Characterization of marginal zone-like B cells and lymphomas believed to originate from marginal zone B cells

PhD thesis

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I dedicate this thesis to my brother, Khalid Ali-Guul Warsame, who passed away while I was writing this thesis.

Oslo, Septemper 2012

Abdirashid A Warsame

Abbreviations

ABC	Activated B-cell like
AID	Activation-induced cytidine deaminase
ALL	Acute lymphoblastic leukemia
API2	Apoptosis inhibitor 2
BAFF(-R)	B-cell activating factor (receptor)
BCL2	B-cell lymphoma 2
BCL6	B-cell lymphoma 6
BCR	B-cell receptor
BL	Burkittt lymphoma
BLIMP1	B-lymphocyte induced maturation protein 1
BLNK	B cell linker protein
CD	Cluster of differentiation
CDKN2A/B	Cyclin-dependent kinase 4 inhibitor A and B
CDR3	Complementarity determining region 3
CLL	Chronic lymphocytic leukemia
c-MYC	Avian myelocytomatosis virus oncogene cellular homolog
CNS	Central nervous system
CSR	Class switch recombination
DC	Dendritic cell
DHBCL	Double hit B-cell lymphoma
DLBCL	Diffuse large B-cell lymphoma
EBF	Early B-cell factor
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
FL	Follicular lymphoma
FOX1	Forkhead-box protein P1
GC	Germinal center
GCB	Germinal center B-cell like
HCV	Hepatitis C virus
HHV8	Human herpes virus 8
HIV	Human immunodeficiency virus
HL	Hodgkin’s lymphoma
HSA	Human serum Albumin
HTLTV1	Human T-lymphotropic virus 1
IARC	International Agency for Research on cancer
IHC	Immunohistochemistry
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgV	Immunoglobulin variable region
IGVH	Immunoglobulin variable region of the heavy chain
IGVL	Immunoglobulin variable region of the light chain
IL	Interleukin
IPSID	Immunoproliferative small intestine disease
IRF4	Interferon regulatory factor 4

IRTA1	Immunoglobulin superfamily receptor translocation-associated 1
ITAM	Immunoreceptor tyrosine-based activation
LMP2	Latent membrane protein 2
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MALT	Mucosa associated lymphoid tissue
MBL	Monoclonal b-cell lymphocytosis
MC	Mixed cryoglobulinemia
MCL	Mantle cell lymphoma
MSBC	Monoclonal small B cells
MUM1	Multiple myeloma oncogene 1
MYD88	Myeloid differentiation primary response gene 88
MZ	Marginal zone
MZL	Marginal zone lymphoma
NHL	Non-Hodgkin's lymphoma
NF-κB	Nuclear factor kappa B
NMZL	Nodal marginal zone lymphoma
PAX5	Paired box protein 5
PCBCL	Primary cutaneous B-cell lymphoma
PCR	Polymerase chain reaction
PLCγ2	Phospholipase C gamma 2
PMBCL	Primary mediastinal B-cell lymphoma
RA	Rheumatoid arthritis
RAG	Recombination activating gene
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
SMZL	Splenic marginal zone lymphoma
SS	Sjögren's syndrome
ssDNA	Single strand DNA
S1P	Sphingosine-1-phosphate
TCR	T-cell receptor
TD	T-cell dependent
T/HLBCL	T-cell/hystocyte-rich B-cell lymphoma
TI	T-cell independent
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha
TRAF	Tumor necrosis factor alpha receptor associated factor
VH	Variable region of the immunoglobulin heavy chain
WB	Western blot
XBP1	X-box binding protein 1

List of articles

Article I

Warsame A, Delabie J, Malecka M, Wang J, Trøen T, Tierens A. **Monocytoid B cells: an enigmatic B-cell subset showing evidence of extrafollicular immunoglobulin gene somatic hypermutation.** *Scand J Immunol*. 2012 May;75(5):500-9

Article II

Warsame A, Aasheim HC, Nustad K, Trøen G, Tierens A, Wang V, Randen U, Dong HP, Heim S, Brech A, Delabie J. **Splenic marginal zone lymphoma with VH1-02 gene rearrangement expresses poly- and self-reactive antibodies with similar reactivity.** *Blood.* 2011 Sep 22;118(12):3331-9

Article III

Trøen G, **Warsame A** and Delabie J

Few CD79B and MYD88 mutations in splenic marginal zone lymphoma (*Manuscript*)

Accepted for publication: ISRN-Oncology, 2013

Article IV

Tierens A, Holte H, **Warsame A**, Ikonomou IM, Wang J, Chan WC, Delabie J. **Low levels of monoclonal small B cells in the bone marrow of patients with diffuse large B-cell lymphoma of activated B-cell type but not of germinal center B-cell type.** *Haematologica*. 2010 Aug;95(8):1334-41

1. General introduction

A full understanding of B-cell lymphoma, a malignancy of B lymphocytes, is not possible without basic knowledge of normal B lymphopoiesis. This introduction will therefore briefly review normal B lymphopoiesis and B-cell lymphomagenesis, in particular marginal zone B-cell lymphomagenesis, the topic of this doctoral thesis.

1.1 Normal B-cell lymphopoiesis

1.1.1 Immunoglobulin gene assembly and B-cell maturation

B cells are important to safeguard the human body against invading pathogens. B-cell development takes place in distinct anatomic sites. At the same time B cells go through distinct differentiation stages that are characterized by the specific structural rearrangement of the B-cell receptor (BCR). The BCR is composed of two identical heavy and light chain heterodimers that are covalently linked with disulfide bridges. Each of these chains contains a variable region that shapes the immunoglobulin domain involved in antigen-binding. Specific antibodies are produced through variable (V), diversity (D) and joining (J) segment rearrangement, also called V(D)J recombination as well as somatic hyprermutation (SHM) and class switch recombination (CSR). During B-cell ontogeny, the repertoire of B cells secreting unique antibodies is generated at two stages; a primary B-cell repertoire is generated in the bone marrow, while a secondary B-cell repertoire is formed in peripheral lymphoid tissue (Figure 1).

1.1.2 The primary B-cell repertoire

The primary B-cell repertoire is generated by a random but ordered recombination of 51 variable (V), 27 diversity (D) and 6 joining (J) segments of the immunoglobulin (*Ig*) heavy chain gene on chromosome 14, and V and J segments of the Igκ or Igλ light chain on chromosome 2 and 22, respectively (MacLennan, 1994). V(D)J rearrangement is mediated by recombination activating gene proteins 1 and 2 (RAG1 and RAG2) during the early stages of B-cell development in the bone marrow. These two proteins recognize short recombination signal sequences (RSS) located adjacent to V, D and J segments (Gellert, 2002). Successful rearrangement of the immunoglobulin heavy and light chain genes leads to synthesis of a complete immunoglobulin IgM molecule. B cells at this stage are called immature B cells. While still in the bone marrow, immature B cells develop further into IgM and IgD expressing cells called mature naïve or virgin B cells. Precursor B cells in the bone marrow that fail to produce functional antibodies or express potentially harmful autoreactive antibodies are eliminated through apoptosis, clonal deletion or receptor editing of the rearranged κ or λ L chain (Rajewsky, 1996). Whereas mature, naïve B cells expressing functional BCRs leave the bone marrow and migrate to secondary or peripheral lymphoid organs, i.e. lymph nodes, tonsils, spleen and mucosa-

associated lymphoid tissues (MALT), where they form B-cell follicles. B-cell follicles are highly specialized structures that comprise distinct micro-anatomical compartments such as germinal center (GC), also known as the follicle center, and the mantle zone. The mantle zone is composed of the lymphocytic corona and the marginal zone in the spleen, extra nodal lymphoid tissue and Peyer's patches. A marginal zone is not usually seen in prepherial lymph nodes (De Wolf-Peeters and Delabie, 1993).

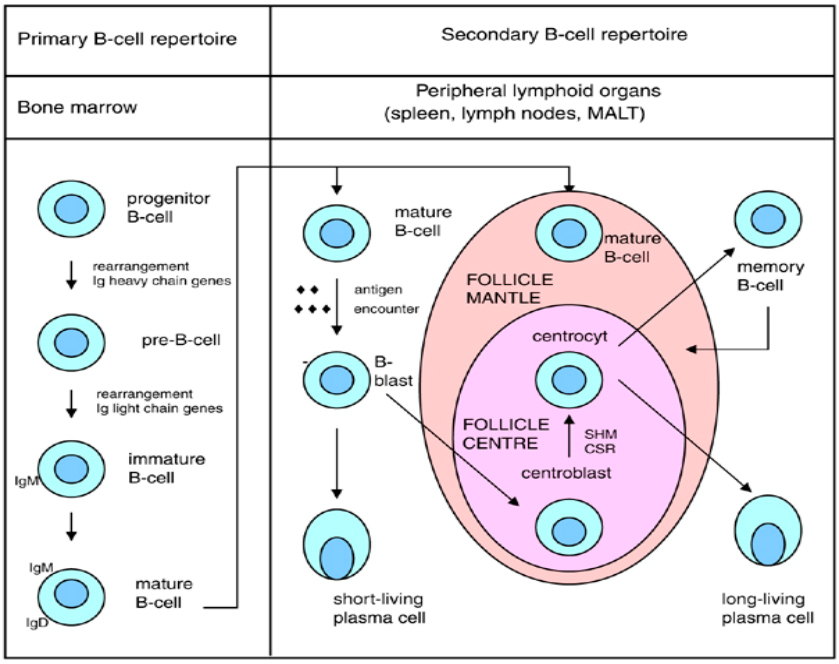

Figure 1. **Schematic representation of the B-cell development**
The formation of the primary B-cell repertoire occurs in the bone marrow, whereas the secondary B-cell repertoire is generated in the secondary lymphoid organs. Abbreviations: CSR (Class switch recombination) SHM (somatic hypermutation), (Reprinted with permission from Sagaert et al., 2007).

1.1.3 The secondary B-cell repertoire

The secondary B-cell repertoire is generated when naïve B cells are recruited into germinal centers upon antigen encounter. Mature germinal centers are divided into dark and light zones and different B-cell subsets are recognized in these zones. A rapidly proliferating subset of B cells known as centroblasts occupy the dark zone of the GC, whereas the resting B cells called centrocytes are seen in the light zone (MacLennan et al., 1997). In the dark zone of the germinal center, antigen-activated B cells clonally expand subsequent to appropriate stimulation by CD4 T cells through CD40- CD40L interactions and dendritic cells (DC). In this zone, rapidly proliferating B cells undergo somatic hypermutation (SHM), a process in which mutations such as single nucleotide exchange, small

deletions or duplications are introduced in the variable region of the immunoglobulin genes. Some of these mutations create amino acid substitutions while other mutations can be silent. Somatic mutations are scattered along the length of the transcribed immunoglobulin variable region (*IgV*) gene. Many mutations are disadvantageous because they reduce the ability of BCR to bind to antigen or may increase auto-reactivity. This leads to apoptosis facilitated by down-regulation BCL2 and other anti-apoptotic molecules (Kuppers, 2005). In contrary, a minor fraction of the GC B cells may acquire mutations that result in increased BCR affinity to antigen. These cells will be positively selected for further differentiation and antibody secretion. The combination of V(D)J rearrangement and somatic hypermutaion result in potential synthesis of more than 10^{12} different BCRs. Antigen-stimulated B cells typically show somatic mutations that are typically concentrated in the complementarity determining regions (CDRs) of the immunoglobulin heavy chain variable (*IGVH*) and light chain variable (*IGVL*) genes. The *IGVH* CDR3 is the most diverse region and encodes the antigen-binding pocket of the *IgV* region. Framework regions show lower mutation rates and need to be considered to provide a structural framework for the variable domain.

Antigen-activated B cells in the GC undergo also second immunoglobulin gene recombination event known as class-switch recombination (CSR) (Harriman et al., 1993). Through the class switching process either the Cμ or Cδ exon of the heavy chain constant (C_H) gene coding for IgM or IgD subclasses, respectively, is replaced by Cγ, Cα or Cε allowing the production of IgG, IgA or IgE, respectively. In human, nine immunoglobulin heavy chain isotypes are found; IgM, IgD, IgE, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. Class switching results in a change in effector functions of the antibody molecule, but this process leaves the antigen binding specificity of the antibody unchanged. B cells expressing high-affinity antibodies for their antigen are positively selected to differentiate to plasma cells or long-lived memory cells and exit from the germinal center into peripheral circulation via the marginal zone. B cells producing unfavorable antibodies such as poly-reactive or non-productive antibodies are eliminated or at least reduced from the repertoire by several checkpoints during B-cell development in the bone marrow and the secondary lymphoid tissues (Tsuiji et al 2006). In spite SHM and CSR events are two separate processes with different molecular signatures, both are strictly dependent on a shared pathway initiated by activation-induced cytidine deaminase (AID). Distinct catalytic domains of the AID protein seem to be functionally required for either SHM or CSR mediation (Muramatsu et al., 2000; Chaudhuri et al., 2007). The germinal center reaction in peripheral lymphoid organs is the principal source of somatic hypermutation (reviewed in Klein and Dalla-Favera, 2008). However, whether somatic hypermutations occur at other microanatomical regions than germinal centers is still controversial (Weller et al., 2001; Weller et al., 2003; Mao et al., 2004; Weller et al., 2004; Weill et al., 2009).

1.2 B-cell subsets

B-cell development goes through two major stages that take place in two functional anatomic sites. At the first differentiation stage, B-cell precursors differentiate from haematopoietic stem cells into naive mature B cells in the bone marrow. At the second stage naive mature B cells differentiate into memory or plasma B cells in secondary lymphoid tissue such as lymph nodes, spleen and mucosa associated lymphoid tissues. Based on the rearrangement and mutation status of the *Ig* genes and expression of a variety of surface markers and intercellular proteins, 5 B-cell subsets have been identified in the bone marrow: Pro-B, Pre-B-I, pre-B-II, immature and mature naïve B cells (reviewed by Perez-Andres et al., 2010). In secondary lymphoid tissue, in addition to the circulating mature naïve B cells, four other B-cell populations have been well characterized: GC B cells, memory B cells, MZ B cells and plasma cells. Table 1 summarizes the major human B-cell subsets and their characteristic features.

The B cell commitment to stem cells is determined by the expression of a set of unique regulatory proteins including, transcription factor PU.1, early B-cell factor (EBF), Ikaros, paired box protein 5 (PAX5) and forkhead-box protein P1 (FOXP1) (Matthias and Rolink, 2005; Medina and Singh, 2005). These transcription factors induce the synthesis of RAG-proteins (RAG1 and RAG2) and terminal-deoxynucleotidyl-transferase (TdT), which initiate the assembly of D and J segments of the immunoglobulin heavy locus (Pelayo et al., 2006). Precursor cells with successfully joined D and J segments are known as Pro-B-cells. Pro-B cells express surface markers including CD22, CD34, but not CD19 at this stage (Perez-Andres et al., 2010). Upon subsequent rearrangement of one of the heavy gene V segments and the D-J_H fragment, the precursor cell express the heavy chain VDJ gene, leading to maturation of Pro-B-I cells into early pre-B cells (Pre-B-I cells) that are phenotypically characterized as $CD10^{hi}CD19^{+}CD22^{+}CD34^{hi}CD38^{+}CD45^{lo}$. Pre-B-I cells give then rise to late Pre-B cells (Pre-b-II cells). Pre-B-II cells display typical $C10^{+}CD19^{+}CD22^{+}CD38^{+}CD45^{+}$ phenotype. Pre-B-II cells express also CD79A (Igα) and CD79B (Igβ). These two proteins, together with the Igμ chain and the surrogate light chain (Vpreb and λ5) form the pre-B cell receptor (pre-BCR). If the pre-BCR is able to bind to the BM microenvironment, Pre-B-II cells are positively selected and further differentiate into immature B cells. Expression of a complete IgM molecule on the cell surface is the hallmark of immature B cells. They further display the $CD10^{lo}CD19^{+}CD20^{+}CD22^{+}CD38^{+}CD45^{+}$ immunophenotype (Perez-Andres et al., 2010). These immature B cells lose CD10 and CD38 expression as they develop into the mature B-cell state (van Lochem et al., 2004). Mature naïve B cells are characterized by expression of IgM^{+}, IgD^{+} and $CD27^{-}$ surface molecules, and they are typically $CD19^{+}CD20^{+}CD22^{+}CD38^{-}$ phenotype.

B-cell stage	Ig gene status	Somatic mutations	Ig production	Immunophenotype
Pro-B cell	Germ line	Pre-B-cell	None	CD22, CD34, TdT
Pre-B-I cell	Joined DJ_H segments	None	μ-Chain	CD19, CD79a, $CD10^{hi}$, CD22, CD34, CD38, $CD45^{lo}$, TdT
Pre-B-II cell	Rearranged VDJ_H	None	Cyto-Igμ-Chain	CD19, CD79a, CD10, CD22, CD38, CD45, TdT
Immature B cell	Rearranged IgH and IgL genes	None	sIgM	CD19, CD79a, $CD10^{lo}$, CD20, CD22, $CD38^{hi}$, CD45, $CD27^{-}$
Mature naïve B cell	Rearranged IgH and IgL genes	None	sIgM and sIgD	CD19, CD79a, CD20, CD23, $CD44^{lo}$, $CD45^{hi}$, $CD10^{-}$, $CD27^{-}$
GC B cell	Rearranged IgH and IgL genes	Mutated	Minimal or absent Ig	CD19, CD79a, $CD20^{hi}$, CD10, BCL-6, $CD27^{het}$, CD38, CD44, $CD45^{hi}$,
Marginal Zone B cell	Rearranged IgH and IgL genes	Mutated and Unmutated	$sIgM^{hi}$ and $sIgD^{lo}$	CD19, CD79a, CD20, CD21, CD27, CD1, $CD23^{-}$
B-1 B cells∗	Rearranged IgH and IgL genes	Mutated and Unmutated	IgM and IgD	∗$CD5^{+/-}$, CD19, CD79a, CD20, CD21, CD23, CD27, $CD43^{hi}$, $CD69^{-}$, $CD70^{-}$
Memory B cell	Rearranged IgH and IgL genes	Mutated	IgG, IgA, IgM IgD	CD19, CD79a, CD20, $CD22^{hi}$, CD27, $CD45^{hi}$
Plasma cell	Rearranged IgH and IgL genes	Mutated	IgG > IgA > IgD	CD19, $CD38^{bright}$, CD138, CD27, CD45

Table 1. **Classification of B cells according to their differentiation status**
∗ B-1 B cells can be subdivided into two subgroups based on the differential expression of CD5; $CD11b^{+}$ $CD5^{+}$ B cells are referred to as B-1a B cells, whereas those that do not express CD5 are termed B-1b B cells.
(References: Sagaert and De Wolf-Peeters, 2003; Perez-Andres et al., 2010; Griffin and Rothstein, 2012)

About 60-70 % of human circulating B-cells are $IgM^+IgD^+CD27^-$ with unmutated *IGVH* genes and represent mature naïve B cells (Klein et al., 1998). The $IgM^+IgD^+CD27^-$ B cells can be divided into a large population of $CD5^-$ IgM^+IgD^+ and a relatively small subset of $CD5^+IgM^+IgD^+$ B cells (Fischer et al., 1997). The CD5–negative $IgM^+IgD^+CD27^-$ B-cell population mature into three B-cell subpopulations, GC-, MZ - and B-1- B cells. Theses subsets are central players of adaptive and non-adaptive immunity. GC- and MZ- B cells are so-called conventional B cells or B-2 cells and are localized in distinctive anatomical sites; follicular B cells are located in the B cell follicles, while MZ B cells are abundant in the splenic MZ. The follicular naïve B cells represent presumed precursors of GC B cells that are involved in T cell-dependent (TD) immune response. MZ and B-1 cells participate in T cell-independent (TI) immune responses (Lanzavecchia and Sallusto, 2007). MZ and B-1b cells express toll-like receptors (TLRs) that identify a variety of microbial TLR ligands such as bacterial polysaccharides (Lanzavecchia and Sallusto, 2007).

About 15% of the peripheral B-cell pool is $CD5^+IgM^+IgD^+CD27^-$. These cells are likely B-1a cells that participate primarily in the TI immune response. At least in mice, B-1a B cells are involved in autoimmune diseases and a large proportion of cells produce auto-antibodies, so-called natural antibodies with polyreactivity (Casali and Notkins, 1989). The main function of B-1a cells is presumably to produce polyreactive IgM auto-antibodies that function as a first-line antigen-independent humoral defense against potentially harmful pathogens. B-cell lymphocytic leukemia (B-CLL or CLL) likely arises from B-1 cells (Kipps, 1989)

About 30-40% of the human peripheral blood cells are $IgM^+IgD^+CD27^+$ memory B cells that are distinguished from naïve B cells by the expression of CD27 on the cell surface (Klein et al., 1998). The vast majority of these $IgM^+IgD^+CD27^+$ B cells show a typically low somatic hypermutation frequency of *IgH* genes. Accordingly these cells were presumed to be post-germinal B cells. It was initially proposed that human memory B cells were composed of three categories according to their Ig isotype, namely switched B cells (mainly IgG^+), IgM^+IgD^- B cells known also as (IgM-only) and a B-cell subset of IgM^+IgD^+. The latter two subsets display a similar phenotype to that of the MZ B-cell subset (i.e. $IgM^+IgD^{+/-}CD21^+CD23^-CD1c^+$ and mainly $CD5^-$). However, MZ B cells primarily participate in the TI immune response, whereas memory IgM and isotype-switched memory B cells are produced in germinal centers during the TD immune response (Weller et al., 2004; Tsuiji et al., 2006). Upon a re-encounter with the original antigen, IgM^+ memory B cells may undergo new rounds of the SHM and isotype-switching resulting in a new batch of IgM and IgG memory B-subsets with high mutation frequency (Bende et al., 2007; Reynaud et al., 2012). Indeed, in patients with persistent infection such as HIV, as high as 60 mutations can be observed in the *IGVH* genes of the HIV-specific memory B cells (Scheid et al., 2009).

However, the human $IgM^+IgD^+CD27^+$ B-compartment is likely more complex than originally thought. It is now known that there is a substantial fraction of memory B cells that lack of CD27 expression (Wirths and Lanzavecchia, 2005). Moreover, several researchers questioned whether CD27 expression

is an unambiguous marker for memory B cells (Weller et al., 2004 Weill et al., 2009). IgM^+CD27^+ B cells are not necessarily generated from germinal centers: for instance, CD40-deficient patients, who lack functional germinal centers, may acquire SHM during the generation of the preimmune repertoire and are exclusively involved in the TI immune response. More recently, Berkawska and co-workers identified 6 memory B-cell subsets (Berkawska et al., 2011). Based on proliferation rate, SHM and CSR characteristics of the *IgH* genes as well as immunophenotype, Berkawska and colleagues purposed that memory B cells are generated through three different maturation pathways. IgG^+CD27^- and IgM^+CD27^+ B-cell subsets are derived from primary GC reactions, IgA^+ $CD27^+$ and IgG^+ $CD27^+$ cells are generated during primary or secondary GC reactions secondary to a TD immune response. In contrast, IgA^+CD27^- and $IgM^+IgD^+CD27^+$ B cells originate mainly from a TI response in a non-GC immune reaction. The naïve B cells that leave the bone marrow circulate through the body via blood and lymph vessels and enter the secondary lymphoid tissues on the search for foreign antigens. If they do no encounter an antigen, they recirculate between the peripheral blood and lymphoid tissue and they die within several days. However, upon encounter with antigen, mature naïve B cells are activated either in TI or TD response. Only a small number of antigens can directly activate naïve B cells by binding to their BCRs and thus may generate a TI response, however, most antigens are TD as they require T-cell help (Sagaert et al., 2007). After antigen encounter, the B cells move to the boundary area between the B-cell zone and T-cell zone in search for T-cell help. This movement is directed by interactions between chemokine ligands CCL19 and CCL21 produced by the stromal cell in the T-cell zone and chemokine-receptor 7 (CCR7), which is rapidly upregulated in B cells in response to antigen activation (Reif et al., 2002). Once the antigen-activated B cell encounters with its cognate T cell at the border of the B- and T-cell zone, it transforms into a B-blast that may take one of the two following pathways (Figure 1): (1) In the extrafollicular pathway, most of B-blasts migrate to the extrafollicular area of the lymph nodes where they proliferate and differentiate into short-living transient plasma cells and later plasma cells, which rapidly secrete low affinity antibodies that serve as the first line defense against pathogens. (2) In the follicular pathway, a few B-blasts will migrate into the primary follicle and form the GC where they rapidly proliferate, undergo SHM and CSR, and differentiate into memory B cells or plasma cells that secret antibody with high affinity. Communication between B cell and T cells is mediated by interaction between CD40, constrictively expressed by B cells and its ligand, CD40L expressed by activated T-cell as well as different cytokines and their respective receptors. These communication signals drive B-cell proliferation and differentiation. Chemokine receptors including CXC5, CC7, and CXCR4 and their respective ligands CXCL13, CC19 (also CCL21) and CXC12 play a pivotal role in the control of activated B cells migration within the GC microenvironments that support their differentiation into plasma cell or GC cells (Gatto and Brink, 2010) .

The molecular mechanisms that regulate differentiation of some GC B cells into plasma B cells or into memory B cells are not fully elucidated. However, recent experiments have revealed that the

differentiation of GC B cells into plasma cells requires the expression of transcription factors including B-lymphocyte induced maturation protein 1(BLIMP1), X-box binding protein 1(XBP1) and interferon regulatory factor 4 (IRF4) (Shapiro-Shelef et al., 2005; Klein and Dalla-Favera, 2008). BLIMP1 represses PAX5 as well as B-cell lymphoma 6 (BCL6) in plasmablasts and plasma cells terminating the differentiation program of B cells (Nera and Lassila, 2006). On the other hand, OX40-OX40L mediated interactions and high expression of the interleukins IL-2, IL-3, IL-6 and IL-10 seem to play a critical role in directing GC B cells to differentiate into memory cells (Liu and Banchereau, 1997).

1.2.1 Marginal zone B cells

Splenic MZ B cells are recognized primarily on the basis of their anatomical location and morphological features. They reside in the secondary lymphoid tissues, especially in the spleen and in Peyer's patches of the gut, where an abundant influx of antigens occurs. They are characterized by abundant clear cytoplasm and pale irregular centrally positioned nucleus (van den Oord et al., 1986; Spencer et al., 1998). These cells have been initially hypothesized to be IgM memory B cells, but subsequent studies indicate that they represent a distinct B cell subset related to mouse MZ B cells (Weill et al., 2009).

Most of our current knowledge on MZ B cells is derived from studies performed in rodents, particularly in mice (reviewed in Pillai et al., 2005; Weill et al., 2009). Human MZ B cells share many surface markers as well as other properties with mouse MZ B cells. Most importantly, as mouse MZ B cells, human MZ B cells are typically $IgM^{+}IgD^{+/-}CD27^{+}CD21^{+}CD23^{-}CD1c^{+}$(mouse cells express the CDd1 isoform) (Spencer et al., 1998; Martin and Kearney, 2001; Weller et al., 2004). The first three markers, namely IgM^{+}, $IgD^{+/-}$ and $CD27^{+}$, are also molecular footprints of IgM memory B cells (not-isotype-switched memory B cells). In addition to BCR signaling, Notch2 and its ligand Delta1 have a critical role in the MZ B cell development both in human and mouse. Despite that mouse and human MZ B cells share common features, it is now known that they also have striking differences regarding their anatomical location as well as diversification properties. Mouse MZ B cells express high levels of S1P1 and S1P3 lysophospho-lipid receptors that permit the cells to be retained in the MZ, whereas their human B cell counterparts lack such an expression and thus recirculate freely between the MZ and blood. Mouse MZ B cells are carrying unmutated IgM molecules and are thought to represent a distinct naïve B-cell population, separate from mature follicular B cells and B1 cells (Dammers et al., 2000). Only a small proportion of mouse MZ B cells have mutated IgM genes, and presumably represent memory B cells. Contrary to rodents, mutational analyses of the rearranged *Ig* genes in human showed that the human MZ B cells are a heterogenous B-cell population displaying equal proportions of mutated and unmutated genes (Dunn-Walters et al., 1995; Tierens et al., 1999; Stein et al., 1999; Weller et al., 2008). It was previously proposed, based on the fact that a large fraction of MZ B cells are IgM^{+} $IgD^{+/-}$ $CD27^{+}$ and harboring mutated *Ig* genes, that the MZ of human spleen is a reservoir for memory B cells. However, new data suggest that *Ig* genes in $IgM^{+}IgD^{+}CD27^{+}$ human MZ

B cells have an approximately twofold lower somatic hypermutation frequency than isotype-switched B cells or so-called true memory B cells (Weill et al., 2009). Additionally, most switched B memory cells express mutated *Ig* genes with highly restricted CDR3 size, while about half of the $IgM^+IgD^+CD27^+$ human B cells (circulating in blood or residing in the spleen) carry *Ig* genes harboring somatic hypermutations and show irregular CDR3 lengths (Weller et al., 2008). These distinct features raise the question whether splenic MZ B cells are IgM memory B cells or belong to a separate lineage, as it has been recognized in mice.

MZ B cells are thought to involve both TI as well a TD immune responses. It has been suggested that MZ B cells, as mouse B-1 B cells, are the source of the most natural antibodies that provide a bridge between innate and adaptive immune responses (Martin and Kearney, 2001). Their unique anatomical location makes MZ B cells well suited to rapidly respond to blood-borne pathogens. Mouse splenic MZ IgM^+CD27^+ B cells have been identified as a part of TI response against blood-borne antigens such as polysaccharide determinants from encapsulated bacteria (Tangye and Good, 2007). Furthermore, it has been shown that inflammation and/or TLR signals can induce TI proliferation and accumulation of somatic hypermutation as well as a basal level expression of AID in mouse immature B cells (Han et al., 2007; Ueda et al., 2007).

In addition to their role in TI antigen response, MZ B cells may also engage in the TD immune response because of their ability to respond to protein antigens and function as antigen-presenting cells (Kraal and Mebius, 2006; Allman and Pillai, 2008). The TD antigen response usually generates a GC reaction leading to antibody-producing plasma cells and memory cells. Alternatively, MZ B cells may perhaps be of a B-cell subset that has pre-diversified its BCR outside GCs in a TD immune response. Indeed, $IgM^+IgD^+CD27^+$ B cells with mutated *Ig* gene are generated in individuals that are unable to develop GCs, despite they have somatic hypermutation in their *Ig* loci (Weller et al., 2001; Weill et al., 2009). Maturation outside the GC is also indicated by studies showing that MZ B-cells may acquire low level SHMs outside the GC (Tierens et al., 1999; Weller et al., 2004). Therefore, a fraction of mutated MZ B cells may have never gone through the GC. As a consequence, it has been proposed that these cells might develop outside the GCs and thus not represent bone fide memory B cells (Weill et al., 2009). However, the one possibility does not necessarily exclude the other, and perhaps MZ B cells with mutations arise in various ways.

1.2.2 Monocytoid B cells

Monocytoid B cells are localized in the peri-sinusoidal area of the lymph node. These cells are especially numerous in various lymphadenopathies caused by infection such as toxoplasma, EBV and HIV. The ontogeny and function of moncytoid B cells are as yet poorly understood. The view that monocyoid B cells might represent the nodal counterpart of splenic MZ B cells has previously been proposed (van den Oord et al., 1986; Nizze et al., 1991). However, the precise relationship between these two B-cell subsets is still debated. The immunophenotype of monocyoid B cells is largely

identical to that of MZ B cells, as both show expression of pan-B-cell markers (CD20, CD79A and PAX5) and lack CD5, CD10 CD23 and CD43. Similar to MZ B cells, a substantial fraction of monocytoid B cells carry rearranged immunoglobulin genes with a variable number of somatic hypermutations (Tierens et al., 1999; Lazzi et al., 2006). Despite these similarities, the negative or low CD27 expression in monocytoid B cells, the absence or low expression of BCL2, CD21 and expression of immunoglobulin super-family receptor translocation-associated 1(IRTA1) can be used to distinguish monocytoid B cells from MZ B cells (Johrens et al., 2005; Falini et al., 2012).

1.3 Lymphoma

1.3.1 Introduction

Malignant lymphomas are tumors of lymphoid cells and they make up an estimated 3-4% of malignant diseases worldwide (Stewart World Cancer Report. Lyon: IARC Press, 2003). Lymphomas are traditionally subdivided into to main groups: Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma ((NHL) (Swerdlow, IARC Press, Geneva, 2008) constituting 20-30% and 70-80 % of all cases, respectively.

Lymphomas, in particular B-cell lymphomas, have more than doubled in frequency during the last decades in the western world, including in Norway as illustrated in figure 2. The reasons for this increase are not certain, but at least some of the increase can be attributed to improved diagnosis, increasing age, lifestyle changes, a better registration and environmental factors.

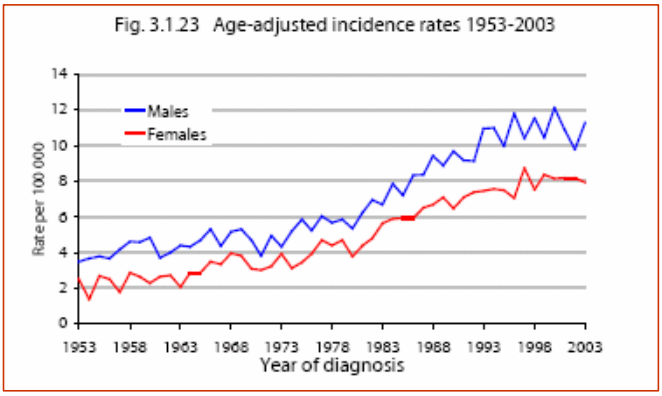

Figure 2. Age-adjusted incidence rates for lymphoma (reproduced from the Norwegian Cancer Registry)

According to current taxonomy of the World Health Organization (WHO), NHL consists of a group of closely related yet heterogeneous diseases, and can be classified on basis of their distinctive histology as well as immunophenotypic, genetic and clinical features (Swerdlow, IARC Press, Geneva, 2008). This WHO classification divides NHL into two main groups; B-cell and T/NK-cell neoplasms. Approximately 95% of the lymphomas are of B-cell origin, while 5% are T-cell malignancies (Kuppers, 2005). About 15 types of B-cell lymphomas are distinguished in the WHO classification. Major B-cell lymphoma subtype are Burkitt's lymphoma (BL), chronic lymphocytic leukemia (CLL or B-CLL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL) and marginal zone lymphoma (MZL) subtypes.

1.3.2 B-cell lymphomas and cell of origin

The WHO classification recognizes distinct B-cell lymphoma types according to the supposed normal B-cell subtype and according to morphology, immunophenotype, genetic abnormalities and clinical characteristics (Swerdlow, IARC Press, Geneva, 2008). The study of somatic hypermutation in the variable heavy (VH) region sequence of the immunoglobulin genes in lymphoma has been of help to identify the cellular origin and developmental stage of a given B-cell lymphoma type (Seto, 2004; Klein and Dalla-Favera, 2010). As such, B-cell lymphoma without somatic hypermutations are recognized as being of pre-germinal center origin, while those with mutations are traditionally believed to be of germinal center or post-germinal center origin (figure 3). Acute lymphocytic leukemia (ALL) and most MCL cases do not show somatic hypermutations. FL, DLBCL, BL and MZL are lymphoma types that show somatic hypermutations and are thus of germinal center or of post-germinal center origin. The demonstration of ongoing hypermutations in immunoglobulin variable regions suggests a still active hypermutation process and thus a germinal center origin whereas fixed somatic hypermutations indicate a post-germinal center origin. A typical example of a lymphoma of germinal center origin is FL. Of interest, some lymphoma types such as CLL and some MZL subtypes, consist of both mutated and unmutated subtypes and may thus not have a uniform cell of origin, according to this scheme.

Alternatively, some lymphomas with somatic hypermutations may arise from non-GC B cells that diversified and acquired somatic mutations in microanatomical sites other than germinal centers as it has been reported that somatic hypermutation can be T-independent and may occur in a non-germinal center pathway (William et al., 2002; Weller et al., 2004; Scheeren et al., 2008).

Gene expression profiling of human B-cell lymphoma and normal B cells has shown to be a more elegant tool to characterize the cellular origin B-cell NHL and has allowed to identify previously unrecognized subsets of B-cell lymphomas. As such, two main subsets of DLBCL have been recognized; one originating from activated B cells and one from germinal center B cells (Alizadeh et al., 2000; Rosenwald et al., 2002).

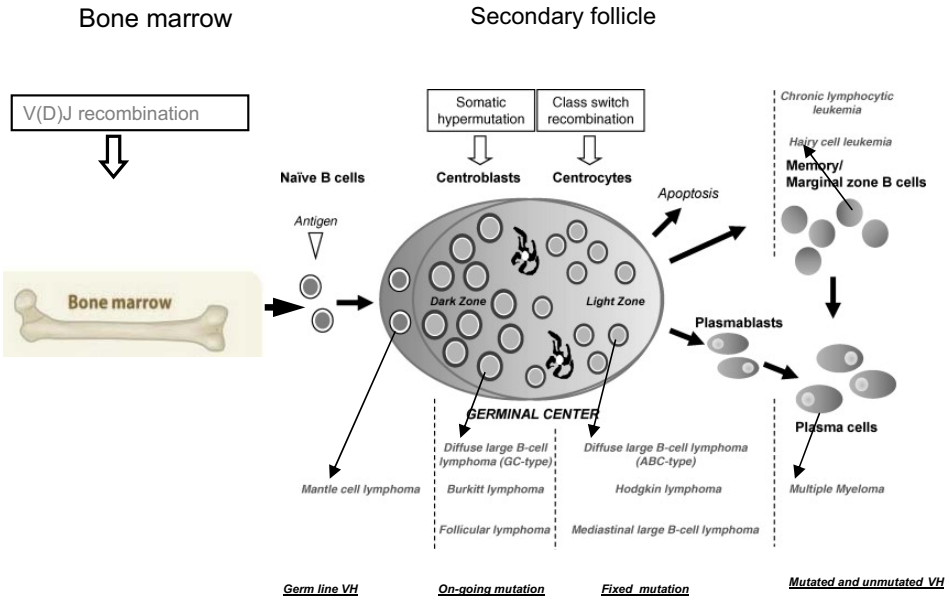

Figure 3. Human B-cell lymphoma and their normal B-cell counterparts

The variable region sequence of immunoglobulin genes (*Ig*) can be used as a marker to identify developmental stages of B-cell lymphoma. ALL and MCL originate from pre-B and naïve B cells respectively and carry unmutated *IGVH* gens. FL and GC-like DLBCL arise from germinal center B cells (the GC stage) and have ongoing mutation in the *IGVH* genes. The activated B- cell subtype of DLBCL carrying *IGVH* genes with fixed mutations is assumed to be of a post-germinal center B-cell origin. Marginal zone lymphoma including MALT lymphoma and splenic marginal zone lymphoma (SMZL) are assumed to originate from post-germinal center and perhaps B cells that acquired mutations in other micro-anatomical sites outside the germinal center (cases with fixed mutations) but also to a lesser extent pre-germinal center (un-mutated cases) B cells. (Adapted and modified with permission from Klein and Dalla-Favera, 2010)

1.3.3 Oncogene translocations in B-cell lymphomas

Approximately 40% of B-cell NHLs show recurrent reciprocal chromosomal translocations that can cause transcriptional deregulation of oncogenes or production of chimeric oncogenic proteins (Aukema et al., 2011). The majority of these translocations arise at the germinal center or post germinal center stage of the B- cell differentiation as a consequence of an aberrant SHM and CSR process. As a consequence, these translocations involve the *Ig* loci as well as a variety of partner proto-oncogenes. The process results in juxtaposition of proto-oncogenes to an immunoglobulin regulatory sequence and leads to deregulated expression of the affected oncogene (Kuppers and Dalla-Favera, 2001). Aberrant translocations can also arise in the bone marrow due to mistakes occurring during the *Ig*-gene rearranging process and result from illegitimate RAG-mediated V(D)J

recombination. Classic examples of these translocations include the t(11;14)/ *BCL1-IgH* translocation in MCL, the t(14;18)/ *BCL2-IgH* translocation in FL, the t(8;14)/ *c-MYC-IgH* translocation in BL and DLBCL as well as the t(3;14)/ *IgH-BCL6* translocation in DLBCL. Translocations involving immunoglobulin loci have also been described in MALT lymphoma and multiple myeloma (Isaacson and Du, 2004; Du, 2007). The consequence of oncogene translocations in B-cell lymphoma are diverse and can involve enhanced proliferation, inhibition of differentiation and apoptosis (Seto, 2004). For example, aberrant expression of BCL2 inhibits apoptosis (Seto, 2004). Constitutive expression of c-*MYC* results in a proliferative advantage and engenders cellular immortalization (Hurlin and Dezfouli, 2004). Constitutive expression of BCL6 inhibits differentiation to plasma or memory B cells but also enhances proliferation (Cattoretti et al., 2005).

While chromosomal translocations involving *Ig* loci are a recurrent theme in B-cell lymphomas, non-*Ig* involving chromosomal translocations are also observed. The t(11;18)(q21;q21) translocation, which fuses *API2* and *MALT1* genes, occurs in a subset of cases of extranodal marginal zone lymphoma (MALT lymphoma) (Akagi et al., 1999; Ye et al., 2003). This translocation results in a functional API2-MALT1 fusion gene. The API2-MALT1 fusion protein binds to both TRAF2 and TRAF6, and thereby enforces activation of NF-κB. In addition, *API2-MALT1* expression is enhanced by NF-κB, resulting in a positive feedback loop (Hosokawa et al., 2005). This results in uncontrolled NF-κB activation. Two other chromosomal translocations detected in MALT lymphoma, albeit less frequently, involve immunoglobulin genes, t(1;14) *IgH-BCL10* and t(14:18)/*IgH-MALT1*. Deregulated expression of BCL10 and MALT1 through result of these translocations also leads to activation of the NF-κB pathway (Nakagawa et al., 2006).

Some oncogene translocations, such as those involving BCL2 or CYCLIN D1, may not be sufficient for malignant transformation as these translocations are also detected in healthy individuals with increasing age (Seto, 2004; Roulland et al., 2006; Lecluse et al., 2009). Multiple genetic hits and/or epigenetic alterations are thus needed to induce lymphoid malignancy.

Apart from causing oncogene translocations, the SHM process also introduces low level somatic mutation in multiple proto-oncogenes genes including *c-MYC, BCL6, PIM1*, *PAX5*, *Rhoh*, and *FAS* (CD95) (Pasqualucci et al., 2001; Nussenzweig and Nussenzweig, 2010). Some of these mutations result in amino acid substitutions and also contribute to lymphomagenesis. However, the pathogenic role of many of these mutations is not yet fully elucidated as they have also been detected in non-malignant germinal center B cells (Liu et al., 2008).

1.3.4 Infection and lymphomagenesis

Although the etiology of each of the lymphoma types is not fully known, bacterial and viral infections, inherited and acquired immunodeficiency, autoimmune disorders, genetic disposition as well as environmental exposure and occupational risk factors have all been implied (Alexander et al., 2007). Infections or chronic inflammation may initiate lymphomagenesis by promoting increased cell proliferation and decreased apoptosis and by blocking differentiation. This increases the risk of subsequent genetic alterations that allow lymphoid cells to transform into malignant cells. Known infectious agents involved in B-cell lymphomagenesis are bacteria, viruses and likely protozoa. Infection-associated lymphomas can be categorized according to the mechanism of lymphoid transformation (Suarez et al., 2006; Engels, 2007). A first mechanism is that in which infection of B cells leads to the expression of viral oncogenes that directly promote high proliferation, decreased apoptosis and that block differentiation. Epstein-Barr virus (EBV), human T lymphotropic virus 1 (HTLTV1) and human herpes virus 8 (HHV8) are examples of B-cell transforming viruses. A second scenario is chronic immune stimulation leading to sustained lymphoid proliferation followed by genetic and epigenetic alternations leading to malignancy represents. *Helicobacter pylori* (*H pylori*) and gastric MALT lymphomagenesis is the best exemplified chronic antigenic stimulation-driven lymphoma. A third mechanism of infection-associated transformation is through decreased immune surveillance, such as for example induced by human immunodeficiency virus (HIV) infection (Carbone, 2003; Engels, 2007).

1.3.5 BCR signaling and lymphomagenesis

Upon encounter with antigen and upon receiving appropriate co-stimulatory signals, mature B cells expressing an antigen-specific BCR become activated and will mature into plasma cells. In addition, naïve mature resting B cells rely on constitutive or tonic BCR signaling for survival (Batten et al., 2000; Kraus et al., 2004; Monroe, 2006). The latter process is less understood than antigen-dependent BCR activation. BCR signaling is initiated when a specific antigen binds to the BCR. Subsequent signal transduction is mediated by other components in the BCR-complex. The BCR signaling complex is a multi-protein structure that is composed of a transmembrane immunoglobulin with the Igα and Igβ heterodimeric signaling subunit, also known as CD79A and CD79B, respectively (Reth, 1989). Engagement of the BCR by antigen results in BCR aggregation and triggers tyrosine phosphorylation of immunoreceptor-based tyrosine activations motifs (ITAMs) of CD79A and CD79B. In return, the activated BCR activates the Src family kinases Lyn, Blk, and Fyn as well as the Syk and Btk tyrosine kinase (reviewed by Dal Porto et al., 2004). This assembly initiates the formation of a 'signalosome' composed of the BCR, the tyrosine kinases, adaptor proteins such as CD19 and BLNK, and signaling enzymes such as phospholipase C g2 (PLCγ2), phosphoinositide 3-kinase (PI3K), and Vav (Dal Porto et al., 2004). The signalosome activates multiple signaling cascades that involve kinases, GTPases, and transcription factors. These cascades result in changes in cell metabolism, gene

expression and cytoskeletal organization. BCR signaling is complex and may lead to various outcomes, including survival, tolerance, apoptosis, proliferation, and differentiation into antibody-producing cells or memory B cells. The outcome of BCR signaling is determined by the maturation state of the cell, the nature of the antigen, the magnitude and duration of BCR signaling, and signals from other receptors such as CD40 and BAFF-R. NF-κB is one of the major transcription factors activated by BCR stimulation. NF-κB is a master transcription factor regulating differentiation, cell growth and apoptosis.

The BCR is also expressed by the majority of B-cell NHL (Kuppers, 2005). The BCR expressed by malignant B cells as for normal B cells is required for cell survival but may also drive proliferation (Kraus et al., 2004). That a functional BCR is of importance for malignant B cells is evidenced by the observation that translocations involving *Ig* loci are overwhelmingly located on the non-productively rearranged *Ig*-gene (de Jong et al., 1989). BCR signaling in both the absence and presence of cognate antigen is of importance in the early stage of lymphomagenesis (Klein and Pasqualucci et al., 2010). In B-cell lymphoma, BCR signaling can act through at least two major molecular mechanisms leading to NF-κB activation. The first mechanism involves constitutive activation of NF-κB signaling through mutations of BCR signaling components. Activating mutations in genes such as *CD79A*, *CD79B* and *CARD11*, down stream of the BCR receptor, lead to constitutional NF-kB activation. Such mutations are present in a substantial fraction of DLBCL but are less frequent or rare in other B-cell lymphomas (Lenz and Staudt, 2010; Staudt, 2010). A second mechanism involves stimulation of the BCR by antigen and is seen in clinically indolent B-cell malignancies. In CLL and MALT lymphoma foreign and/or auto-antigen activates the BCR which together with co-stimulatory signals, leads to NF-κB activation.

Some lymphoma types of B-cell origin do not express the BCR or express the receptor only at low levels (Kuppers, 2005). Typically, classic Hodgkin lymphoma has lost BCR expression due to the introduction of a stop codon or frame shift in the *Ig* variable-region genes. The latter has also been shown in post-transplant lymphoma (Capello et al., 2003). About 40% of cHL and the majority of post-transplant lymphoma are infected by EBV and express the EBV encoded latent membrane protein 2(LMP2). In those lymphoproliferative diseases LMP2 likely replaces the BCR-mediated signaling necessary for survival (Kuppers, 2005).

1.4 Marginal zone lymphoma

Marginal zone lymphoma (MZL) comprises distinct lymphoma types, believed to originate from marginal zone B cells (Isaacson and Jones, 1983). Depending mainly on the organ in which lymphoma arises, the current WHO lymphoma classification recognizes three distinct types of marginal zone lymphomas: nodal marginal zone lymphoma (NMZL) , extranodal marginal B cell zone lymphoma of MALT type known as MALT lymphoma and splenic marginal zone lymphoma (SMZL) arising in lymphoma nodes, mucosal sites and the spleen, respectively (Swerdlow, IARC Press, Geneva, 2008). MZL represents about 8-10% of all lymphoma, and thus MZL is the third most frequent lymphoma type after DLBCL and FL. MZL shares some common immunophenotypic features, but the clinical course and molecular characteristics are different for each of the types.

1.4.1 MALT lymphoma

Extranodal marginal lymphoma of mucosa associated lymphoid tissue (MALT lymphoma) is an extranodal lymphoma comprised of small B cells including centrocyte-like cells, which are resembling small lymphocytes or so-called monocytoid B cells (Isaacson, 2005). MALT lymphoma is the most common MZL. It is a clinically indolent disease and presents at a median age of 60 years (Swerdlow, IARC Press, Geneva, 2008). MALT lymphoma preferentially arises in the stomach and small intestine, but can occur in almost all mucosal tissues such as lacrimal glands, the thyroid, the salivary glands, breast tissue and bronchi. It also occurs in the skin. Interestingly, these organs are under normal circumstances devoid of organized lymphoid tissue (Morse et al., 2001). MALT lymphoma is therefore thought to arise from acquired lymphoid tissue, such as seen with chronic infection or inflammatory disorders. Several infectious agents and inflammatory disorders have been associated with MALT lymphomas, among which the association between gastric MALT lymphoma and *Helicobacter pylori* (*H pylori*) infection is the best characterized.

MALT lymphoma shares immunophenotypic features with normal marginal zone B cells: $CD20^{+}$, $CD21^{+}$, $CD35^{+}$, IgM^{+} $IgD^{+/-}$ (Spencer et al., 1985; Swerdlow, IARC Press, Geneva, 2008). MALT lymphoma does not typically express CD5, CD10 and BCL6.

1.4.2 Gastric MALT lymphoma and *Helicobacter pylori*: a model for chronic infection-driven B-cell lymphomagenesis

Chronic infection with *H pylori*, a Gram-negative bacterium, has been closely associated with gastric B lymphoma of mucosa-associated lymphoid tissue (MALT) lymphoma (Wotherspoon et al., 1991; Isaacson, 1995; Isaacson and Du, 2004). Several lines of epidemiological, clinical and molecular evidence are linking gastric lymphoma to *H pylori* infection. First, the incidence of primary gastric lymphoma is highest in geographical areas where *H pylori* infection is endemic (Doglioni et al., 1992). Second, *H pylori* presence has been shown in gastric biopsy samples of the majority of the gastric lymphoma patients (Wotherspoon et al., 1991). Third, at the early stages of gastric lymphoma,

eradication of *H pylori* infection by antibiotic treatment leads to complete regression of the disease in approximately 75-80% of the patients with this lymphoma (Wotherspoon et al., 1993; Bayerdorffer et al., 1995; Roggero et al., 1995). In addition, reinfection with the same strain of *H pylori* re-induces the growth of the lymphoma (Chang and Parsonnet, 2010). Pooled data analysis from 34 studies of more than 1270 patients with gastric MALT lymphoma showed that antibiotic treatment achieved eradication of infection in 98.3% of cases and that 77.8% of the *H pylori*-cured patients achieved lymphoma remission (Zullo et al., 2009). Fourth, nucleotide sequence analysis of the *IgV* genes from patients with gastric lymphoma supports the concept of the antigen-driven lymphomagenesis. The *IGVH* and *IGVL* genes of gastric MALT lymphoma are heavily mutated, suggesting antigen exposure (Du et al., 1996). However, *H pylori* infection may by itself not be sufficient to cause lymphoma. At least three stages in *H pylori*–driven lymphomagenesis are recognized (Figure 4); the initiation phase or chronic antigenic stimulation stage, the progression phase or immortalization stage and the transformation phase or advanced stage.

In its early stage, gastric MALT lymphoma is a low-grade and antigen-dependent neoplasia. At this stage, lymphoma B cells receive signals through their B-cell receptor leading to activation of the NF-κB signaling pathway that promotes increased survival and reduced apoptosis. Of interest, antibodies derived from gastric MALT lymphoma are not specific for *H pylori* antigens but bind to a variety of foreign as well as auto-antigens, thus showing polyreactive features (Craig et al., 2010). Reactivity with auto-antigens and well as Helicobacter antigens together with CD40-CD40 ligand independent help by T cells allows proliferation of B cells. The latter ultimately give rise to lymphoma through chronic stimulation (Enno et al., 1998; Bende et al., 2005; Mueller et al., 2005). In this early stage, gastric MALT lymphoma can be cured with antibiotic therapy in 75-80% of the cases (Wotherspoon et al., 1993).

With disease progression, additional oncogenic events occur, such as chromosomal translocations, inactivation of tumor repressor genes by mutations or hypermethylation leading to enhanced cell proliferation. At this stage, the disease becomes independent of antigenic stimulation (Farinha and Gascoyne, 2005). Among these changes include hypermethylation of cyclin-dependent kinase 4 inhibitors including *p15* and *p16,* also termed B and A, *CDKN2B* and *CDKN2A*, respectively, inactivate cell-cycle regulating genes, leading to a B-cell clonal expansion (Martinez-Delgado et al., 1997) (figure 4A and 4B). In addition, about 20-25% of gastric MALT lymphoma shows t(11;18), fusing *API2* to *MALT 1* genes or t(3;14) and t(14;18) fusing *BCL10* and *MALT1* genes to the heavy chain of the immunoglobulin locus, respectively (Isaacson and Du, 2004). These changes bypass the requirement of the BCR stimulation for growth.

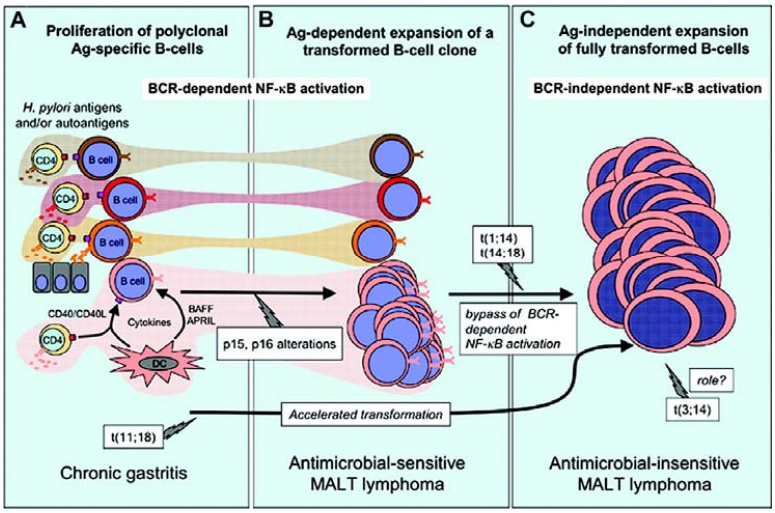

Figure 4. **Infection-associated indirect lymphoid transformation**
Bacterial antigens or autoantigen(s) could indirectly promote B cell proliferation and formation of neoplasia through an uncontrolled, chronic stimulation that may favour the occurrence of still poorly defined secondary genetic and epigenetic changes (reprinted with permission from Suarez et al., 2006).

1.4.3 Ocular adnexal lymphoma and *Chlamydia psittaci*

Ferreri and his colleagues (Ferreri et al., 2004, 2005, 2006A) have proposed an association between *Chlamydia psittaci* (*C psittaci*) infection and ocular adnexal lymphoma, a MALT lymphoma affecting conjunctiva, orbit, eyelid, or lacrimal gland. Subsequent studies have demonstrated that this association is dependent on geographical region (Mulder et al., 2006; Chanudet et al., 2006; Du et al., 2007). Also, *C psittaci* was shown in lymphoma tissue by PCR (Ferreri et al., 2004). *C psittaci* DNA was also detected in peripheral blood mononuclear cells of patients with lymphoma, but not in healthy individuals (Chang et al., 2010). Complete or partial response to antibiotic therapy has also been described (Ferreri et al., 2005). Of interest, one-third of *C psittaci* -negative patients with ocular adnexal lymphoma have also shown tumor regression after treatment with antibiotics (Ferreri et al., 2006B). The latter indicates that perhaps other microbial agents are also involved in the pathogenesis of the lymphoma.

It is assumed that the role of *C psittaci* in lymphomagenesis is quite similar to that of *H pylori* in gastric lymphoma. Very recently, Dagklis *et al*. provided new molecular data supporting relationship between *IGVH* gene repertoire and *C psittaci* infection in ocular adnexal lymphoma (Dagklis et al., 2012). Interestingly, approximately 10% of *IGVH* genes expressed by ocular adnexal lymphomas have auto-reactive properties, indicating an origin of the lymphoma from auto-antigen reactive B cells, at least in a proportion of cases (Dagklis et al., 2012).

1.4.4 Immunoproliferative small intestine disease and *Campylobacter jejuni*

Immunoproliferative small intestine disease **(**IPSID), also known as alpha heavy chain disease (αHC) disease or Mediterranean lymphoma, is a rare form of MALT lymphoma. Excessive production and secretion of truncated immunoglobulin alpha heavy chain fragments lacking immunoglobulin light chain is typical for this lymphoma (Martin and Aldoori, 1994). Of interest, *Campylobacter jejuni* (*C jejuni*) infection is often associated with this lymphoma. Eradication of the infection by antibiotic treatment is strongly suggestive of a causative association between *Campylobacter Jejuni* infection and lymphoma development (Parsonnet and Isaacson, 2004). Up to 40% of lymphomas have shown complete regression with antibiotic treatment (Al-Saleem et al., 2005). Lecuit and colleagues provided further evidence, based on molecular approaches, supporting a link between immunoproliferative small intestinal disease and *C. jejuni* infection (Lecuit et al., 2004). The pathogenesis of IPSID shows many similarities with this of gastric MALT lymphoma (Figure 5) (Parsonnet and Isaacson, 2004).

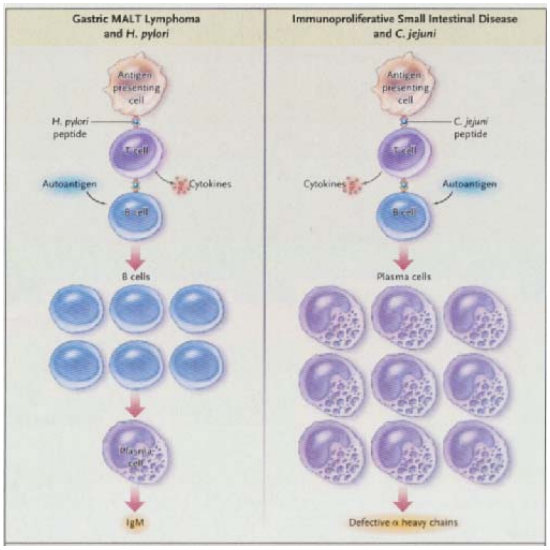

Figure 5. The similarities and differences between the pathogenesis of gastric MALT lymphoma (left panel) and immunoproliferative small intestine disease (right panel). In both lymphomas, the bacterial infection releases auto-antigens and stimulates T cells to produce cytokines, contributing to the proliferation of lymphoid B cells (reprinted with permission from Parsonnet and Isaacson, 2004).

1.4.5 Primary cutaneous B-cell lymphoma and *Borrelia burgdorferi*

MALT lymphoma of the skin has been associated with *Borrelia burgdorferi* infection in two independent studies (Goodlad et al., 2000; Ferreri et al., 2004). Antibiotic treatment with penicillin has been an effective as treatment for these lymphomas (Roggero et al., 1995; Kutting et al., 1997). However, a significant association between the infection and cutaneous MALT lymphoma has not

been found in the USA and Asia (Wood et al., 2001; Li et al., 2003). Therefore, the pathogenesis of primary cutaneous B-cell lymphoma is not yet clearly understood. The molecular genetics of cutaneous MALT lymphoma differs from that of the other MALT lymphomas (Streubel et al., 2004; Schreuder et al., 2005). For instance, the immunoglobulin proteins produced by cutaneous MALT lymphoma lacks rheumatoid factor reactivity. In addition, NF-κB signaling is probably activated through toll-like receptor 2 rather than through the BCR or BAFF as in gastric MALT lymphoma (Cho-Vega et al., 2006).

1.4.6 MALT lymphoma and Autoimmunity

Systemic autoimmune diseases are characterized by immune dysregulation and self-reactive reactivity resulting in damage to cells and tissue. In Western countries, roughly 5% of the population are suffering of some type of autoimmune disorder. Autoimmune disorders have been recognized as risk factors for the development of NHLs. An increased lymphoma incidence has been noted in rheumatoid arthritis (RA) (Thomas et al., 2000; Askling et al., 2005; Smedby et al., 2006), Sjögren's syndrome (SS) (Theander et al., 2006), systemic lupus erythematosus (SLE) (Mellemkjaer et al., 2008; Smedby 2008), celiac disease (Askling et al 2002; Catassi et al., 2002; West et al., 2004), dermatitis herpeteformis (Sigurgeirsson et al., 1994; Askling et al., 2002) and chronic thyroiditis (Askling et al., 2005; Mellemkjaer et al., 2008). Interestingly, autoimmune disorders may also develop secondary to hematologic malignancies (Ehrenfeld et al., 2001).

The nucleotide and amino acid sequence analysis of the *IGVH* repertoire in a comprehensive panel of B-NHLs has shown that more than 40% of salivary gland lymphomas in Sjögren's syndrome expressed BCRs with strong CDR3 homology to that of rheumatoid factor (Bende et al., 2005). Furthermore both *VH1-69* and *VH3-21* gene segments that have been shown to be preferentially used by MALT lymphoma, HCV-associated lymphoma, chronic lymphocytic leukemia and mantle cell lymphoma, respectively, are also used to produce rheumatoid factors in RA (Baecklund et al., 2006). Equally of interest, the synovial tissue of the RA patients contains oligoclonal B-cell proliferations with properties of antigen-driven selection (Kim and Berek, 2000). Perhaps these chronically stimulated auto-reactive B cells are precursor cells for B-cell lymphomas associated with auto-immune disease.

1.4.7 Splenic marginal zone lymphoma

Splenic marginal zone lymphoma (SMZL) is a relatively rare disease comprising 1-2 % of NHL and roughly 20% of MZL. It is mostly, but not always, a clinically indolent lymphoma (Swerdlow, IARC Press, Geneva, 2008). Most patients with SMZL are over 60 years. There is an equal incidence of SMZL in men and women (Matutes et al., 2008). The lymphoma originates in the spleen and spreads to the bone marrow in most cases. Variable numbers of lymphoma cells are also detected in the peripheral blood.

SMZL is assumed to arise from the splenic marginal zone B cells and have a concordant $CD20^{+}$, $CD19^{+}$, $FMC7^{+}$, $CD43^{+/-}$, IgM^{hi}, IgD^{lo}, $CD27^{+}$ $CD5^{-/+}$ immunophenotype (Matutes et al., 2008). Skewed VH gene usage is pronounced in SMZL, with about 30% of cases using the *VH1-2* genes. Also CDR3 regions are very similar in the latter cases (Bikos et al., 2012). This may indicate antigen-stimulation as part of the SMZL lymphomagenesis (Tierens et al., 2003; Arcaini et al., 2009a). Other SMZL are associated with Hepatitis virus (HCV) and also malaria infection has been associated with SMZL in geographic areas where these infections are endemic (Bates et al., 1997, Suarez, et al., 2007). The largest series of cytogenetic analysis of 330 patients with SMZL showed that 72% of SMZL cases exhibit chromosomal aberrations, of which 53% are complex genetic changes (Salido et al., 2010). None of these cytogenetic abnormalities are considered typical of SMZL, with the exception of the 7q32 deletion occurring in 39% of SMZL (Salido et al., 2010). Contrary to MALT lymphoma, chromosomal translocations involving the immunoglobulin loci are rarely found in SMZL.

1.4.8 Hepatitis C Virus and splenic marginal zone lymphoma

HCV is a single strand RNA virus that infects approximately 170 million people worldwide and is one of the major causes of liver disease (Mele et al., 2003). HCV-infection has also been associated with mixed cryoglobulinemia (MC) as well as with the development of NHL. This association was first described by Ferri and his colleges (Ferri et al., 1994). The finding was later supported by a large number of studies including a meta-analysis (Dal and Franceschi, 2006). Of interest, HCV infection is associated with specific NHL types including marginal zone lymphoma, chronic lymphocytic leukaemia and diffuse large B-cell lymphoma (Viswanatha and Dogan, 2007; Arcaini and Bruno, 2010). An oncogenic role of HCV in NHL was strengthened by clinical studies showing complete or partial lymphoma remission following anti-viral treatment (Hermine et al., 2002; Vallisa et al., 2005).
Further evidence directly linking HCV virus infection to NHL came from mutational analysis of the immunoglobulin variable genes showing the preferential usage of the *VH1-69* gene with similar CDR3s and preferential usage of the immunoglobulin light chain Vκ4–34 (also known as κv325) gene in the majority of the HCV-positive marginal zone lymphoma (Marasca et al., 2001). The *VH1-69* antibodies are typically polyreactive (Ivanovski et al., 1998). It has been proposed that HCV stimulates B cells through either interaction between CD81, present on the B-cell surface, and HCV-E2 glycoprotein, the major envelope protein of HCV (Rosa et al., 2005). Alternatively, HCV may activate B cells through interaction between the BCR and HCV-E2 or other HCV antigens. CD81 is a multimeric protein known to associate with the CD19 and CD21 complex on the cell surface which when activated, stimulates proliferation (Weng and Lau, 2005). It has been also reported that antibodies produced by HCV-associated lymphoma B cells react with the viral E2-protein in a manner identical to bone fide human anti-E2 antibody, implicating that the B activation is generated by the dual binding of the viral E2-protein to a cognate BCR as well to the CD81 molecule (Zignego et al., 2012). As proposed for other marginal zone lymphoma types, proliferating polyclonal B cells are

prone to gain subsequent genetic alterations resulting in growth advantage and ultimately lymphoid neoplasia.

1.4.9 Malaria and splenic marginal zone lymphoma

More than 250 million people worldwide suffer from malaria and nearly 1 million deaths are attributed to malaria each year (World Health Organization 2010). Hyperreactive malarial splenomegaly, previously known tropical splenomegaly syndrome is a disease of malaria-endemic regions of Africa (Fakunle et al., 1978). The syndrome is characterized by massive enlargement of the spleen, fever, weight loss, B lymphocytosis and cryoglobulinemia. Evolution of hyperreactive malarial splenomegaly to splenic marginal zone lymphoma has been described, thereby strengthening the possible role of infectious agents in the SMZL development, but its detailed pathogenesis is as yet unknown (Jimmy et la., 1996; Bates et al., 1997; Dascalescu et al., 2004).

1.4.10 Nodal marginal zone lymphoma

Nodal marginal zone lymphoma (NMZL), also called monocytoid B-cell lymphoma, is a primary nodal B-cell neoplasm that morphologically resembles SMZL and extra nodal MZL, but without evidence of splenic or extra nodal involvement, and represents < 2% of all lymphoid neoplasms (Campo et al., 2011). Most cases occur in individuals with a median age of 60-65 years. There is an equal incidence in men and women. A subtype of NMZL occurs in the pediatric age group, which has distinctive clinical and morphological features, and predominantly occurs in males with a ratio of 20:1. Typically, this lymphoma consists of B cells that resemble monocytoid B cells and can be admixed with scattered transformed cells. Most nodal NMZL cells express pan-B-cell markers, CD20, CD79A and PAX5, with coexpression of CD43 in 50% of the cases. Typically, these cells lack expression of CD5, CD23, CD10, BCL6 and cyclin D1, whereas BCL2 expression is positive in most cases (Campo et al., 2011; Arcaini et al., 2009b). Additionally, a recent study showed that IRTA1 is selectively expressed in NMZL and extra nodal MZL but not SMZL, thereby can be used as a valuable diagnostic marker to distinguish NMZL from SMZL (Falini et al., 2012). Some epidemiological studies have reported an association between NMZL and HCV infection (Arcaini et al., 2003; Arcaini et al., 2004). Also, the majority of NMZL patients show somatic hypermutation of rearranged immunoglobulin genes and preferential usage of *VH4-34*, suggesting that antigenic stimulation is involved in the lymphomagenesis (Traverse-Glehen et al., 2005). The most frequent cytogenetic aberration in NMZL is trisomy 3 that occurs in 24% of cases (Rinaldi et al., 2011). However, characteristic translocations or chromosome imbalances have not been described in NMZL.

2. Aim of the study

The overall aim of our studies was to further characterize marginal zone-like B cells and lymphomas believed to derive from marginal zone B cells. The following questions were addressed.

1) Do monocytoid B cells acquire somatic mutations outside the context of the germinal center?
2) Is there evidence for antigen stimulation in lymphomagenesis of splenic marginal zone lymphoma?
3) Can oncogenic mutations be found in genes that regulate NF-κB activation in marginal zone lymphoma?
4) Does diffuse large B-cell lymphoma arise from precursor marginal zone-like B cells?

3. Methodological considerations

3.1 Patient samples and human cell lines

Tissue, bone marrow and blood samples used in this thesis were retrieved from the archive of the department of pathology, the Norwegian Radium Hospital, Oslo University Hospital. The use of patient materials was approved by the Oslo University Hospital institutional review board and the regional ethics committee.

In article I, lymph nodes from 10 patients diagnosed with monocytoid B-cell hyperplasia and of whom freshly-frozen samples were available were studied. Two lymph nodes from these ten cases were selected for microdissection of monocyoid B cells for further analysis. For control studies, peripheral blood leukocytes from a healthy donor were obtained.

In article II, freshly-frozen biopsies from 10 cases of SMZL were selected to produce the antibodies expressed by the lymphoma B cells. In addition, the following cell lines were also used in the study in this article: HEK293 (ATCC), Jurkat (ATCC), OVCAR-8 (a kind gift of professor Reuven Reich, Hebrew University, Jerusalem, Israel), MIA PaCa-2 (ATCC), PANC-1 (ATCC), MIN6 (DMSZ), HEP-G2 (DMSZ), HELA (DMSZ), NALM-6 (DMSZ), KARPAS-422 (DMSZ), SU-DHL-6 (DMSZ) and Daudi (ATCC).

In article III, the same cases of SMZL studied in articles II as well as 13 control cases of marginal zone lymphoma, including 7 nodal, 6 extranodal MALT-type cases, were used to detect potentially oncogenic mutations in the NF-κB signaling pathway.

In article IV, diagnostic biopsies, bone marrow aspirates and/or blood samples from 165 consecutive patients with a primary diagnosis of DLBCL without histological evidence of concurrent no-Hodgkin's lymphomas were examined by routine microscopy of trephines and smear, immunohistochemistry and flow cytometry. Paired sample materials comprising diagnostic lymphoma biopsies as well as bone marrow of 10 of these 165 cases were available for the analysis of rearranged immunoglobulin heavy chain genes.

3.2 DNA and RNA extraction and nucleic acid sequencing

Genomic DNA and total RNA were extracted from paraffin-embedded materials, frozen tissue, blood and bone marrow samples by either TRIzol® Reagent kit (Life Technologies) or Biorobot® EZI DNA reagents and extractor or genomic DNA kit and RNeasy kit protocols (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The DNA and RNA concentration was measured with NanoDrop ND-1000 spectrophotometer (Witeg a.g., Littau Switzerland). The purified DNA and mRNA were amplified with the desired primer sets (one step reverse-transcriptase-polymerase-chain reaction, RT-PCT was performed when RNA is used). The PCR products were either directly sequenced or cloned into the respective expression vector and then sequenced from both orientations with the use of ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems), on an ABI 3100 Genetic Analyzer (Applied Biosystems).

3.3 Antibody Constructs

To produce lymphoma-derived antibodies, we designed and constructed two versatile expression vectors, one for the Ig heavy chain and another for the Ig light chain. These versatile expression vectors can be used for transient as well as stable transfection in mammalian cells. Recombinant *IgH* gene constructs consisted of a CMV promoter, a mouse Ig leader signal sequence, the rearranged *IGVH* gene from the respective SMZL, a human *CHγ1* region, and mouse *CH2-CH3-γ2b* (Fcγ2b) region (supplemental Figure 1 in article II,). The mouse *Fcγ2b* constant region was chosen because of the readily available anti-IgG2b secondary antibodies necessary for in the various tests we performed. Recombinant Ig light chain constructs consisted of a CMV promoter, a mouse Ig leader signal sequence, the rearranged *IGVL* gene from the respective SMZL, and a human Cκ region (supplemental Figure 1 in article II). The negative control construct contained only a mouse Ig *CH2-CH3 γ2b* fragment (Fcγ2b).

The constructs for the heavy chains were made as follows: first, the *IGVH* PCR products were digested with *Bsm*I and *Bsi*WI restriction enzymes and inserted into a pLNOH2 vector that is a derivative of the pcDNA3 vector (Invitrogen), as described by Norderhaug (Norderhaug et al., 1997). Subsequently, the inserted *IGVH* gene together with an upstream leader signal sequence and the downstream human CHγ1 sequence from the pLNOH2 vector were amplified using a forward primer (5′-CCG GGG GAT CCT CAC CAT GGG ATG GAG CTG TAT-3′) and a reverse primer (5′-TGT GGA TCC TTT GTC ACA AGA TTT GGG CTC-3′). This PCR fragment was then inserted in-frame in a pcDNA1-Fc expression vector (Aasheim et al., 2000) between the upstream CMV promoter and the downstream mouse *CH2-CH3-γ2b* gene sequence. The constructs for the light chains were made by inserting the *IGVL* gene fragment into a variant of the pLNOH2 vector containing a CMV promoter, a mouse Ig leader sequence, and a human *Ck* sequence (Norderhaug et al., 1997).

3.4 Transformation of bacterial cells

Transformation is a technique to introduce DNA into bacterial cells, whereas the term transfection is typically used when the process is performed in mammalian cells. Depending on the type of cells to be transformed and the characteristic of DNA to be introduced, there are a number of transformation methods that can be applied including direct uptake, electroporation and microprojectile bombardment. Direct uptake of relatively small molecules is the simplest method to transform *Escherichia coli* (*E. coli*) and can be achieved by adding the construct to bacterial competent cells at a slightly elevated temperature followed by rapid chilling down in an ice-bath. The cells are then grown in medium with $MgSO_4$ and $MgCl_2$. In article II, *IgH* and *IgL* constructs were transformed into TOP10 competent *E. Coli* cells (Invitrogen). For each lymphoma case at least 10 clones were selected for mapping and sequencing. Only the plasmids containing *IgV* sequences that were identical with the patient *IgV* sequences were used for transfection.

3.5 Transfection of mammalian cells

Several methods are used to deliver the desired DNA/plasmids into eukaryotic cells: a biochemical method in which chemical carriers such as cationic lipids or calcium phosphate are used; a physical method where electroporation, microinjection or gene gun are applied and finally, a virus-mediated transduction method in which adenoviruses and retroviruses are used as vectors to deliver large amount of DNA into the host cell.

Two transfection approaches can be used to transfer DNA/plasmid into cells, namely transient transfection and stable or permanent transfection. In transient transfection, plasmid is introduced into cells in order to achieve a temporary but high expression of the target gene. The transfected DNA does not necessarily become integrated into the genome of the host cell with this technique.

In contrast, stable or permanent transfection is usually employed to establish clonal cell lines were the transfected DNA is integrated into the genome of the recipient cell line. The production levels of the recombinant protein following permanent transfection is usually lower than that of transient transfection.

In article II, we were interested in the production of relatively large amounts of a panel of recombinant antibodies, and therefore, transient transfection was the method of choice. Two constructs carrying the respective *IgH* and *IgL* genes were co-transfected into HEK293T-cells by using lipofectAMINE transfection reagents (Invitrogen). The HEK293 cell line is relatively easy to transfect with the lipofectAMINE method and produces high quality recombinant proteins. HEK293T cells were grown in DMEM media (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum at 37º C in a humidified atmosphere with 5% CO_2. The recombinant antibodies were purified from culture supernatant by using protein-A affinity chromatography. The concentration of the purified recombinant proteins was determined by the Bradford assay and the quality of the protein products was confirmed by SDS-PAGE and western blotting with mouse anti-IgG antibody. The yield of the respective recombinant antibodies were satisfactory (0.3 μg/mL) except for one case, case 255 in article II, for which the production was very low compared to other cases despite several optimization attempts. In one case, case 152, we found two different expressed *VL* genes; hence, we produced two different monoclonal antibodies using the same heavy chain and either of the two light chains genes, respectively.

3.6 Flow cytometry

Flow cytometry (FCM) is an established method for the immunophenotypical characterization of cells, and is extensively used in research and clinical settings. Flow cytometry was used in articles II and V. In article II, flow cytometry was used to detect whether the recombinant antibodies bound to auto-antigens. Several cell lines, bone marrow aspirate and peripheral blood samples as well as lymph node cell suspensions were analyzed. Apoptosis assays were also performed to test whether the reactivity of the lymphoma-derived recombinant antibodies increased in apoptotic induced cell lines. Apoptosis

was induced in Jurkat and ovarian carcinoma cell line OVCAR-8 using the alkaloid staurosporine, which is known to induce apoptosis through both caspase-dependent and caspase-independent mechanisms. The apoptotic assay was performed as described previously in detail by Chu and Dong (Chu et al., 2010; Dong et al., 2008).

Briefly, the sample cells to be analyzed by flow cytometry were suspended in a solution of 100 mg/ml human γ-globulin in dilution buffer (PBS with 1% BSA and 12.5 mM Na-Azide) and incubated with antibodies (30 μg/ml) at 4°C for 30 min before staining with phycoerythrine(PE)-linked goat anti-mouse. To examine whether the recombinant antibodies reacted with potential antigens on the cell surface, the cells were pretreated with Trypsin EDTA solution containing 0.5 mg/mL trypsin and 0.22mg/mL EDTA for 30 minutes at 37º C. The control cases were incubated only with PBS before staining. To investigate whether the recombinant antibodies bound to glycosylated epitopes, the cell cultures were incubated overnight with 1μg/mL of tunicamycin (Sigma-Aldri), known to inhibit *N*-glycosylation or were pre-treated with 5 units of *N*-glycosidase and 10 units of *O*-glycosidase (Roche Diagnostics) before staining with the recombinant antibodies.

In article IV, flow cytometry was used to characterize B cells in the bone marrow and blood from patients with DLBCL. This was done by using a panel of antibodies reactive with B lymphocyte surface antigens. Bone marrow aspirates and blood samples were analysed by a stain, lyse and wash method that has been previously described (Rothe and Schmitz, 1996). After pre-treatment, the cells were stained with a panel of commercially obtained primary monoclonal antibodies directly conjugated to fluoresceine thyocyanate (FITC), phycoerythrine (PE), phycoerythrinecyanine 5 (PeCy5), peridinin chlorophyll protein (PerCP), peridinin chlorophyll protein-cy5.5 (PerCP-Cy5.5) or allophycocyanin (APC). Not all markers could be performed on all samples because of the number of cells available for analysis was limited in some samples.

Flow cytometry analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and flow data were analyzed using software programs CellQuest 3.3 (BD Biosciences) and FlowJo 7.2.2 (Tree Star, Ashland, OR, USA). A total number of 10000 or more gated events were analyzed in each sample.

3.7 Immunohistochemistry

Immunohistochemistry (IHC) is used to detect the presence of antigens in cells of a tissue section. It is widely used both in clinical diagnostics as well as in basic research. Antigen-antibody interactions are visualized and examined in a microscope, therefore IHC provides information about the distribution and localization of antigens in tissues and cells. Experimental parameters such as the specificity and the amount antibody used, fixation, antigen retrieval steps as well as well as subjectivity of interpretation may influence the results achieved in IHC staining (Fritschy, 2008). These parameters were therefore controlled where possible.

In article I, immunohistochemistry was performed on paraffin-embedded tissue sections from ten lymph nodes to analyze monocytoid B cell in their microenvironment. Immunohistochemistry was also performed on frozen tissue sections of two lymph nodes, in order to select monocytoid B cells for micro-dissection and PCR analysis. Either the peroxidase-labeled ready-to-use EnVision+ Systems (DakoCytomation, Glostrup, Denmark) or double staining was carried out using a combination the EnVision+ Systems and biotin-streptavidin-peroxidase complex method (ABC) according to the instructions of the manufacturers. Table 4.1 summarizes the anti-bodies used in this study, their source and their respective distribution and function in tissues and cells.

Antibody	**Supplier**	**Distribution in normal tissue**	**Function**
CD3	Novocastra	thymocytes, peripheral T cells; CD3 is a pan T-cell marker	member of immunoglobulin superfamily
CD4	Novocastra	T helper cells (80-90%), granulocytes, macrophages, dendritic cells	serves as co-receptor in MHC class II-restricted antigen induced T cell activation
CD8	Novocastra	cortical thymocytes, mostly cytotoxic T cells, NK cells dendritic cells	recognizes antigens displayed by an antigen presenting cell (APC)
CD21	Novocastra	mature B cells, follicular dendritic cells, some thymocytes, some T cells	part of the B cell co-receptor complex and participates in B cell response to the antigen
CD23	Dako	activated mature B cells expressing IgM or IgD, activated monocytes / macrophages, T cell subsets, platelets, eosinophils, follicular dendritic cells	acts as a B cell growth and activation factor, promoting differentiation into plasma cells
CD27	Dako	memory B cells, NK cells, plasma cells, medullary thymocytes and some T cells	regulates B cell activation and immunoglobulin synthesis, marker of T cell activation
CD35	Dako	erythrocytes, basophils, eosinophils, granulocytes, monocytes, macrophages (some), B cells, T cells (10%), NK cell subset; CD35 is a dendritic cell marker	binds immune complexes coated with C3b or C4b and mediates their transport to and removal by the fixed phagocyte systems of the spleen and liver
CD40	Dako	mature B cells, dendritic cells, macrophages, mast cells, activated monocytes, platelets	plays a central role in germinal center formation, in T-cell mediated immunity and mediates immunoglobulin class switching and the B cell memory cell development
AID	H.Niedobitek	B cells	activation-induced cytidine deaminase, essential for immunoglobulin somatic hypermutation and class-switch
BCL2	Dako	many cell types	member of the B-cell lymphoma 2 large family of proteins that regulate and contribute to programmed cell death or apoptosis
BCL6	Dako	activated B cells (germinal centers)	represses genes that function in lymphocyte differentiation, inflammation and cell cycle control
IgD	Dako	mature B cells	is one of the BCR isotypes; participates antigen binding and BCR signal transduction
Ki-67	Dako	multiple tissues; proliferation marker	plays a role in cell proliferation; essential for cell cycle progression

Table 4.1: antibodies used for immunohistochemistry in article I

In article II, immunohistochemical analysis was performed using the peroxidase-labeled ready-to-use EnVision+ Systems (DakoCytomation, Glostrup) according to the instructions of the manufacturer. The formalin-fixed and paraffin-embedded tissues as well as cell-blocks from human pancreatic carcinoma cell lines MIA PaCa-2 and PANC-1 were cut at 4-5 µm, dried at 60 º C overnight, then stored in a fridge at 4-8 º C until used. Subsequently, the sections were stained with lymphoma-derived recombinant antibodies as primary antibodies. The recombinant antibodies reacted diffusely with ubiquitous antigenes in the cytoplasm and to a lesser degree on the membrane of cells, indicating

poly- and self reactivity. Negative controls were incubated with either the Fcγ2 protein or only washing-buffer instead of primary antibodies. The scoring was assessed by an experienced pathologist. In article IV, immunohistochemical staining was performed on diagnostic biopsies from patients with primary diffuse large B-cell lymphoma without evidence of concurrent non-Hodgkin's lymphoma as well as on bone marrow trephine biopsies. Commercially obtained primary antibodies (table 4.2) and peroxidase-labeled ready-to-use EnVision+ Systems (Dako) were used according to the manufacturer's instructions.

Antigen	**Supplier**	**Distribution in normal tissue**	**Function**
CD5	Novocastra,	thymocytes, almost all T cells, subset of mature B cells	negative regulator of TCR and BCR signaling
CD10	Novocastra	Early-B and T-cell precursors and subset of mature B cells including germinal centers	zinc-dependent cell-surface metallopeptidase that cleaves peptide bonds on the amino side of hydrophobic amino acids, plays a role in B-cell differentiation
CD20	Novocastra	pan B-cell marker but absent in plasma cells	plays a role in creation of a structure similar to an ion channel that allows for the influx of calcium required for cell cycle activation
CD23	Novocastra	activated mature B cells expressing IgM or IgD, activated monocytes /macrophages, T-cell subsets, platelets, eosinophils, follicular dendritic cells	acts as a B-cell growth and activation factor, promoting differentiation into plasma cells
CD30	Dako	ativated B, T and NK cells, monocytes, ranulocytes and some plasma cells	member of tumor necrosis factor family, activates expression of nuclear factor-KB
CD138	Dako and /or Lab Vision,	B cell precursors, plasma cells, stratified squamous epithelium	mediates cell adhesion, growth factors, associated with late stage of B cell differentiation
BCL2	Dako	multiple cell types	member of B-cell lymphoma 2 protein family that contribute to programmed cell death or apoptosis
BCL6	Dako	activated B-cell lymphocytes in the germinal center	represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control
Igλ	Dako	mature and immature B cells	is the light chain domain of the immunoglobulin protein; BCR antigen binding and signal transduction
Igκ	Dako	mature and immature B cells	is the light chain domain of the immunoglobulin protein; BCR antigen binding and signal transduction
IgD	Dako	mature B cells	is one of the BCR isotypes; participates in antigen binding and BCR signal transduction
IgM	Dako	mature and immature B cells	is one of the BCR isotypes; participates in antigen binding and BCR signal transduction
Ki-67	Dako	multiple tissue, a proliferation marker	plays a role in cell proliferation and is essential for cell cycle progression
MUM1	Dako	cells of lymphocytic and melanocytic lineages; absent in centroblasts but upregulated in a subset of centrocytes and plasma cells	MUM1 (multiple myeloma oncogene 1)/IRF4 (interferon regulatory factor 4); plays important roles in cellular proliferation and survival
Cyclin D1	Lab Vision,	multiple tissues	cyclin D1 a proto-ongogen; plays a role in inactivatition of the retinoblastoma protein and promotes progression through the G_1-S phase of the cell cycle

Table 4.2: antibodies used for immunohistochemistry in article IV

3.8 Western blot and enzyme-linked immunosorbent assay

In addition to IHC, reactivity of the recombinant antibodies was tested by WB and ELISA analysis in article II. WB is a useful method to detect the presence and relative amounts of the protein present in different samples, the relative molecular weight (MW), posttranslational modifications of the protein of interest as well as protein-protein interactions. Despite many protocol modifications and new technologies to increase the sensitivity and speed of the method, experimental conditions still require optimizing. Cell lysis conditions, the quality and quantity of the loaded protein, antibody specificity and concentration, the type of the membrane used, the number of times washed, the incubation and exposure time are among the factors that can influence the accuracy of WB results (MacPhee et al., 2010). The Enzyme-Linked Immunosorbent Assay (ELISA) is another technique used for the detection of a protein in a sample based on an immunological reaction. The ELISA method offers two major advantages compared to WB. ELISA is more suitable for screening a large number of samples and provides better quantitative data.

In article II, total cell lysates from four different cell lines, MIN6, HEK293T, HEP-G2 and HELA were analyzed by WB. In brief, cell pellets were washed twice in phosphate-buffered saline (PBS), lysed in appropriate lysis buffer supplemented with inhibitor cocktails targeting phosphatases and proteases, and clarified with centrifugation. 25 μg of the cell lysate in sample buffer with DTT was resolved by SDS-PAGE, and the separated proteins were transferred to a PVDF membrane. To verify even loading of the proteins on the gel and successful transfer of the proteins to the membrane, the membrane was stained with a naphthol–blue black solution. Blocking agents were used to reduce unspecific antibody binding in immunoblotting. First, we used non-fat dry milk in PBS 0.1 % tween as a blocking agent. Since as we observed that the recombinant antibodies bound to epitopes in milk, blocking was subsequently performed with 5% BSA bovine serum albumin in PBS 0.1 % tween. After blocking, the membrane was incubated overnight at 4°C with 3 μg/mL of the respective recombinant antibodies, washed with PBS 0.1 % Tween and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies. Enhanced chemiluminescence (ECL) ready-to-use-kit (GE Healthcare, Fairfield, CT, USA) was used to visualize antibody binding. Recombinant Fcγ2 fragment was used instead of a primary antibody for control experiments.

To validate the polyreactivity of the lymphoma-derived recombinant antibodies, indirect ELISA analyses were performed. Crude cell lysates of HEK293T and PANC1 were prepared as described for Western analysis. A panel of proteins including thyroglobulin (TG), β-galactosidase, insulin, lipopolysaccharide (LPS) human serum albumin (HSA), BSA as well as single strand DNA (ssDNA), either purchased from commercial suppliers or obtained as gift, were tested with the respective recombinant antibodies in ELISA. HRP-linked rabbit anti-mouse Ig was used as secondary antibody. Fish-skin gelatin (Sigma-Aldrich) was used as blocking agent as we found that our monoclonal recombinant antibodies bound to some degree to coated BSA. Appropriate positive and negative

control runs using either the recombinant Fcγ2 protein (homemade), a mouse monoclonal IgG2b-E149 specific for neuron-specific enolase or mouse monoclonal anti-TG IgG1(E41) antibody specific to thyroglobulin (TG) (kind gifts from Dr Elisabeth Paus, Medical Biochemistry Department, the Norwegian Radium Hospital), were included in all ELISA experiments.

4. Results in brief

4.1 Article I: Monocytoid B cells: an enigmatic B-cell subset showing evidence of extrafollicular immunoglobulin gene somatic hypermutation

The presence of somatically hypermutad immunoglobulin *VH* genes in patients with hyper IgM syndrome and X-linked lymphoproliferative diseases, conditions that are characterized by the absence of germinal centers, raises the question whether somatic hypermutation and clonal expansion might take place in micro-anatomical sites outside the germinal center. We investigated whether somatic hypermutation might be acquired in the monocytoid B cell-zone. Monocytoid B cells are a B-cell subset localized in the perisinusoidal area of the lymph node and can be recognized in certain reactive lymph node and lymphoma conditions, including lymphadenitis caused by toxoplasma and HIV infections (Miettinen, 1981; Mohrmann et al., 1991). The ontogeny and function of monocytoid B cells is poorly understood. It has been suggested that monocytoid B cells are closely related to marginal zone B cells (De Wolf-Peeters et al., 1997; Tierens et al., 1999). Accordingly, nodal marginal zone cells may display the morphology of monocytoid B cells (Nathwani et al., 1999; Swerdlow, IARC Press, Geneva, 2008).

Whether monocytoid B cells are post-germinal center B cells or whether these acquire *IGVH* gene somatic hypermutations outside the germinal center had yet not been investigated. To address this, we performed a detailed analysis of clonal expansion, SHM profile rearranged immunoglobulin gene and investigated expression of activation-induced cytidine deaminase (AID) in monocytoid B cells.

Results of the immunohistochemical analysis showed that monocytoid B cells co-express AID and the proliferation-associated marker Ki-67, conditions that are prerequisites for somatic hypermutation and clonal expansion, respectively. We observed that monocytoid B cells microdissected from defined extrafollicular areas shared mutated *IGVH* genes confirming clonal expansion. Importantly, ongoing somatic hypermutation was demonstrated.

The expression of AID as well as the presence of on-going mutations in proliferating monocytoid B cells provides evidence that somatic hypermutation occurred *in situ* in the perisinusoidal area of the lymph node, where the monocytoid B cells are located. Hence our results indicate that somatic hypermutation may occur outside the germinal center, and as consequence, that the presence of somatic hypermutation not necessarily indicates a germinal or post-germinal center cell origin.

4.2 Article II: Splenic marginal zone lymphoma with *VH1-02* gene rearrangement expresses poly- and self-reactive antibodies with similar reactivity

About 30% of SMZL shows a rearranged *VH1-02 IgVH* gene, suggesting that SMZL arises from a B cell that has been selected by an antigen. The objective of this study was to determine the antigen-specificity and reactivity of antibodies expressed by SMZL that display a rearranged *VH1-02* gene. This information is part of a study that seeks to investigate antigen stimulation in SMZL pathogenesis.

First, we characterized *IGVH* and *IGVL* genes of 10 SMZL patients. The complementarity determining regions 3 (CDR3) of the *IGVH* and *IGVL* genes were also analyzed. Interestingly, we found mutated *IGVH* and *IGVL* genes in all of the cases. 6 of 10 cases revealed rearrangement of the *VH1-02*04* gene, and 7 of the 10 cases including, 5 cases with *VH1-02*04* genes, also rearrange the *HD3-3*01* gene. In addition, the amino acid sequence analysis of the CDR3 regions of the cases with the *VH1-02*04* and *HD3-3*01* genes showed remarkably homologous sequences. The CDR3 region is determining for antibody binding. Amino acid sequence alignment of the CDR3 regions from this study with those from previously published cases of SMZL with *VH1-02*04* rearrangement showed similar CDR3 properties. This indicates that the antibodies produced by SMZL with *VH1-02*04* gene rearrangement bind similar epitope(s).

IGVH and *IGVL* genes from 5 SMZL cases with *VH1-02*04* gene rearrangement were amplified and cloned into appropriate expression vectors. Subsequently, lymphoma-derived recombinant monoclonal antibodies of these cases were produced *in vitro*. The reactivity and specificity of those antibodies were tested by flow cytometry, immunohistochemistry, Western blotting and Enzyme-linked immunosorbent assay (ELISA). Of interest, 4 of the 5 recombinant antibodies showed similar reactivity. Poly- and self-reactivity was demonstrated with cytoplasmic, nuclear as well as cell surface antigens in a wide range of cell types, such as epithelial cells from different organs, mesenchymal cells including smooth muscle cells, lymphoid cells as well as macrophages. In addition, these lymphoma-derived recombinant antibodies reacted also with epitopes in milk and in serum. Furthermore, Western blotting revealed that the antibodies recognized multiple proteins. The polyreactivity of the antibodies was further confirmed by ELISA. The recombinant antibodies showed strong but variable binding to a panel of antigens including thyroglobulin (TG), β-galactosidase, insulin, lipopolysaccharide (LPS) human serum Albumin (HSA), BSA and single strand DNA (ssDNA). Of interest, antibody reactivity to antigens expressed on the cell surface induced by apoptosis was not demonstrated, in contrast to findings by others in chronic leukemia cases with *VH1-02* gene rearrangement (Lanemo et al., 2008; Chu et al., 2010). Our results demonstrate that SMZL using the *VH1-02*04* gene express polyreactive antibodies that react with common ubiquitous auto-antigens. This major subset of SMZL arises therefore likely from polyreactive B cells. Polyreactive B cells may be important as the first line of defense against infectious agents before an antigen-selected response can be mounted.

4.3 Article III: Few CD79B and MYD88 mutations in splenic marginal zone lymphoma

NF-κB is a transcription factor that plays a key role in the survival and proliferation of many kinds of B-cell tumors, including SMZL. Signaling from antigen receptors such as BCR, TCR, TLR, interleukin-1, CD40 and TNFα receptor activates the canonical NF-κB pathway. Signaling from the B-cell activating factor (BAFF) receptor and CD40 leads to activation of the alternative NF-κB pathway. Genetic aberrations and various mutations in genes activating the NF-κB signaling pathway have been identified in several B-cell NHL subtypes, including DLBCL with an activated B-cell immunophenotype. Constitutive activation of NF-κB in these lymphomas contributes to lymphoma cell survival and proliferation. Since our results (see article II) indicate that antigen stimulation plays a role in SMZL lymphomagenesis, we investigated the occurrence of activating somatic mutations in genes of the NF-κB pathway in SMZL. Ten samples of SMZL and 13 control cases of other MZL types were subjected to DNA sequence analysis of *CD79A, CD79B, CARD11* and *MYD88* genes. These genes contribute to the activation of the canonical NF-κB pathway. We detected a point mutation in the *CD79B* gene (Y196H) of one of the SMZL cases. Additionally, one point mutation was identified in the MYD88 gene (L265P) of another SMZL case. No mutations were discovered in *CD79A* or *CARD11* genes in SMZL cases. As expected from previous publications, no mutations were identified in the other MZL control cases. Interestingly, the missense mutation detected in CD79B in a SMZL case has been previously reported in the ABC subtype of the DLBCL (Davis et al., 2010). This mutation results in the substitution of the first tyrosine, which is known to be phosphorylation site in the ITAM region, with another amino acid, histidine in our case, leading to constitutively active BCR signaling and activation of the canonical NF-κB pathway. Equally interesting, the L265P point mutation in MYD88, which we detected in onother SMZL case, has also been detected in 9% of gastric MALT lymphomas as well as in 29% of ABC DLBL by Davis *et al*. This mutation occurs at an amino acid residue which is functionally critical in the MYD88 mediated NF-κB and JAK-STAT signaling pathways (Ngo et al., 2011). Indeed, the mutation is found in a highly conserved domain of the *MYD88* gene in all mammalians and the L265P point mutation in MYD88 can cause three dimensional change of the MYD88 protein, leading to a protein complex that spontaneous triggers the phosphorylation of IRAK1 and NF-κB activation as a consequence (Ngo et al., 2011).

The identification of mutations in *CD79B* and *MYD88* genes in some SMZL cases represents additional genetic aberrations of signaling genes that may contribute to the constitutive activation NF-κB. Although few mutations were detected, the results provide evidence that chronic antigen stimulation likely is important for SMZL pathogenesis. However, continuous stimulation through (auto) antigen binding of the BCR is likely of more importance than activating mutations of NF-κB pathway genes.

4.4 Article IV: Low levels of monoclonal small B cells in the bone marrow of patients with diffuse large B-cell lymphoma of activated B-cell type but not of germinal center B-cell type

The objective of this study was to investigate the occurrence of monoclonal small B cells (MSBCs) in the bone marrow of diffuse large B-cell lymphoma (DLBCL) patients and to establish a possible clonal relationship between DLBCL B cells and MSBC in the marrow. DLBCL represents about 30% of all adult non-Hodgkin's lymphoma. The largest entity of this lymphoma is DLBCL not other-wise specified (NOS), which comprises two molecular distinct subtypes based on gene expression profiling; a germinal center B-cell-like (GCB) subtype and activated B-cell-like (ABC) subtype. In addition to DLBCL-NOS, other variants of DLBCL are recognized; primary cutaneous DLBCL of leg-type, primary DLBCL of central nervous system (CNS), primary testicular DLBCL, primary mediastinal B-cell lymphoma (PMLBCL), T-cell/hystocyte-rich B-cell lymphoma (T/HLBCL) and double hit B-cell lymphoma with *BCL-2* and *C-MYC* gene translocations (DHBCL).

We investigated the presence of MSBCs in the bone marrows of 165 patients with primary DLBCL without histological evidence of concurrent non-Hodgkin's lymphoma. The study was performed by multiparameter flow cytometry on bone marrow aspirates combined with morphological examination and immunohistochemical analysis of bone marrow aspirates and bone marrow trephine biopsies.

DLBCL infiltration in marrows was detected in 11 cases of 165 patients (6.7%). Of note, only 3 of 119 cases with DLBCL NOS showed bone marrow infiltration. By contrast, flow cytometry analysis combined with morphological analyses revealed the presence of MSBCs in 24 of 165 cases of DLBCL. Strikingly, we observed a significant different frequency of the MSBC occurrence among the different DLBCL subtypes. Of the 119 patients with DLBCL NOS, 11 of 39 (28.2 %) of cases with ABC and only 3 of 80 (3.7%) cases with GCB subtype showed MSBC in the marrow ($P = 0.0002$). Whereas, the presence of MSBC in the marrow was detected 4 of 8 (50%), 4 of 15 (26.7) and 2 of 3(66.7) patients with primary testicular, CNS and leg-type DLBCL, respectively. No MSBCs were found in marrows of patients with rare DLBCL subtypes, such as PMLBCL, T/HLBCL or DHBCL. MSBCs in 15 of 25 cases showed a non-CLL-like immunophenotype, 3 cases a CLL-like immunophenotype whereas 6 cases were unclassifiable. Because of lack of proper materials, we could not systematically investigate the clonal relationship between MSBC in the marrow and DLBCL. However, identical rearranged *IGVH* genes from MSBCs in the marrow and DLBCL tumor cells from the same patient were successfully identified in one case, suggesting that at least in this case MSBC and DLBC share a common cellular origin. In addition, monoclonal small B cells in the blood were detected in 4 of 5 DLBCL patients with MSBCs in their bone marrow. In those 4 cases, MSBCs in the blood and small MSBCs in the marrow showed a similar immunophenotype.

The results from this study provide evidence that the ABC subgroup of the DLBCL NOS as well as some of the rare DLBCL subtypes with an ABC immunophenotype but not GCB DLBCL are highly associated with the presence of MSBCs in the bone marrow. Whether MSBCs in the bone marrow are precursors of the ABC subtype of DLBCL remains to be further elucidated.

5. Discussion

B cells are prone to malignant transformation because molecular mechanisms that are normally used for antibody diversification to fight against pathogens can result in aberrant chromosomal translocations and oncogenic mutations. Of interest, upon malignant transformation, B-cell lymphomas frequently retain properties of their cell of origin. Therefore, better understanding of B-cell lymphomagenesis requires knowledge of normal B-cell lymphopoiesis. In this regard, the first article of this thesis studies a hitherto enigmatic B-cell subset, the monocytoid B cells. This B-cell subset is likely the cell of origin for the subset of nodal marginal zone lymphoma. In a second article we provide evidence that splenic marginal zone lymphoma likely arises from chronic stimulation of auto- and poly-reactive B cells. In a third study, we demonstrate that only few activating mutations are found in the BCR pathway genes of marginal zone lymphoma, despite activation of NF-κB. The latter is consistent with the hypothesis that marginal zone lymphoma shows no autonomic growth but is dependent on continued chronic antigen stimulation for growth. In a fourth study, we provide evidence that even diffuse large B-cell lymphoma with an activated B-cell phenotype, may arise from circulating small monoclonal B cells, with an immunophenotype reminiscent of germinal zone B cells.

5.1 Somatic hypermutation and clonal expansion of monocyoid B cells

The pattern of somatic hypermutation in *IgV* genes allows us to deduce the ontogeny of B-cell malignancies. It is widely accepted that somatic hypermutation and class switching are the hallmark of the T-dependent immune response and markers for memory B cells. There is also a general consensus that somatic hypermutation of the *IgV* genes principally takes place in germinal centers (MacLennan, 1994; Rajewsky, 1996). However, recent reports proposed that somatic hypermutation can be T-independent thought still occurring in the germinal center (William et al., 2002; Weller et al., 2004; Scheeren et al., 2008; Weill et al., 2009).

To analyze whether somatic hypermutation may occur outside the germinal center, we examined clonal expansion, expression of AID and somatic hypermutation of *IGVH* genes in monocytoid B cells in the lymph node. Monocytoid B cells are poorly characterized B cells. They are mature B cells that typically are present in perisinusoidal area of the lymph node. These cells are especially prominent in certain infections such as toxoplasmosis or viral infections. Whether monocytoid B cells give rise to nodal marginal zone B-cell lymphoma (NMZL), also known as monocytoid B-cell lymphoma, has been suggested but is still debated. We and others have previously demonstrated that monocytoid B cells express rearranged immunoglobulin genes with somatic hypermutations in variable regions (Tierens et al., 1999; Lazzi et al., 2006). In article I, we showed that monocytoid B cells co-express AID and the proliferation-associated marker Ki-67, proteins required for the induction of somatic hypermutation and clonal expansion, respectively. The sequences derived from clusters of the proliferating B cells revealed intraclonal diversification of *IGVH* genes through on-going mutations.

IGVH gene mutational analysis showed characteristic features of somatic hypermutation, such as G/C mutation bias and the presence of deletions as well as a predominance of transitions over transversions. These findings suggest that monocytoid B cells acquire somatic hypermutations *in situ*, i.e. the peri-sinusoidal area of the lymph node where these B cells were isolated from. Several studies have already suggested that somatic hypermutation may occur outside the germinal center both in the human and mouse (Weller 2004; Mao et al., 2004). Patients with hyper-IgM syndrome as well as patients with X-linked lymphoproliferative disease, whom lack germinal centers, do possess $IgM^+IgD^+CD27^+$ circulating B cells with somatically mutated *IGVH* genes suggesting that these B cells develop outside the germinal center (Chu et al., 1995; Weller et al., 2001; Weller et al., 2004). It has been proposed that IgM^+IgD^+ $CD27^+$ B cells acquire somatic mutations during a primary, T-independent pre-diversification process outside the germinal center, as demonstrated in sheep and rabbits (Reynaud et al., 1995; Weill et al., 2004). This proposal was also supported by the finding that murine immature B cells express somatically hypermutated *IGVH* as well as AID (Mao et al., 2004). Contrary to this view, it has been suggested that $IgM^+IgD^+CD27^+$ B cells with mutated *IGVH* genes represent germinal center-derived memory B cells, because the majority of these B cells carry mutated BCL6, the master transcriptional regulator in germinal center B cells and known to be targeted by the somatic hypermutation machinery (Pasqualucci et al., 2003; Seifert and Kuppers, 2009). BCL6 is only expressed in the germinal center B cells and its expression is prerequisite for the acquisition of somatic hypermutation.

Monocytoid B cells are a distinct B-cell population, but show some immunophenotypic similarity with circulating $IgM^+IgD^+CD27^+$ B cells. Indeed, monocytoid B cells are $IgM^+IgD^-CD27^{-/+}$. Whether circulating $IgM^+IgD^+CD27^+$ B cells arise from monocytoid B cells is an interesting question that requires further study.

Our data show that monocytoid B cells may not arise from germinal center or post- germinal center B cells, but may indeed acquire somatic mutations in another way. Whether these mutations are acquired during a pre-diversification process or whether mutations are acquired by T-cell or non-T-cell-dependent antigen stimulation remains to be demonstrated.

5.2 A subset of SMZL expresses *IGVH* genes with unique features

Irrespective of the question where somatic hypermutation occurs in MZL, the vast majority of these lymphomas express IgM antibodies with mutated *IGVH* genes. Furthermore, this disease is strongly associated with chronic antigen stimulation (Suarez et al; 2006). Although the association between *H pylori* infection and extranodal marginal zone lymphoma MALT type is clinically and molecularly well characterized, the pathogenesis of SMZL and NMZL are as yet not well studied. However, antigen stimulation is believed to be important for the pathogenesis of these two latter entities as well. In article II, we studied, albeit indirectly, the possible role of antigenic stimulation in the SMZL development. First, detailed molecular analysis of the immunoglobulin gene variable regions was

performed in 10 well-characterized SMZL patients. Interestingly, 6 of those 10 cases used the same *VH1-02*04* gene, while the remaining four cases used *VH1-18*, *VH*4-59 (two cases) and *VH3-30* genes. At least three of the cases revealed intraclonal sequence variation caused by on-going mutations. Additionally, 7 of these 10 cases, including 5 cases with *VH1-02*04* gene rearrangement, showed also usage of the same *HD3-3*01* gene, whereas the other case with *VH1-02*04* gene rearrangement used the *HD3-9*01* gene which is highly homologous to the *HD3-3*01* gene. However, no bias was detected in *HJ* segment use. Furthermore, the deduced amino acids of these 6 cases with *VH1-02*04* rearrangement showed highly homologous sequences with several conserved or semi-conserved amino acids and exceptionally long CDR3 segments with a relatively high isoelectric point (pI). Our results are quite similar to those that have been reported in previous studies, showing the preferential usage of *VH1-02* gene rearrangement in SMZL (Algara et al., 2002; Tierens et al., 2003**;** Stamatopoulos et al., 2004; Arcaini et al., 2009a). Of interest, there is apparent geographical variation with respect to the preferential usage of *VH1-02* among different study cohorts with the fraction of cases using *VH1-02* rearrangement being remarkably low in the in the Greek series compared to other cohorts (Stamatopoulos et al., 2004). Very recently, a large study of 337 SMZL cases confirmed the preferential usage of the *VH1-02* gene rearrangement, mostly in combination with a *HD3-3* gene rearrangement (Bikos et al., 2012). The characteristics of the CDR3 regions in SMZL with *VH1-02*04* rearrangement reported by Bikos' group are very similar to those of the cases we studied (article II).

A biased repertoire of *IGVH* genes is generally regarded as evidence for antigen selection involving a limited set of antigens or perhaps superantigens in B-cell lymphomagenesis (Stevenson et al., 2001; Kuppers, 2005). Several B-cell malignancies, most notably CLL and MCL are also characterized by both a biased *IGVH* usage as well as stereotyped CDR3 regions. Almost one-third of CLL samples show stereotyped BCRs, whereas 10% of MCL possess stereotyped BCRs (Stamatopoulos et al., 2007; Murray et al., 2008; Hadzidimitriou et al., 2011).

Of interest, CLL cases with *VH1-02* gene show different, short and stereotyped CDR3 sequences compared to SMZL cases with *VH1-02* gene rearrangement (see article II, figure 1). The latter data suggest that the involved antigens in CLL are different than those in SMZL. The restricted immunoglobulin repertoire in SMZL implies that the antibodies expressed by SMZL B cells recognize common antigenic determinants. By consequence, these antigens very likely play a role in SMZL lymphomagenesis.

5.3 SMZL-derived recombinant antibodies indicate involvement of antigen stimulation in SMZL pathogenesis

To further characterize the binding activity of monoclonal antibodies (mAbs) expressed by SMZL cases with *VH1-02*04* rearrangement, we synthesized recombinant antibodies derived from five cases of SMZL and tested their reactivity by several experimental approaches. Interestingly, 4 of the SMZL-derived recombinant antibodies reacted similarly with a wide variety of cell lines as well as normal

and lymphoma tissues, whereas the fifth recombinant mAb failed to bind any antigen at all in all experiments that were performed. Cytoplasmic, nuclear as well as cell surface reactivity was detected in a wide range of cell types, but mostly in epithelial cells from different organs. Of interest, the recombinant antibodies reacted with multiple (auto) antigens. Remarkably, reactivity of the antibodies was drastically reduced or competitively blocked when these antibodies were pre-incubated or diluted with serum or milk, indicating that the antibodies also reacted with antigens that were present in milk and serum. Taken together, these results suggested that SMZL-derived recombinant antibodies were self-reactive and polyreactive. Polyreactivity of SMZL-derived antibodies was further evidenced by ELISA experiments that showed binding of the antibodies to a panel of purified antigens.

Polyreactive antibodies comprise a large fraction of the early B-cell repertoire (Casali and Notkins, 1989; Zhou et al., 2007). Nearly 75% and 55% of the early immature B cells of healthy human individuals express polyreactive and self-reactive BCRs, respectively (Wardemann et al., 2003), but most of these potentially harmful polyreactive auto-antibodies are eliminated from the repertoire at different checkpoints during early B-cell maturation. Thus only 5% of the naïve B-cell compartment retains polyreactivity (Tsuiji et al., 2006).

Natural polyreactive IgM antibodies are likely important for the immediate immune defense against foreign antigens, such as viruses and bacteria, usually through T-cell independent immunity before more specific antibodies are generated. However, polyreactivity can also be detected in antibodies generated through T-cell dependent immunity. Polyreactivity of these antibodies might enhance antibody affinity for antigens where simple homotypic bivalent ligation is not achievable. Immunologic analysis of monoclonal anti-HIV-gp140 antibodies cloned from HIV patients revealed that the affinity of these antibodies for HIV-gp40 was improved through heteroligation to more abundant self-antigens, since homotypic bivalent binding is not feasible. Thus, polyreactive anti-HIV-gp 40 antibodies seem to be positively selected if they bind more strongly to HIV-epitopes than their monoreactive counterparts (Mouquet et al., 2011).

Although amino acid sequence alone cannot determine whether an antibody is polyreactive, the length of the Ig heavy chain CDR3 regions has been correlated with self-reactive and polyreactive antibodies (Ichiyoshi and Casali, 1994; Aguilera et al., 2001). Polyreactive anybodies usually have longer CDR3 segments resulting in increased flexibility of the antigen-binding site with multiple configurations that allows the antibody to recognize a variety of structurally unrelated epitopes (Notkins, 2004). Accordingly, the CDR3 regions of our SMZL cases with *VH1-02* rearrangement were unusually long.

Also antibodies derived from other B-cell lymphoma types including some gastric MALT lymphoma, CLL with unmutated *IGVH* genes as well as HCV-positive SMZL revealed polyreactive features (Hamblin et al., 1999; Carbonari et al., 2005; Craig et al., 2010). Of interest, most of these lymphomas show *VH1-69* gene rearrangement. *VH1-69* encoded antibodies have been shown to display rheumatoid activity (Bende et al., 2005; Baecklund et al., 2006). By contrast, the *VH1-02* encoded antibodies in our study do not show rheumatoid activity.

Whether SMZL with the *VH1-02* gene rearrangement arises from natural antibody producing B cells or from an antigen-selected post-germinal center B cell is yet to be clarified. The presence of somatic hypermutation in SMZL is in favor of the latter hypothesis. However, somatic hypermutation may also occur outside the context of the germinal center as we have demonstrated in article I, and therefore an origin from a natural antibody producing B cell is not excluded. Of interest, the major population of IgM^+ cord blood B cells, about 20% shows *VH1-02* gene rearrangements in contrast to circulating adult IgM^+ B cells that mostly show *VH3* gene rearrangements (Prabakaran et al., 2012). This may be indirect support for an origin of SMZL from natural antibody producing B cells. In addition, the preferential usage of the same *VH1-02(*04)* gene, the presence of somatic hypermutation, highly homologous CDR3 segments with distinctive features and similar pattern of polyreactivity strongly suggest a role for antigen/auto- antigen stimulation in lymphomagenesis in SMZL.

5.4 Somatic mutations in NF-κB pathway regulating genes in SMZL

Up-regulation of the *NF-κB* pathway genes as wells as its target genes have been observed in SMZL (Ruiz-Ballesteros et al., 2005). NF-κB activation promotes proliferation and decreases apoptosis (Davis et al., 2010). The results from article II indicate that antigen stimulation may have contributed to SMZL lymphomagenesis. In study III of this thesis, we analyzed potentially oncogenic mutations in the BCR and MYD88 signaling. MYD88 is a cytosolic adaptor protein that contains an N-terminal death domain and a C-terminal Toll–IL-1R (TIR) domain separated by a short linker sequence. It plays crucial roles in both innate and acquired immune responses through signal transduction initiated by toll-like-receptors (TLRs) and various interleukin-receptors (Medzhitov et al; 1998). The activation of MYD88 signaling originates from interaction between the cytosolic TIR domain of the ligand-induced TLRs and the TIR domain in the MYD88 C-terminal. Upon stimulation, MYD88 recruits and activates IL-1-receptor associated kinase (IRAK) to the TLR-MYD88 complex, forming a TLR-MYD88-IRAK complex. In return, the TLR-MYD88-IRAK complex recruits and activates TNF receptor associated factor-6 (TRAF6) to the TLR-MYD88-IRAK complex, then activated TRAF6 mediates activation of down stream signaling pathways including NF-κB (reviewed in Takeda and Akira, 2004).

We detected a point mutation in the *CD79B* gene (Y196H) in one of the SMZL cases. Additionally, one point mutation was detected in the *MYD88* (L265P) gene in another SMZL case. However, neither *CARD11* nor *CD79A* were found mutated in these 10 SMZL cases. Furthermore, none of the 13 control cases form non-SMZL MZL cases did demonstrate any mutations in the analyzed genes. Interestingly, the site where we detected mutation in the *CD79B* gene in SMZL has been previously described as one of the *CD79B* hotspot mutation motifs in the ABC subtype of DLBCL, and this mutation is detected in almost all of the mutated ABC DLBCL cases (Davis et al., 2010).

While preparing our study III, Yan and colleagues reported mutations either in *CARD11* (8.8%), *TNFAIP3*(13%) or *MYD88* (13%) genes in SMZL. However, mutations in *CD79A* and *CD79B* genes were not detected in these SMZL cases (Yan et al., 2012). *TNFAIP3* codes for the "global" NF-κB

negative regulator A20, and is inactivated by mutations or epigenetic silencing in many lymphoma subtypes, including HL, PMBL and ABC DLBCl, and MALT lymphomas (Schmitz et al., 2009; Compagno et al., 2009; Kato et al., 2009).

Activating somatic mutations in *CD79A*, *CD79B*, *CARD11, MYD88* genes have been previously demonstrated in ABC DLBCL (Lenz et al, 2008; Davis et al., 2010; Ngo et al., 2011). More recently, in a study of 101 SMZL cases, Rossi *et al.* detected that more than one third of SMZL harbour mutually but exclusive somatic mutations in various regulators involved in the canonical (*A20* and *IKB-κB*) as well no-canonical (*BIRC3*, *TRAF3*, *MAP3K14*) NF-κB activation pathways (Rossi et al., 2011).

In conclusion, constitutional activation of NF-κB through activating mutations also contributes to SMZL lymphomagenesis. Whether chronic antigen stimulation increases the risk of activating mutations is an interesting hypothesis but remains to be demonstrated. Of interest, activation of NF-κB is also seen in the two other types of MZL, NMZL and extra-nodal MZL or MALT lymphoma, albeit through different genetic mutations. In MALT lymphoma several oncogene translocations t(1;14)(p22;q32), t(14;18)(p32;q21), t(11;18)(q212;21) result in constitutive activation of the NF-κB pathway (reviewed by Du, 2011). Additionally, genetic lesions inactivating A20 have been detected in translocation-negative MALT lymphoma cases, particularly those from the ocular adnexa, salivary and thyroid glands, resulting prolonged NF-κB activity (Kato et al., 2009; Bi et al., 2011).

5.5 Monoclonal small B cells (MSBC) in the bone marrow of ABC DLBCL: are they precursor cells of this lymphoma?

DLBCL accounts for about 30% of all adult non-Hodgkin's lymphoma. The WHO lymphoma classification recognized several clinically and genetically distinct types of DLBCL (Swerdlow, IARC Press, Geneva, 2008). The largest entity is DLBCL, not other-wise specified (NOS). This lymphoma type is further subdivided into two subtypes with distinct gene expression profiling, germinal center B cell-like (GCB) and activated B cell-like (ABC) subtypes. These two subtypes have the expression signature of normal germinal center B cells and activated B cells, respectively (Alizadeh et al.; 2000; Rosenwald et al., 2002). The GCB subtype is associated with a better prognosis whereas the ABC subtype shows a worse outcome (Rosenwald et al., 2002).

In article IV, we studied the occurrence of MSBC in the bone marrow of 165 consecutive patients with various DLBCL subtypes without histological evidence of concurrent NHL. 119 of these cases were DLBCL NOS type, whereas the rest of the cases were other types and subtypes of DLBCL. We detected MSBCs in 24 of 165 cases of DLBCL. Strikingly, the occurrence of MSBCs in the bone marrow has been observed in 11 of 39 (28.2%) of cases of ABC subtype and in only 3 of 80 (3.7%) of GCB DLBCL subtype. Of note, clinical records of these cases did not show diagnosis of leukemia. The difference in incidence of MSBCs between ABC and GCB of DLBCL NOS was significant (P

value of 0,0002). Interestingly, of the 24 cases with MSBC cells in the marrow, 10 cases showed a discordant immunophenotype between MSBC and the respective DLBCL.

MSBCs in the bone marrow of DLBCL patients are likely similar to those cells present in the peripheral blood of elderly people, also termed as monoclonal B-cell lymphocytosis (MBL) (Ghia et al., 2004; Marti et al., 2005). This is demonstrated in our study by showing that patients with MSBC displayed MBL in their blood with similar immunophenotype.

MBL is defined as the presence of monoclonal B cells in the peripheral blood below 5000 cell/ μL in the absence of small B-cell lymphoma (Hallek et al., 2008; Mowery and Lanasa, 2012). The etiology of MBL is still unknown. However, risk factors including aging, certain chromosomal abnormalities as well as inherited genetic variation in certain genes such as *FARP2*, *IRF4* and *SP140* have been associated with the MBL development (Rawstron, 2009; Crowther-Swanepoel et al., 2010). Antigen stimulation may also contribute to the clonal expansion of MBL, as it has been demonstrated that a high proportion of CLL-like MBL cases show somatically hypermutated *IGVH* genes (Rawstron, 2009).

MBL is heterogeneous in terms of immunophenotype and can be distinguished on the bases of CD5 surface marker expression (Ghia et al., 2004). Only a minor fraction of MBL cases are CD5-negative (termed $CD5^-$ MBL), whereas the majority of MBL cases are CD5-postive ($CD5^+$ MBL). $CD5^+$ MBL cases can be further subdivided into two subtypes based on the expression level of CD20; a non-CLL-like MBL subtype with bright CD20 expression and a CLL-like MBL subtype with low levels of CD20 expression (Marti et al., 2005). The latter subtype shares with CLL certain characteristic features including high expression of the CD23 surface marker and weak or no expression of CD79B as well as surface immunoglobulins. Most importantly, CLL-like MBL shares genetic feature with CLL. The CLL-like MBL subtype is the most frequent MBL type, especially among the elderly and can be detected in up to 7% of individuals older than 75 years (Ghia et al., 2004).

Of interest, of 24 DLBCL patients with MSBC in their bone marrow, only 3 had MSBC with a CLL-like immunophenotype, whereas 15 had MSBC with a non-CLL-like immunophenotype. The remaining 6 patients had MSBC with a non-classifiable immunophenotype. This is in contrast to the published frequencies of MBL immunophenotypes in normal population in which the CLL-like immunophenotype dominates (Ghia et al., 2004; Rawstron, 2009).

We could not systematically investigate the clonal identities of MSBC and DLBCL because of lack of proper materials for this analysis. The analysis was attempted in 10 sample pairs of the bone marrow trephines and matched DLBCL biopsies that were available for analysis. Despite several attempts, immunoglobulin gene rearrangement analysis was only successful in one case (patient 11). The monoclonal B cells in this case showed a CLL-like immunophenotype. Interestingly, the rearranged *IGVH* genes of MSBC and the DLBCL tumor of this patient displayed an identical *IGVH* sequence, including the clone-specific CDR3 region, proving the similar clonal origin of MSBC and DLBCL.

The identification of MBL as a precursor lesion of CLL has been well documented in recent years and the MBL progression to CLL has been estimated a rate of 1.1% per year (Rawstron, 2009). However, the overwhelming majority of MBL does not progress to CLL.

Our study demonstrates that at least some MSBC may progress to DLBCL. To understand the nature of MSBC in the bone marrow of DLBCL and MBL in the peripheral blood, we will prospectively collect bone marrow and the peripheral blood of DLBCL patients, isolate the MSBC from the bone marrow and blood, perform genetic analysis and compare results with these obtained in DLBCL.

6. Concluding remarks and future perspectives

B-cell lymphomas arise from B cells at distinct stages of maturation and differentiation. The majority of SMZL carries mutated immunoglobulin genes and accordingly it has been postulated that SMZL originates from post-germinal center B cells. However, our data as well as that from others suggests that the somatic hypermutation process is not restricted to the germinal center micro-environment, as evidenced by somatic hypermutation occurring in monocytoid B cells in the perisinusoidal lymph node area. Therefore, the mere presence of somatic hypermutation in the rearranged immunoglobulin genes cannot be taken as final proof for germinal center or post-germinal center cell origin for B-cell lymphoma. Impact of somatic hypermutations on *IGVH* genes can be detected in about 87% of SMZL (Bikos et al., 2012). Irrespective of the maturation stage of its cell of origin, it can safely be assumed that somatic hypermutation is proof of antigen exposure in SMZL. Our study of SMZL confirms a role of antigenic stimulation in SMZL lymphomagenesis. In spite of the self and poly-reactive nature of SMZL-derived antibodies, reactivity with a defined external pathogen, perhaps infectious, is not excluded. Infections have been demonstrated in various MALT lymphoma subtypes. The search for an infectious origin of SMZL should therefore be continued.

Similar to several other B-cell lymphoma types, SMZL lymphoma is characterized by oncogenic mutations that constitutively activate the NF-κB signaling pathway (Compagno et al., 2009; Lenz et al, 2008; Davis et al., 2010; Ngo et al., 2011), and we identified some additional potentially oncogenic mutations affecting that pathway. The identification of such mutations may lead to the development of targeted therapies for patients suffering from SMZL.

Finally, we found the presence of MSBC in the bone marrow of a large fraction of patients with the ABC subtype of DLBCL NOS but not of GCB subtype of DLBCL NOS. This finding provides further evidence that the ABC and GCB DLBCL subtypes have distinct pathogeneses. At least in one ABC DLBCL case, the rearranged *IGVH* gene of the monoclonal small B cells and the respective tumor biopsy showed identical *IGVH* gene sequences, indicating that MSBC may be a direct precursor of the ABC subtype of DLBCL. However, the relationship between MSBC and the ABC DLBCL needs to be further investigated. We hypothesize that MSBC arise through chronic antigen stimulation, may acquire oncogenic mutations and ultimately lead to the development of ABC DLBCL. This hypothesis will require future studies.

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ERRATA

A) Typographical errors;

1. page 8, line 6; changed from associated tissue to associated lymphoid tissue
2. page 8, line 7; changed from b-cell to B-cell
3. page 9, after line 14; a new line containing "*accepted for publication: ISRN-Oncology, 2013*" is inserted:
4. page 11, line 19; changed from occupy he dark zone to occupy the dark zone
5. page 13, line 20; changed from the D-J_H point to and the D-J_H fragment
6. page 16, line 2; changed from CD40-dificient to CD40-deficient
7. page 19, line 2; changed from fraction monocytoid to fraction of monocytoid
8. page 23, line 1; changed from examples of translocations to examples of these translocations
9. page 24, line31; changed from (ITAMS) to (ITAMs)

B) Reference numbering in article 3 (**Few CD79B and MYD88 mutations in splenic marginal zone lymphoma),** the following number corrections were made.

Page 3, line 7; changed from18 to 15
Page 3, line 9; changed from 15 to 16
Page 4, line 3; changed from 13 to 14
Page 5, line 7; changed from 16 to17
Page 5, line 8; changed from 17 to15
Page 5, line 11; changed from 15 to16

C) References and the reference list of the thesis manuscript were corrected.

In the main text, the names of authors are now corrected (for instance, La-Favera (Riccardo Dalla-Favera) is changed to Dalla-Favera, Van Den Oord is changed to van den Oord or Maclennan is changed to MacLennan, etc). References from the reference list that were not referred to in the main text are now added to the latter, and vice versa.

I

II

III

Few CD79B and MYD88 mutations in splenic marginal zone lymphoma.

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Key words: mutations, NF-κB signaling, lymphoma

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Abstract

In this study we have examined the mutation status of genes involved in the NF-κB signaling pathway. DNA sequence analysis of four genes was performed: CD79A, CD79B, CARD11 and MYD88 that are activated through BCR signaling or Toll-like and interleukin signaling. A single point mutation was detected in the CD79B gene (Y196H) in one of ten SMZL cases. Additionally, one point mutation was identified in the MYD88 gene (L265P) in another SMZL case. No mutations were revealed in CD79A or CARD11 genes in these SMZL cases. Neither were mutations detected in these four genes studied in 13 control MZL samples. Interestingly, the two cases with mutations of CD79B and MYD88 showed increased numbers of immunoblasts spread among the smaller and typical marginal zone lymphoma cells. Although SMZL shows few mutations of NF-κB signaling genes, our results indicate that the presence of these mutations is associated with a higher histological grade.

Introduction

Marginal zone lymphoma (MZL) is a non-Hodgkin lymphoma that likely develops from B-lymphocytes in the marginal zone of secondary lymphoid tissue. There are three subtypes of marginal zone B-cell lymphoma (MZL)[1] : nodal, extra-nodal and splenic marginal zone lymphoma, arising in the lymph node, mucosa and the spleen, respectively. Splenic marginal zone lymphoma (SMZL) is an indolent, low grade B-cell lymphoma primarily characterized by splenomegaly with variable involvement of lymph nodes, bone marrow, peripheral blood and other organs. It accounts for less than 1% of non-Hodgkin lymphoma[2]. The normal splenic marginal zone contains both memory B cells and naive B cells. In parallel, unmutated as well as mutated immunoglobulin heavy chain genes are found[3-5].

Gene expression profiling has revealed aberrant NF-κB signaling in several lymphoma types such as diffuse large B-cell lymphoma (DLBCL)[6], [7]Hodgkin Lymphoma[8] and SMZL[9]. NF-κB is a transcription factor that regulates different cellular processes, such as cell growth and survival[10] and is activated when normal B-cells respond to antigen. In one subtype of DLBCL, of activated B-cell origin (ABC), the constitutively activated NF-κB signaling is due to mutations of important B-cell receptor (BCR) signaling genes[11]. These include mutations of the gene for caspase recruitment domain-containing protein 11 (CARD11)[12] and

mutations and/or deletions of an essential signaling subunit of BCR, CD79[13]. Of interest, Ngo et al.[14] have described oncogenically active MYD88 mutations, an adaptor gene involved in Toll-like receptor signaling leading to NF-κB activation in both ABC DLBCL (29 %) and in MALT lymphoma (9%), however SMZL were not studied. Interestingly, Rossi et al. demonstrated mutations and copy number alterations of genes such as TNFAIP3, IKBKB, BIRC3, TRAF3 and MAP3K14, involved in both the canonical and non-canonical NF-κB pathways in about 20% of SMZL[15.] These authors found no mutations in CARD11 and MYD88. However, in a more recent study both CARD11 mutations (8.8 %) and MYD88 mutations (13 %) have been detected in SMZL [16]. The reason for the discrepancies between the two studies is unclear. The aim of this study was to analyze somatic mutations in additional genes, specifically CD79A and B, and study CARD11 and MYD88 gene mutations in our collection of SMZL cases.

Methods

Tissue samples of 10 cases of SMZL and 13 control cases of other MZL types, 7 nodal and 6 extranodal MALT-type, were selected from the archives of the Department Pathology at The Norwegian Radium Hospital, Oslo University Hospital, Norway. All studied cases were reviewed to confirm diagnoses. The study was approved by the Regional Committee for Research Ethics.

DNA was isolated from frozen tissue using the EZ1 tissue kit (Qiagen, Hilden, Germany). PCR was carried out using AmpliTaq Gold polymerase (Applied Biosystem, Weiterstadt, Germany) according to the supplier's instructions and using the following conditions: 94°C for 5 min followed by 34 cycles of denaturation 30s at 94°C, annealing 30s at 60°C (or 58°C) and extension 45 s at 72°C. PCR primers were used as described in table 1. The primer pair for CD79A (exon 4 and 5, NM_001783), CD79B (exon 5 and 6, NM_000626) and MYD88 (part of exon 5, NM_002468) were designed using Primer-BLAST. The primer pairs used for CARD11 exon 5-10 (NM_032415) were as described in Lenz et al. (exon 5-10 is equal to exon 3-8 in their publication). The PCR products were verified on an Agilent 2100 Bioanalyzer (Agilent Technologies) and an aliquot of the products was directly sequenced from both ends on a 3130 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany) using BigDye Terminator v1.1 (Applied Biosystems). A single nucleotide polymorphism, in one amino acid position (265) of the MYD88 gene was detected using PCR and SNaPshot

multiplex kit (Applied Biosystems) and analyzed with both forward and reverse primers by 3130 Genetic Analyzer and GeneMapper 4.1 Software (Applied Biosystems).

Results

A point mutation in the CD79B gene (Y196H) was detected in one SMZL case whereas a MYD88 gene (L265P) mutation was detected in another case. The CD79B mutation affected the first tyrosine of the CD79B immunoreceptor tyrosin-based activation motif (ITAM). No mutations were detected in the CD79A gene or in the CARD11 gene in any of the SMZL cases (Table 1). Neither were mutations detected in all of the genes studied in the 13 control MZL samples.

Of interest, the two SMZL cases with mutations of CD79B and MYD88, respectively, showed increased numbers of immunoblasts spread among the smaller and typical marginal zone lymphoma cells (Fig. 1).

Discussion

In the present study we found a low frequency of mutations in four NF-κB-signaling genes studied. Out of 10 cases of SMZL, only one mutation was found in CD79B and one mutation in MYD88 and none in CD79A and CARD11. This is in contrast to ABC DLBCL were CD79B mutations were detected in 21 %, MYD88 mutation (L265P) in 29 % and CARD11 mutations in 10 % of the cases [12-14]. However, no CD79B mutations were detected in 16 extranodal MZL cases (gastric MALT lymphoma) analyzed in the same study. The latter is in accordance with the data of our study. Davis et al [13] found a similar low frequency of MYD88 mutation (9 %) in gastric MALT lymphomas as we found in our SMZL cases.

The mutation detected in CD79B affects a critical residue; the first tyrosine in the ITAM is replaced by another amino acid. Interestingly, this mutation is also found in almost all of the mutated ABC DLBCL cases. In ABC DLBCL this mutation increases the surface BCR expression, inhibits Lyn kinase activity and enhances activation of NF-κB[13]. The MYD88 L265P mutation detected in one of the SMZL cases occurs at a residue that is important for protein complex assembling and supports cell survival by activating NF-κB and JAK-STAT signaling pathways [14]. Although the somatic origin of the two mutations detected in our study could not be verified, these mutations have been shown by others to be functionally

significant. It is therefore likely that mutations leading to amino acid substitutions demonstrated in two of the SMZL samples represent somatic mutations.

A recent report has described mutations of multiple genes that cause deregulation of NF-κB in DLBCL [11]. Of these the NF-κB negative regulator TNFAIP3 (A20) shows inactivation mutations and/or deletions in about 30 % of patients, thus contributing to NF-κB activation. More importantly, A20 is also inactivated in 19 % if MZL, however with a frequency of only 8 % in SMZL [16]. A low mutation frequency in the four NF-κB-related genes studied is also reported by a recent study of a large series of SMZL [17]. Out of 101 cases of SMZL no mutations or copy number abnormalities of CARD11 and MYD88 were revealed, while CD79A and B were not studied. However, out of the other 20 genes studied, 5 of these genes showed mutations in 20 % of SMZL cases. Yan and colleagues [15] found a similar level of MYD88 mutation in (13 %) as in our study, however no mutations were detected in CD79A or CD79B in their series of 57 SMZL cases.

The cases with CD79B mutation and MYD88 mutation, respectively, showed increased numbers of immunoblast-like cells compared with the cases without mutation. This observation is of interest and might indicate that both mutations are associated with higher histological grade and therefore with risk of transformation. However, larger series with clinical follow-up data are needed to confirm the latter.

In this study we found few activation mutations in four NF-κB-signaling genes, including for the first time in CD79B. It is therefore likely that yet other genetic aberrations of signaling genes may contribute to the constitutive activation of NF-κB in SMZL.

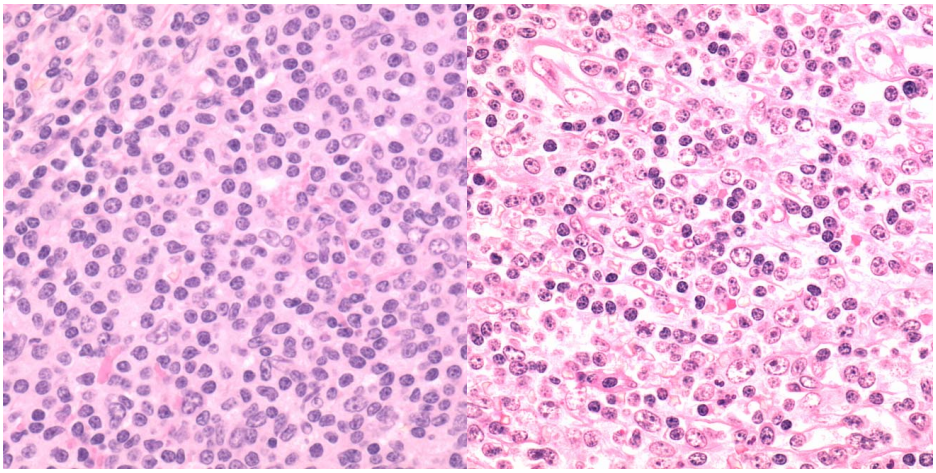
Table 1: Detected mutations in CD79A, CD79B, CARD11 and MYD88.

Gene analyzed	SMZL cases Detected mutations/ cases analyzed	Other MZL cases Detected mutations/ cases analyzed
CD79A (exon 4 and 5)	0/10	0/13
CD79B (exon 5 and 6)	1/10	0/13
CARD11 (exon 5-10)	0/10	0/13
MYD88 (one hotspot)	1/10	0/13

Figure legend

Figure 1. The left panel illustrates the lymphoma histology of a SMZL case without mutations in CD79A, CD79B, CARD11 or MYD88. The spleen shows homogenous infiltration with small cells with abundant clear cytoplasm (H&E, 400X). The right panel illustrates the lymphoma histology case with a CD79B mutation. The spleen shows infiltration with small lymphoid cells as well as with larger immunoblast-like cells (H&E, 400X).

Figure 1



Supplementary Table 1. Primer pairs used for amplification and sequencing of CD79A, CD79B, CARD11 and MYD88 genes in SMZL.

	FORWARD	REVERSE
CD79A exon 4	cat cca gga ggg tct gaa ag	ccc taa cac aac tgc ccc ta
CD79A exon 5	agg tgt cag ggt gct gat gt	ccc act ggg gga ata tga ct
CD79B exon 5	tct tgc aga atg cac ctc ac	gca gcg tca cta tgt cct ca
CD79B exon 6	tac gag gta agg aga ggg gc	aga caa atg gca gct ctg gt
CARD11 exon 5	gtc acc ctg gcg gag tag cc	cag tgc ctc gtg ggc aga gt
CARD11 exon 6	ctg gag aag gtt tct tgg agc	aca ccc tgg cag gtt cat c
CARD11 exon 7	ccc agg ccc tca tct ggt tg	ccc agg ata cgc cca agc aa
CARD11 exon 8	tcc cct atg tta cct ggt ctg tag tg	gcc tgt gac ttc caa aaa agc c
CARD11 exon 9	cct cag tgc cct cat ctg taa aat g	caa agg aca agg agc cat tca ttg
CARD11 exon 10	agc gag tcg cag gat ttc ca	cca gaa gcc tgg gag gag ga
MYD88 PCR	tgc agg tgc cca tca gaa gcg	cag aca gtg atg aac ctc agg atg c
MYD88 SNaPshot	ccc ccc ccc c agg tgc cca tca gaa gcg ac	cct tgt act tga tgg gga tc

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Supplementary figure legends

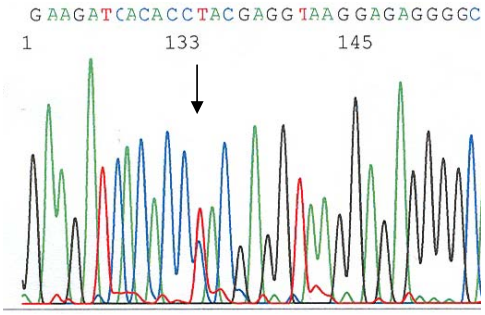
Supplementary Figure S1

Electropherogram illustrating mutation Y196H of the CD79B gene in case of SMZL.

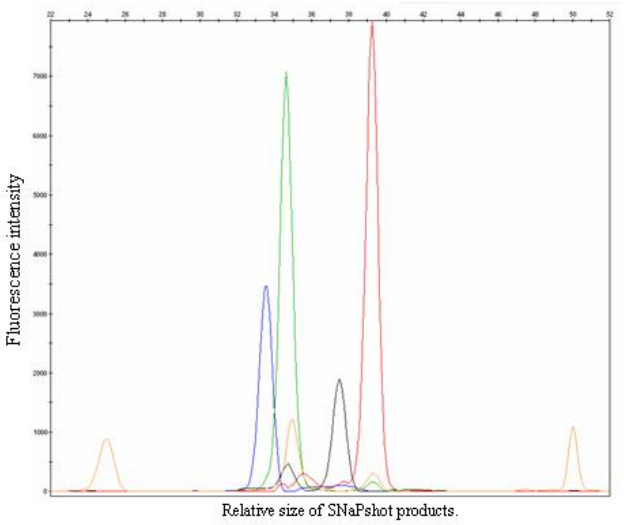
Supplementary Figure S2

Electropherogram of SNaPshot products illustrating the heterozygous loci detected in the MYD88 gene (L265P). The plot shows the relative fluorescence intensity versus the measured size in nucleotides of the products relative to the GeneScan-120 LIZ internal size standard (orange peaks). Bases are represented by the following colours: T = red, wild type; C = black, mutated; A = green, wild type G = blue, mutated.

Supplementary Figure S1.



Supplementary FigureS2.



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