Heparan sulfate proteoglycans in B cell maturation and myeloma plasma cell survival
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General introduction
Introduction

The immune system serves to protect the host against pathogens such as bacteria, viruses, and parasites. On a functional basis, it can be divided in two branches: the innate and the adaptive immune system. Although these are usually regarded as two separate systems, the collaboration is considerable. Cells from the innate immune system are immediately available to combat a wide range of pathogens without requiring prior exposure, and acts as a first line of defense. In contrast, the adaptive immune system will provoke a highly specific immune response. In addition, it will develop an immunological memory, which will provide a faster and stronger immune response upon stimulation with the same antigen (Ag). The most important players during an adaptive immune response are the B and T lymphocytes. While T lymphocytes are mainly involved in the destruction of the infected cells, and in the regulation of the immune response, B lymphocytes are the producers of immunoglobulins (Igs). These Igs specifically recognize and bind Ag, leading to the clearance of the organisms or cells that express that specific Ag.

The development and maturation of B and T lymphocytes occurs in specialized lymphoid structures. Early B and T cell development takes place in the bone marrow and the thymus, respectively, which are also known as the primary lymphoid organs. After the development into immature B and T cells, these lymphocytes will enter the circulation and migrate to the secondary lymphoid organs, such as the spleen and lymph nodes, but also the mucosal-associated lymphoid tissue and Peyer’s patches. These specialized lymphoid structures allow B and T cells to further mature and eventually differentiate into immune-competent cells, ready to battle host endangering invaders. The secondary lymphoid organs are crucial for long-term defense against the pathogens because it is in these structures that immunological memory can be formed.

Lymphoid organs are localized at strategic positions throughout the body. The following section describes the development and positioning of these organs, which is subject of study in some of the chapters in this thesis.

Lymphoid organ development and function

The thymus

In mice, the morphogenic events of early thymus organogenesis occur between embryonic day 10 (E10) and E13.5. The cell types required for initiation of organogenesis are present by E10, and the earliest phase of organogenesis culminates in the formation of two primordia, thymic and parathyroid, each surrounded by a condensing mesenchymal capsule. By E13.5 the parathyroid and thymus are separated into physically distinct organs. The thymus develops bilaterally as a result of interactions between the third pharyngeal pouch endoderm and surrounding neural crest mesenchyme. This will eventually result in the formation of a bilobular thymus, positioned at the midline on top of the heart, which subsequently becomes populated by thymocytes (Manley and Blackburn, 2003). Although marked progress has been made in identifying the molecular regulators of
thymic organogenesis (Chisaka et al., 1991; Hetzer-Egger et al., 2002; Peters et al., 1998; Wallin et al., 1996; Xu et al., 2002), the signaling pathways that control early thymic development remain to be fully identified. The development and maturation of the thymus primordium and its subsequent population by T cell precursors is dependent on several soluble factors, including fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF)-BB and chemokines, including CCL21 and CCL25 (Khan et al., 2008; Tsai et al., 2003). The mature thymic epithelium is complex, with two major compartments, the cortex and the medulla, each containing several functionally distinct thymic epithelial-cell (TEC) types, which appear between E12 and E14. Pro thymocytes develop from bone marrow hematopoietic stem cells and migrate to the thymus where they mature. The different TEC subsets generate discrete intrathymic microenvironments each specialized in mediating a particular aspect of thymocyte maturation and development (Anderson et al., 2001; van Ewijk et al., 2000; Ritter and Boyd, 1993). Consistent with this model, T cell development is characterized by the progression through several phenotypically distinct stages, defined as double negative (DN), double positive (DP) and single positive (SP) based on expression of the co-receptors CD4 and CD8. T cells at these different stages of development occupy distinct spatially restricted domains in the adult thymus (Lind et al., 2001; Plotkin et al., 2003; Prockop and Petrie, 2000), indicating that differentiation occurs concomitantly with a highly ordered migration. Thymocytes and TECs are in close contact throughout this differentiation program. Once matured, the T cells migrate to the periphery to contribute to the adaptive immune system.

The spleen

The spleen anlage, in mice, is detectable at approximately E10.5-11, as progenitor cells start to condense within the dorsal mesogastrium, adjacent to the stomach and dorsal of the pancreas primordia. At this stage, the spleen anlage is surrounded by the splanchnic mesodermal plate, which is supportive and crucial during this early developmental stage (Green MC, 1967). The formation and outgrowth of the spleen from E12 onwards, depends on the commitment of the mesenchymal cells and the subsequent colonization by hematopoietic and red blood cells (Godin et al., 1999; Mebius et al., 1997; Sasaki and Matsumura, 1988). The first cells that colonize the spleen are progenitors of the erythroid and myeloid lineages; at E14.5, after these progenitors have entered, the first hematopoietic stem cells populate the spleen. Ultimately, the spleen is organized as a tree of branching arterial and arteriolar vessels that end in a venous sinusoidal system. The organ is surrounded by a fibrous capsule of connective tissue, from which trabeculae emerge, that support the larger vasculature. The smaller branches of the arterial supply are sheathed by lymphoid tissue, which forms the white pulp of the spleen. In rodents, some of the smallest arterial branches terminate in the marginal sinus, the space between the white pulp and the surrounding marginal zone, whereas others traverse the marginal zone to form the venous system of the red pulp. By its location in the circulatory system and the unusual structure of its lymphoid compartments, the spleen is a unique lymphoid organ (Mebius and Kraal, 2005).
Once fully developed, the spleen combines the innate and adaptive immune system, and functions as the largest filter of the blood. The highly specialized structure of the venous system of the red pulp provides the spleen with the capacity to filter the blood and to remove old erythrocytes (Mebius and Kraal, 2005). Within the lymphoid sheaths T- and B cells are localized in separate compartments. T cells are found in the T-cell zone, also known as the periarteriolar lymphoid sheaths (PALS), and B cells are localized in B cell follicles (Mebius and Kraal, 2005). The T- and B cells entering the spleen are guided by the chemokines CXCL13 and CCL21/CCL19, respectively, to their destined locations. In addition, the white pulp has a surrounding marginal zone, containing cells with specialized functions, including marginal zone macrophages and marginal zone B cells. Whereas the white pulp is mainly involved in the adaptive immune system, the outer marginal zone is involved in both innate and adaptive immunity, through its specific macrophage populations and marginal zone B cells. The macrophages can directly bind and internalize pathogens via their specialized (pattern recognition) receptors, after which the pathogen is targeted to the lysosomes for degradation (Geijtenbeek et al., 2002). Interestingly, after Ag encounter, the marginal zone B cells can either directly differentiate into IgM-secreting plasma cells or gain the capacity to function as antigen presenting cells (APCs). To function as an APC, they migrate towards the white pulp, where they activate naive CD4+ T cells (Attanavanich and Kearney, 2004). The entry of APCs, including marginal zone B cells, but also blood dendritic cells, into the white pulp is an important step in the initiation of an adaptive immune response. In the section B cell development, antigen specific differentiation and malignant transformation, the adaptive, or humoral response will be further discussed.

Taken together, the spleen is an organ that accommodates the efficient phagocytosis of old erythrocytes and the capture and destruction of pathogens via the innate immunity, and supports the induction of adaptive immune responses.

**Lymph nodes**

The earliest event in lymph node (LN) development is the generation of the lymphatic vasculature. The mammalian lymphatic vasculature has a venous origin and is derived from primitive lymph sacs scattered along the body axis of the embryo. It has been generally accepted, that LNs originate from these embryonic lymph sacs (Sabin, 1909; Srinivasan et al., 2007), however, very recently it has been demonstrated that the initial formation of the mammalian LN anlage can occur without the support of lymph sacs. Further progression into clusters of hematopoietic cells requires the presence of normal lymph sacs (Vondenhoff et al., 2009).

The second stage in LN development (Fig. 1) depends on the population of the lymphoid primordia by circulating hematopoietic cells (Moore, 2004). Interactions between these cells and mesenchymal stromal cells are necessary for the survival and further development of both cell types and for the outgrowth of the primordium (Vondenhoff et al., 2007; Mebius, 2003). Over the past decade, it has become clear that the signals for LN development emanate from lymphoid tissue inducer cells (LTi cells). These cells express lymphotoxin-α1β2 (LTα1β2) and trigger the lymphotoxin β receptor (LTβR) expressed on stromal cells (Adachi et al., 1997; Adachi et al., 1998; Finke et al., 2002; Honda et al., 2001; Mebius, 2003; Yoshida et al., 1999;
Yoshida et al., 2002). This results in the production of the homeostatic chemokines CCL19, CCL21, and CXCL13, and the increased expression of adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), necessary for the attraction and retention of additional LTi cells, and other hematopoietic cells, such as B and T lymphocytes that will also contribute to the hematopoietic-mesenchymal cell interaction (Ansel et al., 2000; Cupedo et al., 2004; Dejardin et al., 2002; Luther et al., 2003; Muller et al., 2003; Ohl et al., 2003; Rennert et al., 1998). Consequently, the primordium develops into a functional LN (Fig. 1). These LNs collect the extracellular fluid from the tissues and return it to the blood. The extracellular fluid from tissues will enter the LN via the

**Figure 1. General scheme of the development of secondary lymphoid organs.** Step 1-3 occur between embryonic day 9 (E9) and E18, which consists of the initial phase of the development of the lymphoid structure, the subsequent attraction of lymphocytes and the formation of early follicles. Lymphoid tissue inducer cells (LTi) interact with lymphoid tissue organizer cells (LTo), resulting in a positive feedback loop. The secretion of chemokines, including CXCL13, CCL21 and CCL19, attract additional LTi cells and other lymphocytes (step 3), and induces the expression of the adhesion molecules VCAM-1 and ICAM-1 on the LTo cells via the lymphotixn-beta receptor (LTβR). The clustering will eventually organize into follicles, which is completed 3-5 days after birth (step 4). At this stage the T and B cells are separated, with the B cells in follicles containing follicular dendritic cells (FDC). For more detail see text. Adapted and modified from Randall et al., 2008.
afferent lymphatic vessels, which carry cells bearing Ags from sites of infection. In these secondary lymphoid organs, where B cells are localized in follicles surrounded by T cells in the paracortical areas, an adaptive immune response can occur and immunological memory can be generated.

**The bone marrow**

Although the embryonic development of the bone marrow (BM) is not investigated in this thesis, it is an important primary lymphoid organ. The BM constitutes the site of hematopoiesis, the generation of the cellular elements of blood, including red blood cells, monocytes, polymorphonuclear leukocytes, and platelets. The BM is also the site of B cell development and is the source of stem cells that give rise to T cells upon migration to the thymus. All hematopoietic cell lineages are derived from the same progenitor cell or precursor cell, identified as the hematopoietic stem cell (HSC). In addition, the BM provides specialized survival niches for normal BM plasma cells, but also multiple myeloma cells. In the following sections, the role of the BM in B cell development and (malignant) plasma cell survival will be further described.

**B cell development, antigen-dependent differentiation and malignant transformation**

**B cell development and antigen-dependent differentiation**

B cells represent the humoral component of the adaptive immune system. In mammals, the development of B cells is initiated in the bone marrow. Here, committed common lymphoid progenitors differentiate into immature B cells, a process that is antigen-independent. An important process during early B cell development is the rearrangement of the variable (V), diversity (D), and joining (J) gene segments of the heavy chain (IgH) gene locus (Alt et al., 1987; Tonegawa, 1983). This allows the B cell to express the pre-B cell receptor (pre-BCR), which is composed of the rearranged IgH chain complexed to a surrogate light chain (Melchers et al., 1994). Successful expression of a functional pre-BCR is indispensable for survival and further maturation of the B cell. Signaling via the pre-BCR induces the rearrangement of the V and J gene segments of the light chain (IgL) gene locus (ten Boekel et al., 1995; Constantinescu and Schlissel, 1997). Proper rearrangement and successful complexation with the IgH chain allows the B cell to express a BCR of the IgM isotype, which is required for (tonic) survival signals (Melchers et al., 1994). These IgM expressing B cells are the immature bone marrow B cells, which are further characterized by the expression of CD19, B220, and HSA.

Newly formed, immature B cells complete their maturation after migrating to the periphery (Allman et al., 1992; Allman et al., 1993), and are named transitional cells. According to Loder and colleagues these transitional cells can be divided in transitional 1 (T1) B cells, which are IgM<sup>H</sup>CD21<sup>−</sup>CD23<sup>−</sup>B220<sup>−</sup>IgD<sup>lo/bright</sup> and T2 B cells, being IgM<sup>H</sup>CD21<sup>−</sup>CD23<sup>−</sup>B220<sup>−</sup>IgD<sup>bright</sup> (Loder et al., 1999). Eventually the transitional B cells will develop into mature follicular B cells in the secondary lymphoid organs, including the spleen and lymph nodes. The mature B cell production rates are primarily a reflection of the degree to which immature bone marrow cells successfully
navigate the transitional pools, which fully depends on the expression of a functional, non-autoreactive BCR (Loder et al., 1999).

Upon challenge with antigen mature B cells can undergo antigen-specific B cell differentiation, which requires multiple interactions with T cells and follicular dendritic cells (FDC), and with the extracellular matrix. These interactions take place in the germinal center (GC), a specialized microenvironment within the B cell follicles of the secondary lymphoid organs. Two distinct areas in the GC can be distinguished, known as the dark and light zone. Stromal-derived factor-1 (SDF-1 or CXCL12), which is more abundant in the dark zone than in the light zone, attracts centroblasts that have high expression of CXCR4. In contrast, CXCR5 in combination with B lymphocyte chemokine (BLC or CXCL13) helps direct the centrocytes to the light zone, this occurs after downregulation of CXCR4 (Allen et al., 2004). Within the dark zone B cells undergo rapid clonal expansion and somatic hypermutation of their immunoglobulin genes by activation-induced cytidine deaminase (AID), an enzyme that creates deliberate mutations in DNA, replacing cytidines by uracils, resulting in a CG to AT transition (Arakawa et al., 2002; Yoshikawa et al., 2002). These B cells, or centrocytes, than migrate towards the light zone of the GC. Here, they re-encounter Ag, presented as immune complexes by FDC, and undergo affinity selection. Dependent on the strength of the BCR signal the B cell clones will rapidly expand (high affinity) or go into apoptosis (low affinity). As a consequence of isotype switching of the IgH genes to IgG, IgE or IgA, the antibodies produced are better adapted to their specific effector function. The stimulatory signals will eventually lead to the differentiation of the centrocytes into either antibody-producing plasma cells, or memory B cells, which upon antigen recall cause a rapid, more specific secondary
immune response (Kraal et al., 1982; Arpin et al., 1995; Malisan et al., 1996). After re-encounter of the antigen by memory B cells, they will either directly differentiate into plasma cells, or will do so after they have re-entered a germinal center reaction (Bende et al., 2007).

**Plasma cell function and survival**

Plasma cells can be short-lived or long-lived, and long-lived plasma cells constitute an independent compartment of “immunological memory” (Slifka et al., 1998). Plasma cells are no longer responsive to antigen and are resting in terms of proliferation. Whether a plasma cell is short-lived or becomes a long-lived plasma cell is determined by poorly defined molecular mechanisms and by survival signals from its environment (Hoyer et al., 2004). Nevertheless, when B cells have differentiated into plasma cells after a successful germinal center reaction, they are programmed to home to the bone marrow (Fig. 2). Here, the long-lived or bone marrow plasma cells lie in close proximity to non-dividing mesenchymal stromal cells, expressing vascular cell-adhesion molecule-1 (VCAM-1) and high levels of SDF-1, key to their survival (Tokoyoda et al., 2004). In addition, besides SDF-1, bone marrow plasma cells mainly require the supporting signals of BAFF, APRIL, and IL6 (Hideshima et al., 2002; O’Connor et al., 2004). The frequency of VCAM-1 positive, SDF-1-expressing stromal cells is approximately 1% of all bone marrow cells in mice and the physiological frequency of plasma cells among bone marrow cells of adult humans and mice is also found to be around 1% (Fig. 2). Thus, the stromal cells put protective plasma cell memory in the context of the volume of the bone marrow, which is apparently a physiological necessity to prevent hypergammaglobulinaemia and plasmacytosis (Tokoyoda et al., 2004 and 2010). Within this specialized environment, or survival niche, where plasma cells synthesize huge amounts of systemic protective antibodies, they can survive for many years.

**Malignant transformation of B cells**

The classification of human lymphoproliferative disorders has steadily evolved since their recognition by Thomas Hodgkin, already in 1832 (Hodgkin, 1832). The characteristics displayed by B cell tumors are often reminiscent of that of normal B cells, allowing for the classification of distinct subcategories (Fig. 3). During development and differentiation, DNA-modifying processes that many B cells undergo, involve double stranded DNA-breaks and the induction of mutations introduced by the recombination activating genes-1 (Rag-1) and -2 (Rag-2) and AID (Kuppers, 2005; Shaffer et al., 2002). In combination with rapid clonal expansion these processes predispose to malignant transformation. Although sophisticated mechanisms are developed to repair unwanted DNA damage, errors still do occur. These errors in B cell-specific DNA remodeling processes, often taking place in the Ig genes, may lead to translocation to the Ig locus. As a result, the powerful enhancers, which normally control BCR expression, are juxtaposed to genes that play important roles in survival, cell cycle regulation, or proliferation. This can ultimately lead to the formation of different B cell tumors (Fig. 3), which are classified based on morphology, configuration of the BCR, and expression of membrane proteins and large scale gene profiling (Swerdlow et al., 2008).
B cell homing and lymphoma dissemination

The orchestration of a systemic immune response is critically dependent on coordinated lymphocyte migration and recirculation. This homing capacity guides lymphocyte subsets to the appropriate specialized microenvironments (Pals et al., 2007). Lymphocyte homing is a multi-step process that requires chemotaxis and cell adhesion, coupled with strategies to overcome physical barriers. At the molecular level, it is regulated by adhesion molecules in concert with chemokines and facilitated by intrinsic molecular programs that allow 'amoeboid' shape change of lymphocytes. These properties allow highly effective lymphocyte traffic between different tissue compartments. In case of malignant transformation, however, the fact that lymphocytes are 'licensed to move' forms a serious threat to the organism, since it allows rapid tumor dissemination irrespective of the conventional anatomic boundaries limiting early spread in most other types of cancer. The dissemination...
patterns often reflect basic rules of lymphocyte homing, explaining the striking tissue-specific distribution of, for example B cell lymphomas and multiple myeloma (MM) (Pals et al., 2007).

**Chemotaxis and chemokine signaling**

Chemotaxis is the process of induced directional migration towards a gradient of chemokines, and determines the eventual localization of different sub-sets of normal lymphocytes, but also of malignant lymphoma and multiple myeloma cells. In particular, the role of SDF-1 in lymphocyte trafficking is well established. SDF-1 was originally identified as a growth-stimulatory factor for pre-B cells, and is a potent chemotactic factor for different cell types (Ansel and Cyster, 2001). SDF-1 is constitutively expressed in the BM by bone marrow stromal cells (Ma et al., 1998), and retains multipotent hematopoietic progenitors and pre-pro-B cells in specific niches of the BM (Tokoyoda et al., 2004). Interestingly, mice lacking either SDF-1 or CXCR4, the cognate receptor for SDF-1, have a nearly absent B cell lymphopoiesis in the BM, and die perinatally due to cardiac and vascular defects (Ma et al., 1998). Finally, SDF-1 induces plasma cell migration, and CXCR4 is required for accumulation of long-lived, but also malignant plasma cells in the BM (Hargreaves et al., 2001).

Several proteins involved in the signaling pathways underlying chemokine-induced lymphocyte adhesion and migration have been identified. These proteins include kinases, adapter proteins and small GTPases. Of the small GTPases, members of the Rho family have been studied extensively, in particular regarding their capacity to regulate the cytoskeletal rearrangements (Ridley, 2001; Etienne-Manneville and Hall, 2002) required for cell polarity and motility. Members of the Ras family of small GTPases, like Ras and Rap1, and the less well studied small GTPase Ral, have been found to be involved in chemotaxis and cell motility (Feig, 2003; McLeod et al., 2002; Suzuki et al., 2000; Weber et al., 2001). The Ral GTPases are highly homologous to Ras GTPases (Chardin and Tarvitian, 1986), with 58% identity. Ral proteins have been implicated in controlling a multitude of cellular processes, including proliferation, survival, endo- and exocytosis, cytoskeletal rearrangements, migration, gene transcription and transformation (Feig, 2003). Two Ral GTPases, RalA and RalB, have been identified that show 85% identity. The main difference between RalA and RalB is found in their C-terminal variable regions and causes different subcellular localization of the two isoforms, resulting in different functions (Lim et al., 2005; Shipitsin and Feig, 2004). Despite the high degree of sequence homology, the affinity for effectors also varies between the two Ral isoforms (Shipitsin and Feig, 2004).

Interestingly, Ras mutations have been found in multiple myeloma (MM). In addition, it was demonstrated that Ras is activated in MM cells by numerous signals provided by the BM microenvironment. Importantly, Ral was found to be a critical mediator of Ras-induced tumorigenesis (Feig, 2003). Knockdown of RalA, but not of RalB expression, impaired the ability of human cancer cells to form tumors in immunodeficient mice (Lim et al., 2005). Although recent studies have clearly demonstrated an important function for Ral in cancer, the role of Ral in MM cells remains to be established.
Multiple myeloma

Multiple myeloma (MM) is a common B cell neoplasm, which is characterized by clonal expansion of malignant plasma cells in the bone marrow (BM), accompanied by renal failure, pancytopenia, and osteolytic bone destruction. MM is often preceded by a pre-malignant stage termed monoclonal gammopathy of undetermined significance (MGUS), defined by the presence of serum monoclonal protein at less than 30 g/L, fewer than 10% of plasma cells in bone marrow, and the absence MM features (Kuehl and Bergsagel, 2002). Although significant progression in the treatment of MM has been made over the last decade, the disease is still incurable with a median survival of 3-5 years. MM accounts for ~2% of all cancer deaths and nearly 20% of deaths caused by hematological malignancies (Kuehl and Bergsagel, 2002). Like in many B cell malignancies, in MM cells chromosomal translocations place oncogenes under transcriptional control of enhancer regions near the IgH gene locus (Kuehl and Bergsagel, 2002). These tumors are nonhyperdiploid, and mostly have one of five recurrent IgH translocations involving CCND1, CCND3, FGFR3 and MMSET, and C-MAF, contributing to the disease in approximately 40% of the patients (Table 1). The remaining hyperdiploid tumors have multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, and infrequently one of the five translocations. A common rearrangement that often occurs in late stage MM involves the misregulation of the c-myc gene (Shou et al., 2000). In addition, virtually all MM and MGUS tumors have deregulated and/or increased expression of cyclin D1, D2, or D3 (Table 1), providing an apparent early, unifying event in pathogenesis (Bergsagel and Kuehl, 2005).

Table 1. Most frequently found translocations in MGUS and MM with cyclin-D status. Abbreviations: D, diploid; H, hyperdiploid; NH, nonhyperdiploid

<table>
<thead>
<tr>
<th>Primary translocation</th>
<th>Gene at breakpoint</th>
<th>D-cyclin</th>
<th>Ploidy</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20q11</td>
<td>mafB</td>
<td>D2</td>
<td>NH</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>None</td>
<td>NH</td>
<td>2</td>
</tr>
<tr>
<td>6p21</td>
<td>CCND3</td>
<td>D3</td>
<td>NH</td>
<td>3</td>
</tr>
<tr>
<td>16q23</td>
<td>c-maf</td>
<td>D2</td>
<td>NH</td>
<td>5</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>D1 &amp; D2</td>
<td>H</td>
<td>6</td>
</tr>
<tr>
<td>4p16</td>
<td>FGFR3/MMSET</td>
<td>D2</td>
<td>NH &gt; H</td>
<td>15</td>
</tr>
<tr>
<td>11q13</td>
<td>CCND1</td>
<td>D1</td>
<td>D, NH</td>
<td>16</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>D2</td>
<td>H, NH</td>
<td>17</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>D1</td>
<td>H</td>
<td>34</td>
</tr>
</tbody>
</table>

Like normal antibody-producing plasma cells, during all stages of intramedullary MM, the cells are dependent on the BM environment or niche, where cytokines and growth factors, like IL6, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin growth-factor-1 (IGF-1), stromal cell-derived factor-1 (SDF-1), hepatocyte growth factor (HGF), and WNTs, amongst others, are produced by BM stromal cells or by MM cells themselves (Fig. 4) (Derksen et al., 2002; Derksen et al, 2003; Kuehl and Bergsagel, 2002; Seidl et al., 2003). Equally important is the direct physical contact of the MM cells with BM stromal cells via integrins and other adhesion molecules, which in turn can mediate growth and survival, and trigger para- and autocrine cytokine production (Fig. 4) (Damiano et al., 1999; Uchiyama et al., 1993; Esteveand Roodman, 2007). Recently, it was demonstrated that overexpression of c-maf, a frequent oncogenic event in MM, induces the
expression of integrin β7. β7 can heterodimerize with either α4 or αE, which can bind to MadCAM-1 and E-cadherin respectively. Induction of β7 increased the adhesion to E-cadherin on stromal cells, resulting in the expression of VEGF, and promoting MM cell proliferation and survival (Hurt et al., 2004). Interestingly, research over the past decades points towards a versatile and pivotal role for the pleiotropic heparan sulfate proteoglycan (HSPG) syndecan-1, being a key regulator in the interaction of MM cells with the BM microenvironment (Fig. 4) (Derksen et al., 2002; Khotskaya et al., 2009; Mahtouk et al., 2006, 2007; Yang et al., 2002, 2007).

**Heparan sulfate proteoglycans**

**Heparan sulfate proteoglycans**

Heparan sulfate proteoglycans (HSPGs) are proteins with covalently attached, unbranched polysaccharide heparan sulfate (HS) chains, which in the native form consist of alternating N-acetylated glucosamine (GlcNAc) and D-glucuronic acid (GlcA) units (Esko and Lindahl, 2001; Esko and Selleck, 2002; Lindahl et al., 1998).

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**Figure 4. Interaction of multiple myeloma cells with the bone marrow microenvironment.** A schematic overview of a multiple myeloma (MM) cell interacting with different components of the bone marrow (BM) environment. MM cells migrate into the BM, directed by the stromal cell-produced chemokine SDF-1. Once in the BM, MM cells are provided with an array of auto- and paracrine soluble survival and proliferation inducing factors. In addition, the MM cell can interact with the extracellular matrix, or stromal cells, via several integrins or the heparan sulfate proteoglycan (HSPG) syndecan-1. By producing factors that inhibit osteoblast differentiation and enhancing osteoclast activity, patient will develop osteolytic bone disease. For more detail see text.
These macro-molecules are expressed in all mammalian tissues as extracellular matrix components or as cell-membrane-bound proteins. Three major families of proteoglycan core proteins have been characterized; the membrane spanning syndecans (four members) (Bernfield et al., 1992), the glycosylphosphatidylinositol-linked glypicans (six members) (Filmus and Selleck, 2001), and the basement membrane proteoglycans perlecan, agrin, and collagen XVIII (Cole and Halfter, 1996; Iozzo et al., 1994). Several other HSPGs are known as well, such as betaglycan and CD44v3 (Andres et al., 1991; van der Voort et al., 1999). The different core proteins are expressed in a cell-type-specific, and temporal and spatial regulated manner, and their expression correlates with different physiological responses in cells (Kato et al., 1994). HSPGs can function via both their core protein and the attached HS chains (Fig. 5 and 6). For example, the syndecan-1 core protein can...
interact with, and regulate the activity of integrin adhesion molecules (Beauvais et al., 2004; Kramer and Yost, 2003; Morgan et al., 2007; Yoneda and Couchman, 2003). The cytoplasmic domain also contains peptide sequences that can bind

Figure 6. Multiple functions of cell membrane heparan sulfate proteoglycans. A schematic representation of a heparan sulfate proteoglycan (HSPG) at the cell surface (i.e., syndecan), after the HS chains have been polymerized and modified. In each model, depending on the cellular context, cell surface receptor ligands and intracellular signals can correspond to different proteins. For example, cell surface receptors could include c-MET, VEGF-R or FGF-R; receptor ligands could be HGF, VEGF or FGF; and intracellular signals could be PKB, MAPK, Rac, Rho, Ral or FAK. a. Ternary complex formation. Syndecan associates with a cell-surface receptor and its ligand and generates intracellular signals. Extracellular matrix (ECM) engagement of integrins, independently of syndecans, may potentiate the syndecan-mediated signals. b. Multimeric complex formation. Cell-surface receptors for chemoattractive, repulsive or growth factors are central to the formation of complexes with their ligands, syndecans, and integrins and ECM molecules. Cooperative intracellular signals are generated from each receptor, initiating a coordinated cellular response. c. Syndecan-mediated ligand-presentation in trans. A syndecan molecule on an adjacent cell binds to and localizes a soluble ligand to allow interaction with its receptor (possibly in complex with an integrin). Such interactions could initiate both syndecan and integrin signaling events. d. Syndecan-mediated ligand-presentation in cis. Syndecans, viewed as pioneering molecules, bind to and localize chemoattractive, repulsive or growth factors to allow interaction with their receptors and possibly integrins on the same cell. Collaborative intracellular signals might be elicited from both syndecans and integrins in such a model, but might also require simultaneous interaction with ECM molecules. Adapted and modified from Morgan et al., 2007.
the cytoskeletal proteins, which can serve as substrates for cellular kinases. Thus, syndecans may act as signaling molecules (Rapraeger, 2000). On the other hand, HSPGs can bind and present proteins via their HS chains, of which the binding capacity and specificity is determined by enzymatic modifications (Esko and Lidahl, 2001; Esko and Selleck, 2002). Hence, HSPGs act as multifunctional scaffolds regulating important biological processes including cell adhesion and migration, tissue morphogenesis, organogenesis, and angiogenesis (Bishop et al., 2007; Esko and Selleck, 2002).

**Heparan sulfate synthesis and modification: assembly of ligand binding sides**

The synthesis of the heparan sulfate glucosaminoglycan side chains of proteoglycans proceeds in three steps (Fig. 5). The first step is the chain initiation. The synthesis starts after translation of a core protein and transfer of xylose from UDP-xylose by xylosyltransferase-I and/or –II to specific serine residues within the proteoglycan core protein. The subsequent attachment of two D-galactose residues by galactosyltransferase-I and –II and GlcA by glucuronosyltransferase-I completes the formation of the core protein linkage tetrasaccharide (Esko and Lidahl, 2001; Esko and Selleck, 2002). The second step, chain elongation or polymerization, can only take place once the tetrasaccharide linker is present (Esko and Lindahl, 2001). This polymerization reaction is carried out by enzymes from the exostin (EXT) family, with a major role for exostin-1 (EXT-1) and EXT-2 (Zak et al., 2002). These EXTs are thought to function as a hetero-oligomeric complex to polymerize the HS chain. It should be noted that while reduced function in either co-polymerase results in the bone exostosis phenotype, complete loss of either enzyme results in total loss of full chain HS and is lethal at gastrulation (Lin et al., 2000). Finally, the unbranched HS chains undergo a third step, which consists of a complex series of processing reactions involving GlcNAc deacetylation and sulfation by the N-deacetylase/N-sulfotransferases (NDST), epimerization of GlcA by glucuronyl C5-epimerase (GLCE), and subsequent O-sulfation by three different O-sulfotransferases (HS2ST, HS3ST and HS6ST), which exhibit different isoforms (Lindahl et al., 1998; Esko and Selleck, 2002). In contrast to heparin, HS chains are only partly modified, with the modifications made in clusters, resulting in a polysaccharide chain having regions that are highly sulfated interspersed with nearly unmodified regions. These highly modified domains provide specific docking sites for a large number of bio-active molecules, and are usually defined by their GlcNAc N-sulfation, indicated by NS, NS/NA, or NA, representing areas with (NS or NA/NS) or without (NA) N-sulfation (Fig. 5). Binding of these ligands, such as growth factors and cytokines, serves a variety of functions, ranging from immobilization, protection, and concentration to distinct modulation of biological functions (Fig. 6) (Belenkaya et al., 2004; Bishop et al., 2007; Ruoslahti and Yamaguchi, 1991). After synthesis, HS GAGs are known to preferentially bind to the consensus sequences BBXB and BBBXXB, where B is a basic amino acid like Lys, Arg, or His. Many, if not all chemokines contain such a consensus motif. For example, the SDF-1 BBXB HS-binding motif is located in the first β-strand of the protein. Interestingly, SDFγ, a splice variant of SDF-1, is extended by a highly cationic carboxy-terminal domain that encompass four overlapping BBXB HS-binding motifs, making it a paradigm of chemoattractant proteins (Rueda et al., 2008).
Heparan sulfate proteoglycans in health and disease
Genetic defects in man and studies with genetically modified animals have clearly demonstrated the importance of HSPGs and their correct modification in (embryonic) development, normal physiology, and disease (Bishop et al., 2007; Celie et al., 2008; Merry et al., 2001; Ringvall et al., 2000). HSPGs and HS-modifying enzymes are implicated in a wide variety of human diseases, including cancer, and are essential for normal development. An X-linked condition characterized by pre- and postnatal overgrowth with visceral and skeletal anomalies, known as Simpson-Golabi-Behmel syndrome, is caused by the loss of glypican-3 (Pilia et al., 1996). In contrast, point mutations or larger genomic rearrangements in glypican-6, lead to autosomal-recessive omodysplasia, a genetic condition characterized by short-limbed short stature, craniofacial dysmorphism, and variable developmental delay (Campos-Xavier et al., 2009). Mutations in the HS co-polymerases exostin-1 (EXT1) and/or EXT2 are responsible for another bone phenotype, characterized by limb deformity, limb malalignment, limb length discrepancy, and short stature (Ahn et al., 1995; Stickens et al., 1996). Recently, it was demonstrated that methylation-associated repression of several HS 3-O-sulfotransferase genes contributes to the invasive phenotype of H-EMC-SS chondrosarcoma cells (Bui et al., 2010). On the contrary, mice deficient in syndecan-1 expression resist Wnt-1-induced tumorigenesis of the mammary gland (Alexander et al., 2000). Furthermore, manipulating the expression of glypican (Dally) in D. Melanogaster, has resulted in diverse developmentally abnormal phenotypes, including affected eye development and impaired wing formation (Takeo et al., 2005; Tsuda et al., 1999). Finally, mice deficient for different HS-modifying enzymes reveal a wide variety of developmental abnormalities, ranging from renal agenesis, pulmonary hypoplasia, and skeletal malformations to neonatal death or even disruption of gastrulation (Li et al., 2003; Lin et al., 2000; Merry et al., 2001; Ringvall et al., 2000).

Mouse models
Animal models constitute a powerful research tool in biological science. In this thesis we applied several mouse models to understand the role of HSPGs and HS-modifying enzymes in lymphoid organogenesis, and in the development, migration, adhesion, proliferation and survival of normal and malignant B cells. In the next sections these mouse models are described.

Heparan sulfate glucuronyl C5-epimerase knockout (Glce<sup>−/−</sup>) mice
Glucuronyl C5-epimerase (Glce) converts D-glucuronic acid (GlcA) into L-iduronic acid (IdoA). Unlike GlcA, IdoA can adopt several pyronase ring conformations. Therefore, this conversion into IdoA will lead to increased HS chain flexibility that is essential for proper ligand binding and apositioning (Esko and Lindahl, 2001; Esko and Selleck, 2002; Jia et al., 2009). All identified protein-binding HS epitopes contain IdoA. Targeted disruption of Glce in mice (Glce<sup>−/−</sup>), resulted in structurally altered HS lacking IdoA and exhibiting a highly distorted sulfation pattern (Li et al., 2003). Interestingly, however, Glce-deficient mice reach birth, with only a number
of developmental, and organ specific defects, including kidney agenesis, skeletal malformation, and poorly inflated lungs, leading to early neonatal death. Hence, major early developmental events known to critically depend on heparan sulfate apparently proceed normally, even in the absence of IdoA (Li et al., 2003). Assessment of HS-growth factor interaction by nitrocellulose filter trapping revealed a strikingly diminished binding of fibroblast growth factor-2 (FGF2) and glia-derived neurotropic factor (GDNF) to Glce-/- HS, whereas FGF10 binding appeared to be normal. As a consequence, FGF2 stimulation of Glce-deficient mouse embryonic fibroblasts resulted in defective signaling as measured by downstream ERK phosphorylation (Jia et al., 2009). These findings demonstrate the importance of HS-modification by Glce in the regulation of growth factor-specific cellular events. This prompted us to investigate the role of Glce in lymphoid organ development, and in the development and antigen-specific differentiation of B cells, since many cytokines, growth factors, and chemokines involved in immunological processes have HS-binding domains.

**Rag-2-γc-/- mice**

The recombination activating genes, Rag-1 and Rag-2, are essential for rearrangement of both the B cell and T cell receptor. Therefore, defects in either one of these genes results in the inability to generate peripheral B and T cells. When, in addition, the common gamma chain for the interleukin receptors 2 and 7 is lost, as in Rag-2-γc-/- mice, NK cells also do not develop (Colluci et al., 1999). As a consequence, these mice permit highly reproducible engraftment of mouse and human hematopoietic cells and cell lines (Legrand et al., 2008, Rozemuller et al., 2008; Traggiai et al., 2004). This provided a model in which we could transfer fetal liver hematopoietic stem and progenitor cells of the neonatal lethal Glce-/- and wild type mice to study the role of glucuronyl C5-epimerase in B cell development and antigen-dependent differentiation. In addition, the Rag-2-γc-/- mice are also highly permissive to grafting of human MM cells, which allowed us to investigate the role of HS (EXT1) in the growth and survival of MM in vivo.

**Cre-recombinase expressing mice and conditional gene expression**

Conditional gene targeting based on excision or inversion of LoxP-flanked DNA segments by Cre-recombinase is a powerful tool to study gene function. Conditional gene targeting in mice allows the introduction of targeted mutations in a cell type-specific and/or inducible way. This is most commonly achieved by flanking a DNA segment with recognition sequences for a site-specific recombinase, and expressing the recombinase from a transgene controlled in a cell type-specific or inducible manner (Fig. 7, left panel). Bacteriophage-derived Cre-recombinase and its 34-base pair target sequences called LoxP sites are mostly used in this type of experiment (Branda and Dymecki, 2004; Rajewsky et al., 1996). Since its introduction about 15 years ago, this approach has become increasingly popular in biomedical research and of course also in immunology, for both in vivo and in vitro analysis of gene function, either knocked-out (flanked exon) or overexpressed (flanked stop-codon preceeding the gene of interest) (Schmidt-Supprian and Rajewsky, 2007). A big advantage of the site-specific recombinase technology is that it can be applied to induce null mutations in discrete cell types in vivo, bypassing the embryonic lethality associated with many germline null
alleles (Branda and Dymecki, 2004). To investigate the biology of a wide range of immune cells in vivo, it is also common practice to generate conditional transgenic mice. However, the classical approach to produce transgenic mice is labor intensive and time consuming. First, a transgenic mouse containing a \( \text{LoxP} \)-flanked DNA segment has to be generated by targeting of embryonic stem (ES) cells. Secondly, most ES cell lines used in gene targeting are on a 129 genetic background, thus, if a mutation on the C57BL/6 background is desired, which is the most commonly used mouse strain in immunology, the mutant mice have to be crossed nine times or more to C57BL/6 mice to establish it on that strain. Finally, these mice have to be crossed with mice expressing Cre-recombinase in a cell type specific (e.g. CD19-Cre) or inducible way (Fig. 7, left panel) (Bucholtz, 2008).

The recent development of the retrogenic mouse model (Fig. 7, right panel), however, allows for the rapid generation of transgenic mice, preventing tedious genetic engineering and ES targeting and selection (Holst et al., 2006; Nakagawa et al., 2006). In this system, a gene of interest is introduced into hematopoietic stem or progenitor cells by retroviral infection. The infected cells are subsequently injected into irradiated BL/6 or immunodeficient mice, e.g. \( \text{Rag}-2^{-/-} \eta_{c}^{-/-} \) mice, which eventually
will lead to the development and differentiation of a donor-derived, transgenic, immune system (Holst et al., 2006; Nakagawa et al., 2006). Interestingly, when a viral vector is used containing regulatory sequences from for example the B cell-specific CD19 or T cell-specific CD4 gene (Fig. 7, right panel), even cell type specific expression (e.g. in B and T cells, respectively) of a gene of interest can be achieved in a much more rapid way (Marodon et al., 2003). A drawback of the retrogenic mouse model is that it can only be applied to the hematopoietic system. Nonetheless, it is a sophisticated technique to rapidly study genes, involved in immunology, and hematological malignancies, such as lymphoma and multiple myeloma. The two methods to obtain conditional expression in, for example B cells, stimulated us to develop an approach to further simplify the conditional expression of a gene of interest in the hematopoietic compartment. This method (chapter 6) could ultimately lead to rapid analysis of tumor suppressor- or oncogenes, underlying for example MM development, without the need to generate classical conditional mice, or retro- or lentiviral vectors containing regulatory sequences or enhancers.

**Aim and outline of this thesis**

The aim of the studies described in this thesis was to investigate the contribution of heparan sulfate proteoglycans (HSPGs) in embryonic lymphoid organogenesis, and the development, migration, adhesion, proliferation and survival of normal and malignant B cells. Lymphoid organogenesis is essential for the development of functional T- and B cells that supply defense against pathogens and toxins, and provides immunological memory. **Chapter two** reveals the contribution of the heparan sulfate modifying enzyme glucuronyl C5-epimerase to the development of primary and secondary lymphoid organs, and shows that modification of heparan sulfate (HS) is essential for proper binding of instructive morphogens and chemokines involved in these processes. **Chapter three** describes the role of glucuronyl C5-epimerase in the development, maturation and antigen-dependent differentiation of B-lymphocytes and the contribution of HS-modification in APRIL-mediated survival of plasma cells. High expression of the HSPG syndecan-1 is characteristic of terminally differentiated B cells, that is plasma cells, and their malignant counterpart multiple myeloma (MM). **Chapter four** shows that the HS chains attached to syndecan-1 are essential for the growth of MM, both in vitro and in vivo. This was demonstrated by applying inducible RNAi-mediated knockdown of EXT1, a co-polymerase indispensable for HS biosynthesis. In addition, the appendix of chapter four examines the impact of EXT1 knockdown on drug-induced cell death of MM, and shows that loss of HS expression renders the MM cells more susceptible to the treatment of the chemotherapeutics lenalidomide and bortezomib. SDF-1α, a chemokine with HSPG-binding potential, has a major role in the adhesion, migration, survival and retention of normal and malignant B cells. **Chapter five** demonstrates that the small GTPase Ral is activated in response to SDF-1α and mediates migration of both B cells and MM cells, suggesting that Ral is involved in B cell homeostasis, trafficking, and function, as well as homing of MM cells to the bone marrow.
Chapter six describes a single retroviral vector-based method to rapidly generate conditional retrogenic mice. This novel mouse model could provide new opportunities to gain insight into the development and function of an array of hematopoietic cell lineages, and the pathogenesis of hematological malignancies, such as multiple myeloma. Finally, chapter seven summarizes and discusses the results presented in this thesis, and provides suggestions for further studies and potential therapeutic applications of these findings for the treatment of MM patients.

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General introduction


