Defining the role of CD47 and SIRP\(\alpha\) in murine B cell homeostasis

Shrikant Shantilal Kolan
Dedicated to my Family
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Abstract

B cell development is a highly organized process, which commences in the fetal liver during embryogenesis and in the bone marrow (BM) after birth. Surface IgM+ immature B cells emigrate from the BM via the blood stream to the spleen and finally differentiate into conventional mature follicular B (FoB) cells and marginal zone (MZ) B cells. Conversely, some sIgM+ immature B cells can also mature into IgD+ FoB cells in the BM.

The ubiquitously expressed cell surface glycoprotein CD47 and its receptor signal regulatory protein α (SIRPα) are members of the immunoglobulin superfamily. Both individually and upon their interaction, CD47 and SIRPα have been found to play important role in the homeostasis of T lymphocytes or CD8+ conventional dendritic cells (cDCs) in secondary lymphoid organs. However, their role in regulating B cell homeostasis has remained unknown.

The present study describes important roles of CD47 and SIRPα in B cell homeostasis. Lack of SIRPα signaling in adult SIRPα mutant (MT - cytoplasmic domain deletion) mice resulted in an impaired B cell maturation in the BM and spleen, which was also reflected in the blood. In the BM and spleen of SIRPα MT mice, reduced numbers of semi-mature IgD+IgM$^{hi}$ follicular type-II (F-II) and mature IgD+IgM$^{lo}$ follicular type-I (F-I) B cells were observed, while earlier BM B cell progenitors or splenic transitional B cells remained unaltered. In SIRPα MT mice, maturing B cells in BM and spleen were found to express higher levels of the pro-apoptotic protein BIM and contained an increased level of apoptotic cells.

In contrast to that for FoB cells, the splenic MZ B cell population was increased with age in SIRPα MT mice without showing an increased level of activation markers. Immunohistochemical analysis revealed an increased follicular localization of MZ B cells in the spleens of SIRPα MT mice. In addition, MZ macrophages and marginal metallophilic macrophages were not restricted to their normal position in SIRPα MT spleens. Interestingly, CD47-deficient (CD47-/-) mice mimicked the FoB cell phenotype observed in SIRPα MT mice and had a reduced number of FoB cells in the BM, blood and the spleen at 5-6 months of age, but not in younger mice. Similar to SIRPα MT mice, CD47-/- mice also displayed an increased number of splenic MZ B cells. Sera form both mouse strains did not show any signs of an increased production of autoantibodies or antinuclear antigens.

BM reconstitution experiments identified a requirement for non-hematopoietic SIRPα signaling for normal B cell maturation in the BM and
to maintain normal numbers and retention of MZ B cells in the splenic MZ. On the contrary, hematopoietic SIRPα signaling appeared to be important for FoB cell maturation in the spleen. Interestingly, hematopoietic SIRPα was required for normal MZ retention of MZ macrophages while normal distribution of metallophilic macrophages required non-hematopoietic SIRPα signaling.

Collectively, these findings revealed an important role of CD47 and of SIRPα signaling in B cell homeostasis in different lymphoid organs.
## Abbreviations

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<tr>
<td>CLP</td>
<td>Common lymphoid progenitors</td>
</tr>
<tr>
<td>IL-7R</td>
<td>Interleukin-7 receptor</td>
</tr>
<tr>
<td>RAG-1/RAG-2</td>
<td>Recombinase activating gene 1 and 2</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>MZ B</td>
<td>Marginal zone B cells</td>
</tr>
<tr>
<td>FoB</td>
<td>Follicular B cells</td>
</tr>
<tr>
<td>T1/2/3</td>
<td>Transitional type 1/2/3</td>
</tr>
<tr>
<td>MZM</td>
<td>Marginal zone macrophages</td>
</tr>
<tr>
<td>MMM</td>
<td>Marginal metallophilic macrophages</td>
</tr>
<tr>
<td>SIRPα</td>
<td>Signal regulatory protein α</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor-κB ligand</td>
</tr>
<tr>
<td>ANA</td>
<td>Antinuclear antigens</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cell</td>
</tr>
<tr>
<td>ASC</td>
<td>Antibody secreting cell</td>
</tr>
<tr>
<td>F-I</td>
<td>Follicular type-I cell</td>
</tr>
<tr>
<td>F-II</td>
<td>Follicular type-II cell</td>
</tr>
<tr>
<td>T-D</td>
<td>T cell-dependent</td>
</tr>
<tr>
<td>T-I</td>
<td>T cell-independent</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>FRC</td>
<td>Fibroblastic reticular cell</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>PALS</td>
<td>Periarteriolar lymphoid sheath</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>MAdCAM-1</td>
<td>Mucosal addressin cell adhesion molecule 1</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular adhesion molecule 1</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1 phosphate</td>
</tr>
<tr>
<td>MRC</td>
<td>Marginal reticular cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-cell activating factor</td>
</tr>
<tr>
<td>MARCO</td>
<td>Macrophage receptor with collagenous structure</td>
</tr>
<tr>
<td>CXCL-13</td>
<td>CXC-chemokine ligand 13</td>
</tr>
<tr>
<td>SP</td>
<td>Surfactant protein</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
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List of original publications

The papers will be referred to in the text by their roman numerals.


III. **Kolan SS**, Lejon K and Oldenborg P-A. The Integrin Associated Protein CD47 Modulates Murine B cell Maturation. (Manuscript)
Background

Immunity, the immune system and immune cells

In day to day life, human beings get exposed to a diverse array of infectious agents such as bacteria, viruses and other microbes in different ways. These infectious organisms can cause serious damage to the infected host and in the worst case scenario they might be life threatening as well. To combat these organisms, mammalians have developed a complex and multifaceted immune system which keeps the host healthy by preventing infections.

The immune system is made up of a network of cells, tissues and organs, which work in tandem to protect the body. The immune system can be divided into two parts depending on the defense mechanisms which ensures host survival:

- Natural or innate immunity
- Acquired or adaptive immunity

Innate immunity refers to a nonspecific immune response and offers a first line of defense against infectious agents without any discrimination between them. These mechanisms include physical barriers at the surface of the body (e.g. skin), secreted molecules and specialized immune cells. The cellular components of the innate immune system are derived mainly from myeloid progenitors and include monocytes, macrophages, dendritic cells (DCs) and mast cells, as well as neutrophil, basophil and eosinophil granulocytes. All these specialized innate immune cells have the capacity to recognize evolutionarily conserved features of the foreign antigens and to quickly become activated to combat these invading antigens.

In contrast to innate immunity, the adaptive immune system is highly specific and generates immunological memory after an initial exposure to pathogens, which leads to an enhanced immune response to successive encounters with that antigen. Cells of the adaptive immune system come from the lymphoid lineage and are grouped into B lymphocytes (B cells) and T lymphocytes (T cells), each containing several subsets. A third lymphoid cell type is the natural killer (NK) cell, which is functionally considered to be part of the innate immune system. In addition, there are also more innate-like subsets of B cells (i.e. B1 cells and MZ B cells), which are described in more detail below.
The innate and adaptive immune systems form one integrated defence network and maximizes host defense. Macrophages and DCs recognize the conserved microbial molecular signatures via germline-encoded pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and present them to specific lymphocytes of the adaptive immune system to initiate defensive responses. In contrast to macrophages and DCs, lymphocytes recognize discrete antigenic epitopes in a specific but temporally delayed manner through antigenic receptors, the B cell receptor (BCR) or the T cell receptor (TCR), on their surfaces. These lymphocytes bind to invading pathogens, or molecules produced by them, and respond by proliferation and differentiation into memory or effector cells with an increased lifespan.

**Lymphoid organs**

The lymphoid system is traditionally divided into primary and secondary lymphoid organs. Primary lymphoid organs are the sites where lymphocytes are generated from progenitor cells and differentiate into mature or semi-mature naïve lymphocytes. In mammals, the bone marrow (BM) is the primary organ for B cell development, although the prenatal site of B cell differentiation is the fetal liver. The other primary lymphoid organ is the thymus, the site for development of thymic-derived lymphocytes, i.e. T cells. In this organ lymphoid progenitor cells, derived from the BM, develop into functional mature T cells (Crivellato et al 2004).

Mature or semi-mature T and B cells exit primary lymphoid organs and are transported via the bloodstream to the secondary lymphoid organs, where they become activated after encountering the antigens. Secondary lymphoid organs include the lymph nodes [which are clustered at sites of armpits, neck, groin and along the small intestine, and collect antigen from the tissues], the spleen [which collect antigens from the bloodstream], and mucosa-associated lymphoid tissue or MALT [consist of Peyers patches (PP), mesenteric lymph nodes (mLN) and skin-associated lymphoid tissue (SALT), which collect antigen from the respiratory, gastrointestinal and urogenital tracts].

**Primary lymphoid organs**

**Bone Marrow**

The BM is a soft tissue, which lies within the hollow interior of large bones. There are two different types of BM, the yellow BM rich in fat and the red BM maintaining hematopoiesis. The red BM supports the self-renewal and
differentiation of hematopoietic stem cells (HSCs) into mature blood cells. Even though all bones of the body contain marrow, the hip bones (ileum), long bones (femur, humerus) and sternum are the most active sites of hematopoiesis. BM stromal cells create distinct micro environmental niches within the BM that provide support for hematopoiesis and development of specific hematopoietic lineages. Mesenchymal stem cells are also present in the central sinus of the BM and have the capacity to form various cells of the body, including myocytes (forming muscles), osteoblasts (forming the bones), chondrocytes (forming cartilages) and other cells. It is widely accepted that mature myeloid cells, B cells and mature antibody secreting B cells (plasma cells; PC) can home to the BM.

**Thymus**

The thymus is located just behind the sternum in the upper part of the chest and is the primary lymphoid organ where T cells mature. T cell precursors travel via the blood from the BM to the thymus. Immature T cells, known as thymocytes pass through defined stages of development in the thymic microenvironment and finally mature into functional T cells. Mature thymocytes expressing either CD4 or CD8, also called single positive cells, leave the thymus via blood vessels and finally mature in the periphery where these naïve T cells explore antigens present in secondary lymphoid organs like the spleen or lymph nodes.

**Secondary lymphoid organs**

**Lymph nodes**

The lymph nodes are small, encapsulated, bean-shaped structures in which a network of stromal cells harbors lymphocytes and antigen-presenting cells (APCs) such as macrophages and DCs. Structurally, a lymph node can be divided into a cortex, a paracortex, and a medulla. The outermost layer, the cortex, contains lymphocytes (B cells), follicular dendritic cells (FDCs) and macrophages arranged in follicles. T cells congregate in the paracortical region. The medulla is the innermost layer and is populated by cells of the lymphoid lineage, including antibody-secreting plasma cells. Lymph nodes are well organized to encounter antigens, since they are connected to both lymphatic vessels and blood vessels. Lymphocytes enter the lymph node from the bloodstream in the paracortical area, while lymph containing antigens or activated DCs drains into the node through afferent lymphatic vessels and percolates through the lymph node. This enables antigens and APCs to come into contact with lymphocytes and to activate them. Activated
lymphocytes then exit the node through efferent lymphatic vessels and finally enter the bloodstream.

**Spleen**

The spleen is a large secondary lymphoid organ positioned behind the stomach in the abdominal cavity. In the spleen, blood-borne pathogens (antigens) are recognized and processed by different immune cells to eventually elicit an immune response. As the biggest lymphoid organ in the body, and also the biggest filter of the blood, the spleen contains approximately 25% of the total lymphocytes in the body. Histologically, the spleen is divided into three principal regions: the red pulp, the white pulp, and the marginal zone (MZ) (Fig. 4). The erythrocyte-rich red pulp area is physically located outside of the white pulp and is concentrated with macrophages which are highly phagocytic and remove aged or damaged erythrocytes (Mebius and Kraal 2005). The white pulp is organized as a T cell zone (peripheral lymphocyte sheath, PALS), and a B cell zone (follicle) (Allman et al 2004, Allman et al, 2008). The MZ, located in between the white pulp and the red pulp area, where a large fraction of the blood percolates into the spleen and it is important for sensing, trapping and responding to blood-borne antigens (Mebius and Kraal 2005).

**Mucosa-associated lymphoid tissue (MALT)**

It has been documented that about half of the lymphocytes in the immune system are present in the MALT. MALT is present along the surfaces of all mucosal tissues, but is abundant in the digestive, respiratory and urinary tracts, which are constantly exposed to potentially harmful microorganisms and therefore require their own system of antigen capture and presentation to lymphocytes [reviewed in (Cesta 2006)]. For example, Peyer’s patches (PPs) are round or oval shaped, small masses of lymphatic tissue found throughout the ileum region of the small intestine. Morphologically, PPs are separated into three main domains: the follicular area, the interfollicular area and follicle associated epithelium. The follicular and the interfollicular area of PPs consist of the lymphoid follicles with a germinal center (GC) containing FDCs, proliferating B cells and macrophages (Jung et al 2010). The structure of adult PPs closely resembles that of lymph nodes, with T and B cells segregating in distinct organized areas [reviewed in (Cesta 2006)].

**Mature B cell populations**

B cells play a central role in the adaptive immune response by producing antibodies against different kinds of antigens. Antibodies are soluble
proteins produced by differentiated B cells, i.e. plasma cells (PCs) or antibody secreting cells (ASCs), in response to foreign antigens (e.g. viruses or bacteria). These antibodies permeate through the body fluids to bind, neutralize and finally eliminate pathogens from the body with the aid of phagocytic cells. Based upon function and cell surface marker expression, naïve B cells are traditionally classified into three distinct mature populations: follicular (FoB); marginal zone (MZ) and B1 B cells. Some of the unique features of FoB, MZ B and B-1 cells are summarized in Table 1.

**FoB cells**

Naïve FoB cells circulate through lymph and blood, thus moving in between different lymphoid organs such as lymph nodes, Peyer's patches and the spleen. In the murine spleen, approximately 90% of the mature B cells are FoB cells, and are located in the splenic B cell follicles of the white pulp. FoB cells are comparatively long-lived (t1/2 approximately 4.5 months) (Hao and Rajewsky 2001). In secondary lymphoid organs, B cell follicles are always adjacent to T cell zones and this arrangement permits activated FoB cells and activated T helper cells to migrate towards each other and interact at the interface between these two areas. Therefore, FoB cells are mainly involved in T cell-dependent (T-D) immune responses to protein antigens, resulting in the production of highly specific antibodies [reviewed in (Allman and Pillai 2008)]. In the BM, FoB cells are positioned in organized aggregates around the BM sinusoids and can respond to blood borne pathogens in a T cell-independent (T-I) manner (Cariappa et al 2005).

**MZ B cells**

MZ B cells are larger cells and less abundant than the FoB cells (~5% of splenic B cells are MZ B cells), and are principally located in the splenic MZ (further described below). The close proximity to the rich blood flow of the MZ sinuses allows MZ B cells to respond quickly to blood-borne antigens [reviewed in (Mebius and Kraal 2005)]. MZ B cells differ from FoB cells in terms of their phenotype, function and location. Unlike FoB cells, MZ B cells do not leave the spleen and migrate to other organs. However, MZ B cells have been found to continuously shuttle between the splenic marginal zone and the B cell follicles, a behavior which is regulated by their responses to sphingosine 1-phosphosphate (S1P) and CXC-chemokine ligand 13 (CXCL-13) (further described below) (Cinamon et al 2008).
Table 1: Types and characteristics of mature B cells

<table>
<thead>
<tr>
<th>Functional property</th>
<th>FoB cells</th>
<th>MZ B cells</th>
<th>B1 B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Localization</strong></td>
<td>Spleen, lymph node, bone marrow</td>
<td>Splenic MZ</td>
<td>Peritoneal cavity, pleural cavity, spleen</td>
</tr>
<tr>
<td><strong>T-D response</strong></td>
<td>+++</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>T-I response</strong></td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Response to TLR-ligands</strong></td>
<td>Mild proliferation</td>
<td>Strong proliferation</td>
<td>Strong proliferation</td>
</tr>
<tr>
<td><strong>BCR response</strong></td>
<td>Proliferation</td>
<td>Death</td>
<td>Death</td>
</tr>
<tr>
<td><strong>Half life</strong></td>
<td>Comparatively low*</td>
<td>Long</td>
<td>Long</td>
</tr>
<tr>
<td><strong>Blood circulation</strong></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Ag presentation in vitro</strong></td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* In comparison with MZ B cells.


MZ B cells are long lived and they might survive as long as the host. Several studies have shown that the levels of MZ B cells are likely to be maintained either by self-renewing MZ B cells themselves or by cycling precursors (Hao and Rajewsky 2001). Functionally, MZ B cells respond rapidly to T-I antigens, but some studies have demonstrated that MZ B cells are also potent activators of naïve CD4+ T cells (Attanavanich and Kearney 2004). The fact that MZ B cells exhibit an activated phenotype and express a BCR biased against T-I bacterial antigens, enable these cells to rapidly proliferate and differentiate into short-lived IgM-secreting plasma cells (Lopes-Carvalho and Kearney 2004). Principally MZ B cells secrete antibodies of the IgM and IgG3 isotypes (Gunn and Brewer 2006).
**B1 B cells**

The B1 B cells represent only a minor fraction of total B cells, but constitute the major B cell population in the peritoneal and pleural cavities. B-1 cells have an IgM$^{hi}$IgD$^{lo}$CD23$^{lo}$B220$^{lo}$ phenotype. Unlike splenic B1 cells, peritoneal B1 cells express CD11b (Hardy and Hayakawa 2001). Two different subsets of B1 B cells can be phenotypically distinguished: CD5$^+$ B-1a cells and CD5$^-$ B-1b cells [reviewed in (Baumgarth et al 2010)]. B1 B cells are effectors of innate immunity and mainly respond to T-I type-2 (TI-2) antigens along with MZ B cells (Martin and Kearney 2000). B1a B cells are thought to develop from fetal liver precursors with a restricted BCR repertoire [reviewed in (Droshkind and Montecino-Rodriguez 2007)], whereas B1b cells may originate from B2 cells in a T-I manner and represent a specialized type of IgM memory cells (Alugupalli et al 2004). After activation, B1B cells from peritoneal and pleural cavities rapidly migrate into gut lymphoid tissues or spleen, where they lose CD5 expression and differentiate into antibody secreting plasma cells (Bikah et al 1996, Hippen et al 2000). Furthermore, B1 cells have self renewal potential [reviewed in (Baumgarth et al 2010)].

**Murine B cell development**

In both human and mice, B cells develop from pluripotent hematopoietic stem cells (HSCs) in the liver during fetal development and in the bone marrow after birth. The differentiation of precursor cells along the developmental pathway from B lineage-committed progenitors to the competent mature B cells is characterized by changes in the expression pattern of different surface markers (Fig. 1) (Nunez et al 1996; Hardy and Hayakawa 2001). However, since the work within this thesis has been focused on mouse models, I will in the following sections focus on murine B cell development and homeostasis.

**B cell development in the bone marrow**

The development into either T or B cells segregates after the common lymphoid progenitor (CLP) stage (Fig. 1), although CLPs still posses T (Allman et al 2003), NK (Kouro et al 2002) and DC (Karunsky et al 2003) potential. B cell progenitor downstream of the CLP is referred as a pre-pro B cells or fraction A (Fr. A). The two most commonly used nomenclatures to describe B cell development are the Philadelphia nomenclature (Hardy et al 1991, Hardy and Hayakawa 2001) and the Basel nomenclature (Osmond et al 1998). The major difference between these two is that instead of naming each developmental stage (Basel nomenclature), Hardy and colleagues
Figure 1. Bone marrow B cell development. B cells originate from hematopoietic stem cells (HSCs) in the bone marrow and differentiate into common lymphoid progenitors (CLPs) through several intermediate steps which are not depicted in this figure. CLPs further differentiate sequentially into precursor progenitor (Pre-pro B/Fr.A), progenitor (pro/Fr. B/C) B cells, precursor (pre/Fr.D) B cells and finally immature B cells (Fr.E).

named these developmental stages as fractions A (Fr. A) to F (Fr. F) (Fig. 1). Pre-pro (Fr. A) B cells express the B-lineage marker B220 (CD45R) along with many B-lineage-restricted genes with the exception of CD19 (Li et al 1996, Rumfelt et al 2006) and account for about 1% of the total nucleated BM cells (Hardy et al 1991). Pre-pro B cells display residual T-lineage potential (Martin et al 2003), but lack myeloid potential (Mansson et al 2008). After the level of pre-pro B cells, the next characterized precursors are the pro-B cells (Fr. B/C cells), which gain expression of CD19 (Rolink et al 1993; Rumfelt et al 2006). At this stage, D_H to J_H rearrangement is initiated at the immunoglobulin heavy chain (IgH) locus by up-regulating recombination activating gene 1 and 2 (Rag-1 and Rag-2) followed by recombining their variable (V) gene segments to previously rearranged D_H-J_H segments at the IgH locus (Mombaerts et al 1992; Shinkai et al 1992). Once IgH rearrangement is completed, the cell is classified as a pre-B cell (Fr.D) and it expresses the pre-BCR in which the rearranged μ heavy chain (μH) is combined with the surrogate light chain (VpreB, λ5) and an accessory signaling molecule (Igα, Igβ) to form the pre-B cell receptor (pre-BCR)
Pre-B cells re-express Rag1/2 and undergo immunoglobulin light chain (IgL) rearrangement (Ehlich et al 1994). IgLs are encoded by two gene loci, the IgLκ and the IgLλ locus. First, rearrangement takes place at the IgLκ locus and only if recombination was unproductive the segments at the IgLλ locus are rearranged (Geier and Schlissel 2006). Successful IgL rearrangement results in expression of the complete BCR composed of 2 heavy chain, 2 light chains and a functional signaling molecule ιγC and ιβ (also known as CD79A and CD79B). Expression of the pre-BCR is a crucial checkpoint in early B cell development, where newly generated μHCs are tested for functionality. Only pre-B cells expressing functional μHC are allowed to differentiate further into immature (Fr. E) B cells (Brouns et al 1993; Kline et al 1998). At the immature B cell stage, the BCR is tested for auto-reactivity and functionality. Potentially auto-reactive B cells and B cells expressing a non-functional μHC can be rescued by a mechanism called receptor editing (Nemazee and Weigert 2000). This mechanism involves rearrangement of the second IgLκ or the IgLλ locus to produce a non-autoreactive functional BCR (Edry and Melamed 2004; Nemazee and Weigert 2000). Functionally, non-autoreactive immature (Fr. E) B cells express high levels of IgM and low expression of IgD (Loder et al 1999). These sIgM+ immature B cells complete their development into mature naïve B cells in the BM itself or spleen (Loder et al 1999, Cariappa et al 2006).

**B cell maturation in the bone marrow**

Original stathmokinetic studies on B cell maturation suggested that immature B cells emigrate out of the BM and reach the spleen for further maturation into FoB or MZ B cells (Loder et al 1999). More recently, it has been shown that B cells of the follicular IgD^hi_ phenotype may home to the BM and can participate in T-I IgM responses by interacting with blood-borne pathogens (Cariappa et al 2005)

Since immature B cells are generated in the BM, it was rational to think that B cell maturation could also take place in the BM itself. Indeed, in 2006 Cariappa and colleagues showed that naïve B cells mature simultaneously in the BM and the spleen (Cariappa et al 2006). That study suggested that newly generated IgD^- IgM+ immature B cells that mature within the BM first develop into IgD^lo_ IgM^hi_ transitional 1 (T1) B cells, followed by becoming semi-mature IgD^+_ IgM^hi_ B cells (corresponding to splenic follicular type-II cells; F-II) and then fully mature IgD^+_ IgM^lo_ B cells (corresponding to splenic follicular type-I cells; F-I). These IgD^hi_ B cell have easy access to the circulatory system and can freely enter and exit the BM (Fig. 2) (Cariappa et al 2006).
Figure 2: B cell maturation in the BM. Immature B cells (Fr. E) generated in the BM may migrate to the spleen for further maturation, or mature within BM itself. In the BM, this maturation proceeds via transitional 1 (T1) cells, follicular type II (F-II) and finally follicular type I (F-I) B cells (Cariappa et al 2006).

Extrinsic factors affecting BM B cell development

BM stromal cells create specialized niches and affect proliferation and differentiation of B lineage cells by providing requisite factors essential for B cell development. Several studies have shown the importance of CXC-chemokine ligand 12 (CXCL-12), interleukin-7 (IL-7), FLT3 ligand (FLT3L) and stem cell factor (SCF) in B cell development in vivo (Nagasawa 2006, Tokoyoda et al 2004). CXCL12 was the first soluble factor that was reported to be important for the earliest identifiable B cell precursor, the pre-pro B cell (Nagasawa 2000). The primary cytokine regulator of murine B cell development is IL-7, which is produced by stromal cells in primary or secondary lymphoid organs. IL-7 acts by binding to a heterodimeric complex of its receptor IL-7Rα and the common gamma chain (IL-2Rγ) [(reviewed in Nagasawa 2006)]. In the B cell lineage, IL-7Rα expression is restricted to early lymphoid cells and is expressed from the CLP stage to the pre-B cell stage of development (Kondo et al 1997). Mice deficient in IL-7 or the IL-7Rα chain have severe defects in the development of T and B cells, but not that of DC or NK cells. These mutant mice exhibited normal numbers of pre-pro B cells in the BM, but severely reduced numbers of pro-B cells, pre-B cells and more differentiated B cells, indicating that IL-7 plays a crucial role at the
pro-B cell stage (Peschon et al 1994; Von-Freeden Jeffry et al 1995). Mice
over expressing IL-7 (IL-7Tg) show a massive expansion of the FoB cells
without altering the MZ B cell compartment of the spleen, indicating that the
FoB and MZ B cell compartments are independently regulated by IL-7

**Peripheral B cell development and maturation**

In adult mice (6-8 weeks old), about 1-2×10^7 sIgM+ immature B cells are
generated daily in the BM (Osmond 1991, Osmond 1993). Some of the
immature B cells that survived after negative selection enter the spleen
(Rolink et al 1998) while the remaining surviving cells mature within the BM
(Cariappa et al 2006). Immature B cells emigrated from the BM enters the
mature B cell pool through different stages of development (Fig. 3) (Förster
and Rajewsky 1990). Immature B cells leave the BM via the blood and from
there enter the spleen through the terminal branches of central arterioles
and the marginal sinuses into the outer zone of the periarteriolar lymphoid
sheaths (PALS) (Liu 1997). In the spleen, the immature B cell compartment
can be subdivided into three distinct subsets called transitional 1 (T1), T2
and T3 B cells. As compared to the mature B cell population, transitional B
cells are short lived and more sensitive to anti-IgM induced apoptosis
(Allman et al 2001; Rolink et al 1998). These heterogeneous transitional cells
are short lived (t_{1/2} = 2-4 days), express CD93, alothugh with different levels,
and are thought to be direct precursors of the mature B cell compartment
(Rolink et al 1998, Förster and Rajewsky 1990) (Fig. 3). The non-
recirculating T1, or newly formed (NF), B cells are CD19+ IgM^{high} IgD^{low} CD21-
CD23- CD93+ and localized at the outer limits of the PALS. T1 cells undergo
negative selection to eliminate strongly self-reactive cells, while the
remaining T1 B cells further differentiate into T2 B cells that reside into the
follicles. These T2 B cells still carry markers of immaturity but are able to
recirculate. The survival of CD19^{+} IgM^{high} IgD^{high} CD21^{high} CD23^{+} CD93^{+} T2 B
cells requires survival signals from the BCR, as well as signals from a
receptor that binds the TNF-family member B cell-activating factor (BAFF)
(Liu 1997, Loder et al 1999). A population of CD93^{+} T3 B cells with the
phenotype B220^{+} CD19^{+} CD21^{int} CD23^{+} IgM^{low} IgD^{+} has been described. This
population has been identified as an anergic B cell population as these T3 B
cells were found unable to give rise to mature B cells (Fig. 3) (Teague et al

It is widely accepted that T2 B cells further differentiate into either
conventional mature FoB cells or MZ B cells, which both lack expression of
CD93. On the way to become MZ B cells, T2 B cells first develop into
marginal zone precursor (MZP) B cells, defined by the expression of B220+ CD19+ CD23+ CD21hi IgMhigh IgDhigh CD1dhi.

Phenotypically, MZ B cells are CD19+ B220+ CD23−/low IgDlow CD21hi IgMhi CD1dhi CD9+, while FoB cells are B220+ CD19+ CD21mid CD23+ IgMlo IgDhi CD1dlow CD9− (Fig. 3) [reviewed in (Pillai and Cariappa 2009)]. FoB cells represent the bulk of the mature B cells and can be subdivided into semi-mature sIgMhigh CD21mid follicular type II (F-II) and mature IgMlow CD21low follicular type I (F-I B) cell subset. F-I cells represent two thirds of the FoB cells, whereas F-II cells constitute the remaining one third (Cariappa et al 2007). Whether one refers to FoB or MZ B cells, they are both called mature naïve B cells.

**Figure 3. Schematic representation of B cell development in the spleen.** Immature B cells emigrate from the BM and reach the spleen via blood, where different transitional stages (T1/T2) primarily lead to differentiation into either mature naïve FoB cells or MZ B cells.

- **Immature B**: B220– CD19+ CD23– CD21– CD9–
- **T1**: B220– CD19+ CD23– CD21– CD9–
- **T2**: B220– CD19+ CD23+ CD21– CD9–
- **T3**: B220– CD19+ CD23+ CD21– CD9–
- **FoB**: B220– CD19+ CD23+ CD21mid CD9–
- **MZP-B**: B220– CD19+ CD23+ CD21mid CD9–
- **MZ B**: B220– CD19+ CD23+ CD21high CD9−

**Cell fate decision in the development of FoB or MZ B cells**

The development of T2 B cells into either FoB or MZ B cells depends on the activities of different signaling molecules. Consequently, mutations affecting BCR signaling strength, nuclear factor κ light chain enhancer of activated B cells (NFκB) signaling, BAFF-R signaling, or NOTCH signaling have each been found to influence lineage choice. Several studies indicate that a lower BCR signal strength favors MZ B cell development, whereas stronger BCR signals favor FoB cell development [reviewed in (Pillai and Cariappa 2009)]. NFκB and BAFF-R signaling are also critical for the FoB versus MZ B cell
lineage decision. NFκB activation can be achieved by two different pathways; the canonical or the non-canonical NFκB pathway, where canonical NFκB signaling is required for MZ B cell development but not for FoB cell development (Cariappa et al 2000). BAFF-R signaling can elicit both the canonical and non-canonical NFκB pathway although it mainly activates non-canonical signaling important for B cell survival (Claudio et al 2002). Mice devoid of NOTCH signaling components like NOTCH2 (Saito et al 2003), recombination signal binding protein for immunoglobulin kappa J region (RBP-Jκ) (Tanigaki et al 2002), notch ligand Delta-like 1 (DL1) (Hozumi et al 2004) or Mastermind-like 1 (MAML1) (Oyama et al 2007) all demonstrated a reduced number of MZ B cells. On the contrary, increased NOTCH signaling in mice results in larger MZ B cell populations, such as in mice lacking the Msx2-interacting nuclear target protein (MINT) (Kuroda et al 2003). A B cell-specific conditional deletion of Notch2 was shown to result in a decreased number of MZ B cells, while the FoB and B1 cell compartments developed normally (Saito T et al 2003). As expected, mutations of other proteins related to BCR signaling, such as CD19, also influence the MZ B cell compartment [reviewed in (Pillai and Cariappa 2009)].

**B cell activation and differentiation**

When mature naïve FoB cells recognize foreign antigens via their BCRs, these antigens get internalized, processed and presented on MHC-class II molecules, after which the B cell will migrate to the periphery of the B cell follicle (T-B cell boundary) to interact with CD4+ T cells. After antigen encounter and reception of T cell help, antigen activated B cells can either differentiate into short-lived plasma cells or enter the germinal center (GC) reaction (Jacob et al 1991). It has been proposed that B cells with high affinity undergo extrafollicular plasma cell-differentiation, while cells with weaker affinity move towards the GC. Plasma cells formed in extrafollicular regions are non-migratory, have a shorter life span of about 3 days and express low affinity antibody mainly of the IgM class [reviewed in (King et al 2008)]. MZ B cells responding to a TI-2 antigen move to the red pulp for proliferation and plasmablast differentiation (Lopes-Carvalhio and Kearney 2004).

**The germinal center (GC)**

GCs represent areas of a unique collaboration between proliferating antigen-specific B cells, T follicular helper cells and the specialized follicular DCs that constitutively occupy the central follicular zones of secondary lymphoid organs like the spleen, Peyer’s patches or lymph nodes. The primary function
of GCs is to produce the high affinity antibody-secreting plasma cells and memory B cells that ensure sustained immune protection and rapid recall responses against previously encountered foreign antigens [reviewed in (Allen et al 2001)]. In GCs, FoB cells undergo massive proliferation which is accompanied by affinity maturation and class-switch recombination (CSR). B cell clones positively selected within GCs differentiate into memory B cells or long-lived plasma cells. Plasma cells exit diminishing GCs 10-14 days later and present somatically mutated, high affinity BCRs and express switched immunoglobulin isotypes. Emerging memory B cells can persist independently of antigenic stimulation without secreting antibodies for longer time and express high affinity BCRs. These cells can rapidly respond to a second antigen encounter. Plasma cells which have undergone GC reactions build the pool of long-lived plasma cells that migrate either to the BM, the intestinal mucosa or sites of inflammation [reviewed in (De Silva and Klein 2015)].

The splenic MZ

**MZ structure**

The MZ is an area where blood from the circulation enters the splenic tissue in a way that allows specialized immune cells present in the MZ to detect pathogens or dying cells. Apart from MZ B cells, other immune cells present in the murine MZ are marginal metallophilic macrophages (MMM), marginal zone macrophages (MZM), natural killer (NK) cells and DCs, all enmeshed within a network of non-hematopoietic stromal cells (Kraal and Mebius 2005). The marginal sinus is located in the inner part of the MZ, closest to the white pulp, and is composed of sinus lining vascular endothelial cells, under which mucosal addressin cell adhesion molecule-1 (MAdCAM-1)+ marginal reticular cells (MRCs) and MMMs can be found (Kraal and Mebius 2005, Katakai 2008, 2012) (Fig. 4). MZM are localized at the outer boundary of the MZ, adjacent to the red pulp, and interact with MZ B cells. DCs are also localized in the vicinity of MZM and MZ B cells (Kraal and Mebius 2005). Maturation of the micro-architecture of the MZ and the development of MZ B cells appears to go hand in hand. Proper development of the MZ and the MZ B cell compartment is delayed until about 2 years after birth in humans and 3-4 weeks after birth in rodents [reviewed in (Cerutti et al 2013)]

**Marginal zone macrophages (MZMs)**

MZMs play an important role in immune surveillance due to their strategic location in the MZ and their high phagocytic capacity [reviewed in (Mebius...
et al 2004)]. MZMs are supposed to be generated from monocytes that habitat in the MZ in response to the chemokines CCL19 and CCL21. These two chemokines are produced by gp38+ stromal cells present in the PALS and also known to be important for T cell homing into the latter region (Ato et al 2004). It’s widely accepted that both B cells and MZMs are central for the integrity and proper function of the MZ. In the absence of B cells during ontogeny, the MZMs and MMMs, as well as MAdCAM-1+ MRCs are absent in the MZ (Dingjan et al 1998).

Figure 4: Schematic representation of the splenic MZ. The central arteriole branches into a terminal arteriole that releases blood into the marginal sinus. Blood that passes through get filtered by MZ resident B cells (purple), macrophages (pink) and DC (green) before reaching the red pulp area. Marginal metallophilic macrophages (blue) are present on the inner part of the sinus while follicular B cells (green) and T cells (red) are present in the white pulp area of the spleen.


This macrophage population expresses scavenger receptor-A (SR-A), macrophage receptor with collagenous structure (MARCO) (Elomaa et al 1995) and C-type lectin, specific intercellular adhesion molecule-3-grabbing nonintegrin-related 1 (SIGN-R1) recognized by the ERTR-9 antibody (Dijkstra et al 1985). MARCO or bacteria-binding receptor (Elomaa et al 1995) is expressed constitutively on MZMs while SIGN-R1 is highly expressed on a subpopulation of MZMs (Kang et al 2003). SIGN-R1, on MZM was shown to bind the capsular polysaccharide of Streptococcus pneumoniae and Mycobacterium tuberculosis (Kang et al 2003; Koppel et al
2004) as well as gram-negative bacteria like *Escherichia coli* (*E*.coli) and *Salmonella typhimurium* (Nagaoka et al 2005). MARCO recognizes broad range of microbial antigens including *E*.coli and *Staphylococcus aureus* (Elomaa et al 1995; Karlsson et al 2003). In mice, MZMs are not one homogenous population, since at least two types of MZMs (MARCO⁺SIGN⁻R1⁻ and MARCO⁺SIGN⁻R1⁺) with different phenotypes and function are present. It has been shown that the MARCO⁺SIGN⁻R1⁺ subset disappear in the absence of MZ B cells while the MARCO⁺SIGN⁻R1⁻ subset remain in the MZ (You et al 2009).

**Marginal metallophilic macrophages (MMMs)**

MMM is a subset of macrophages identified by their expression of CD169/sialoadhesion/Siglec-1 (recognized by the anti-CD169 antibody clone MOMA-1) (Munday et al 1999). These macrophages are present at the inner margin of the MZ and the lymphocyte-rich white pulp, but are also present under the subcapsular sinus in the lymph nodes (Nakamura et al 2002). These macrophages are thought to carry antigens into the lymphocyte compartment (katakai et al 2008).

**Splenic non-hematopoietic stromal cells**

Several non-hematopoietic, fibroblast-like, stromal cell populations can be found in the splenic MZ and white pulp, all with a capacity to influence the structure and function of the spleen. MZ fibroblastic reticular cells (FRC) are present in the MZ outside the marginal sinus, express VCAM-1 and ER-TR7, and exhibit phenotypes different from that of stromal cells in the red or white pulp, such as playing an important role in antigen delivery [reviewed in (Mueller and Germain 2009)]. A newly identified stromal cell subset, called marginal reticular cells (MRCs), is present as a thin layer just beneath the vascular endothelial cells on the follicle-side of the marginal sinus. MRCs express MAdCAM-1, CXCL13 and RANKL, but not CCL21 (Katakai et al 2008). Finally, the B cell follicle harbors CR1/CD35⁺ FDCs and the T cell area contains gp38⁺ FRCs. Interestingly, BAFF required for normal splenic B cell homeostasis and survival was suggested to be provided by FDCs. However, recent findings instead suggest that BAFF produced by gp38⁺ FRCs and not FDCs mediates this important function (Cremasco et al 2014).

**The migration and retention of MZ B cells**

Unlike FoB cells, MZ B cells express higher levels of α1β2 (LFA-1) and α4β1 (VLA-4) integrins (Lu and Cyster 2002). Binding of these integrins to their ligands, intercellular adhesion molecule 1 (ICAM-1) and vascular cell
adhesion molecule (VCAM-1), respectively, on stromal cells has been shown to be critical for the retention of MZ B cells in the MZ (Lu and Cyster 2002). As already mentioned, MZ B cells have been found to shuttle between the MZ and the B cell follicle in response to S1P and CXCL13 (Cinamon et al 2004). S1P is present at high concentrations in the blood and stimulates migration of MZ B cells into the MZ (Cinamon et al 2004). This requires the S1P-receptor S1P1, since mice lacking this receptor or treatment of mice with the S1P-receptor antagonist FTY720, results in dislocalization of MZ B cells into B cell follicles (Brinkmann and Lynch 2002, Matloubian et al 2004). A direct intercellular interaction between MZM and MZ B cells, mediated by MARCO on the MZM and a so far unknown ligand on MZ B cells, has also been shown to mediate retention of MZ B cell in the MZ (Karlsson et al 2003). Deletion of SH2-containing inositol-5 phosphate (SHIP) in myeloid cells has been shown to induce MARCO+ MZMs to relocate to the red pulp area with a subsequent loss of MZ B cells from the MZ (Karlsson et al 2003). Thus, the physical presence of MZMs in the MZ does also mediate retention of MZ B cells. A role of MOMA-1+ MMMs in regulating localization of MZ B cells has also been established in a study where mice lacking one of the receptors for S1P, S1P3, were found to have a widened distribution of MMMs and an increased follicular localization of MZ B cells (Girkontaite et al 2004).

Further evidence for an intimate relationship between macrophages and B cells in the MZ comes from studies involving depletion of either MZ B cells or MZM in adult mice. Such studies have reported a loss of the corresponding cell population or morphological disruptions of MZ. Depletion of macrophages, including MZM, by in vivo liposome treatment has been found to result in a subsequent loss of the B cells in the MZ (Van Rooijen et al 1989). An over-expression of the TNF family member CD70 has been shown to induce a gradual loss of splenic B cells along with a loss of the MZM pool (Nolte et al 2004). In the same study, BCR+/− mice lacking B cells showed impairment in MZM development (Nolte et al 2004). It has also been found that lack of CD19 in B cells leads to a loss in of MZ B as well as MZMs (You et al 2010). However, CCL-19/CCL-21 deficient plt/plt mice show a strong reduction of MZM but maintained normal numbers and localization of MZ B cells in the spleen, suggesting that lack of MZM may not always result in dislodgement of MZ B cells (Ato et al 2004).

**MZ B cells and auto-antibodies**

Autoimmune diseases are complex and multifactorial and lead to the breakdown of tolerance towards self-molecules. Lymphocytes (T and B cells) are the main perpetrators in autoimmune diseases due to expression of antigenic receptors with the potential to recognize self-molecules. In the
avoidance of autoimmunity, selection of lymphocytes is very crucial, but in the case of MZ B cells the multi-reactive specificities are allowed to persevere [reviewed in (Lopes-Carvalho and Kearney 2004)]. This strengthens a role of MZ B cells in initiating autoimmunity by recognizing self molecules. The MZ B cell population was observed to be expanded in the SLE model (NZB×NZW) F1 mice, where high levels of anti-nuclear antibodies directed against double stranded (ds) DNA was found (Wither et al 1998). BAFF transgenic mice also display an expanded MZ B cell population and developed SLE like symptoms (Mackay et al 1999). Thus, MZ B cells are potent producers of auto-antibodies causing SLE when provided with survival signals from BAFF.

The family of signal regulatory proteins (SIRPs)

The signal regulatory proteins (SIRPs) belong to the immunoglobulin (Ig) family of cell surface glycoprotein and consist of SIRPα (CD172a/SHPS-1/P84), SIRPβ (CD172b) and SIRPγ (CD172g/SIRPβ2) (Fig. 5). These SIRP family members show high sequence similarity and similar extracellular structures, but they differ extensively in their cytoplasmic regions [reviewed in (Barclay and Brown 2006)]. SIRPα, a 110-120 kDa monomeric protein and a prototypic member of the SIRP family, is expressed mainly by myeloid phagocytic cells (i.e. granulocytes, monocytes, macrophages and dendritic cells), stromal cells and neurons, but not by lymphoid cells (Barclay and Brown 2006; Seiffert et al 1999; Koskinen et al 2013; Paper I). In its extracellular region, SIRPα consists of three Ig domains, one distal Ig variable (IgV) or NH-2 terminal V-set domain and two Ig constant (IgC) domains. The cytoplasmic region of SIRPα consists of four tyrosine phosphorylation sites that form two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [reviewed in (Barclay and Brown 2006)]. Upon external stimuli, ITIM-associated tyrosine residues are phosphorylated and mediate recruitment and activation of the Src homology region 2 domain-containing phosphatases 1 or 2 (SHP-1 or SHP-2), which is thought to be important for SIRPα-mediated downstream signaling (Kharitonenkov et al 1997).

This SIRPα ITIM tyrosine phosphorylation can be triggered through ligation by the SIRPα ligands CD47 (Oldenborg et al 2000), the surfactant proteins A or D (SP-A or SP-D) (Gardai et al 2003), or by integrin-mediated adhesion (Johansen et al 2007, Tsuda et al, 1998). SHP-1 is primarily expressed in hematopoietic and epithelial cells and negatively regulates various functions of these cells. On the contrary, SHP-2 is more ubiquitously expressed and generally contributes to positive regulation of cell signaling (Neel et al 2003, Oldenborg 2004). The second SIRP family member, SIRPβ, has a very short
Figure 5: Members of the signal regulatory protein family and their interactions. CD47 is a ligand for SIRPα and SIRPγ while the ligand for SIRPβ is not known. Surfactant protein A (SP-A) can trigger tyrosine phosphorylation of SIRPα. SIRPβ can signal through DNAX activation protein 12 (DAP12).


cytoplasmic tail of 6 amino acids and generates positive signals via the adaptor protein DNAX activation protein of 12 kDa (DAP12). DAP12 contains a single immunoreceptor tyrosine-based activation motif (ITAM), which upon phosphorylation binds spleen tyrosine kinase (SYK) or T cell receptor ζ-chain associated protein kinase (ZAP70), leading to signal transmission (Tomasello et al 2000). Unlike the other SIRP family members, SIRPγ is expressed by T cells but not by myeloid cells. Structurally, SIRPγ has a short cytoplasmic tail that lacks signaling motifs, which makes SIRPγ non-signaling in nature (Brooke et al 2004).

Ligands of the SIRP-family members

The ubiquitously expressed cell surface glycoprotein CD47 is a known ligand for SIRPα in mice (Brown et al 1990, Jiang et al 1999), rats (Vernon-wilson
et al 2000) and humans (Seiffert et al 1999). CD47 is also a ligand for SIRPγ, but it does not interact with SIRPβ (Seiffert et al 1999, Brooke et al 2004). Apart from CD47, SIRPα has also been shown to interact with the soluble collectin family surfactant proteins SP-A and SP-D, which are highly expressed in the lungs (Fig. 5) (Gardai et al 2003). Potentially, these surfactant proteins can block the binding of CD47 to SIRPα, as all three proteins can bind to the same region of SIRPα. The interaction between SIRPα and these surfactant proteins can generate an inhibitory signal to prevent inflammatory activation of alveolar macrophages (Gardai et al 2003).

**CD47**

CD47 (Integrin associated protein/IAP) consists of a highly glycosylated extracellular IgV-like domain, a hydrophobic penta-spanning transmembrane domain, and an alternatively spliced cytoplasmic tail (Lindberg et al 1993). Besides SIRPα and SIRPγ, CD47 also associate with integrins (Brown et al 2001) and binds the soluble extracellular matrix protein thrombospondin 1 (TSP-1) (Gao et al 1996). TSP-1 plays an important role in regulating cell migration, T cell and platelet activation, and in stimulating leukocyte adhesion and phagocytosis (Brown et al 2001). Thus, CD47 interacts with integrins in cis (Lindberg et al 1996), or with SIRPα (Jiang et al 1999, Seiffert et al 1999, Vernono-Wilson et al 2000), SIRPγ (Brooke et al 2004) or TSP-1 (Gao et al, 1996) in trans at the cell surface (Fig. 6). These interactions transduce intracellular signals through heterotrimeric Gαi proteins that become associated with CD47 (Gao et al, 1996). In neurons, ligation of CD47 by SIRPα has also been shown to promote activation of Cdc42, a member of the Rho family of small GTP-binding proteins. Moreover, CD47 has been implicated in diverse physiological processes, including axon development (Miyashita et al 2004), cell migration (Liu et al 2001; Jaiswal et al 2009) and T cell or DC activation (Sarfati et al 2008).

**Importance of the CD47-SIRPα interaction**

As mentioned, CD47 serves as a counter receptor for SIRPα where the Ig domain of CD47 binds to the N-terminal IgV domain of SIRPα, which enables bidirectional signaling by each of these two receptors (Fig. 6) (Jiang et al 1999, Latour et al 2001). The best characterized system regulated by the CD47-SIRPα interaction is the ability of SIRPα to inhibit host cell phagocytosis. This system is based on the interaction between CD47 on a host cell and SIRPα on a phagocytic cell (macrophage or DC). The original finding was that fresh, unopsonized CD47−/− red blood cells (RBCs)
transferred to wild-type recipient mice were rapidly eliminated by splenic macrophages (Oldenborg et al 2000).

**Figure 6: The CD47-SIRPα signaling complex.** The interaction between CD47 and SIRPα can induce bidirectional signaling. Upon binding of CD47, the ITIMs present in the cytoplasmic domain of SIRPα get phosphorylated and mediate recruitment and activation of tyrosine phosphatases SHP-1 and SHP-2.


In a similar manner, later studies showed that other viable CD47-deficient cells (leukocytes or platelets) were rapidly eliminated by dendritic cells or macrophages when transferred into wild-type recipients (Blazar et al 2001, Olsson et al 2005). Thus, CD47 can act as a marker of self in the interaction between normal host cells and phagocytic cells, and CD47-mediated SIRPα engagement on the phagocyte generates an inhibitory or “do not eat me” signal. This inhibitory signal has been found to inhibit pro-phagocytic activation via FcγR, complement receptors and low density lipoprotein receptor-related protein 1 (LRP1), but not SR-mediated phagocytosis (Oldenborg et al 2001, Olsson et al 2005, Gardai et al 2005, Olsson and Oldenborg 2008, Nilsson et al 2012). It has been proposed that the molecular explanation of CD47-SIRPα-mediated phagocytosis inhibition involves SHP-1-mediated dephosphorylation of non-muscle myosin type IIA, which prevents the formation of a phagocytic cup (Tsai and Discher 2008). Recent findings have shown that targeting of CD47-SIRPα-mediated phagocytosis inhibition could be a possible way of treating cancer. CD47 has been found to be overexpressed in multiple human cancers, such as chronic myeloid leukemia (CML) (Jaiswal et al 2009), acute myeloid leukemia (AML) (Majeti et al 2009), multiple myeloma (MM) (Danielsen et al 2007),
acute lymphoblastic lymphoma (ALL) (Chao et al 2011), bladder cancer (Chan et al 2009) and other solid tumors (Willingham et al 2013). This over expression of CD47 may enable tumor cells to escape innate immune surveillance through evasion of phagocytosis, by promoting inhibition of phagocytosis and tumor survival. This process occurs through binding of CD47 on tumor cells to SIRPα on phagocytes (Chao et al 2012, Jaiswal et al 2009, Majeti et al 2009). Therefore, therapeutic blocking of the CD47-SIRPα pathway in combination with therapeutic monoclonal antibodies specific for tumor antigens (IgG-opsonization) seems to be a promising strategy for cancer therapy (Chao et al 2012, Barclay and Van den Berg 2014).

Besides its phagocytosis-inhibitory function, ligation of SIRPα has also been shown to induce macrophage production of nitric oxide, which is normally an activating signal (Alblas et al, 2005). Furthermore, the interaction between CD47 and SIRPα also facilitates trans-endothelial and trans-epithelial migration of monocytes and granulocytes (Liu et al 2001; de Vries et al 2002).

**Phenotypes of SIRPα-mutant and CD47-/- mice**

Both CD47-/- mice and mice expressing a mutant form of SIRPα (lacking most of the cytoplasmic domain and unable to bind SHP-1; SIRPα-MT mice) develop mild thrombocytopenia (Yamao et al 2002, Olsson et al 2005). In contrast, the RBC numbers are normal in both CD47-/- and SIRPα-MT mice (Lindberg et al 1996, Yamao et al 2002), which could be explained by an increased erythropoiesis (Ishikawa-Sekigami et al 2006). Both these transgenic mouse strains were also found to have a reduced number of splenic CD4+CD8- DCs (Hagnerud et al 2006, Saito et al 2010, and Van et al 2006), and studies of BM chimeric mice have demonstrated that SIRPα signaling in CD4+CD8- DCs is important for development of this DC subset. On the contrary, CD47 expression by both non-hematopoietic and hematopoietic cells was required for normal homeostasis of splenic CD4+CD8- DCs. The interaction of CD47 and SIRPα is also thought to play an important role in splenic T cell homeostasis, since spleens of CD47-/- and SIRPα-MT mice have a marked reduction in the area of the T cell zone and a reduced number of CD4+ T cells. Using BM chimeric mice, it was found that hematopoietic SIRPα signaling was required to maintain a normal splenic T cell zone (Sato-Hashimoto et al 2011). It has also been found that BM stromal cells express SIRPα that its tyrosine phosphorylation is dependent on CD47, and that SIRPα is required for BM stromal cell osteoblastic differentiation, bone formation and RANKL production (Koskinen et al
Collectively, the CD47-SIRPα signaling system has been found to play an important role in regulating host cell phagocytosis, DC and T cell homeostasis, as well as bone homeostasis.
Specific Aims

Despite vast amounts of research on the biological functions of CD47 and SIRPα in the immune system, the knowledge of a possible role of these proteins in regulating B cells has been limited. Therefore, the aims of the present thesis were to investigate if SIRPα and/or CD47 could:

- be involved in regulating B cell development, B cell maturation or B cell homeostasis.
- more specifically be involved in regulating MZ B cells and/or other cells of the splenic MZ.
Materials and methods

For detailed descriptions of the methods, please refer to the materials and methods sections of paper I-III.

Mice (paper I-III)

Male and female C57BL/6 Ly5.2 SIRPα-MT mice, lacking most of the SIRPα cytoplasmic domain (Ingaki et al 2000), or their homozygous wild-type littermates, were used at different ages. CD47−/− Balb/c mice, or their homozygous wild-type littermates, were from our own breeding colony. The mice were back-crossed to Balb/c (Jackson) for 16 or more generations and were maintained in a pathogen-free and healthy environment according to local guidelines. C57BL/6 Ly5.1 mice were from Taconic’s, Italy. All the experiments were performed in strict compliance with relevant Swedish and institutional laws and guidelines and were approved by the Umeå Research Animal Ethics Committee (A14-12). All injections in mice were made under isofluoran anesthesia. Animals were euthanized by CO2 asphyxiation and cervical dislocation before organs were surgically removed for further analysis. In BM reconstitution experiments, mice were followed daily to assure that the weight loss did not exceed 10% or that the animals suffered from dehydration. Reconstituted animals were kept in IVC-cages to reduce the risk of infection.

Flow Cytometry or FACS (I-III)

Single cell suspensions were prepared from BM by flushing femur and tibia with phosphate buffered solution (PBS) solution, using 27-G needles or from spleens by mechanical disruption. Further, cell suspensions were filtered using a 70μm cell strainer (I-III) and, following FcγR-block, surface antigens were labeled with fluorescently labeled antibodies as described in the respective materials and methods sections. Samples were analyzed using either FACS Calibur or LSR II (BD biosciences) and by using Cell Quest or FACSDiva software (BD biosciences). Peripheral blood was obtained by vein puncture, collected into heparinized capillary tubes and diluted into PBS + 5 mM EDTA. After RBC lysis, samples were incubated with fluorochrome-conjugated antibodies followed by addition of fluorescent beads (Countbright absolute counting beads, Life Technologies) to calculate absolute cell numbers (II). Samples were analyzed by FACS Calibur and Cell Quest software (BD biosciences).
Immunohistochemistry (II & III)

Cryosections (8µm) from spleens were incubated with antibodies against MOMA-1 or MARCO (II) or IgD (III) followed by Alexa 594 anti-rat IgG. For double staining, sections were incubated with biotin-conjugated anti-CD1d (II) or IgM (III) followed by fluorophore tyramide and analyzed by laser scanning confocal microscopy using leica-LCS software and Adobe Photoshop CS4.

Immunization and ELISA (II)

Mice were immunized i.v. with 10 µg TNP-Ficoll or TNP-LPS. Sera were prepared seven or eight days later and serial serum dilutions were analyzed for TNP-specific IgM or IgG3 by ELISA.

Autoantibody or Anti-nuclear antigen tests (ANA) (II & III)

Serum levels of anti-nuclear antigens (ANA) (Alpha Diagnostic International) and anti-ds-DNA antibodies were detected in younger or older mice, using an ELISA plate reader and Softmax Pro software. Younger (3-4 month old) or older (14-16 month old) wild-type or SIRPα-MT (II) or 5 month old wild-type or CD47−/− (III) mice were used for this analysis.

In vitro B cell stimulation assays (II)

Single cell suspensions of spleens were prepared and, following FcγR-block, surface antigens were labeled with fluorescently labeled antibodies as described in respective materials and methods sections (I & II). MZ B (II) or FoB cells (I) were sorted to more than 95% purity on a FACS ARIA-III cell sorter. Sorted MZ B cells were incubated for 3 days at 1×10^5 cells/well (II). Supernatants were collected and analyzed for IgM production by ELISA.

Bone Marrow Reconstitution (I & II)

Irradiated mice, either Ly5.2 wild-type or SIRPα-MT, or Ly5.1 wild-type mice, received injection of isolated BM cells from Ly5.1 wild-type mice or Ly5.2 wild-type or SIRPα-MT, respectively. Twenty weeks after BM transplantation, peripheral blood, BM and spleen were subjected to flow cytometric analysis as described in respective material and method sections.

Bone marrow stromal cell culture (I)

BM cells were isolated by flushing femurs and tibiae of 16 wks old wild-type or SIRPα MT mice. The cells were cultured in 60cm² culture dishes (Nunc)
with α-MEM media with 10% FCS (Invitrogen), L-glutamine and antibiotics (Sigma Aldrich) for 12 days. After 12 days, cells were lysed for RNA isolation (Koskinen et al 2013).

**Quantitative Real-time PCR (I)**

Isolation of total RNA and synthesis of cDNA from mRNA from BM stromal cells was done as previously described (Koskinen et al 2013). Using the standard curve method, the relative expression of rankl in BM stromal cells was calculated based on the threshold cycle (Ct) values of rankl gene (Taqman gene expression assay) and the Ct values of the endogenous control β-actin by using Taqman ABI PRISM 7900 HTSequence Detection system. mRNA was extracted from FACS sorted splenic FoB cells (CD19<sup>+</sup>CD21<sup>int</sup>CD23<sup>hi</sup>; >95 % purity) using RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. Total RNA (200 ng) was reverse transcribed using High capacity cDNA reverse transcription kit (Applied Biosystems) and Bcl-2 or BIM (Bcl2l11) mRNA levels were determined by using a ViiA 7 instrument (Applied Biosystems). Ct values were used to calculate relative values and were normalized to that of β-actin. The following Taqman gene expression assays (Applied Biosystems) containing pre-designed primers and probes were used: Bcl2, (Mm00477631_m1); BIM (Bcl2l11) (Mm00437796_m1); β-actin (NM_007393.1).

**Intracellular staining of B cells (I)**

In brief, splenic or BM B cells were stained with Abs as mentioned in the figures, fixed with fresh 2% paraformaldehyde and permeabilized with 0.1 % Triton X100. After washing, cells were stained with Alexa 647 conjugated anti-BIM mAb (Novus biotech 151-149) and washed twice with FACS buffer. All the samples were analyzed using FACS LSR II (BD biosciences) and by using FACSDiva software (BD biosciences).

**Cell cycle analysis (I)**

Single cell suspensions were prepared from BM or spleens. Erythrocyte-free cell suspensions were labeled with antibodies as indicated in the figures. To 0.5 ml of cell suspension with a cell concentration of 5×10⁵ cells/ml, 1μl of vibrant dye cycle ruby stain (final stain concentration 5μM) was added. After incubation at 37° C for 15-30 minutes in dark, samples were analyzed using FACS LSR II (BD biosciences) and by using FACSDiva software (BD biosciences).
Apoptosis assay (I)

Splenocytes were prepared by mechanical disruption of spleens, and single cell suspensions were prepared from BM by flushing femurs and tibiae of 16 wks old wild-type or SIRPα MT mice. Cells were incubated with unlabeled anti-CD16/CD32 (2.4G2) for 15 mins to block FcγR, followed by antibodies as indicated in the figures. Further, cells were labeled with FITC-conjugated Annexin-V and the percentage of B cell undergoing apoptosis was determined by flow cytometry FACS LSR II (BD biosciences) and by using FACSDiva software (BD biosciences).

Statistical analysis (I-III)

Statistical analyses were performed using Students t-test for unpaired analyses. Significance levels are indicated as *P<0.05, **P<0.01 and ***P<0.001.
Results

Role of SIRPα and CD47 in regulating blood B cell numbers

In adult mice, immature B cells can be found in the BM, the blood and the spleen (Allman et al 2001). Mature follicular B cells not only home to the spleen, but circulate through the body in the blood and lymph and enter other peripheral lymphatic organs. Blood normally contains sIgM hi CD23-lo immature B cells, recently released from the BM, along with recirculating mature B cell which based on their surface expression levels of IgM and CD23 can be differentiated into sIgM hi CD23 hi semi-mature/follicular type-II (F-II) B cells or sIgM lo/int CD23 hi mature/follicular type-I B cells (F-I) (Paper I, Fig 1B).

Lack of SIRPα signaling results in a reduced number of circulating B cells

A previous study demonstrated that the frequencies of B cells or T cells, out of total lymphocytes, were similar in the blood of 6-12 weeks old wild-type and SIRPα-MT mice (Sato-Hashimoto et al 2011). In the present work, we confirmed that the frequency of CD19+ B cells was similar in 12 weeks old wild-type and SIRPα-MT mice (Paper I, Fig 1A). However, when analyzing the absolute numbers of different B cell subsets in the blood of 12 weeks old wild-type or SIRPα-MT mice, based on the surface expression of IgM and CD23 (Paper I, Fig 1B), we observed significant reductions in total CD19+ B cells (30% reduction), immature B cells (43% reduction), F-II B cells (45% reduction) and mature F-I B cells (30% reduction) in SIRPα-MT mice, as compared with that in wild-type mice (Paper I, Fig 1C). This blood B cell deficit of SIRPα-MT mice was further manifested at 20 weeks of age (Paper I, Fig 1D). To investigate if loss of SIRPα signaling affected other lineages of the hematopoietic system, we analyzed T cells and myeloid lineage cells in the blood of 16 weeks old wild-type or SIRPα-MT mice. This analysis revealed no difference in the numbers of neutrophils or monocytes while the number of T cells was significantly reduced in SIRPα-MT mice (Paper I, Fig 1E). Thus, lack of SIRPα signaling appeared to be of importance to maintain normal numbers of circulating B and T cells in adult mice.

Lack of CD47 expression results in a reduced number of circulating B cells

Since CD47 can function as a ligand for SIRPα, we next analyzed the same B cell subsets in the blood of 20 weeks old wild-type or CD47-/- mice and found no difference in the fraction of CD19+ B cells among the lymphocytes (Paper III, Fig 4A), similar to the phenotype of SIRPα-MT mice. However, CD47-/-
mice also manifested a significant reduction in the absolute numbers of total CD19+ B cells (32% reduction) and mature F-I B cells (46% reduction), while no significant difference was observed among immature or F-II B cells, when comparing CD47− and wild-type mice (Paper III, Fig 4B).

**Role of SIRPα and CD47 in regulating bone marrow B cell maturation**

**Impaired B cell maturation in the BM of SIRPα-MT mice**

To examine the role of SIRPα signaling in the development of earlier B lineage cells, we analyzed the BM of SIRPα-MT mice and their wild-type controls. The total number of nucleated BM cells was found to be similar in 6-28 weeks old SIRPα-MT mice and their age-matched wild-type controls (Paper I, Suppl. fig. 1A). However, a significant reduction in the B220+ B cell population was observed in 13 weeks old SIRPα-MT mice, as compared to that in wild-type mice of the same age (Paper I, Fig 2A). Despite this, no differences in the absolute numbers of early BM B lineage cells were found when comparing the amounts of Hardy fractions A (B220+ IgM− CD43hi CD19−), B-C (B220+ IgM− CD43hi CD19+), or D (B220+ IgM− CD43lo CD19+) in wild-type and SIRPα-MT mice (Paper I, Suppl. fig. 1B).

In adults, roughly two thirds of all sIgM+ immature B cells generated in the BM are released into the blood and migrate to the spleen for further maturation. However, a study by Cariappa et al. firmly established that the remaining immature B cells mature in the BM (Cariappa et al 2006). Newly generated IgD- IgM+ immature B cells that mature within the BM first develop into IgDlo IgMhi transitional 1 (T1) B cells, followed by becoming semi-mature IgD+ IgMhi B cells (corresponding to splenic follicular type-II cells; F-II) and then fully mature IgD+ IgMlo B cells (corresponding to splenic follicular type-I cells; F-I) (Paper I, Fig 2B). In the same study, NF/T1 (IgDlo IgMhi) cells were described as a separate B cell population with slightly longer half-life than immature B cells and they represent a distinct stage of B cell development (Cariappa et al 2006). SIRPα-MT mice were found to have significantly reduced numbers of T1, F-II and F-I B cells in their BM, as compared with that in wild-type controls (Paper I, Fig 2C). When comparing the proportions of the same BM B cells subsets, no significant difference in the fractions of immature or T1 B cells was observed, while F-II and F-I B cells were significantly reduced in SIRPα-MT mice (40% and 55% reduction, respectively), as compared with that in wild-type mice (Paper I, Fig 2D).
Impaired BM B cell maturation in CD47-/ mice

To examine, if CD47-/ mice mimicked the phenotype of SIRPα-MT mice, we analyzed BM cells from 5 months old wild-type or CD47-/ mice. CD47-/ mice exhibited equal numbers of total BM cells and total BM B220+ B cells, as compared with age-matched wild-type controls (Paper III, Fig 3A & 3B). In the analysis of BM B cells of CD47-/ or wild-type mice, we delineated different BM B cell populations by using the difference in surface expression of B220 and IgM. By this classical method, pre-pro B cells (Hardy Fr.A-D) can be defined as B220+ IgM-, immature B cell as B220low IgM+ and mature B cell as B220hi IgM+ (Paper III, Fig 3C). In these analyses, the proportions and absolute numbers of pre-pro/ (Fr.A-D) B cells or immature B cells did not differ significantly between wild-type and CD47-/ mice (Paper III, Fig 3D-E). However, the proportions and absolute numbers of recirculating mature B cells were significantly reduced in CD47-/ BM, as compared with that in wild-type BM (Paper III, Fig 3D-E).

SIRPα and CD47 are required to maintain normal numbers of splenic FoB cells

Reduced numbers of FoB cells in the spleens of SIRPα-MT mice

Once blood sIgM+ immature B cells have entered the spleen, they develop into transitional B cells before finally differentiating into either mature follicular or MZ B cells (Loder et al 1999). When we investigated the B cell subsets in SIRPα-MT or wild-type spleens, lack of SIRPα signaling was found to have profound effects on splenic FoB and MZ B cell homeostasis (the MZ B cell phenotype will be described in a separate section below). As previously described, SIRPα-MT mice displayed a mild splenomegaly, which is the result of an expansion of the red pulp area due to increased extramedullary erythropoiesis (Ishikawa-Sekigami 2006). In spleens of 13 weeks old SIRPα-MT mice, we observed a significant reduction in the total number of B220+ B cells (Paper I, Fig 3A) and significant reductions in the B220+ CD21int IgD+ IgMhi F-II B cell and B220+ CD21int IgD+ IgMlo F-I B cell subsets (Paper I, Fig 3B).

Characterization of transitional B cell subsets in SIRPα-MT spleens

Transitional cells are thought to be the direct precursors of the mature naïve B cell compartment. In order to analyze splenic transitional B cell subsets, we adopted a gating strategy based on differential expression of CD21, CD23 and sIgM (Sundström and Lejon 2007). T1 cells were considered as B220+ CD21-/low CD23+ IgMhigh, T2 cells as B220+ CD21int CD23+ IgMhigh and T3 B cells as B220+ CD21int CD23low IgMlow. In flow cytometric analyses, we found
no significant differences in the absolute numbers of splenic T1-T3 B cell subsets in SIRPα MT mice as compare to that in wild type controls. Interestingly, only the fraction of T3 B cells was significantly increased in SIRPα MT mice as compare to that in wild-type controls (Fig 7, unpublished data).

Figure 7. Flow cytometric analysis of splenic transitional B cell subsets in wild-type or SIRPα-MT mice. T1 B cells, T2 B cells and T3 B cells were identified based on the differential expression of CD21, IgM, CD23 and B220 (Sundström and Lejon 2007).

Delayed impairment of splenic FoB cell maturation in CD47-/- mice

Analysis of spleens from 3 to 10 months old wild-type or CD47-/- mice showed no significant differences in the number of total B220+ B cells in any age group (Paper III, Fig 1A). However, the fraction of B220+ CD23hi CD21int FoB cells was significantly reduced in 5 and 10 months old CD47-/- mice, but not in 3 months old mice, as compared with that in age-matched wild-type control mice (Paper III, Fig 1B-D).
An increased rate of apoptosis could explain the reduced numbers of mature FoB cells in the BM and spleen of SIRPα-MT mice

One possible explanation behind reduced B cell numbers in SIRPα-MT mice could be a reduced survival. Several studies have reported the importance of pro-survival proteins (e.g. Bcl-2 and Bcl-XL) and pro-apoptotic proteins (e.g. BIM) in regulating B cell survival (Tischner et al 2010). In the present study, we did not observe any difference in the expression of Bcl-2 and BIM at the mRNA level in FACS-sorted CD19^{+} CD21^{int} CD23^{hi} mature splenic FoB cells of wild-type or SIRPα-MT mice (Paper I, Fig 3C). However, the pro-apoptotic activity of BIM can also be regulated at the posttranslational level in lymphocytes (Jorgensen et al 2007). Therefore, we investigated if an increased BIM protein level could be detected in BM or splenic B cells of wild-type or SIRPα-MT mice. Immature B and T1 B cells in the BM, as well as splenic F-II and F-I B cells, of SIRPα-MT mice showed an increased level of BIM protein, as compared with that in wild-type mice (Paper I, Fig 2E & 3D). In addition, significantly increased amounts of annexin V^{+} apoptotic immature B, T1 and F-II B cells were found in the BM of SIRPα-MT mice, as compared with that in wild-type BM (Paper I, Fig 2F). In the spleens of SIRPα-MT mice, a significant increase in the fraction of apoptotic F-II B cells was observed, as compared with that in wild-type mice (Paper I, Fig 3E). In contrast, no differences were found in the cell cycle activity among these B cell subsets in the BM or spleen when comparing wild-type and SIRPα-mutant mice (Paper I, Suppl Fig 3). Thus, a reduced number of maturing B cells in the BM and spleen of SIRPα-MT mice appeared to correlate with an increased rate of B cell apoptosis.

Role of SIRPα and CD47 in regulating splenic MZ B cells

SIRPα-MT spleens harbor an increased amount of MZ B cells which accumulate in the B cell follicles

In the analysis of splenic B_{220}^{+} CD23^{hi} CD21^{int} FoB cells and B_{220}^{+} CD23^{lo} CD21^{hi} MZ B cells (Paper II, Fig 1A), a decreased FoB/MZ B cell-ratio was observed in the spleens of 13 and 28 weeks old SIRPα-MT mice, as compared with that in age-matched wild-type spleens (Paper II, Fig 1B). However, this difference was not observed in 8 weeks old mice (Paper II, Fig 1B). Interestingly, in 13 or 28 weeks old SIRPα-MT mice, the fractions as well as the absolute numbers of MZ B cells per spleen were significantly increased, as compared with that in age-matched wild-type controls (Paper II, Fig 1C & 1D). In addition, the use of other markers typical for MZ B cells, such as CD1d, confirmed the MZ B cell phenotype of SIRPα-MT mice (Fig 8).
addition to the increased amounts of MZ B cells found in SIRPα-MT mice, immunohistochemical analysis showed that a large fraction of the CD1d^{hi} MZ B cells were localized in the B cell follicles of SIRPα-MT spleens (Paper II, Fig 3A).

**Normal chemotactic migration towards S1P or CXCL13 in SIRPα-MT MZ B cells**

MZ B cells were once thought to be sessile, however, recent work has shown that MZ B cells are migratory and shuttle between the MZ and B cell follicles of the spleen (Cinamon et al 2008). Reports by Cinamon and colleagues have demonstrated that the S1P receptor S1P₁ is required for MZ B cells to remain lodged in the MZ, and a reduction in S1P₁ receptor expression causes MZ B cells to leave the MZ and move into the follicle in a CXCL-13 dependent manner (Cinamon et al 2004). To determine if the increased follicular localization of SIRPα-MT MZ B cells was due to a defect in chemotactic response to S1P or CXCL-13, we investigated the in vitro migration of MZ B cell towards S1P or CXCL-13. These experiments however showed that the migration to either S1P or CXCL-13 was similar in wild-type and SIRPα-MT MZ B cells (Paper II, Fig 3C).

**Response to T-I antigens in SIRPα-MT MZ B cells**

MZ B cells are well known for their ability to respond to TI antigens by rapidly generating an IgM specific antibody response (Pillai et al 2005). Given that SIRPα-MT mice exhibited an increase amount of MZ B cells, we determined their TI immune responses in vivo. Despite an increased amount
of MZ B cells, the antigen-specific IgM and IgG3 antibody titers in response to TI-1 (TNP-LPS) or TI-2 (TNP-Ficoll) were only slightly increased in SIRPα-MT mice, as compared to that in wild-type controls (Paper II, Fig 2A-D). To investigate if this slightly increased immune response to T-I antigens in SIRPα-MT mice was an intrinsic property of SIRPα-MT MZ B cells, FACS sorted wild-type or SIRPα-MT MZ B cells were stimulated with different doses of LPS (1 or 10μg/ml) for 3 days in vitro. Interestingly, an equal IgM production was observed in wild-type and SIRPα-MT MZ B cells in response to either dose of LPS (Paper II, Fig 2E-F).

No signs of an autoimmune phenotype in SIRPα-MT MZ B cells

Although the knowledge on the role of MZ B cells in autoimmunity is limited, studies in BAFF transgenic or New Zealand black × New Zealand white (NZB/W) F1 mice have indicated an involvement of these cells in the development of autoimmunity (Mackay et al 1999; Atencio et al 2004). To investigate any signs of autoimmunity in SIRPα-MT mice, sera were investigated for the presence of increased levels of anti-ds-DNA antibodies or anti-nuclear antigens (ANA). These analyses showed no signs of increased autoantibody or ANA levels in SIRPα MT mice up to 16 months of age, as compared with that in wild-type controls (Paper II, Fig 1I-J). Mouse MZ B cells express high levels of the activation markers MHC-class II, CD80 and CD86 (Pillai et al 2005). The expression levels of these activation markers were analyzed in wild-type and SIRPα-MT MZ B cells and were found to be expressed at similar levels, with the exception of CD40 which showed a slightly reduced expression in SIRPα MT MZ B cells (Paper II, Fig 1E-H).

CD47/- mice show a delayed accumulation of MZ B cells with normal localization in the MZ

CD47/- mice were also examined to investigate if these mice also display a MZ B cell phenotype similar to that in SIRPα-MT mice. Indeed, we observed a significant reduction in the FoB/MZ B cell-ratio in 5 and 10 months old CD47/- mice (Paper III, Fig 1E) indicating skewed proportions of MZ B and FoB cells at an older age. Next, we analyzed spleens of CD47/- mice by immunohistochemistry and found normal distributions of IgD<sup>hi</sup>/IgM<sup>lo</sup> FoB and IgD<sup>lo</sup>/IgM<sup>hi</sup> MZ B cells (Paper III, Fig 2 A, B, D, E). In addition, MOMA-1<sup>+</sup> MMMs were correctly positioned in the spleens of CD47/- mice (Paper III, Fig 1 C-F). Thus, MZ B and FoB cells as well as MOMA-1<sup>+</sup> MMMs showed a normal localization in CD47/- spleens.
Different requirements for SIRPa signaling in the hematopoietic or non-hematopoietic compartments to maintain normal B cell numbers

To investigate if the B cell phenotype observed in SIRPα-MT mice was due to the lack of SIRPα signaling within the hematopoietic or non-hematopoietic compartments, we reconstituted either Ly5.2 wild-type or SIRPα-MT mice with Ly5.1 wild-type BM cells, or Ly5.1 wild-type mice with Ly5.2 wild-type or MT BM cells.

SIRPa signaling within the non-hematopoietic compartment is required to maintain normal B cell numbers in the blood and normal B cell maturation in the BM

At 20 weeks post BM transfer, Wt → SIRPα-MT BM-chimeric mice had a significant reduction in total CD19+ B cells, as well as F-II and mature/F-I B cells in the blood (Paper I, Fig 4A), and a significant reduction in IgD+IgMhi F-II and IgD+IgMlo F-I B cells in the BM (Paper I, Fig 4C), as compared with that in Wt → Wt or SIRPα-MT → Wt BM-chimeric mice (Paper I, Fig 4D-F). Interestingly, the IgD-IgM+ immature B cell population remained unaffected in the BM of SIRPα-MT mice reconstituted with wild-type BM (Paper I, Fig 4C). Thus, SIRPα signaling within the non-hematopoietic compartment appeared to be required to maintain normal numbers of circulating B cells and normal B cell maturation in the BM. We also observed a significant reduction in the number of T cells in the blood of SIRPα-MT recipients of wild-type BM, while the numbers of neutrophils or monocytes remained unchanged, as compared with that in wild-type recipients of wild-type BM (Paper I, Fig 4B). In contrast, no differences were found in the numbers of blood T cells, neutrophils or monocytes in wild-type mice receiving either wild-type or SIRPα-MT BM (Paper I, Fig 4E). Thus, SIRPα-signaling within the non-hematopoietic compartment appeared to be important to maintain normal numbers of mature B cells in the BM and blood.

SIRPa signaling within the hematopoietic compartment is required to maintain normal B cell numbers in the spleen

In marked contrast to that in the BM or blood, Wt → SIRPα-MT BM-chimeric mice did not show any significant reduction in splenic F-II or F-I B cells (Paper I, Fig 6A). Even though circulating B cells and B cell maturation in the BM appeared to be normal in SIRPα-MT → Wt chimeras, splenic F-II or F-I B cell numbers were reduced to a similar extent as that seen in naïve SIRPα-MT mice (Paper II, Fig 6B). Thus, in marked contrast to that observed in the BM or blood, hematopoietic SIRPα signaling was required to maintain normal B cell maturation in the spleen.
**SIRPα signaling within the non-hematopoietic compartment is required to maintain normal MZ B cell numbers and normal MZ B cell retention in the MZ**

To understand if the MZ B cell phenotype observed in SIRPα MT mice was due to the lack of SIRPα signaling within the hematopoietic or non-hematopoietic compartment, Wt → Wt, SIRPα-MT → Wt or Wt → SIRPα-MT BM-chimeric mice were analyzed. In Wt → Wt and SIRPα-MT → Wt chimeras, MZ B cells numbers remained similar (**Paper II, Fig 4A**). However, we observed a 60% increase of MZ B cells in Wt → SIRPα-MT chimeras, as compared with that in Wt → Wt chimeras (**Paper II, Fig 4B**). In Wt → Wt or SIRPα-MT → Wt chimeric mice, MZ B cells were normally positioned in the splenic MZ (**Paper II, Fig 4C-D**). In contrast, MZ B cells of Wt → SIRPα-MT chimeras were localized normally to the MZ, but were also to a large extent found to be localized into the B cells follicles (**Paper II, Fig 4C-D**). These findings therefore identified a requirement for non-hematopoietic SIRPα signaling to maintain normal numbers and correct positioning of MZ B cells.

In summary, the BM reconstitution experiments identified a requirement of non-hematopoietic SIRPα signaling for normal FoB cell maturation in the BM, to maintain normal blood B cell numbers, and to maintain normal numbers and retention of MZ B cells in the splenic MZ. On the contrary, hematopoietic SIRPα signaling appeared to be important for FoB cell maturation in the spleen.

**Disturbed positioning of MZM and MMM in SIRPα-MT spleens**

In mice, correct positioning of MZ B cells to the MZ requires the interaction between an unknown ligand present on MZ B cells and the scavenger receptor MARCO on MZM (Karlsson et al 2003). In naïve SIRPα-MT spleens, MARCO⁺ MZMs were not restricted to the MZ, but also appeared to be redistributed into the red pulp areas (**Paper II, Fig 3A**). Similarly, MOMA-1⁺ MMMs were neither present in their normal location, but found to extend into the MZ (**Paper II, Fig 3B**). Analysis of the distribution of MOMA-1⁺ MMMs and MARCO⁺ MZMs in BM chimeric mice showed that MOMA-1⁺ MMMs had a broader distribution in the MZ of Wt → SIRPα-MT spleens, similar to that in naïve SIRPα-MT mice (**Paper II, Fig 4D**). In marked contrast, MARCO⁺ MZMs were localized normally to the MZ in spleens of Wt → SIRPα-MT chimeras, but had a disorganized distribution in the spleens of SIRPα-MT → Wt chimeras (**Paper II, Fig 4C**). Thus, hematopoietic SIRPα was required for accurate MZ retention of MZMs while normal distribution of MMMs required non-hematopoietic SIRPα signaling.
Discussion

The size of the mature B cell compartment is controlled by the amount of immature B cells that complete differentiation in the periphery and the average lifespan of mature B cells. The homeostatic mechanisms that control the mature B cell pool are poorly understood due to the complex environments of the BM or spleen. B cell development proceeds through the ordered differentiation of a B cell from committed precursors in the BM and ends with the generation of mature naïve B cells in the spleen or the BM itself (Cariappa et al 2006). Substantial amounts of data suggest that neither commitment to the B lineage, nor successful passage of BM B cells through different stages of development (Fr. A–D), are coupled to peripheral B cell numbers. It has been suggested that about 30% of the normal number of BM precursors is enough to maintain a normal size of the B cell pool in the periphery (Gaudin et al 2004). The mechanisms that regulate early B cell differentiation are fairly well described but the factors regulating B cell maturation are still largely unknown. The fact that studies of SIRPα-MT mice had shown that signaling through this receptor was important to maintain normal numbers of splenic T and DCs (Sato-Hashimoto et al 2011; Saito et al 2010) raised the question whether B cell homeostasis could be regulated by SIRPα signaling as well.

SIRPα is not required for early B cell differentiation in the BM

SIRPα is expressed by BM stromal cells and regulate their functional differentiation to osteoblasts (Koskinen et al 2013). In addition, the receptor is expressed by most myeloid lineage cells (Seiffert M et al 1999). Despite this, SIRPα-MT mice have normal numbers of myeloid cells in the blood (Sato-Hashimoto et al 2011, Paper I), suggesting that lack of SIRPα signaling in the BM may not influence hematopoiesis and blood cell homeostasis. It was therefore quite remarkable that SIRPα-MT mice had reduced numbers of B and T cells in the blood, despite the fact that these two cell types do not express SIRPα. However, the reduced number of circulating B cells was not due to an impaired early B cell differentiation, since analysis of SIRPα-MT BM showed no difference among Hardy fractions (A-D), when compared with that in wild-type mice. The amounts of immature B cells that complete their maturation in the periphery also determine the size of the mature B cell pool (Gaudin et al 2004).
B cell maturation is inhibited in the absence of SIRP\(\alpha\) signaling

Mature B cells generated from BM progenitors remain in equilibrium, suggesting that they are under strict homeostatic control. About two thirds of all sIgM\(^+\) immature B cells exit the BM and migrate to the spleen, whereas the remaining immature B cells continue their maturation within the BM (Cariappa et al 2006). In the spleen, immature B cells pass through two transitional stages (T1/T2) and further differentiate into long lived IgM\(^{hi}\) IgD\(^{hi}\) follicular type II B cells (F-II) that can mature into IgD\(^{hi}\) IgM\(^{low}\) (F-I) B cells. In the BM, immature B cells differentiate through a T1 stage to give rise to F-II and finally IgD\(^{hi}\) F-I B cells. The presence of IgD\(^{hi}\) B cells in the BM, often considered as recirculating B cells, has been recognized for decades (Osmond et al 1994). In the present study, lack of SIRP\(\alpha\) signaling in adult SIRP\(\alpha\)-MT mice resulted in an impaired B cell maturation in both the BM and spleen, evident by a strong reduction in semi-mature IgD\(^+\)IgM\(^{hi}/F\)-II and mature IgD\(^+\)IgM\(^{lo}/F\)-I B cells. While SIRP\(\alpha\)-MT mice also exhibited a significant reduction in T1 BM B cells, which are early precursors for maturing F-II and F-I B cells in BM, SIRP\(\alpha\)-MT mice had no significant reduction among splenic transitional B cells (T1/T2). Thus, lack of SIRP\(\alpha\) signaling had significant consequences for the ability to maintain normal levels of mature FoB cells.

Different requirements of hematopoietic and non-hematopoietic SIRP\(\alpha\) signaling to maintain FoB cell maturation in the BM and spleen

In contrast to that in B or T cells (Sato-Hashimato et al 2011, Paper I), SIRP\(\alpha\) is expressed by both non-hematopoietic stromal cells (Koskinen et al 2013) and hematopoietic cells (myeloid cells) (Matozaki et al 2009). This is important since non-hematopoietic stromal cells play an important role in lymphocyte development in both primary and secondary lymphoid organs (Mueller and Germain 2009). Consequently, SIRP\(\alpha\) signaling in stromal cells of the BM or spleen could therefore theoretically be of importance to regulate B cell maturation. However, the findings of the present work were rather unexpected, since they showed a strong dependence of non-hematopoietic SIRP\(\alpha\) signaling to maintain normal numbers of F-II and F-I B cells in the BM and blood, while SIRP\(\alpha\) signaling within the hematopoietic compartment was required to maintain normal B cell maturation in the spleen.

Although little is known regarding how B cell maturation is regulated by stromal cell factors, recent findings still strongly support an important role of SIRP\(\alpha\) signaling to maintain normal stromal cell function. Evidence for
this comes from the finding that BM stromal cell SIRPα tyrosine phosphorylation is required for stromal cell osteoblastic differentiation and function (Koskinen et al. 2013). In addition, SIRPα-MT mice have a marked reduction of the splenic stromal gp38+ FRC population, which in turn was found to contribute to reduced mRNA levels of CCL-21, CCL-19 and IL-7 in spleens of these mice (Sato-Hashimoto et al. 2011). Finally, the present study also showed that BM stromal cells of SIRPα-MT mice have a strongly reduced expression of RANKL in vitro, as compared with that in wild-type stromal cells. Although B cells express the RANKL-receptor RANK, the latter finding is not likely to explain the impaired B cell maturation in the BM of SIRPα-MT mice, since it has been found that B cell-specific deletion of RANK has no affect on B cell differentiation, maturation or function (Perlot and Penninger 2012).

It is however interesting to note, that the reduction of splenic gp38+ FRCs in SIRPα-mutant mice was found to be due to lack of hematopoietic SIRPα signaling (Sato-Hashimoto et al. 2011). In addition, recent findings have demonstrated that the presence of FRCs, but not that of FDCs, is required to provide enough BAFF to maintain normal numbers of mature B cells in secondary lymphoid organs (Cremasco et al. 2014). It is therefore possible that, since hematopoietic SIRPα was required to maintain normal numbers of mature B cells in the spleen, a reduced number of FRCs in SIRPα-MT spleens could well explain the impaired maturation of splenic B cells in these mice.

**Impaired FoB cell survival in the absence of SIRPα signaling**

Lymphocytes require survival signals to be maintained in the periphery, and the availability of such signals determines the size of the peripheral lymphocyte pool. Thus, mutations affecting either the production or survival of mature B cells can influence the number of B cells in the spleen (Rolink et al. 1999). Even though signaling through the BCR is important for maintenance of mature splenic B cells, it is not the only requirement. It is well known that members of the Bcl-2 family of proteins interact with each other to control the survival of cells (Bouillet et al. 1999). In many types of cells, over expression of anti-apoptotic proteins (e.g. Bcl-2) inhibits cell death, while over expression of pro-apoptotic proteins (e.g. BIM) promotes cell death. BIM is one of the proteins that affect the life expectancy of lymphocytes (Bouillet et al. 1999; Tischner et al. 2010). One possible explanation behind the reduced B cell numbers in SIRPα-MT mice could therefore be a reduced survival of these cells. Despite the fact that the gene expression of Bcl-2 and BIM were similar in wild-type and SIRPα-MT FoB cells, we observed a significant increase in BIM at the protein level in BM
immature and T1 B cells, as well as in splenic F-II and F-I B cells in SIRPα-MT mice. One possible explanation behind this discrepancy could be that the pro-apoptotic activity of BIM is also regulated at the posttranslational levels (Jorgensen et al. 2007). Supporting the hypothesis of a reduced survival among maturing B cells in SIRPα-MT mice was the finding of an increased amount of Annexin V+ apoptotic BM immature, T1 and F-II B cells, as well as splenic F-II B.

In mice, the cytokine IL-7 is considered indispensable for the survival of not only T cells (Creredig and Rollink 2012) but also B cells (Patton et al. 2014). Despite the fact that mature B cells do not express IL-7R, IL-7 has been suggested to indirectly control the survival of splenic FoB cells, where a hematopoietic, IL-7Ra+, Rag-1-dependent non-B cell could be required for normal survival of splenic FoB cells (Patton et al. 2014). Interestingly, SIRPα signaling has been found to be involved in regulating IL-7 in the spleen, since IL-7 gene expression is reduced in spleens of naïve SIRPα-MT mice and normal IL-7 expression requires SIRPα signaling in the hematopoietic compartments (Sato-Hashimoto et al. 2011). Thus, when IL-7 is limiting, the B cells might be occasionally deprived of this pro-survival cytokine and thus more likely to die. Indeed, IL-7-deficient mice are lacking mature B cells (Von Freeden-Jeffry et al. 1995). However, mature B cells accumulate more rapidly in IL-7 deficient mice if they also lack BIM, since lack of BIM promotes the survival of mature B cells, and BIM and IL-7 has therefore been shown to work together to control the survival of B cell precursors and the number of mature B cells (Oliver et al. 2004). Thus, an impaired IL-7 mediated splenic B cell survival is another possible mechanism behind the observed phenotype in SIRPα-MT mice.

In conclusion, a reduced number of maturing B cells in the BM and spleen of SIRPα-MT mice appeared to be associated with an increased rate of B cell apoptosis and strengthens the importance of SIRPα signaling in B cell survival.

**Non-hematopoietic SIRPα signaling influences splenic MZ B cell numbers and their positioning**

The MZ of the spleen is known to be the site where blood-borne antigens first enter the spleen and MZM, in partnership with MZ B cells, are particularly important in the host defense against TI pathogens. The MZ can be seen as a major intersection for the homing of lymphocytes to their respective zone and as a sorting station for TI and TD pathogens. Therefore the positioning of the cellular components that makes up this region can be seen as critical (Kraal and Mebius 2006).
A remarkable finding of the present study was the significantly increased population of splenic MZ B cells despite a decrease in FoB cells. MZ B cells constitutively express elevated levels of surface MHC class II, CD86 and CD80 molecules, which attributes for their robust antigen-presenting activity (Oliver et al 1999). However, the expression levels of all these markers were not increased in SIRPα-MT MZ B cells, indicting a normal activation level of these cells. The natural ability of MZ B cells to rapidly respond to TI antigens or blood-borne antigens conceptually places MZ B cells within a unique position in the immune system (Martin et al 2001). Mice deficient of MZ B cells are also deficient in T-I immune responses. The immune response to TNP-LPS or TNP-Ficoll was only slightly enhanced, despite the fact that SIRPα-MT mice exhibited a dramatic increase in the MZ B cell population. One possible explanation for this observation could be the increased follicular localization of MZ B cells of SIRPα-MT mice, which could reduce their exposure to these antigens.

In marked contrast to that for splenic FoB cells, we found that the increased number of MZ B cells, and their relocalization to the B cell follicles, was dependent on lack of SIRPα signaling in the non-hematopoietic compartment. The exact mechanisms behind non-hematopoietic regulation of MZ B cell number and positioning are still not fully understood. However, the correct localization of MZ B cells in the MZ is governed by many factors which include, integrin mediated adhesion to ligands on non-hematopoietic cells (Lu and Cyster 2002), interaction with MZMs (Karlsson et al 2003), expression of the S1P1 receptor (Cinamon et al 2004) and the splenic microenvironment. MZ B cells are under the influence of the CXCL13 chemokine gradient, responsible for recruiting them into the B cell follicles, and signaling generated by S1P1, and S1P3, which are receptors for S1P abundantly present in the blood and stimulating MZ B cell migration towards the MZ (Cinamon et al 2004). Therefore, one logical hypothesis for a follicular localization of splenic MZ B cells in SIRPα-MT mice could be the enhanced migration of these cells in response to CXCL13, or reduced migration in response to S1P. However, we found that SIRPα-MT MZ B cells migrated equally well in response to either S1P or CXCL13 in vitro, which ruled out this hypothesis.

Another distinct mechanism that has been suggested for retention of MZ B cells in the MZ is the interaction between MZMs and MZ B cells (Karlsson et al 2003). Interestingly, in naïve SIRPα mutant spleens MARCO+ MZM were no longer strictly organized in the marginal zone, but instead appeared to be localized into the red pulp area. However, studies in BM chimeric mice showed that an increased follicular localization of MZ B cells required lack of non-hematopoietic SIRPα, whereas the disturbed localization of MZMs was
only observed in the absence of hematopoietic SIRPα signaling. Therefore, the aberrant MZ B cell localization in SIRPα-MT mice was unlikely mediated by the lack of MZMs in the MZ.

The MOMA-1+ MMMs, normally positioned as a thin layer of macrophages between the marginal sinus and the B cell follicle, were found to be present in the whole MZ of naïve SIRPα-MT spleens. This widened distribution of MMMs was found to result from the lack of SIRPα signaling in the non-hematopoietic compartment. It is interesting to note, that mice deficient in the S1P receptor S1P3 also showed a widened distribution of MMMs and an increased localization of MZ B cells into the follicles (Girkontaite et al 2004). Thus, although this hypothesis needs further investigation, it is possible that an abnormal distribution of MMMs in SIRPα-MT spleens could explain why these mice have an increased amount of MZ B cells in B cell follicles.

It has been suggested that in a lymphopenic mice, such as xid or IL-7Rα deficient mice (where FoB cells are severely diminished), B cells preferentially acquire and maintain an innate like B cell phenotype such as MZ B cells (Martin and Kearney 2002). It is therefore tempting to speculate that the reduced number of mature FoB cells could result in an increased population of MZ B cell in SIRPα-MT mice. In addition, it has been hypothesized that FoB cells can give rise to MZ B cells (Bhaskar 2005). Thus, it is possible that there is an enhanced differentiation of FoB cells into MZ B cells in SIRPα-MT mice, although this hypothesis needs further investigation. Hao and Rajewsky have demonstrated that the half-life of MZ B cells is longer than that of FoB cells, and that unlike FoB cells, MZ B cells are self-renewing (Hao and Rajewsky 2001). Thus, another explanation for the accumulation of MZ B cells in SIRPα mutant mice with increasing age could be that these self-renewing MZ B cells preferentially survive more, and thus their frequency relative to the FoB cell pool increases slowly with age.

Overall, SIRPα appears to be a negative regulator of MZ B cell development and a positive regulator of FoB cell development.

Could CD47 be required for SIRPα-mediated regulation of B cell homeostasis?

It is known that the extracellular region of SIRPα interacts with CD47 and that this constitutes a cell-cell communication system (Barclay and Brown 2006). Thus, the CD47-SIRPα interaction has been shown to play an important role in the regulation of phagocytosis (Oldenborg 2000), DC homeostasis (Hagnerud S et al 2006, Saito Y et al 2010, and Van VQ et al 2006) and T cell homeostasis (Sato-Hashimoto et al 2011). In contrast to the
relatively restricted distribution of SIRPα, CD47 is expressed by virtually all cells including a variety of hematopoietic cells (Barclay and Van den Berg 2013). B cells express CD47, but in contrast they do not express SIRPα (Barclay and Van den Berg 2014). Given this, it was obvious to look for a B cell phenotype also in CD47−/− mice.

Indeed, CD47−/− mice were found to have reduced numbers of mature B cells in BM and blood, a reduction in splenic FoB cells, and an increase in the MZ B cell population. Thus, CD47−/− mice appeared to have a phenotype fairly similar to that observed in SIRPα-MT mice (Paper I & II). However, some differences were also observed when comparing these two genetically manipulated mouse strains: First, a loss of mature splenic FoB cells and an increase in MZ B cells was observed at a higher age in CD47−/− mice, as compared with that in SIRPα-MT mice. Second, splenic MZ B cells and MOMA-1+ MMMs were normally positioned in the MZ of CD47−/− mice, which contrasts that found in SIRPα-MT mice (Paper I & II).

It is important to note that in CD47−/− mice there is a complete lack of the CD47 protein, while SIRPα expression and function is normal. On the contrary, SIRPα-MT mice have normal expression of CD47 and express the extracellular domain of SIRPα, while the cytoplasmic domain is deleted which leads to the complete lack of SIRPα signaling (Sato-Hashimoto et al 2011). Therefore, since B cell do not express SIRPα, the phenotype of SIRPα mutant mice must be related to effects that are indirectly caused by the lack of SIRPα signaling in other hematopoietic or non-hematopoietic cells. As discussed in paper I and paper II, non-hematopoietic SIRPα signaling is required for normal B cell maturation in the BM, the blood and to maintain normal numbers of MZ B cells in the splenic marginal zone. On the contrary, hematopoietic SIRPα signaling appeared to be important for FoB cell maturation in the spleen (Paper I). If the CD47-SIRPα interaction is the only way SIRPα signaling could be involved in regulating B cell homeostasis, lack of CD47 should result in a phenotype mimicking that of the SIRPα-MT mice. However, SIRPα signaling can also be induced by CD47-independent mechanisms such as integrin activation (Johansen and Brown 2007). This might explain the observed differences in the B cell phenotype of CD47−/− and SIRPα-MT mice and at what age they occur.

As mentioned, both CD47−/− and SIRPα-MT mice accumulate splenic MZ B cells although at different ages, and MZ B cells have been suggested to be self-renewing and live longer than FoB cells (Hao and Rajewsky 2001). It is therefore interesting that CD47 has also been shown to regulate apoptosis, and CD47−/− cells are more resistant to Fas (CD95)-induced apoptosis (Manna et al 2003; 2005). Thus, it is tempting to speculate that the
accumulation of MZ B cell in CD47−/− mice at an increasing age is due to a defect in MZ B cell apoptosis, although this attractive hypothesis requires further investigation.

In conclusion, deficiency of CD47 results in a late onset B cell phenotype presenting as a reduction in mature FoB cells with a subsequent increase of the MZ B cell population. However, this phenotype was not associated with an altered B cell distribution in the spleen or increased levels of autoantibodies in aged mice.
Conclusions

The major findings of this thesis can be summarized as follows:

- Lack of SIRPα signaling in SIRPα MT mice does not affect early B cell differentiation, but results in an impaired FoB cell maturation in the BM and spleen.

- SIRPα signaling seems to be important for the survival of maturing FoB cells, since such cells in BM or spleen of SIRPα MT mice expressed higher levels of the pro-apoptotic protein BIM and contained an increased number of apoptotic cells.

- In contrast to that for FoB cells, SIRPα signaling seems to be important to inhibit the accumulation of MZ B cells, since SIRPα MT mice had an increased number of this B cell subset with increasing age.

- The fact that SIRPα MT mice showed altered distributions of splenic MZ B cells, MARCO+ MZMs and MOMA-1+ MMMs, together suggest that SIRPα signaling regulates the retention of MZ B cells, as well as the distribution of MZMs and MMMs, in the MZ.

- Using bone marrow chimeric mice, this study identified the following requirements for hematopoietic or non-hematopoietic SIRPα signaling in regulating B cell homeostasis, and in regulating the positioning of cells in the splenic MZ:
  
  o **non-hematopoietic SIRPα signaling** was required to maintain normal B cell maturation in the BM, to maintain normal numbers and retention of MZ B cells, and for normal positioning of MOMA-1+ MMMs.

  o **hematopoietic SIRPα signaling** appeared to be important for FoB cell maturation in the spleen and for normal positioning of MARCO+ MZMs.

- **CD47/−** mice partially mimicked the phenotype of SIRPα MT mice and exhibited reduced numbers of mature FoB cells with subsequent increase of the MZ B cell population. However, this phenotype was occurring at a higher age than that in SIRPα MT mice, and **CD47/−** mice had normal splenic positioning of MZ B cells and MOMA-1+ MMMs.
Overall, the data in the present thesis has uncovered a hitherto unknown role of SIRPα signaling in regulating B cell homeostasis. However, further investigation is needed to fully understand if CD47 binding is involved in mediating these important effects of SIRPα signaling, or if CD47 has other separate roles in regulating B cell homeostasis. Based on the findings in this thesis, the following model can be proposed for the different requirements of SIRPα signaling in B cell homeostasis (Fig. 9).

Figure 9: A model for the requirement of SIRPα signaling to maintain B cell subsets in different immunological organs.
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