myeloid, endothelial and mesenchimal cells *in vitro*[214,215].

Additional evidence indicates that, in the presence of medium containing endothelial growth factors, circulating $CD34^+CD45^+CD14^+$ cells differentiate into endothelial cells that also express some DC molecules[213,216]. In keeping with these data, one would be tempted to speculate that, during adult life, SLCs could derive from a circulating $CD34^+CD45^+CD14^+$ myeloid-endothelial-stromal precursor capable to home to the spleen, perhaps in response to chemotactic signals provided by resident stromal cells. Our data do not clarify this possibility, but show that SLCs express the receptor for G-CSF, a colony-stimulating factor produced by splenic stromal cells (S. Bascones and A. Cerutti; unpublished data). Besides stimulating the survival, activation and differentiation of granulocytes and their precursors, signals from G-CSF-R may guide the migration and enhance the proliferation of some endothelial cells.

Consistent with their possible origin from a precursor with stromal potential, SLCs expressed vimentin and SMA, two molecules of mesenchimal and smooth muscle cells that form intermediate filaments and stress fibers, respectively[191]. Remarkably, SLCs expressed CD10, a neutral endopeptidase associated not only with some mesenchimal cells, but also with precursors of lymphoid and myeloid cells. This finding suggests that SLCs may retain some plasticity in their developmental potential. Accordingly, SLCs progressively down-regulated CD8α and MR expression and concomitantly enhanced both morphological and phenotypic

endothelial features after exposure to endothelial growth factors such as VEGF (data not shown).

Collectively, these findings are consistent with an origin of SLCs from a fetal or circulating multipotent progenitor and may additionally reflect the persistence of developmental plasticity in mature SLCs. This plasticity may allow SLCs to dynamically shape the structure and function of the splenic sinusoidal network in response to antigenic stimuli. In the presence of appropriate growth factors, including G-CSF or M-CSF, SLCs might also retain the capability to differentiate into macrophages, which were indeed detected in close proximity to SLCs around sinusoidal vessels from both fetal and adult spleens. Ongoing studies are addressing these possibilities through strategies involving the isolation of splenic $CD34^{+}CD45^{+}CD14^{+}$ progenitor cells from adult spleens and the culture of either progenitor cells or mature SLCs with different combinations of VEGF, GM-CSF, G-CSF and/or M-CSF.

SLCs show dominant myeloid features

In addition to endothelial and stromal features, SLCs expressed a prominent myeloid phenotype. The myeloid nature of SLCs is consistent with their strong expression of CD8α, a TCR co-receptor originally thought to be exclusively associated with cytotoxic T cells. These cells usually express heterodimers of CD8α and CD8β, but a large body of literature shows that homodimers of CD8α characterize myeloid cells, including a mouse subset of splenic DCs specialized in the cross presentation of antigens[217]. The function of CD8α in these DCs remains unknown. In humans, circulating DCs with cross-

presenting function lack CD8α but typically express CD141[218], a thrombin-interacting molecule also detected on SLCs.

Unlike endothelial cells but similar to splenic macrophages, SLCs expressed multiple germline-encoded PRRs that mediate antigen capture and internalization, including the C-type lectin receptor family members MR[219]. Remarkably, MR recognizes carbohydrate residues associated with pathogenic determinants such as CPS on microbes as well as ABO blood group antigens on erythrocytes[190,220,221]. This implies that SLCs could use MR to detect and clear not only bacteria, but also altered erythrocytes present in the sinusoidal circulation. Similar to macrophages, SLCs may also use MR to enhance the phagocytosis of IgG-coated antigens through FcγRs[222].

Besides MR, SLCs expressed another member of the C-type lectin receptor family known as DEC-205[219]. In DCs, DEC-205 facilitates the processing of internalized antigens and the presentation of peptide-HLA-II complexes to $CD4^+$ T helper cells[170]. Given their strategic perisinusoidal position, SLCs might use DEC-205 to capture, internalize and process circulating antigens into intracellular degradative compartments for the initiation of immune responses by $CD4^+$ T helper cells.

Alternatively and similar to MR and some FcγRs, DEC-205 may convey internalized antigen to non-degradative intracellular compartments that subsequently recycle on the cell surface for the presentation of native antigen to B cells[223]. A similar non-degradative pathway may involve the CLR, langerin, and the scavanger receptors CD36 and CD163. Besides functioning as microbial sensors, these

PRRs recognize apoptotic cells and the hemoglobin-binding protein haptoglobin, respectively, and may thus allow SLCs to clear apoptotic leukocytes and senescent erythrocytes. Consistent with this possibility, earlier ultrastractural studies show that SLCs contain erythrocytes and hemoglobin degradation products, including intracellular deposits of iron[224].

Despite sharing numerous microbial sensors, SLCs are likely to play a complementary immunological roles with respect to other cellular components of the splenic innate immune system, including macrophages and DCs. Indeed, SLCs expressed a unique combination of PRRs that distinguishes them from DCs and macrophages. By establishing unique reciprocal interactions and recruiting other receptors such as CD8α and CD141, this unique array of PRRs may confer specific immune surveillance, effector and regulatory functions to SLCs. Regulatory molecules such as SIRP-α may further contribute to the functional specificity of SLCs. This inhibitory receptor is a signal-transducing regulatory protein that attenuates pro-inflammatory signals emanating from PRRs, including TLRs. In partial agreement with an earlier study[72], we found prominent SIRP-α expression in both SLCs and splenic macrophages, but weak SIRP-α expression in conventional endothelial cells.

Compared to endothelial cells, SLCs showed stronger expression of TLR8 and TLR9. SIRP-α may mitigate pro-inflammatory signals generated by these TLRs in response to nucleic acids released by microbes and damaged/apoptotic cells[225]. Along the same lines, SLCs may also use SIRP-α to attenuate pro-inflammatory signals generated

by TLR4, an LPS receptor expressed by SLCs together with its co-receptor CD14. SLCs also expressed FcγRIII (CD16), FcγRIIA (CD32a) and FcαR (CD89) receptors, which may facilitate the clearance of antigens opsonized by IgG or IgA. Also these pro-inflammatory FcRs may require constraining signals from SIRP-α.

Consistent with its dominant homeostatic function, SIRP-α has been shown to regulate the migration and phagocytosis of myeloid cells by interacting with a ubiquitous protein known as CD47[226–228]. The interaction of SIRP-α with CD47 likely occurs in the context of signals from multi-receptor complexes on SLCs and may regulate functions unrelated to microbial recognition, including the transit of erythrocytes across sinusoidal vessels. Indeed, decreased CD47 expression on senescent erythrocytes may attenuate inhibitory signals emanating from CD47-SIRP-α interaction, thereby instructing SLCs to initiate phagocytosis.

The attenuation of inhibitory signals from SIRP-α might also cause activation of FHOD1, an actin nucleator positioned downstream of the small GTPase RhoA and the kinase ROCK. Besides inducing the phagocytic machinery in SLCs, activation of FHOD1 may trigger the contraction of SLCs, which in turn could facilitate the mobilization of leukocytes dwelling in the sinusoid. The putative contractile function of SLCs may be consistent with their abundant expression of SMA, a protein that also plays a critical role in the cytoskeletal remodeling required for the contraction of smooth muscle cells[191].

Consistent with their prominent myeloid phenotype, SLCs showed a gene expression profile resembling that of splenic macrophages rather than that of endothelial cells. Indeed, gene ontology analysis revealed that SLCs were enriched in functional gene categories associated with innate immune responses, microbial sensing, microbial recognition and cytokine production. For example, SLCs showed higher expression of transcripts encoding MYD88, IRAK3, IRAK4 and BTK, which are signaling proteins associated with TLR. Consistent with their elevated expression of TLR3, TLR8, TLR8 and TLR9, SLCs were also enriched in transcripts encoding IRFs, a group of IFN-related transcription factors typically induced by nucleic acid receptors of the TLR family. Additional IFN-related factors included the IFN-α receptor subunit IFN-αR2, the IFN-αR-induced transcription factor STAT2, and the antiviral protein Mx2, OAS1 and OAS2. Remarkably, SLCs also highly expressed transcripts encoding recognition systems different from TLRs, including the NLR inflammasome-associated proteins NAIP, AIM2 and PYCARD and the DNA sensor TREX1. These and other PRRs are usually expressed by myeloid cells actively engaged in microbial recognition, which points to a prominent role of SLCs as immune sentinels within the splenic sinusoidal system.

Compared to endothelial cells, SLCs were also enriched in gene products encoding receptors and signaling proteins involved in endocytosis and phagocytosis, including FcγRIIA, SYK, PLCG2, DOCK2, ITPR1, PIK3CG, PAK1, GRB2 and ACTR2. Accordingly, SLCs were able to efficiently phagocytose serum-coated beads as well as whole bacteria through a cytocalasin D-sensitive process that

involved actin polymerization. This finding represents the first functional demonstration that SLCs indeed have phagocytic activity.

Moreover, SLCs contained abundant transcripts encoding proteins involved in antigen presentation, including various HLA-II molecules and TAP2. This observation reinforces the possibility that, in principle, SLCs may function as antigen-presenting cells similar to DCs and macrophages. Whether or not this function actually contributes to the activation of splenic $CD4^+$ T helper cells and the initiation of TD antibody production by splenic follicular B cells remains to be established.

SLCs activate MZ B cells to enhance CSR and antibody production

SLCs with typical endothelial-myeloid features, including co-expression of CD8α, MR and vWF, were detected in humans and rhesus macaques, but not in mice and rats. This species specificity suggests a recent emergence of SLCs during evolution and may reflect an adaptation of higher primates to the development of an open splenic circulation promoting the slow percolation of arterial blood through the PFZ and red pulp of the spleen via sinusoidal vessels. Together with the sessile nature of human MZ B cells, this peculiar vascular anatomy may facilitate the interaction of SLCs with MZ B cells as they transit through sinusoidal vessels to gain access to the systemic circulation. During this process, SLCs may interact with some antigenically activated MZ B cells to form extrafollicular foci of plasma cell differentiation and antibody production. Consistent with

this possibility, some B cells and plasma cells were detected in SLC-containing areas of the PFZ and red pulp of the spleen.

Under steady state condition, extrafollicular foci of MZ B cell activation and plasma cell differentiation may lead to the formation of pre-immune (also known as natural) antibodies directed to various highly conserved microbial determinants, including canonical TI antigens. Consistent with this possibility, SLCs expressed BAFF and APRIL, two CD40L-related factors usually produced by macrophages, DCs and other myeloid cells[156,203]. In addition to driving B cell and plasma cell survival signals, BAFF and APRIL enhance antibody responses to TI antigens by activating MZ B cells[66,156,177,203]. Similar to macrophages, SLCs constitutively secreted both BAFF and APRIL and further increased BAFF secretion after exposure to LPS. Similar to the coagulation protein vWF, APRIL showed a predominant perinuclear expression pattern in SLCs, which may reflect the accumulation in Weibel-Palade bodies. These storage organelles may release their content in response to antigen-induced signals, including cytokines and TLR ligands.

Previous studies from our and other groups indicate that BAFF and APRIL cooperate with both antigen and cytokines to stimulate TI antibody responses[156]. These responses involve the engagement of TACI on MZ B cells by BAFF or APRIL. Our findings suggest that SLCs may activate this TI pathway to enhance CSR and antibody production in MZ B cells[2,66,229–232]. Indeed, we found that MZ B cells expressed more TACI than follicular B cells and efficiently responded to SLCs by undergoing IgM secretion as well as CSR from IgM to IgG

and IgA through a process involving sequential induction of the DNA-editing enzyme AID and plasma cell differentiation.

Of IgG and IgA subclasses, IgG1, IgG3 and IgA2 were preferentially induced by SLCs through a mechanism that appeared to involve both contact-dependent and contact-independent signals. While intercellular contact was required to induce plasma cell differentiation and IgG secretion, these processes additionally involved the secretion of IL-6 by SLCs. Among contact-dependent signals, BAFF may play an important role in plasma cell differentiation[67] and indeed a transmembrane form of BAFF was detected on SLCs. Additional contact-dependent signals may be provided by the adhesion molecules ICAM-1 and VCAM-1 on SLCs. Of note, SLCs also released TGF-β, which may explain the induction of contact-independent IgA secretion by MZ B cells exposed to SLC-derived conditioned medium. Ongoing studies are ascertaining the individual contribution of cytokines and membrane-bound molecules to the activation of MZ B cells by SLCs.

SLCs deliver chemotactic and survival signals for MZ B cells and plasma cells

Previous studies show that, unlike plasma cells in the bone marrow, plasma cells in the spleen express CD11a (or LFA-1), an integrin that interacts with ICAM-1 on stromal cells. This interaction facilitates the retention and survival of plasma cells within stromal niches in extrafollicular areas of the spleen[183]. Having shown that SLCs express CD54, SLCs may form additional extrafollicular niches for splenic plasma cell survival. Accordingly, we observed the presence of some plasma cells expressing CD138 in close proximity to SLCs. As

discussed earlier, SLCs appeared to promote plasma cell differentiation and survival through both contact-dependent and contact-independent signals, including the cytokine IL-6. SLCs enhanced the release of IL-6 in response to TLR ligands and IL-6 played a key role in the differentiaton and survival of plasma cells emerging from activated MZ B cells. Interestingly, IL-6 has been shown to cooperate with APRIL to promote plasma cell survival in bone marrow and intestinal niches containing eosinophils as well as other “nurse-like” cells[178,183,233]. SLCs express APRIL in addition to IL-6 and thus may take advantage of cooperative signals from these two cytokines to promote plasma cell survival. Consistent with this possibility, we found that splenic plasma cells expressed BCMA, a receptor that drives survival signals in response to APRIL[234].

Interestingly, SLCs also expressed CXCL10 (or IP-10), a macrophage-derived chemokine that cooperates with IL-6 to drive the differentiation and survival of plasma cells generated during TI (but also TD) antibody responses[179]. CXCL10 delivers chemotactic and survival signals via CXCR3, a receptor usually expressed by activated NK and T cells[235]. We found that splenic MZ B cells highly expressed CXCR3 and effectively migrated across a chemotactic field established by CXCL10. Thus, SLCs may form CXCL10-expressing extrafollicular niches to promote both the recruitment of MZ B cells and the survival of MZ B cell-derived plasma cells.

We recently found that the PFZ of the spleen contains activated neutrophils that release BAFF, APRIL and other cytokines to support CSR and antibody production under homeostatic conditions[66]. These neutrophils are partially controlled by survival, activation and

recruitment signals provided by GM-CSF and possibly CXCL8 (or IL-8), two factors released by perifollicular type-3 ILCs. Additional neutrophil-attracting signals may originate from perifollicular SLCs, which indeed expressed CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8. These chemokines are all involved in the chemotaxis of myeloid cells, including neutrophils[236,237]. Besides playing a role in TI antibody responses, SLCs along with macrophages, DCs, ILCs and neutrophils may generate recruitment and survival niches also for memory B cells and plasma cells emerging from TD antibody responses. This becomes particular relevant in consideration of the functional plasticity of MZ B cells, which permits them to participate in both TI and TD antibody responses.

Possible clinical implications

Splenic MZ B cells recognize microbial CPS and thus generate antibody-mediated protection against encapsulated bacteria, including *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitides*. Currently available unconjugated and protein-conjugated CPS-containing vaccines against these pathogens show a variable efficacy in relationship to the age and immunological state of the recipient. In general, conjugated vaccines show a better efficacy, but have higher costs of preparation, which limits their broad use in developing countries. Because of these limitations and the emergence of bacterial strains unresponsive to conjugated vaccines, there is an urgent need to develop novel, more efficacious and less expensive vaccine strategies.

By showing that SLCs deliver helper signals to MZ B cells and their plasma cell progeny, our findings indicate that SLCs could be targeted

by adjuvants to enhance vaccine-induced antibody responses. These adjuvants may take advantage of antigen delivery strategies capable to specifically target SLCs. One possible strategy could involve antibodies to CD163, which may offer the additional advantage of delivering antigen to splenic macrophages. Antibodies to DEC-205 or MR could be an additional possibility and may offer the advantage of delivering antigen to splenic DCs. Similar to recent studies involving targeting of antigen to DC-SIGN on DCs[238], antigens could also be modified to facilitate their interaction with CLR family members expressed by SLCs, including MR. These and other SLC-targeting vaccination strategies could be pre-clinically tested in rhesus macaques, because the spleen of these primates contains SLCs.

Chapter VI

Conclusions

1. SLCs are unique endothelial-myeloid cells of the splenic innate immune system characterized by perisinusoidal topography, endothelial morphology, and hybrid $CD8\alpha^{+}CD14^{+}CD34^{-}CD45^{-}MR^{+}vWF^{+}$ phenotype.

2. SLCs form a mature $CD8\alpha^{+}MR^{+}vWF^{+}$ sinusoidal meshwork in the PFZ and red pulp areas of the spleen after birth.

3. SLCs are specific to humans and non-human primates, but cannot be detected in rodents.

4. SLCs express a macrophage-like gene expression profile that includes microbial sensors, scavanger receptors, interferon-related molecules, and molecules regulating phagocytosis and antigen presentation.

5. SLCs express multiple germline-encoded receptors involved in the recognition of conserved microbial products and initiation of innate immune responses, including TLRs, the C-type lectin receptors MR and DEC-205, and the scavanger receptor CD163.

6. SLC express innate B cell-helper factors usually expressed by myeloid cells, including BAFF and APRIL.

7. SLCs induce CSR from IgM to IgA and IgG as well as plasma cell differentiation and antibody production by stimulating MZ B cells through both contact-dependent and contact-independent signals, including IL-6, a cytokine usually expressed by myeloid cells.

8. SLCs release CXCL10 as myeloid cells do and may use this chemokine to induce extrafollicular recruitment of MZ B cells expressing the CXCL10 receptor CXCR3.

9. SLCs form extrafollicular niches that deliver survival signals to splenic plasmablasts and plasma cells originating from MZ B cells.

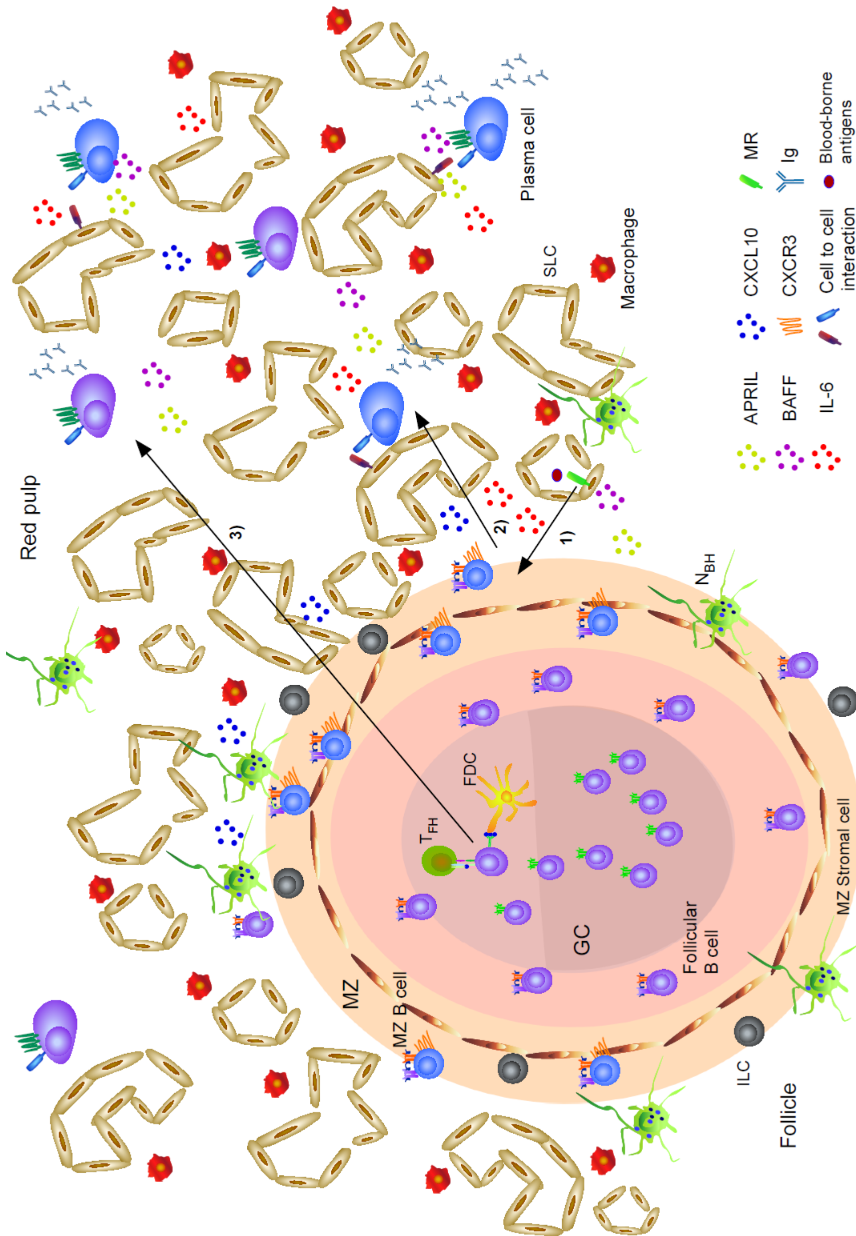


Figure 7.1 Model depicting the hypothetical functions of SLCs in the spleen. (1) Sinusoidal vessels in the PFZ and red pulp of the spleen include SLCs characterized by unique endothelial-myeloid features, including abundant expression of microbial sensors (TLRs, NLRs, C-type lectin receptors, scavanger receptors) and Fc receptors (FcγRs, FcαRI, FcεRI).

These receptors enable SLCs to sense circulating microbial and autologous molecular patterns associated with soluble antigens or immunocomplexes bound to red blood cells. After recognizing antigen, SLCs initiate macrophage-like innate immune responses that entail phagocytosis, activation of IFN-dependent defensive pathways, release of B cell-helper factors, and presentation of native antigen to MZ B cells and possibly other recirculating splenic B cell subsets, including germinal center-derived memory B cells. SLCs may also internalize and process antigen to present HLA-II-peptide complexes to $CD4^{+}$ T cells. Of note, SLCs may coordinate all these defensive activities with nonspecific macrophages, which are indeed positioned in close contact with SLCs. Pro-inflammatory signals generated by SLCs through TLRs and FcγRs may be mitigated by SIRP-α, an inhibitory receptor on SLCs that binds to CD47 on red blood cells and other antigen-transporting cells. SLCs may further constrain inflammation by generating additional inhibitory signals through CLRs (e.g., MR) and scavanger receptors (e.g., CD163) in the context of antigen recognition. **(2)** After sensing blood-borne antigens, SLCs would release CXCL10, which promotes the recruitment of MZ B cells by binding to CXCR3. Moreover, SLCs release BAFF, APRIL and IL-6, which may cooperate with BCR (Ig receptor) and TLR ligands to induce survival, activation, CSR and plasma cell differentiation signals in MZ B cells. In addition, IL-6 promotes the survival of fully differentiated plasma cells emerging from either low-affinity MZ B cells during TI antibody responses or high-affinity memory B cells during TD antibody responses. **(3)** TD responses recruit either follicular or, less frequently, MZ B cells and involve the activation of a germinal center reaction that selects high-affinity memory B cells through a complex pathway requiring T_{FH} cells and FDCs. SLCs might further increase MZ B cell activation and plasma cell survival by co-opting neutrophils with B cell-helper activity (N_{BH} cells). SLCs would recruit these neutrophils by releasing CXCL8 (IL-8) and other CXCL chemokines in response to microbial

signals[66]. Importantly, the recruitment and activation of neutrophils is further coordinated by ILCs through the release of GM-CSF and CXCL8. By releasing BAFF, APRIL and other B cell-helper factors, neutrophils would cooperate with SLCs, ILCs and stromal cells to generate multi-component niches for the activation, differentiation and survival of both MZ B cells and plasma cells[67]. Besides generating pre-immune (or natural) antibodies to circulating commensal and self-antigens under homeostatic conditions, these splenic niches would enhance the production of post-immune antibodies produced in response to immunization or infection.

ANNEX 1

References

1. Klein Klouwenberg, P. & Bont, L. Neonatal and infantile immune responses to encapsulated bacteria and conjugate vaccines. *Clin. Dev. Immunol.* **2008,** 628963 (2008).

2. 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. *Nat. Immunol.* **1,** 31–36 (2000).

3. Weller, S., Reynaud, C.-A. & Weill, J.-C. Vaccination against encapsulated bacteria in humans: paradoxes. *Trends Immunol.* **26,** 85–89 (2005).

4. González-Fernández, A., Faro, J. & Fernández, C. Immune responses to polysaccharides: lessons from humans and mice. *Vaccine* **26,** 292–300 (2008).

5. Weill, J.-C., Weller, S. & Reynaud, C.-A. Human marginal zone B cells. *Annu. Rev. Immunol.* **27,** 267–285 (2009).

6. Parra, D., Takizawa, F. & Sunyer, J. O. Evolution of B Cell Immunity. *Annu. Rev. Anim. Biosci.* **1,** 65–97 (2013).

7. Cooper, M. D. & Alder, M. N. The evolution of adaptive immune systems. *Cell* **124,** 815–822 (2006).

8. Galli, S. J., Borregaard, N. & Wynn, T. a. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat. Immunol.* **12,** 1035–1044 (2011).

9. Martin, F. & Kearney, J. F. Marginal-zone B cells. *Nat. Rev. Immunol.* **2,** 323–335 (2002).

10. Alt, F., Blackwell, T. & YancoPouLos, G. Development of the primary antibody repertoire. *Science (80-.).* **238,** 1079–1087 (1987).

11. Maizels, N. Immunoglobulin gene diversification. *Annu. Rev. Genet.* **39,** 23–46 (2005).

12. Shapiro-Shelef, M. & Calame, K. Regulation of plasma-cell development. *Nat. Rev. Immunol.* **5,** 230–242 (2005).

13. Herzog, S., Reth, M. & Jumaa, H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat. Rev. Immunol.* **9,** 195–205 (2009).

14. Cheng, P. C., Dykstra, M. L., Mitchell, R. N. & Pierce, S. K. A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J. Exp. Med.* **190,** 1549–1560 (1999).

15. Matsuuchi, L. & Gold, M. R. New views of BCR structure and organization. *Curr. Opin. Immunol.* **13,** 270–277 (2001).

16. Melchers, F. The pre-B-cell receptor: selector of fitting immunoglobulin heavy chains for the B-cell repertoire. *Nat. Rev. Immunol.* **5,** 578–584 (2005).

17. Stavnezer, J. Antibody class switching. *Adv. Immunol.* **61,** 79–146 (1996).

18. Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M. & Dwek, R. a. The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu. Rev. Immunol.* **25,** 21–50 (2007).

19. Ehrenstein, M. R. & Notley, C. a. The importance of natural IgM: scavenger, protector and regulator. *Nat. Rev. Immunol.* **10,** 778–786 (2010).

20. Grönwall, C., Vas, J. & Silverman, G. J. Protective Roles of Natural IgM Antibodies. *Front. Immunol.* **3,** 66 (2012).

21. Chen, K. & Cerutti, A. The function and regulation of immunoglobulin D. *Curr. Opin. Immunol.* **23,** 345–352 (2011).

22. Chen, K. *et al.* Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. *Nat. Immunol.* **10,** 889–898 (2009).

23. Schroeder, H. W. & Cavacini, L. Structure and function of immunoglobulins. *J. Allergy Clin. Immunol.* **125,** S41–52 (2010).

24. Nimmerjahn, F. & Ravetch, J. V. Fcgamma receptors as regulators of immune responses. *Nat. Rev. Immunol.* **8,** 34–47 (2008).

25. Shakib, F. & Stanworth, D. R. Human IgG subclasses in health and disease. (A review). Part II. *Ric. Clin. Lab.* **10,** 561–580

26. Pan, Q. & Hammarstrom, L. Molecular basis of IgG subclass deficiency. *Immunol. Rev.* **178,** 99–110 (2000).

27. Cerutti, A. *et al.* Regulation of mucosal IgA responses: lessons from primary immunodeficiencies. *Ann. N. Y. Acad. Sci.* **1238,** 132–144 (2011).

28. Woof, J. M. & Russell, M. W. Structure and function relationships in IgA. *Mucosal Immunol.* **4,** 590–597 (2011).

29. Cerutti, A., Chen, K. & Chorny, A. Immunoglobulin responses at the mucosal interface. *Annu. Rev. Immunol.* **29,** 273–293 (2011).

30. Shan, M. *et al.* Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science* **342,** 447–453 (2013).

31. Bax, H. J., Keeble, A. H. & Gould, H. J. Cytokinergic IgE Action in Mast Cell Activation. *Front. Immunol.* **3,** 229 (2012).

32. Gould, H. J. *et al.* The biology of IGE and the basis of allergic disease. *Annu. Rev. Immunol.* **21,** 579–628 (2003).

33. Panda, S., Zhang, J., Tan, N. S., Ho, B. & Ding, J. L. Natural IgG antibodies provide innate protection against ficolin-opsonized bacteria. *EMBO J.* **32,** 2905–2919 (2013).

34. Figueroa, J. E. & Densen, P. Infectious diseases associated with complement deficiencies. *Clin. Microbiol. Rev.* **4,** 359–395 (1991).

35. Ochsenbein, a. F. *et al.* Control of Early Viral and Bacterial Distribution and Disease by Natural Antibodies. *Science (80-.).* **286,** 2156–2159 (1999).

36. Matter, M. S. & Ochsenbein, A. F. Natural antibodies target virus-antibody complexes to organized lymphoid tissue. *Autoimmun. Rev.* **7,** 480–486 (2008).

37. Shibuya, A. *et al.* Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes. *Nat. Immunol.* **1,** 441–446 (2000).

38. Lutz, H. U. *et al.* Naturally occurring anti-band-3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes. *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7368–7372 (1987).

39. Cerutti, A., Cols, M. & Puga, I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat. Rev. Immunol.* **13,** 118–132 (2013).

40. Berland, R. & Wortis, H. H. Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol.* **20,** 253–300 (2002).

41. Soldevila, G., Raman, C. & Lozano, F. The immunomodulatory properties of the CD5 lymphocyte receptor in health and disease. *Curr. Opin. Immunol.* **23,** 310–318 (2011).

42. Duan, B. & Morel, L. Role of B-1a cells in autoimmunity. *Autoimmun. Rev.* **5,** 403–408 (2006).

43. Donze, H. H. *et al.* Human peritoneal B-1 cells and the influence of continuous ambulatory peritoneal dialysis on peritoneal and peripheral blood mononuclear cell (PBMC) composition and immunoglobulin levels. *Clin. Exp. Immunol.* **109,** 356–361 (1997).

44. Dorshkind, K. & Montecino-Rodriguez, E. Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential. *Nat. Rev. Immunol.* **7,** 213–219 (2007).

45. Jefferson, T., Ferroni, E., Curtale, F., Giorgi Rossi, P. & Borgia, P. Streptococcus pneumoniae in western Europe: serotype distribution and incidence in children less than 2 years old. *Lancet Infect. Dis.* **6,** 405–410 (2006).

46. Berkowska, M. a *et al.* Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* **118,** 2150–2158 (2011).

47. Spencer, J., Perry, M. E. & Dunn-Walters, D. K. Human marginal-zone B cells. *Immunol. Today* **19,** 421–426 (1998).

48. Hendricks, J. *et al.* Class-switched marginal zone B cells in spleen have relatively low numbers of somatic mutations. *Mol. Immunol.* **48,** 874–882 (2011).

49. Van de Pavert, S. a & Mebius, R. E. New insights into the development of lymphoid tissues. *Nat. Rev. Immunol.* **10,** 664–674 (2010).

50. Randall, T. D., Carragher, D. M. & Rangel-Moreno, J. Development of secondary lymphoid organs. *Annu. Rev. Immunol.* **26,** 627–650 (2008).

51. Turley, S. J., Fletcher, A. L. & Elpek, K. G. The stromal and haematopoietic antigen-presenting cells that reside in secondary lymphoid organs. *Nat. Rev. Immunol.* **10,** 813–825 (2010).

52. MacLennan, I. C. Germinal centers. *Annu. Rev. Immunol.* **12,** 117–139 (1994).

53. Batista, F. D. & Harwood, N. E. The who, how and where of antigen presentation to B cells. *Nat. Rev. Immunol.* **9,** 15–27 (2009).

54. Mebius, R. E. & Kraal, G. Structure and function of the spleen. *Nat. Rev. Immunol.* **5,** 606–616 (2005).

55. Chadburn, a. The spleen: anatomy and anatomical function. *Semin. Hematol.* **37,** 13–21 (2000).

56. Sagaert, X. & De Wolf-Peeters, C. Classification of B-cells according to their differentiation status, their micro-anatomical localisation and their developmental lineage. *Immunol. Lett.* **90,** 179–186 (2003).

57. Cesta, M. F. Normal structure, function, and histology of the spleen. *Toxicol. Pathol.* **34,** 455–465 (2006).

58. Bronte, V. & Pittet, M. J. The spleen in local and systemic regulation of immunity. *Immunity* **39,** 806–818 (2013).

59. Steiniger, B., Barth, P., Herbst, B. & Crocker, P. The species specific structure of microanatomical compartments in the human spleen: strongly sialoadhesin positive macrophages occur in the perifollicular zone, but. *Immunology* **92,** 307–316 (1997).

60. Timens, W., Boes, A. & Poppema, S. Human marginal zone B cells are not an activated B cell subset: strong expression of CD21 as a putative mediator for rapid B cell activation. *Eur. J. Immunol.* **19,** 2163–2166 (1989).

61. Steiniger, B., Timphus, E.-M., Jacob, R. & Barth, P. J. CD27+ B cells in human lymphatic organs: re-evaluating the splenic marginal zone. *Immunology* **116,** 429–442 (2005).

62. Kraal, G. & Mebius, R. New insights into the cell biology of the marginal zone of the spleen. *Int. Rev. Cytol.* **250,** 175–215 (2006).

63. Martin, F., Zhou, T., Kearney, J. J. F., B, B. & Balázs, M. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity* **17,** 341–352 (2002).

64. Steiniger, B. & Barth, P. The Perifollicular and Marginal Zones of the Human Splenic White Pulp Do Fibroblasts Guide Lymphocyte Immigration ? *Am. J. Pathol.* **159,** 501–512 (2001).

65. Pack, M. *et al.* DEC-205/CD205+ dendritic cells are abundant in the white pulp of the human spleen, including the border region between the red and white pulp. *Immunology* **123,** 438–446 (2008).

66. Puga, I. *et al.* B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat. Immunol.* **13,** 170–180 (2012).

67. Magri, G. *et al.* Innate lymphoid cells integrate stromal and immunological signals to enhance antibody production by splenic marginal zone B cells. *Nat. Immunol.* **15,** 354–364 (2014).

68. Hirasawa, Y. & Tokuhiro, H. Electron microscopic studies on the normal human spleen: especially on the red pulp and the reticulo-endothelial cells. *Blood* **35,** 201–212 (1970).

69. Pusztaszeri, M. P., Seelentag, W. & Bosman, F. T. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *J. Histochem. Cytochem.* **54,** 385–395 (2006).

70. Buckley, P. J., Dickson, S. A. & Walker, W. S. Human splenic sinusoidal lining cells express antigens associated with monocytes, macrophages, endothelial cells, and T lymphocytes. *J. Immunol.* **134,** 2310–2315 (1985).

71. Giorno, R. Unusual structure of human splenic sinusoids revealed by monoclonal antibodies. *Histochemistry* **81,** 505–507 (1984).

72. Ogembo, J. G. *et al.* SIRPα/CD172a and FHOD1 are unique markers of littoral cells, a recently evolved major cell population of red pulp of human spleen. *J. Immunol.* **188,** 4496–4505 (2012).

73. Korkusuz, P., Dağdeviren, A. & Aşan, E. Immunophenotypic analysis of human spleen compartments. *Ann. Anat.* **184,** 431–441 (2002).

74. Cols, M. *et al.* Stromal endothelial cells establish a bidirectional crosstalk with chronic lymphocytic leukemia cells through the TNF-related factors BAFF, APRIL, and CD40L. *J. Immunol.* **188,** 6071–6083 (2012).

75. Krieken, J. H. J. M. Van & van Krieken, J. H. Histopathology of the spleen in non-Hodgkin's lymphoma. *Histol. Histopathol.* **5,** 113–122 (1990).

76. Steiniger, B., Bette, M. & Schwarzbach, H. The open microcirculation in human spleens: a three-dimensional approach. *J. Histochem. Cytochem.* **59,** 639–648 (2011).

77. Steiniger, B., Stachniss, V., Schwarzbach, H. & Barth, P. J. Phenotypic differences between red pulp capillary and sinusoidal endothelia help localizing the open splenic circulation in humans. *Histochem. Cell Biol.* **128,** 391–398 (2007).

78. Steiniger, B., Ulfig, N., Risse, M. & Barth, P. J. Fetal and early post-natal development of the human spleen: from primordial arterial B cell lobules to a non-segmented organ. *Histochem. Cell Biol.* **128,** 205–215 (2007).

79. Vellguth, S. The development of the human spleen. *Cell Tissue Res.* 579–592 (1985).

80. Wilkins, B. S., Green, A., Wild, a E. & Jones, D. B. Extramedullary haemopoiesis in fetal and adult human spleen: a quantitative immunohistological study. *Histopathology* **24,** 241–247 (1994).

81. Mebius, R. E., Nolte, M. A. & Kraal, G. Development and function of the splenic marginal zone. *Crit. Rev. Immunol.* **24,** 449–464 (2004).

82. Vale, A. M. & Schroeder, H. W. Clinical consequences of defects in B-cell development. *J. Allergy Clin. Immunol.* **125,** 778–787 (2010).

83. Dunn-Walters, D. K. & Ademokun, A. a. B cell repertoire and ageing. *Curr. Opin. Immunol.* **22,** 514–520 (2010).

84. Nuñez, C. *et al.* B cells are generated throughout life in humans. *J. Immunol.* **156,** 866–872 (1996).

85. Nagasawa, T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat. Rev. Immunol.* **6,** 107–116 (2006).

86. Matthias, P. & Rolink, A. G. Transcriptional networks in developing and mature B cells. *Nat. Rev. Immunol.* **5,** 497–508 (2005).

87. Hagman, J. & Lukin, K. Early B-cell factor “pioneers” the way for B-cell development. *Trends Immunol.* **26,** 455–461 (2005).

88. Marie-Cardine, A. *et al.* Transitional B cells in humans: characterization and insight from B lymphocyte reconstitution after hematopoietic stem cell transplantation. *Clin. Immunol.* **127,** 14–25 (2008).

89. Pillai, S. & Cariappa, A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat. Rev. Immunol.* **9,** 767–777 (2009).

90. Schiemann, B. *et al.* An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* **293,** 2111–2114 (2001).

91. Mackay, F. & Schneider, P. Cracking the BAFF code. *Nat. Rev. Immunol.* **9,** 491–502 (2009).

92. Cariappa, A., Pillai, S., General, M., Cancer, H. & Medical, H. Antigen-dependent B-cell development. *Curr. Opin. Immunol.* **14,** 241–249 (2002).

93. Martin, F. & Kearney, J. F. Positive Selection from Newly Formed to Marginal Zone B Cells Depends on the Rate of Clonal Production, CD19, and btk. *Immunity* **12,** 39–49 (2000).

94. Thomas, M. D., Srivastava, B. & Allman, D. Regulation of peripheral B cell maturation. *Cell. Immunol.* **239,** 92–102 (2006).

95. Wen, L. *et al.* Evidence of marginal-zone B cell-positive selection in spleen. *Immunity* **23,** 297–308 (2005).

96. Tanigaki, K. *et al.* Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat. Immunol.* **3,** 443–450 (2002).

97. Hozumi, K. *et al.* Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat. Immunol.* **5,** 638–644 (2004).

98. Tan, J. B. *et al.* Lunatic and manic fringe cooperatively enhance marginal zone B cell precursor competition for delta-like 1 in splenic endothelial niches. *Immunity* **30,** 254–263 (2009).

99. Scheeren, F. A. *et al.* T cell-independent development and induction of somatic hypermutation in human IgM+ IgD+ CD27+ B cells. *J. Exp. Med.* **205,** 2033–2042 (2008).

100. Descatoire, M. *et al.* Identification of a human splenic marginal zone B cell precursor with NOTCH2-dependent differentiation properties. *J. Exp. Med.* **211,** 987–1000 (2014).

101. Weller, S. *et al.* IgM+IgD+CD27+ B cells are markedly reduced in IRAK-4-, MyD88-, and TIRAP- but not UNC-93B-deficient patients. *Blood* **120,** 4992–5001 (2012).

102. Capolunghi, F. *et al.* CpG drives human transitional B cells to terminal differentiation and production of natural antibodies. *J. Immunol.* **180,** 800–808 (2008).

103. Tierens, a, Delabie, J., Michiels, L., Vandenberghe, P. & De Wolf-Peeters, C. Marginal-zone B cells in the human lymph node and spleen show somatic hypermutations and display clonal expansion. *Blood* **93,** 226–234 (1999).

104. Dunn-Walters, D. K., Isaacson, P. G. & Spencer, J. Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells. *J. Exp. Med.* **182,** 559–566 (1995).

105. Seifert, M. & Küppers, R. Molecular footprints of a germinal center derivation of human IgM+(IgD+)CD27+ B cells and the dynamics of memory B cell generation. *J. Exp. Med.* **206,** 2659–2669 (2009).

106. Weller, S. *et al.* Somatic diversification in the absence of antigen-driven responses is the hallmark of the IgM+ IgD+ CD27+ B cell repertoire in infants. *J. Exp. Med.* **205,** 1331–1342 (2008).

107. Agematsu, K. *et al.* Absence of IgD-CD27(+) memory B cell population in X-linked hyper-IgM syndrome. *J. Clin. Invest.* **102,** 853–860 (1998).

108. Weller, S. *et al.* CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1166–1170 (2001).

109. Willerford, D. M., Swat, W. & Alt, F. W. Developmental regulation of V(D)J recombination and lymphocyte differentiation. *Curr. Opin. Genet. Dev.* **6,** 603–609 (1996).

110. Gellert, M. V(D)J recombination: RAG proteins, repair factors, and regulation. *Annu. Rev. Biochem.* **71,** 101–132 (2002).

111. Van der Burg, M. Ordered recombination of immunoglobulin light chain genes occurs at the IGK locus but seems less strict at the IGL locus. *Blood* **97,** 1001–1008 (2001).

112. Alt, F. W. *et al.* Ordered rearrangement of immunoglobulin heavy chain variable region segments. *EMBO J.* **3,** 1209–1219 (1984).

113. Tonegawa, S. Somatic generation of antibody diversity. *Nature* **302,** 575–581 (1983).

114. Li, A. *et al.* Utilization of Ig heavy chain variable, diversity, and joining gene segments in children with B-lineage acute lymphoblastic leukemia: implications for the mechanisms of VDJ recombination and for pathogenesis. *Blood* **103,** 4602–4609 (2004).

115. Fugmann, S. D. Form follows function - the three-dimensional structure of antigen receptor gene loci. *Curr. Opin. Immunol.* **27C,** 33–37 (2014).

116. Berberich, I., Shu, G. L. G. & Clark, E. E. A. Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. *J. Immunol.* **153,** 4357–4366 (1994).

117. Klein, U. & Dalla-Favera, R. Germinal centres: role in B-cell physiology and malignancy. *Nat. Rev. Immunol.* **8,** 22–33 (2008).

118. Schaerli, P. *et al.* CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J. Exp. Med.* **192,** 1553–1562 (2000).

119. Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* **192,** 1545–1552 (2000).

120. Nurieva, R. I. *et al.* Bcl6 mediates the development of T follicular helper cells. *Science* **325,** 1001–1005 (2009).

121. Johnston, R. J. *et al.* Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* **325,** 1006–1010 (2009).

122. Linterman, M. A. *et al.* IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J. Exp. Med.* **207,** 353–363 (2010).

123. 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).

124. Iciek, L. A., Delphin, S. A. & Stavnezer, J. CD40 cross-linking induces Ig epsilon germline transcripts in B cells via activation of NF-kappaB: synergy with IL-4 induction. *J. Immunol.* **158,** 4769–4779 (1997).

125. Davies, L. C., Jenkins, S. J., Allen, J. E. & Taylor, P. R. Tissue-resident macrophages. *Nat. Immunol.* **14,** 986–995 (2013).

126. Hauser, A. E. *et al.* Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns. *Immunity* **26,** 655–667 (2007).

127. Muramatsu, M. *et al.* Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102,** 553–563 (2000).

128. Rogozin, I. B. & Kolchanov, N. a. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. *Biochim. Biophys. Acta* **1171,** 11–18 (1992).

129. Di Noia, J. M. & Neuberger, M. S. Molecular mechanisms of antibody somatic hypermutation. *Annu. Rev. Biochem.* **76,** 1–22 (2007).

130. Haynes, B. F., Kelsoe, G., Harrison, S. C. & Kepler, T. B. B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. *Nat. Biotechnol.* **30,** 423–433 (2012).

131. Kolar, G. R., Mehta, D., Pelayo, R. & Capra, J. D. A novel human B cell subpopulation representing the initial germinal center population to express AID. *Blood* **109,** 2545–2552 (2007).

132. Allen, C. D. C., Okada, T., Tang, H. L. & Cyster, J. G. Imaging of germinal center selection events during affinity maturation. *Science* **315,** 528–531 (2007).

133. Victora, G. D. *et al.* Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* **143,** 592–605 (2010).

134. Allen, C. D. C. *et al.* Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat. Immunol.* **5,** 943–952 (2004).

135. Liu, Y. J. *et al.* Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. *Eur. J. Immunol.* **21,** 1905–1910 (1991).

136. Klein, U. *et al.* Transcriptional analysis of the B cell germinal center reaction. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2639–2644 (2003).

137. Vinuesa, C. G., Sanz, I. & Cook, M. C. Dysregulation of germinal centres in autoimmune disease. *Nat. Rev. Immunol.* **9,** 845–857 (2009).

138. Martinez-Valdez, H. *et al.* Human germinal center B cells express the apoptosis-inducing genes Fas, c-myc, P53, and Bax but not the survival gene bcl-2. *J. Exp. Med.* **183,** 971–977 (1996).

139. Hao, Z. *et al.* Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity* **29,** 615–627 (2008).

140. Cerutti, A., Cols, M. & Puga, I. Activation of B cells by non-canonical helper signals. *EMBO Rep.* **13,** 798–810 (2012).

141. Weller, S. *et al.* Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* **104,** 3647–3654 (2004).

142. Castigli, E. *et al.* TACI and BAFF-R mediate isotype switching in B cells. *J. Exp. Med.* **201,** 35–39 (2005).

143. Dunnick, W., Hertz, G. G. Z., Scappino, L. & Gritzmacher, C. DNA sequences at immunoglobulin switch region recombination sites. *Nucleic Acids Res.* **21,** 365–372 (1993).

144. Kinoshita, K., Honjo, T. & Chemistry, M. Unique and unprecedented recombination mechanisms in class switching. *Curr. Opin. Immunol.* **12,** 195–198 (2000).

145. Stavnezer, J., Guikema, J. E. J. & Schrader, C. E. Mechanism and regulation of class switch recombination. *Annu. Rev. Immunol.* **26,** 261–292 (2008).

146. Chaudhuri, J. & Alt, F. W. Class-switch recombination: interplay of transcription, DNA deamination and DNA repair. *Nat. Rev. Immunol.* **4,** 541–552 (2004).

147. Ehrenstein, M. R., Rada, C., Jones, A. M., Milstein, C. & Neuberger, M. S. Switch junction sequences in PMS2-deficient mice reveal a microhomology-mediated mechanism of Ig class switch recombination. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 14553–14558 (2001).

148. Defrance, T. *et al.* Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J. Exp. Med.* **175,** 671–682 (1992).

149. Zan, H., Cerutti, A., Dramitinos, P., Schaffer, A. & Casali, P. CD40 engagement triggers switching to IgA1 and IgA2 in human B cells through induction of endogenous TGF-beta: evidence for TGF-beta but not IL-10-dependent direct S mu-->S alpha and sequential S mu-->S gamma, S gamma-->S alpha DNA recombination. *J. Immunol.* **161,** 5217–5225 (1998).

150. Geha, R. S., Jabara, H. H. & Brodeur, S. R. The regulation of immunoglobulin E class-switch recombination. *Nat. Rev. Immunol.* **3,** 721–732 (2003).

151. Wood, N. *et al.* IL-21 effects on human IgE production in response to IL-4 or IL-13. *Cell. Immunol.* **231,** 133–145

152. Pène, J. *et al.* Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. *J. Immunol.* **172,** 5154–5157 (2004).

153. Brière, F., Servet-Delprat, C., Bridon, J. M., Saint-Remy, J. M. & Banchereau, J. Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. *J. Exp. Med.* **179,** 757–762 (1994).

154. Castigli, E. *et al.* CD40 ligand/CD40 deficiency. *Int. Arch. Allergy Immunol.* **107,** 37–39

155. Gulino, A. V. & Notarangelo, L. D. Hyper IgM syndromes. *Curr. Opin. Rheumatol.* **15,** 422–429 (2003).

156. Litinskiy, M. B. *et al.* DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat. Immunol.* **3,** 822–829 (2002).

157. Teng, G. & Papavasiliou, F. N. Immunoglobulin somatic hypermutation. *Annu. Rev. Genet.* **41,** 107–120 (2007).

158. Muramatsu, M. *et al.* Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J. Biol. Chem.* **274,** 18470–18476 (1999).

159. Maul, R. W. & Gearhart, P. J. AID and somatic hypermutation. *Adv. Immunol.* **105,** 159–191 (2010).

160. Besmer, E., Gourzi, P. & Papavasiliou, F. N. The regulation of somatic hypermutation. *Curr. Opin. Immunol.* **16,** 241–245 (2004).

161. Fagarasan, S. T-Independent Immune Response: New Aspects of B Cell Biology. *Science (80-.).* **290,** 89–92 (2000).

162. Fehr, T. *et al.* T-independent activation of B cells by vesicular stomatitis virus: no evidence for the need of a second signal. *Cell. Immunol.* **168,** 184–192 (1996).

163. Bachmann, M. F., Hengartner, H. & Zinkernagel, R. M. T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: role of antigen patterns in B cell induction? *Eur. J. Immunol.* **25,** 3445–3451 (1995).

164. O'Neill, L. a J., Golenbock, D. & Bowie, A. G. The history of Toll-like receptors - redefining innate immunity. *Nat. Rev. Immunol.* **13,** 453–460 (2013).

165. Bekeredjian-Ding, I. & Jego, G. Toll-like receptors--sentries in the B-cell response. *Immunology* **128,** 311–323 (2009).

166. Vinuesa, C. G. & Chang, P.-P. Innate B cell helpers reveal novel types of antibody responses. *Nat. Immunol.* **14,** 119–126 (2013).

167. Steiniger, B., Timphus, E. M. & Barth, P. J. The splenic marginal zone in humans and rodents: an enigmatic compartment and its inhabitants. *Histochem. Cell Biol.* **126,** 641–648 (2006).

168. Prabagar, M. G. *et al.* SIGN-R1, a C-type lectin, enhances apoptotic cell clearance through the complement deposition pathway by interacting with C1q in the spleen. *Cell Death Differ.* **20,** 535–545 (2013).

169. Hartnell, a. Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. *Blood* **97,** 288–296 (2001).

170. Mahnke, K. *et al.* The Dendritic Cell Receptor for Endocytosis, DEC-205, Can Recycle and Enhance Antigen Presentation via Major Histocompatibility Complex Class II-positive Lysosomal Compartments. *J. Cell Biol.* **151,** 673–684 (2000).

171. Scapini, P., Bazzoni, F. & Cassatella, M. a. Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils. *Immunol. Lett.* **116,** 1–6 (2008).

172. Bernasconi, N. L., Onai, N. & Lanzavecchia, A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* **101,** 4500–4504 (2003).

173. Bryant, V. L. *et al.* Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J. Immunol.* **179,** 8180–8190 (2007).

174. Cinamon, G. *et al.* Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat. Immunol.* **5,** 713–720 (2004).

175. Xu, W. *et al.* Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. *Nat. Immunol.* **8,** 294–303 (2007).

176. He, B. *et al.* The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. *Nat. Immunol.* **11,** 836–845 (2010).

177. Avery, D. T. *et al.* BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. *J. Clin. Invest.* **112,** 286–297 (2003).

178. Jego, G. *et al.* Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity* **19,** 225–234 (2003).

179. Xu, W. *et al.* Macrophages induce differentiation of plasma cells through CXCL10/IP-10. *J. Exp. Med.* **209,** 1813–1823 (2012).

180. Toellner, K. M., Gulbranson-Judge, A., Taylor, D. R., Sze, D. M. & MacLennan, I. C. Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B cell activation. *J. Exp. Med.* **183,** 2303–2312 (1996).

181. García De Vinuesa, C. *et al.* Dendritic cells associated with plasmablast survival. *Eur. J. Immunol.* **29,** 3712–3721 (1999).

182. Mueller, S. N. & Germain, R. N. Stromal cell contributions to the homeostasis and functionality of the immune system. *Nat. Rev. Immunol.* **9,** 618–629 (2009).

183. Ellyard, J. I., Avery, D. T., Mackay, C. R. & Tangye, S. G. Contribution of stromal cells to the migration, function and retention of plasma cells in human spleen: potential roles of CXCL12, IL-6 and CD54. *Eur. J. Immunol.* **35,** 699–708 (2005).

184. Burke, jS & Simon, G. Electron Microscopy of the Spleen. *Am. J. Pathol.* **58,** 451–469 (1970).

185. Gentleman, R. C. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5,** R80 (2004).

186. Ritchie, M. E. *et al.* A comparison of background correction methods for two-colour microarrays. *Bioinformatics* **23,** 2700–2707 (2007).

187. Smyth, G. K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3,** Article3 (2004).

188. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc.* **57,** 289–300 (1995).

189. He, B. *et al.* HIV-1 envelope triggers polyclonal Ig class switch recombination through a CD40-independent mechanism involving BAFF and C-type lectin receptors. *J. Immunol.* **176,** 3931–3941 (2006).

190. Martinez-Pomares, L. & Hanitsch, L. Expression of mannose receptor and ligands for its cysteine-rich domain in venous sinuses of human spleen. *Lab. Investig.* 1238–1249 (2005). doi:10.1038/labinvest.3700327

191. 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).

192. Udroiu, I. Evolution of sinusal and non-sinusal spleens of mammals. *Hystrix It J Mamm* **17,** 99–116 (2006).

193. Duijvestijn, A. M. *et al.* High endothelial differentiation in human lymphoid and inflammatory tissues defined by monoclonal antibody HECA-452. *Am. J. Pathol.* **130,** 147–155 (1988).

194. Streeter, P. R., Rouse, B. T. & Butcher, E. C. Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biol.* **107,** 1853–1862 (1988).

195. Mebius, R. E., Streeter, P. R., Michie, S., Butcher, E. C. & Weissman, I. L. A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits CD4+ CD3- cells to colonize lymph nodes. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11019–11024 (1996).

196. López-Fraga, M., Fernández, R., Albar, J. P. & Hahne, M. Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase. *EMBO Rep.* **2,** 945–951 (2001).

197. Bossen, C. *et al.* Mutation of the BAFF furin cleavage site impairs B-cell homeostasis and antibody responses. *Eur. J. Immunol.* **41,** 787–797 (2011).

198. Rondaij, M. G., Bierings, R., Kragt, A., van Mourik, J. A. & Voorberg, J. Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **26,** 1002–1007 (2006).

199. Bossen, C. & Schneider, P. BAFF, APRIL and their receptors: structure, function and signaling. *Semin. Immunol.* **18,** 263–275 (2006).

200. Thompson, J. S. *et al.* BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science* **293,** 2108–2111 (2001).

201. He, B., Qiao, X. & Cerutti, A. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. *J. Immunol.* **173,** 4479–4491 (2004).

202. Pone, E. J. *et al.* BCR-signalling synergizes with TLR-signalling for induction of AID and immunoglobulin class-switching through the non-canonical NF-κB pathway. *Nat. Commun.* **3,** 767 (2012).

203. Balázs, 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).

204. Manis, J. J. P., Tian, M. & Alt, F. W. F. Mechanism and control of class-switch recombination. *Trends Immunol.* **23,** 31–39 (2002).

205. Pillai, S., Cariappa, A. & Moran, S. T. Marginal zone B cells. *Annu. Rev. Immunol.* **23,** 161–196 (2005).

206. Sneed, T. B., Stanley, D. J., Young, L. A. & Sanderson, R. D. Interleukin-6 regulates expression of the syndecan-1 proteoglycan on B lymphoid cells. *Cell. Immunol.* **153,** 456–67 (1994).

207. Tanaka, Y., Saito, Y. & Gotoh, H. Vascular architecture and intestinal hematopoietic nests of two cyclostomes, Eptatretus burgeri and ammoncoetes of Entosphenus reissneri: a comparative morphological study. *J. Morphol.* **170,** 71–93 (1981).

208. Jønsson, V. Comparison and definition of spleen and lymph node: a phylogenetic analysis. *J. Theor. Biol.* **117,** 691–699 (1985).

209. Weiss, L. Transmural cellular passage in vascular sinuses of rat bone marrow. *Blood* **36,** 189–208 (1970).

210. Barnhart, M. I. & Lusher, J. M. The human spleen as revealed by scanning electron microscopy. *Am. J. Hematol.* **1,** 243–264 (1976).

211. Fujita, T., Kashimura, M. & Adachi, K. Scanning electron microscopy (SEM) studies of the spleen--normal and pathological. *Scan. Electron Microsc.* 435–444 (1982). at <http://www.ncbi.nlm.nih.gov/pubmed/7167759>

212. Weibel, E. R. Fifty years of Weibel-Palade bodies: the discovery and early history of an enigmatic organelle of endothelial cells. *J. Thromb. Haemost.* **10,** 979–984 (2012).

213. Asahara, T. Isolation of Putative Progenitor Endothelial Cells for Angiogenesis. *Science (80-.).* **275,** 964–966 (1997).

214. Kuwana, M. & Okazaki, Y. Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. *J. Leukoc. Biol.* **74,** 833–845 (2003).

215. Hristov, M. & Weber, C. Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. *J. Cell. Mol. Med.* **8,** 498–508 (2004).

216. Harraz, M., Jiao, C., Hanlon, H. D., Hartley, R. S. & Schatteman, G. C. CD34- blood-derived human endothelial cell progenitors. *Stem Cells* **19,** 304–312 (2001).

217. Joffre, O. P., Segura, E., Savina, A. & Amigorena, S. Cross-presentation by dendritic cells. *Nat. Rev. Immunol.* **12,** 557–569 (2012).

218. Villadangos, J. A. & Shortman, K. Found in translation: the human equivalent of mouse CD8+ dendritic cells. *J. Exp. Med.* **207,** 1131–1134 (2010).

219. Aderem, a & Underhill, D. M. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* **17,** 593–623 (1999).

220. Lee, S. J. *et al.* Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science* **295,** 1898–1901 (2002).

221. Hakomori, S. Antigen structure and genetic basis of histo-blood groups A, B and O: their changes associated with human cancer. *Biochim. Biophys. Acta* **1473,** 247–266 (1999).

222. Chavele, K. *et al.* Mannose receptor interacts with Fc receptors and is critical for the development of crescentic glomerulonephritis in mice. *J. Clin. Invest.* **120,** 1469–1478 (2010).

223. Bergtold, A., Desai, D. D., Gavhane, A. & Clynes, R. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. *Immunity* **23,** 503–514 (2005).

224. Jiskoot, P. M. C., Halsey, C., Rivers, R., Bain, B. J. & Wilkins, B. S. Unusual splenic sinusoidal iron overload in sickle cell/haemoglobin D-Punjab disease. *J. Clin. Pathol.* **57,** 539–540 (2004).

225. Kong, X.-N. *et al.* LPS-induced down-regulation of signal regulatory protein {alpha} contributes to innate immune activation in macrophages. *J. Exp. Med.* **204,** 2719–2731 (2007).

226. Baba, N. *et al.* CD47 fusion protein targets CD172a+ cells in Crohn's disease and dampens the production of IL-1β and TNF. *J. Exp. Med.* **210,** 1251–1263 (2013).

227. Legrand, N., Huntington, N. D., Nagasawa, M., Bakker, A. Q. & Schotte, R. Functional CD47/signal regulatory protein alpha (SIRPα) interaction is required for optimal human T- and natural killer- (NK) cell homeostasis in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 13224–13229 (2011).

228. Matozaki, T., Murata, Y., Okazawa, H. & Ohnishi, H. Functions and molecular mechanisms of the CD47-SIRPalpha signalling pathway. *Trends Cell Biol.* **19,** 72–80 (2009).

229. Roussilhon, C. *et al.* Long-term clinical protection from falciparum malaria is strongly associated with IgG3 antibodies to merozoite surface protein 3. *PLoS Med.* **4,** e320 (2007).

230. McLay, J. *et al.* Gamma 3 gene-disrupted mice selectively deficient in the dominant IgG subclass made to bacterial polysaccharides. II. Increased susceptibility to fatal pneumococcal sepsis due to absence of anti-

polysaccharide IgG3 is corrected by induction of anti-polysa. *J. Immunol.* **168,** 3437–43 (2002).

231. Tarkowski, A. *et al.* Immunization of humans with polysaccharide vaccines induces systemic, predominantly polymeric IgA2-subclass antibody responses. *J. Immunol.* **144,** 3770–3778 (1990).

232. Carson, P. J., Schut, R. L., Simpson, M. L., O'Brien, J. & Janoff, E. N. Antibody class and subclass responses to pneumococcal polysaccharides following immunization of human immunodeficiency virus-infected patients. *J. Infect. Dis.* **172,** 340–345 (1995).

233. Chu, V. T. *et al.* Eosinophils are required for the maintenance of plasma cells in the bone marrow. *Nat. Immunol.* **12,** 151–159 (2011).

234. Ellyard, J. I. *et al.* Antigen-selected, immunoglobulin-secreting cells persist in human spleen and bone marrow. *Blood* **103,** 3805–3812 (2004).

235. Jones, D. & Benjamin, R. The chemokine receptor CXCR3 is expressed in a subset of B-cell lymphomas and is a marker of B-cell chronic lymphocytic leukemia. *Blood* **95,** 627–632 (2000).

236. Kobayashi, Y. The role of chemokines in neutrophil biology. *Front. Biosci.* **13,** 2400–2407 (2008).

237. Godaly, G. *et al.* Neutrophil recruitment, chemokine receptors, and resistance to mucosal infection. *J. Leukoc. Biol.* **69,** 899–906 (2001).

238. Sánchez-Navarro, M. & Rojo, J. Targeting DC-SIGN with carbohydrate multivalent systems. *Drug News Perspect.* **23,** 557–572 (2010).

ANNEX 2

Abbreviations

AID	Activation-induced cytidine deaminase
ADCC	Antibody-dependent cellular cytotoxicity
APE	Apurinic/apyrimidinic endonuclease
APRIL	A proliferation-inducing ligand
BAFF	B-cell–activating factor of the TNF family
BAFF-R	BAFF receptor
BCMA	B-cell maturation antigen
BCR	B-cell receptor
BTK	Bruton’s tyrosine kinase
CDR	Complementarity-determining region
CFM	Confocal fluorescence microscopy
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CLRs	C-type lectin receptors
CPS	Capsular polysaccharide
CSR	Class switch recombination
DAPI	4’-6-diamidine-2’-phenylindole
DCs	Dendritic cells
DLL1	Delta-like protein 1

DSBs	Double-strand breaks
FcR	Immunoglobulin receptor
FDCs	Follicular dendritic cells
FHOD1	Formin homology 2 domain containing 1
FRC	Fibroblastic reticular cells
GC	Germinal center
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HSCs	Hematopoietic stem cells
IFA	Immunofluorescence analysis
IFN	Interferon
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IHC	Immunohistochemistry
IL	Interleukin
ILCs	Innate lymphoid cell
IRF	Interferon-regulatory factor
ITAM	Immune receptor tyrosine-based activation motif
LPS	Lipopolysaccharide
LT	Lymphotoxin
MBL	Mannose-binding lectin
MCs	Myeloid cells
MHC	Major histocompatibility complex

MR	Mannose receptor
MRC	Marginal reticular cells
MZ	Marginal zone
N_{BH}	B helper neutrophils
NF-κB	Nuclear factor-kappa B
NK	Natural killer
PALS	Periarteiolar lymphoid sheath
PAMPs	Pathogen associated molecular patterns
PAX5	Paired box protein 5
PFZ	Perifollicular zone
PRRs	Pattern recognition receptors
RAG	Recombination activation gene
RSSs	Recombination signal sequences
S	Switch regions
SIgA	Secretory IgA
SHM	Somatic hypermutation
SIRP-α	Signal regulatory protein α
SLCs	Sinusoid lining cells
SMA	Smooth muscle actinin
TACI	Calcium-modulating cyclophilin ligand interactor
TCR	T-cell receptor
TD	T-cell–dependent

T_{FH}	T follicular helper
TI	T-cell independent
TNF	Tumor necrosis factor
TLRs	Toll-like receptors
UNG	Uracil DNA glycosylase
V(D)J	Variable diversity and joining gene segments
vWF	von Willebrand factor

ANNEX III

Publications

1. Harrak Y, **Barra CM**, Bedia C, Delgado A, Castaño AR, Llebaria A. Aminocyclitol-substituted phytoceramides and their effects on iNKT cell stimulation. *ChemMedChem.* 2009 Oct; 4(10):1608-13.

2. Harrak Y, **Barra CM**, Delgado A, Castaño AR, Llebaria A. Galacto-configured aminocyclitol phytoceramides are potent in vivo invariant natural killer T cell stimulators. *J Am Chem Soc.* 2011 Aug 10; 133(31):12079-84.

3. Cerutti A, Cols M, Gentile M, Cassis L, **Barra CM**, He B, Puga I, Chen K. Regulation of mucosal IgA responses: lessons from primary immunodeficiencies. *Ann N Y Acad Sci.* 2011 Nov; 1238:132-44.

4. Puga, I., Cols, M., **Barra, C.M.**, He, B., Cassis, L., Gentile, M., Comerma, L., Chorny, A., Shan, M., Xu, W., Magri, G., Knowles, D.M., Tam, W., Chiu, A., Bussel, J.B., Serrano, S., Lorente, J.A., Bellosillo, B., Lloreta, J., Juanpere, N., Alameda, F., Baró, T., De Heredia, C.D., Torán, N., Català, A., Torrebadell, M., Fortuny, C., Cusí, V., Carreras, C., Diaz, G.A., Blander, J.M., Farber, C.-M., Silvestri, G., Cunningham-Rundles, C., Calvillo, M., Dufour, C., Notarangelo, L.D., Lougaris, V., Plebani, A., Casanova, J.-L., Ganal, S.C., Diefenbach, A., Aróstegui, J.I., Juan, M., Yagüe, J., Mahlaoui, N., Donadieu, J., Chen, K., Cerutti, A. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nature Immunology* 2012, 13 (2): 170-180.

5. Kerzerho J, Yu ED, **Barra CM**, Alari-Pahissa E, Girardi E, Harrak Y, Lauzurica P, Llebaria A, Zajonc DM, Akbari O, Castaño AR. Structural and functional characterization of a novel nonglycosidic type I NKT agonist with immunomodulatory properties. *J Immunol.* 2012 Mar 1;188(5):2254-65.

6. Cols M, **Barra CM**, He B, Puga I, Xu W, Chiu A, Tam W, Knowles DM, Dillon SR, Leonard JP, Furman RR, Chen K, Cerutti A. Stromal endothelial cells establish a bidirectional crosstalk with chronic lymphocytic leukemia cells through the TNF-related factors BAFF, APRIL, and CD40L. *J Immunol.* 2012 Jun 15;188(12):6071-83.

7. Magri G, Miyajima M, Bascones S, Mortha A, Puga I, Cassis L, **Barra CM**, Comerma L, Chudnovskiy A, Gentile M, Llige D, Cols M, Serrano S, Aróstegui JI, Juan M, Yagüe J, Merad M, Fagarasan S, Cerutti A. Innate lymphoid cells integrate stromal and immunological signals to enhance antibody production by splenic marginal zone B cells. *Nature Immunology* 2014 Feb 23.