UiT
THE ARCTIC UNIVERSITY OF NORWAY

Faculty of health sciences, Institute of Medical Biology, Immunology Research group

Advanced flow cytometry to study signaling pathways and predict outcome in B cell malignancies

—

Egil Støre Blix

A dissertation for the degree of Philosophiae Doctor – September 2014

Table of Contents

ACKNOWLEDGEMENTS 3

LIST OF PUBLICATIONS 4

ABBREVIATIONS 5

1. INTRODUCTION 6

1.1 The immune system 6
1.1.1 Early B-cell development in the bone marrow 7
1.1.2 B-cell maturation in the germinal centre 10
1.1.3 B cell receptor signaling in normal B cells 12
1.1.4 CD40 co-receptor signaling 13

1.2 B cell malignancies 14
1.2.1 Cell of origin in B cell malignancies 15
1.2.2 Small lymphocytic lymphoma/chronic lymphocytic leukemia 17
1.2.2.1 Characteristics 17
1.2.2.2 Prognostic markers in small lymphocytic lymphoma/chronic lymphocytic leukemia 17
1.2.2.3 B cell receptor and co-receptor signaling in small lymphocytic lymphoma/chronic lymphocytic leukemia. 19
1.2.3 Marginal zone lymphoma 20
1.2.3.1 Characteristics 20
1.2.3.2 Prognostic markers in marginal zone lymphoma 20
1.2.3.3 B cell receptor and co-receptor signaling in marginal zone lymphoma 21
1.2.4 Multiple myeloma 21
1.2.4.1 Characteristics 21
1.2.4.2 Prognostic markers in multiple myeloma 22
1.2.4.3 Angiogenesis and endothelial progenitor cells in multiple myeloma 22
1.2.5 Defect signaling in tumor-infiltrating T cells in lymphoma 24
1.2.6 Risk adapted therapy in B cell malignancies 24

2. AIMS OF THESIS 26

3. MAIN RESULTS 27

4 GENERAL DISCUSSION 30

4.1 Methodological consideration 30
4.1.1 Patient population 30
4.1.2 Description of phospho-specific flow cytometry 31
4.1.3 Advantages and limitations of phospho-specific flow cytometry 33
4.1.4 Rationale for choosing phospho-flow in exploring signaling in lymphoma samples 36
4.1.5 Rare cell analysis by flow cytometry 37
4.1.6 Limitations of patient cohort and outcome prediction 37

4.2 Flow cytometry to study signaling pathways and predict outcome in B cell malignancies 38
4.2.1 Basal BCR signaling in lymphoma cells 38
4.2.2 Activated BCR signaling in lymphoma cells 40
4.2.3 Activated CD40 signaling in lymphoma cells 44

4.2.3 Bone marrow angiogenesis in multiple myeloma 46

5. CONCLUSION 48

6. REFERENCES 50

PAPERS I-III..... 49

Acknowledgements

The work for this thesis was carried out at Department of Immunology, Institute of Medical Biology at University of Tromsø. The work was also founded by a grant from Helse Nord. Many people have been important to me during my years as a PhD student.

I am greatly thankful to my main supervisor Anne Husebekk for sharing your vast experience in immunology and transfusion medicine with me and for your scientific and practical support. It has have been invaluable for my work. I appreciate your enthusiasm, your patient belief in my work and ability to inspire and that you always have time to discuss my work although I know you have more than a tight schedule.

I wish to thank my co-supervisor Arne Kolstad. You have been an important mentor and inspiration in the fast-evolving field of oncology. I appreciate your ability to formulate the right and important questions during our work with protocols or when we are interpreting results and for your resoluteness and encouragement during this work.

I also wish to thank my co-supervisor June Myklebust for sharing your impressive knowledge regarding scientific methodology and lymphoma biology with me. I really appreciate your constructive guidance, clear-thinking and for answering the many hundred emails giving invaluable advises and guidance to me during all phases of our experiments.

Special thanks to my other co-authors, Jonathan Irish, Jan Delabie, Lise Forfang, Anne Tierens, Anders Kildal, Eirin Bertelsen, and Anders Waage who have contributed to data collection and interpretation together with critical manuscript reading and valuable suggestions. I especially wish to thank Jonathan Irish for sharing his vast scientific experience with me.

I wish to thank Eirin Bertelsen, Maria Therese Ahlen and Tor Stuge at Department of Immunology together with Goran Kauric and Sylvi Johansen at Department of Immunology and Transfusion Medicine for excellent scientific teaching and friendly smiles.

I appreciate all my fantastic colleagues at Department of Oncology in Tromsø. Your enthusiasm, humor, professionalism and concern for your all your colleagues well-being provides an atmosphere of excellence at the department. I am grateful to the head of department Tone Nordøy for her support during my PhD. I wish to thank Tom Dønnem and Sigve Andersen for your good spirit and helpful statistical supervising.

Finally, and most of all, I wish to thank my wife Bodil for long talks, a warm smile, happy laugh and for your love. Very special thanks to my fantastic children Bendik, Signe and Alfred for your great smiles, warm hugs and all the happiness. “Més que un club”.

Tromsø, September 2014

List of publications

This thesis is based on the following papers. The papers will be referred to by their Roman numerals.

Paper I:

Blix, E. S., J. M. Irish, A. Husebekk, J. Delabie, L. Forfang, A. M. Tierens, J. H. Myklebust, and A. Kolstad. 2012. Phospho-specific flow cytometry identifies aberrant signaling in indolent B-cell lymphoma. *BMC Cancer* 12:478.

Paper II:

Blix, E. S., J. M. Irish, A. Husebekk, J. Delabie, A. M. Tierens, J. H. Myklebust, and A. Kolstad. 2012. Altered BCR and CD40 signalling are associated with clinical outcome in small lymphocytic lymphoma/chronic lymphocytic leukaemia and marginal zone lymphoma patients. *Br J Haematol* 159:604-608.

Paper III:

Blix, E. S., Kildal, A. B., Bertelsen, E., Waage, A., Myklebust, J. H., Kolstad, A. and Husebekk, A. Content of endothelial progenitor cells in autologous stem cell grafts predict survival after transplant in multiple myeloma (submitted)

Abbreviations

AID	activation-induced cytidine deaminase
ALDH	aldehyde dehydrogenase
ASCT	autologous stem cell transplant
BCR	B cell receptor
s- β_2M	serum-β_2-microglobulin
CD40L	CD40 ligand
CLL	chronic lymphocytic leukemia
D	diverse gene segments
DLBCL	diffuse large B cell lymphoma
EPC	endothelial progenitor cell
ERK	extracellular signal-regulated kinase
FDC	follicular dendritic cells
FL	follicular lymphoma
GC	germinal center
HSC	hematopoietic stem cells
H_2O_2	hydrogen peroxide
J	joining gene segments
Ig	immunoglobulin
IL	interleukin
IGHV	immunoglobulin heavy chain variable region
ISS	international staging system
ITAM	immunoreceptor tyrosine-based activation motifs
MFI	median fluorescence intensity
MM	multiple myeloma
MZL	marginal zone lymphoma
NHL	non-Hodgkin lymphoma
NK	natural killer cells
OS	overall survival
P	phosphorylated
PBMC	peripheral blood mononucleated cells
PBSC	peripheral blood stem cells
PI3K	phosphatidylinositol 3-kinase
PMA	phorbol 12-Myristate 13-Acetate
PTK	protein tyrosine kinase
ROS	reactive oxygen species
SFK	src family kinases
SHM	somatic hypermutation
SLL	small cell lymphocytic lymphoma
TNF	tumor necrosis factor
V	variable gene segments
VEGF	vascular endothelial growth factor
ZAP-70	zeta-chain-associated protein kinase 70

1. Introduction

1.1 The immune system

The main function of the immune system is to protect an individual from infectious diseases. Often, infections with a pathogen remain localized within the body and is controlled and terminated by the first part of the immune system named the innate immune system. Innate immunity consists of different components. The first task in innate immunity is to recognize foreign pathogen. Both soluble proteins like complement proteins and C-reactive protein together with pattern recognition receptors like Toll-like receptors and NOD proteins have the ability to distinguish foreign pathogens from human cell components (1). Complement and C-reactive protein attached to pathogens mediate phagocytosis and destruction of pathogens by a process called opsonization. The next step in the defense of innate immunity is mediated by effector cells or complement proteins which either engulf opsonized pathogens like bacteria, destruct bacteria cell-membrane or kill virus-infected cells (2).

Natural killer (NK) cells have traditionally been defined as effector lymphocytes of innate immunity with cytolytic functions aimed to kill virus-infected cells (2). More recently, a more broadened view of NK cells has emerged. NK cells are now recognized to express a repertoire of activating and inhibitory receptors that is calibrated to ensure self-tolerance while allowing efficacy against pathogens and tumor development. Furthermore, NK cells can also mount a form of antigen-specific immunologic memory, thereby exert functions that are attributes of both innate and adaptive immunity, blurring the traditionally functional borders between these two parts of the immune system (3).

The second arm of the immune system is termed the adaptive immune system. It is composed of B and T lymphocytes with unique capability to improve pathogen recognition during infection and to evolve long-lasting memory cells specific for a given pathogen. Collectively, lymphocytes recognize a vast majority of pathogens by means of their highly diverse antigen-receptors, immunoglobulin’s (Ig) on B lymphocytes surface and T-cell receptor on T lymphocytes surface. An enormous

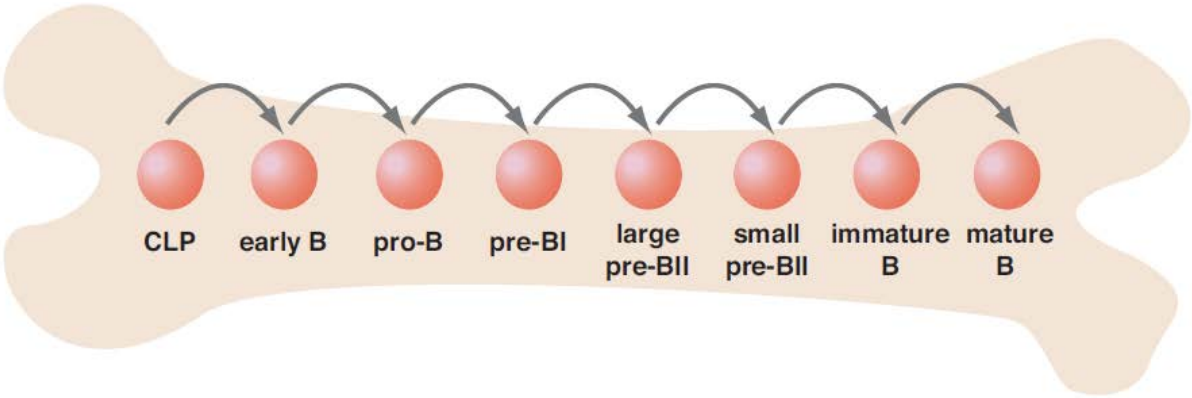
diversity of antigen-receptors within the B and T-cell population is created through somatic gene rearrangement and somatic mutations in antigen receptor genes. A given pathogen is only recognized by a small subset of B and T lymphocytes. However, clonal expansion of the given subset of activated lymphocytes provides an individual with efficient adaptive immune response to any antigen. An important difference between the two components of adaptive immunity is how B and T cells are activated by antigen. The Ig on B cells can recognize whole molecules or an intact pathogen, while the antigen receptor of T cells can only recognize small peptide fragments bound to major histocompatibility complex located on the cell surface. B cells with Ig that recognize foreign antigen can be stimulated by $CD4^+$ T helper cells to differentiate into a terminal stage in a B cell lifecycle called plasma cells. They have unique property to produce and secrete antibodies. A young, naïve B cells is under an enormous pressure. B cells which fail to enter follicles within lymph nodes will die by apoptosis within a few days while B cells that gain access to a primary follicle inside lymph nodes without antigen-activation will live for average 100 days. Only B cells activated by antigen that also receive help from T cells have ability to differentiate into long-lasting memory B cells or antibody-secreting plasma cells (2). Separate and apart from the conventional population of B cells, termed B-2, are B-1 cells that constitutively and spontaneously secrete natural antibody of IgM and IgA isotype. Natural antibodies appear in the absence of infection or immunization and recognize phosphorylcholine on gram-positive bacteria but also on other bacterial pathogens, apoptotic cell membranes, and oxidized lipids (4). Identification of B-1 cells emerged from earlier identification that malignant cells of human and mouse B cell leukemia and lymphomas expressed the T cell antigen CD5 (5, 6). However, it is now clear that CD5 is not an exclusive marker of B-1 cells in human as CD5 is expressed on pre-naive, transitional, and activated B-2 cell populations (7-9).

1.1.1 Early B-cell development in the bone marrow

B cells are generated from hematopoietic stem cells (HSC) in the bone marrow. In 1991, Hardy et al. utilized the innovations in flow cytometry with four-color flow cytometry, new monoclonal antibodies and live cell sorting to characterize four distinct cell subsets (fractions A-D) within the mouse B cell precursor compartment of

HSC (10). B progenitor cells transits through distinct developmental steps in bone marrow characterized by surface marker expression and rearrangements of the B cell receptor (BCR). The current consensus is that human B lineage–restricted cells pass through an early B ($CD34^{+}CD19^{-}CD10^{+}$), pro-B ($CD34^{+}CD19^{+}CD10^{+}$), large pre-BI ($CD34^{+}CD19^{+}CD10^{+}$), large pre-BII ($CD34^{-}CD19^{+}CD10^{+}$) and finally small pre-BII cell ($CD34^{-}CD19^{+}CD10^{+}$) development pathway (Figure 1) (11). CD133, a five-transmembrane glycoprotein, is a positive marker of human cord blood-derived $CD34^{-}$ HSC (12), and human $CD133^{+}CD34^{-}$HSC contains primitive precursors of $CD34^{+}$ cells with NOD/SCID mice repopulating capacity (13).

Naïve B cells require continued expression of functional BCR to survive (14, 15). B cell progenitors rearrange their Ig genes to construct a functional BCR. During early stages of B cell differentiation recombination takes place to assemble the variable (V), diversity (D) and joining (J) gene-segments of the Ig genes. An early pro-B cell must express recombination-activating genes (RAG) and other DNA-modifying enzymes to cut paste and add different gene segments to the final Ig gene. Different stages in B cell development are accompanied by gene rearrangements to make functional Ig heavy and light chains of the BCR. First, rearrangement of the D-J gene segments of the Ig heavy chain locus is initiated at the common-lymphocyte progenitor or pro-B cell stage followed by V-DJ heavy chain rearrangements at the late-pro B cell stage. If successful, this gives rise to the Igμ chain that is expressed on the cell surface in the form of the pre-B cell receptor (pre-BCR) at the large pre-B cell stage. Pre-BCR signaling is necessary to induces clonal proliferation, downregulation of pre-BCR components and recombination of Ig light chain genes. Light chain gene rearrangements in small pre-B cells result in the expression of a final BCR on immature B cells that is composed of two Igμ chains and two Igκ/λ chains. During B-cell development, the transcription factor Pax5 is responsible to switch on B cell exclusive genes coding for Igα, Igβ (described later on page 14) and the BCR co-receptor molecule CD19 (2). CD19 expression marks transition from pre-pro-B cells to pre-B cells (16).



	CLP	early B	pro-B	pre-BI	large pre-BII	small pre-BII	immature B	mature B
CD34	+	+	+	-	-	-	-	-
CD10	+	+	+	+	+	+	+	-
IL-7Rα	+	+	+	-	-	-	-	-
CD19	-	-	+	+	+	+	+	+
CD79a	-	+	+	+	+	+	+	+
TdT	-	-	+	-	-	-	-	-
RAG	-	-	+	+	-	+	+	-
Vpre-B	-	+	+	+	+	-	-	-
μH	-	-	+/-	+	+	+	+	+
pre-BCR	-	-	-	-	+	-	-	-
IgH	GL	DJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H
κL	GL	GL	GL	GL	GL	V_LJ_L	V_LJ_L	V_LJ_L
cycling	-	-	-	+	+	-	-	-
Pax-5	-	-	+	+	+	+	+	+
sIgM	-	-	-	-	-	-	+	+
sIgD	-	-	-	-	-	-	-	+

Figure 1. Model of early stages of human B cell development (11). Reprinted by permission from Annual Review of Immunology.

Early B cell development takes place in a complex bone-marrow micro-environment with mature blood cells and their hematopoietic stem cell precursors in close contact with medullary vascular sinuses (17). Vascular, mesenchymal and bone cells contribute to cellular niches which are functional compartments inside the bone marrow that control cell numbers by providing signals that regulate cell self-renewal,

differentiation or quiescence (18). Osteoblasts and osteoclasts within bone marrow niches are important for B cell development as inhibition of these cells results in decrease or relocalization of early B cell progenitors to spleen (19, 20). Bone marrow endothelial cells express cell-surface molecules that aid in the transit of hematopoietic stem cells and mature B cells between bone marrow and secondary lymphoid organs (21). Furthermore, they express several factors known to stimulate hematopoiesis like granulocyte colony-stimulating factor (G-CSF) and stem cell factor (KIT ligand) (22). Hematopoietic stem cells are also dependent on close contact with CXCL12 (SDF-1) positive mesenchymal cells (23). The chemokine CXCL12 (SDF-1) is essential for early B cell (pre-pro B cells) development (24), and for homing of plasma cells to the bone marrow (25, 26). Furthermore, B cell maturation are promoted by interleukin (IL)-7-secreting stromal cells and sinusoidal endothelial cells (27). IL-7 is central to hematopoietic stem cell decisions to commit maturation to the B cell lineage, to give signals that support proliferation and survival of B cell progenitors and maturation during the pro-B to pre-B cell transition (28). Together with the pre-B cell receptor, IL-7 receptor is essential in proliferation and survival of pre-B cells.

1.1.2 B-cell maturation in the germinal centre

Immature B cells with a BCR that do not recognize self-antigen survive and migrate to secondary lymphoid organs as lymph nodes, spleen or mucosa associated lymphoid tissue. A naïve B cell that encounter antigen presented by specialized dendritic cells move to the T cell area of lymphoid tissue due to up-regulation of chemokine receptors. Interaction between the B cell co-receptor CD40 and CD40 ligand (CD154) expressed by activated $CD4^+$ T cells initiate the germinal-centre (GC) reaction. Activated B cells can either develop directly to antibody-secreting plasma cells or move to a primary follicle consisting of recirculation IgM^+IgD^+ B cells together with antigen-presenting follicular dendritic cells (FDC). Here, the activated B cells proliferate and after few days, the secondary follicle consisting of central zone of rapidly proliferating B cells with IgM^+IgD^+ cells. This structure is now called the GC. Surrounding the GC are naïve B cells forming the mantle zone. Naïve B cell become activated to centroblasts that undergo clonal expansion in the dark zone of GC (2). During centroblast proliferation, somatic hypermutation (SHM) introduces randomly

modifications of the variable region of the Ig light and heavy chain of BCR to achieve enhanced antigen binding. SHM can result in improved but also reduced affinity for antigen Importantly, only the B cells which express BCR with improved antigen affinity will survive as they will outcompete the other B cells for binding to immune complexes attached to FDCs, and receive survival signals. SHM is dependent on the enzyme activation-induced cytidine deaminase (AID) that converts cytosine to uracil and is only made by proliferating B cells (2). AID is up-regulated by the transcription factor BCL-6 which has high expression in GC B cells and is essential for GC formation, inhibition the DNA-damage response and thus allowing for SHM and inducing cell proliferation (29). The activity of this enzyme and consequent repair of damaged DNA is the basis for SHM and isotype switch in GC B cells. Centroblasts then differentiate to centrocytes in the light zone of the GC. A subset of centrocytes undergo isotype switch which change the heavy chain class from IgM or IgD to IgG, IgA or IgE thereby alter how captured antigen is eliminated or to which location of the body the antibody are delivered (2). Traditionally, B cells differentiation was postulated to be in the unilateral direction from dark to light zone of the GC, however recent studies have shown that B cells can move in both directions during differentiation and proliferation within the GC. Antigen-selected centrocytes with ability to bind antigen finally differentiate to become memory cells or plasma cells (30, 31). Little is known about the homeostasis of memory B cells and long-lived plasma cells, which is of central importance to the quality of an immune response (29). The gene-expression changes required for an activated B cell to differentiate into a plasma cell are regulated by the coordinated activity of a small group of transcription factors. These factors can be divided into those that promote the B cell program (Pax5, BCL-6 and Bach2) and others such as Blimp-1 and Xbp1, which control plasma cell differentiation (29). A proposed target of BCL-6 is the gene encoding Blimp-1, and BCL-6 mediated Blimp-1 suppression inhibits plasma cell differentiation in the GC (32). In the beginning of the GC reaction, an activated B cell must make a differentiation decision to become an extrafollicular plasmablast, to acquiring the early adoption of a memory phenotype or to entering the GC. The signals that turn off BCL-6 to allow differentiation into either a memory B cell (Blimp-1^{-}Pax5^{+}) or a plasma cell (Blimp-1^{+}Pax5^{-}) are unclear (29). There are two waves of memory B cell development, one preceding and independent of the GC and the other being the

traditional GC-derived variety (33). Homing of plasma cells to bone marrow is dependent on expression of the chemokine receptors CXCR4 and CCR10 (34).

1.1.3 B cell receptor signaling in normal B cells

The BCR is a dimer with two identical heavy-chain and two identical light-chain Ig subunits covalently coupled by disulfide bridges. The BCR complex also includes the subunits Igα (CD79a) and Igβ (CD79b), which contain cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) (35). Crosslinking of BCR by antigens initiates phosphorylation of tyrosines within the ITAM in the cytoplasmic tails of CD79a and CD79b mediated by different Src family kinases (SFKs) including FYN, BLK, HCK, FGR, LCK and LYN tethered to the inside of the plasma membrane close to the BCR (36-39). SYK is then recruited to phosphorylated ITAMs, which results in autophosphorylation and phosphorylation by Src kinases of multiple tyrosine residues and subsequent activation of SYK (Figure 2) (40-42).

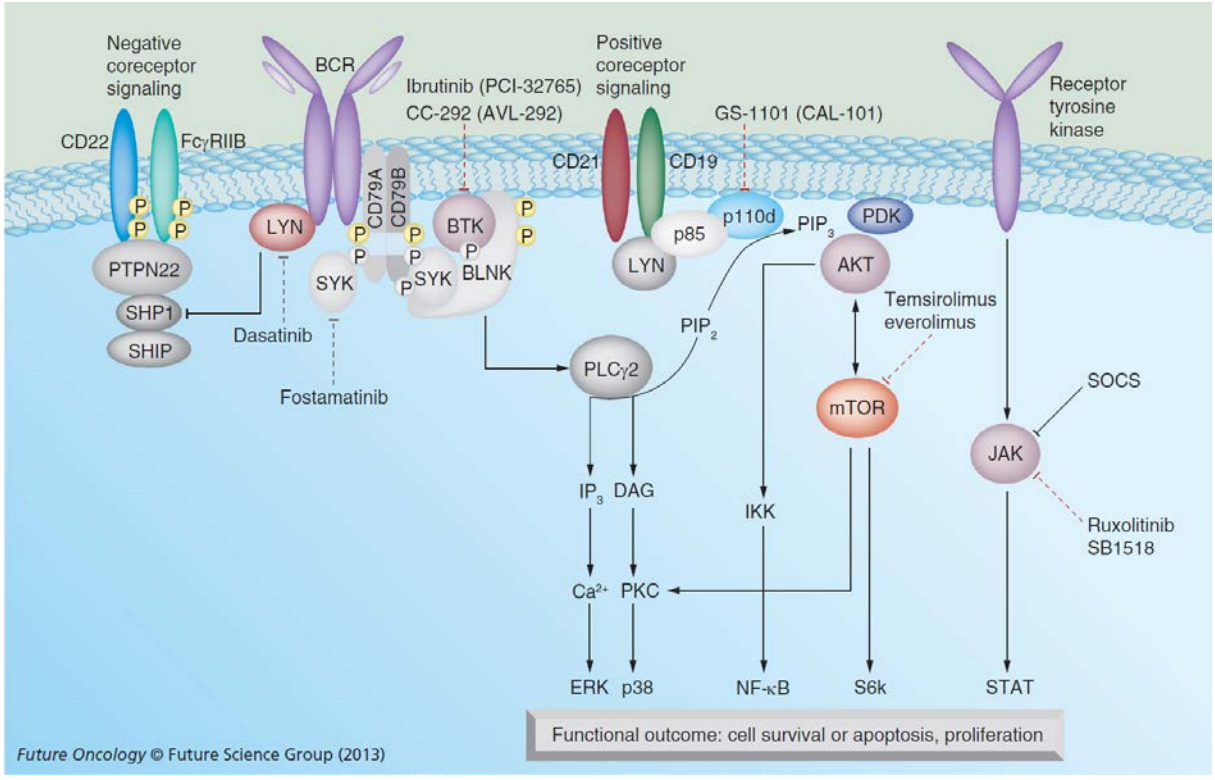


Figure 2. BCR signaling in normal B cells. The solid inhibition signal represents proteins sending an off signal and the dashed inhibition signal represents drugs inhibiting kinases (41).

This activation initiates the coordinate assembly of the signalosome, composed of several adaptor molecules and kinases, including B-cell linker protein (BLNK), Bruton

tyrosine kinase (BTK), phosphatidylinositol 3-kinase (PI3K), Vav and PLCγ (43-45). After PLCγ is activated, it further hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2), resulting in generation of the second messengers phosphatidylinositol-3,4,5-triophosphate (IP3) and diacylglycerol (DAG) (46), and to release of Ca^{2+} and activation of PKC (46). This signaling cascade phosphorylates and activates a complex (CARD11/BCL10, MALT1) which eventually activates the NF-κB pathway (47-49), and the mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-JUN NH_2-terminal kinase and p38 (50). Regulation of BCR signaling is mediated by phosphatases that dephosphorylate the Igα and Igβ ITAMs. Notably, as H_2O_2 inactivates phosphatases it has been proposed to regulate signals generated through the BCR (51).

BCR expression and signaling is a fundamental property of viable B cells as loss of BCR results in death of peripheral B cells (14, 52). Maintenance and survival of normal B cells depends on both ligand-independent BCR activation and ligand-induced BCR activation (15, 53-55). Activation of naïve B cells and clonal expansion require antigen-induced BCR signaling and co-receptor signaling (56). Hence, BCR signaling is intimately connected with B cell survival throughout development (57). BCR activation induces intracellular signaling of several pathways regulating multiple cellular processes, including differentiation, proliferation, apoptosis and cell migration in normal B cells. This includes BCR induced activation of the RAF-MEK-ERK pathway which is important in proliferation of mature B cells (58, 59). BCR induced NF-κB activation is responsible for the survival and activation of B cells (48, 49). In mature B cells, antigen-induced BCR signaling drives B cell proliferation and expansion. Somatic hypermutation results in further selection of the B cell repertoire with increased antigen affinity of the BCR governed by increased Ca^{2+} dependent BCR signaling (60). The balance of these signals determines the B-cell fate (61).

1.1.4 CD40 co-receptor signaling

The B-cell co-stimulatory receptor CD40 is type I transmembrane glycoprotein of the tumor necrosis factor (TNF) receptor superfamily. CD40 is expressed constitutively on normal and most malignant B cells, dendritic cells, and macrophages. Interaction between CD40 on B cells and CD40L (CD154) on T helper cells activates CD40 (62).

CD40 stimulation result in the activation of a variety of phosphoproteins, including NF-κB, p38, c-jun N-terminal N-kinase (JNK), PI3K, ERK, and the Src family kinases SYK, Lyn, and Fyk. CD40 signaling has been shown to induce the production of cytokines and chemokines, including IL-2, IL-6, IL-7, IL-10, IL-12, IL-15, IL-17, IFNγ, lymphotoxin-a (TNF-β), and TNF-α which regulate important functions in immunity like isotype switching and antigen presentation (62). The importance of CD40 stimulation and subsequent signaling is illustrated by patients with x-linked hyper-IgM syndrome who have defect in CD40L resulting in no formation of GC and defects in antibody class-switch (63-67). The CD40 - CD40L interaction initiates B cell differentiation and proliferation when B cells interact with T cells in the T cell area and furthermore, transition from centroblasts to centrocytes in GC through NF-κB signaling resulting in down regulation of BCL-6 (68). Hence, CD40 signaling is central in normal B cell development.

1.2 B cell malignancies

The great antibody repertoire created during normal B cell development by VDJ rearrangement of Igs and the later SHM and isotype switch processes during the GC reaction, comes with the risk of developing B-cell malignancies. A hallmark of many types of B-cell malignancies is chromosomal translocations involving one of the Ig loci and a proto-oncogene (69). In addition to translocations, chromosomal abnormalities like deletions and amplifications of DNA sequences are frequent among B cell malignancies. Hence, about 95% of the mature lymphoid neoplasms are of B-cell origin as compared to T- or NK-cell malignancies (70). More than 25 different sub-types of B-cell lymphoma are distinguished in the current World Health Organization lymphoma classification. B-cell lymphoma is sub-classified into Hodgkins lymphoma and non-Hodgkins lymphomas (NHL) where Burkitt lymphoma, Diffuse large B cell lymphoma (DLBCL), Follicular lymphoma (FL) Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), Marginal zone lymphoma (MZL) and Mantle cell lymphoma (MCL) are some of the most prevalent subtypes (71). The classification of B-cell neoplasms is important regarding pathogenesis, patients counseling and treatment options. The different sub-types of B-cell malignancies have highly variable clinical behaviors, and therefore require adapted treatments (69). Examples of this include SLL/CLL, MZL and Multiple

myeloma (MM) and it is focused especially on those diseases in the following. In US, an assessment of lymphoid neoplasms diagnosed during 1992-2001 according to the internationally recognized World Health Organization (WHO) lymphoma classification (2001) estimated incidence rate of 0.97, 5.17 and 5.29 per 100 000 person-years for MZL, CLL/SLL and MM, respectively (72).

1.2.1 Cell of origin in B cell malignancies

Knowledge about the cellular origin of human B-cell malignancies can provide identification of key transforming events and guide for targeted therapies. The cell of origin in SLL/CLL is controversial. It is uncertain if malignant cells arise from a defined subset of B cells, like naïve, marginal zone or $CD5^+$ B cells. There is evidence that SLL/CLL with mutated Ig variable heavy region (IGHV) arise from antigen-experienced post-GC memory B cells (73). The opponent SLL/CLL cells with unmutated IGHV have a gene expression pattern more similar to post-GC memory B cells than to naive or CD5+ B cells (74), and there is evidence that GC reactions may also generate some memory B cells with low somatic mutation load (75). In conclusion, there are evidence that also SLL/CLL cells with unmutated IGHV stem from antigen-experienced B cells that acquired features of memory B cells (Figure 3) (73). However, the origin of unmutated CLL cells are controversial as the malignant cells may arise from circulating, naïve B cells (76). Recently, it has been shown that unmutated CLL cells derives from unmutated mature $CD5^+$ B cells and mutated CLL cells derives from a distinct, previously unrecognized $CD5^+CD27^+$ post–GC B cell subset (77). Unsupervised multiparametric analyses pointed to $CD5^+$ B cells as the normal B cell subset with the most similar gene expression to CLL. Both unmutated and mutated B-CLL cells was highly similar to normal $CD5^+$ B cells, which are mostly IGHV unmutated. However, a distinct subset of $CD5^+CD27^+$ B cells has somatically mutated IGHV genes. The $CD5^+CD27^+$ B cell subset carried mutations in BCL6 as a specific hallmark of B cells undergoing hypermutation in the GC. BCL6 is only highly transcribed in GC B cells and strong transcription of a gene is essential for somatic hypermutation (78, 79).

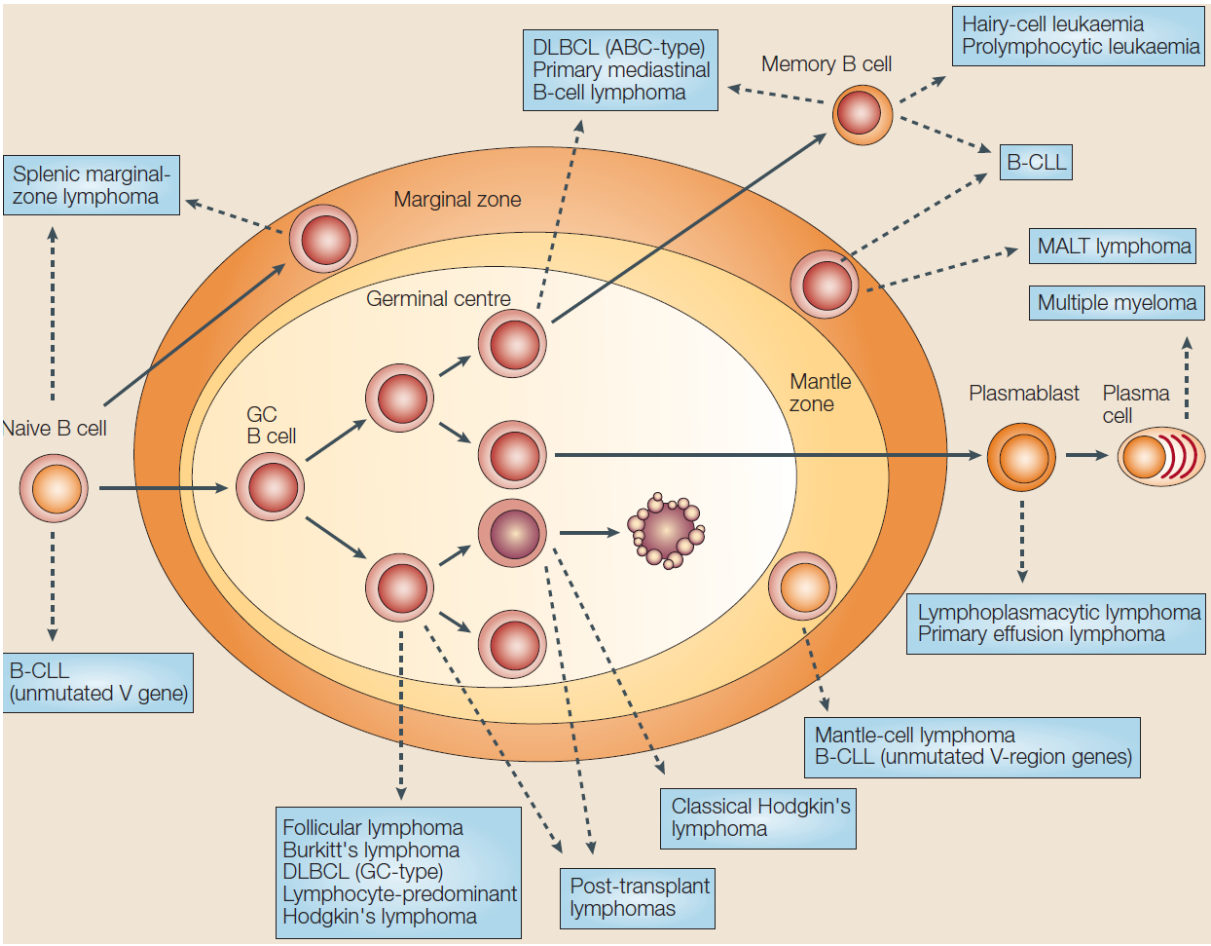


Figure 3. Cellular origin of human B-cell lymphoma. Mechanisms of B-cell lymphoma pathogenesis (69). Reprinted by permission from Nature Publishing Group.

In MZL, gene expression analysis of non-splenic MZL indicates an origin from memory B-cells (80). A subset of splenic MZL cells are $IgM^{+}IgD^{+}$ which supports a naive B-cell origin while others had mutated IGHV genes consistent with a memory B-cell origin (81). There are evidence that MM cells arises from post-GC B cells (69, 82, 83). The variable region of Ig gene sequences are somatically hypermutated (84, 85). In addition, MM cells have undergone class switch recombination, as the secreted monoclonal component in MM cells are of IgG, IgA, IgE or IgD and only rarely of the IgM isotype (86). This provides further evidence for post-germinal origin of malignant MM cells. In conclusion, a common feature of malignant B cells from SLL/CLL, MZL and MM is origin from post-GC B cells.

1.2.2 Small lymphocytic lymphoma/chronic lymphocytic leukemia

1.2.2.1 Characteristics

SLL/CLL is most often an indolent lymphoid malignant disease characterized by accumulation of monoclonal $CD5^+$ B cells in primary and secondary lymphoid tissues. The term SLL is restricted to the non-leukemic form of CLL, but with the same tissue morphology and immunophenotype as CLL. A typical case of SLL/CLL can be diagnosed based on morphological features alone. The characteristic immunophenotype of CLL cells are dim expression of surface Igs and CD20. The cells express B-cell associated antigens like CD19, CD20 and CD79a, together with CD5, CD22, CD23, CD43 and CD11c. A $CD5^+CD23^+$ phenotype are regarded as essential in the diagnosis of CLL (87, 88). There are 170-200 new patients with CLL each year in Norway (89), with an incidence rate of 3.6 new patients per 100 000 person-years (90).

1.2.2.2 Prognostic markers in small lymphocytic lymphoma/chronic lymphocytic leukemia

Morphology, immunophenotype and gene expression are relatively homogenous among patients with SLL/CLL, in contrast to the highly variable clinical course (91-93). Clinical staging systems have been developed for assessment of the extent of the disease in individual CLL patient at time of diagnosis which can serve as a tool for medical decisions regarding follow-up and time of treatment (94, 95). Binet staging is common in Europe and divides patients into three risk groups. Group A have no anemia, no thrombocytopenia and less than three involved lymph-node areas. Group B have no anemia, no thrombocytopenia and three or more involved lymph-node areas while group C patients have anemia and/or thrombocytopenia. Low grade disease have median expected survival over 10 years in contrast to patents in the high risk group with expected median survival about 2 years (92, 96). However, these staging systems fail to predict clinical course and prognosis in patients diagnosed with early stage disease (91, 92). Some patients which initially have signs of low grad disease over time progress to more aggressive disease (97). It is therefore of special interest to identify biological risk factors in order to identify the subset of patients who early in disease will need therapy.

Several molecular prognostic markers have been identified in SLL/CLL patients. Genomic aberrations like 11q deletions, 17p deletions and TP53 mutations have implications on response to chemotherapy and overall survival (OS) (96). In addition, properties of the BCR have impact on prognosis in SLL/CLL. Patients can be divided into two groups depending on if the lymphoma B cells have somatic mutation in IGHV or not (98). In patients whose B-lymphoma cells have unmutated IGHV, the disease tends to have a more aggressive course compared to patients with B-CLL cells with mutated IGHV genes (99-101). B-CLL cells with unmutated IGHV genes often have a relative high expression of 70-kD ξ-associated protein (ZAP-70), comparable to the expression in normal T cells. This is in contrast to CLL B-cells with mutated IGHV genes which have lower levels of ZAP-70 (102-104). High ZAP-70 expression on B-CLL cells determined by flow cytometry is a negative prognostic marker in CLL (105, 106). ZAP-70 is a cytoplasmic protein tyrosine kinase (PTK) originally identified in T cells that mediate T cell receptor signaling after it is recruited to and activated by the phosphorylated ITAMs. B cells generally lack ZAP-70, but instead use SYK, a related PTK to mediate BCR signaling. Similar to ZAP-70, SYK is recruited to the phosphorylated ITAMs and becomes phosphorylated. Phosphorylated ZAP-70 induces downstream signaling pathways PLC-γ and Ras signaling (107). The lymphocyte differentiation marker CD38 is an important enzyme for the regulation of calcium signaling and the cell's energy transfer homeostasis (108). High CD38 expression on B-CLL cells determined by flow cytometry is a negative prognostic marker in CLL patients (99). However, in multivariate analysis, CD38 expression did not improve the predictive power of either ZAP-70 expression or IGHV mutational status (105). To summarize, the association between IGHV mutation, ZAP-70 expression and clinical outcome demonstrates that BCR signaling have a central role in B-CLL cell survival. There are recently developed new drugs targeting pathways downstream of BCR. This includes targeting of SYK with fostamatinib (109), BTK with ibrutinib (110) and PI3Kδ with idealisib (111).

1.2.2.3 B cell receptor and co-receptor signaling in small lymphocytic lymphoma/chronic lymphocytic leukemia.

BCR expression and signaling is a fundamental property of both normal and malignant B cells (69). Several lines of evidence support this. Translocations into the Ig-loci almost always finds place on the non-productively rearranged Ig loci (112), anti-idiotypic antibody treatment of FL did not result in emergence of BCR-negative lymphoma clones (113, 114), and there are only rare occurrence of BCR negative lymphomas despite on-going somatic hypermutations in the BCR (115-117), reviewed in (69). Constitutive activation of BCR signaling by various mechanisms has been shown in B lymphoma cells, giving these lymphoma cells a growth-advantage (118). The BCR from malignant cells in CLL patients has different ability to become activated in vitro. Stimulation of BCR on B-CLL cells results in a heterogeneous response in terms of phosphorylation of SYK, PLCγ and activation of Ca^{2+} response (119-122). The CLL B-cells generally have reduced response to ligation of the BCR complex, and there are several explanations for this finding, including lower expression of surface Ig, inadequate levels of SYK or mutated co-receptor CD79b (122-125). Stimulation of the BCR by anti-IgM elicits Ca^{2+} response in some patients, but not all, and there were no clear association between the level of IgM and response to anti-IgM response (121). Surface IgM expression varies considerably among primary CLL samples, with a subset of patients having markedly decreased IgM expression on the malignant cells (126-128). Anti-IgM stimulation in primary CLL samples results in global tyrosine phosphorylation mainly in unmutated IGHV in contrast to mutated IGHV CLL samples (129-131). Expression of ZAP-70 in B-CLL cells enhances IgM signaling with increase of phosphorylated (p)-SYK, p-BLINK, p-PLCγ and Ca^{2+} (132). This finding was independent of ZAP-70 kinase activity, indicating that ZAP-70 acts as an adaptor protein facilitating BCR signaling independent of its kinase activity (107).

CD40 signaling have implications in B cell malignancies as stimulation of CD40 on normal and B-CLL induces B7 (CD80) expression necessary for alloantigen simulation of T-cell receptor (133). CD40 signaling in normal B cells as well as lymphoma cell line involves activation of NF-κB (134, 135) and p38 (136). This

finding is of interest in B cell malignancies as CD40 induced signaling in B-CLL cells results in up-regulation of NF-κB, activation of anti-apoptotic pathways (137, 138), and induced drug resistance (139). Altogether, BCR and co-receptor signaling connects to malignant B-CLL cells survival and proliferation.

1.2.3 Marginal zone lymphoma

1.2.3.1 Characteristics

MZL is an indolent B-cell malignancy consisting of three different subtypes; extranodal marginal zone lymphoma of mucosa associated tissue (MALT), splenic marginal zone lymphoma and nodal marginal zone lymphoma. MALT is the largest subgroup, representing approximately 70% of MZL (140) and (141)(page 214-17). Splenic MZL accounts for approximately 20% of all MZL (140, 142) and (141)(page 185-7). Patients with splenic MZL typically presents with enlarged spleen, enlarged abdominal lymph nodes and bone marrow involvement, while liver or leukemic involvement occurs only in a subset of patients. Nodal MZL accounts approximately 10% and by definition the disease represents nodal disease without involvement of spleen or extranodal sites (140) and (141)(page 218-19). The malignant MZL cells express the B cell associated antigens CD19, CD20, CD22, CD79a and CD79b. The phenotype of the tumor cells is $CD3^-$, $CD5^-$, $CD10^-$, $CD11c^-$ $CD23^-$ and $CD43^{-/+}$. The lymphoma cells are most frequently IgM^+ (143). The incidence of MZL in Norway is not known.

1.2.3.2 Prognostic markers in marginal zone lymphoma

MZL is often an indolent disease, but a subgroup of MZL patients has signs of aggressive disease. Patients with extranodal, nodal, and splenic subtypes of MZL have a five-year OS rates ranging from 87 to 93% (140, 144). Clinical staging can provide prognostic information. In a MZL cohort including all three subtypes, male sex, B-symptoms and serum-β2-microglobulin (s-β_2M) correlated to decreased recurrence-free survival (140). In splenic MZL, hemoglobin, platelet count, high lactate dehydrogenase level and extrahilar lymphadenopathy were independently associated with lymphoma-specific survival (145). Cytogenetic parameters can affect

prognosis in MZL as patients with splenic-MZL with and without TP53 mutation have median OS of 68 and 140 months, respectively (146).

1.2.3.3 B cell receptor and co-receptor signaling in marginal zone lymphoma

It is limited information regarding BCR signaling in MZL. However, the association between hepatitis C and splenic MZL and helicobacter pylori and mucosa associated lymphoma provides evidence for importance of BCR signaling in MZL genesis (147, 148). In Splenic MZL mutation in CARD11 which links BCR and NF-κB signaling are reported (149). ZAP-70 expression is only occasionally detected in MZL (150) and there are reported that splenic MZL cells express both mutated and unmutated IGHV, without any association with clinical features or outcome (151). Evidence of the importance of BCR signaling in MZL have recently appeared as MZL patients have response with reduced tumor mass after treatment with an inhibitor of PI3Kδ signaling (152).

1.2.4 Multiple myeloma

1.2.4.1 Characteristics

MM is a hematologic malignant disorder affecting plasma cells of B cell origin. The hallmark of MM is clonal proliferation of malignant plasma cells in the bone marrow microenvironment, monoclonal protein in the blood or urine and associated organ dysfunction (153). The malignant MM cells typically express CD138 (syndecan-1) and CD38. MM cells rarely express CD19 and also lack other B-cell-associated antigens, such as CD20, CD22, and CD24, although CD20 and CD45 can be positive in a subset of myeloma cases (154). It accounts for approximately 18% of all lymphoid neoplasms (70). MM was documented by Dr. Samuel Solly in 1844 who described a 39-year-old woman with fatigue and multiple fractures (155). At autopsy the bone marrow was found to be replaced by a red substance. He postulated an inflammatory character of the disease and described “that it commences with a morbid action of the blood-vessels.” This observation can be interpreted as an early description of the role of angiogenesis in MM pathophysiology (156). The incidence rate of MM in Norway was 7.60 / 100 000 (2011) (157).

1.2.4.2 Prognostic markers in multiple myeloma

MM patients have a very variable clinical course; patients may face rapidly progressive disease or experience a more favorable indolent course of disease. High dose chemotherapy with autologous stem cell transplantation (ASCT) has been the standard of care for younger patients (153, 158). A cohort of MM patients all treated with ASCT had highly variable clinical outcome as five-years OS ranged from 72%, 62% and 41% in low, intermediate and high risk groups, respectively (159). There are several prognostic markers which add information about patients outcome. One of the most important host factors is age as MM patients with younger age have a favorable survival (160, 161). The most accepted clinical staging system is international staging system (ISS) which combines albumin and s- β_2M, where the former provides information about host and the latter information regarding disease activity and tumor burden (162). The most important tumor characteristics are genetic aberrations and gene expression profiles, were translocation t(4:14) and t(14:16) together with deletion 17p13 have been shown to be associated with adverse survival (163-165). A combination model with ISS and genetic aberrations has been demonstrated to be able to stratify MM patients into low, intermediate and high risk groups (159, 166, 167). Better risk stratification have implications for a risk-adapted management for MM patients treated with ASCT as there are need for new treatment strategies to the subset of patients with the most adverse prognosis (159).

1.2.4.3 Angiogenesis and endothelial progenitor cells in multiple myeloma

Disease progression in MM is accompanied by increase in bone marrow angiogenesis (168, 169). This is in contrast to DLBCL where estimating the role of angiogenesis in regard to clinical outcome has shown different results (170, 171). Bone marrow angiogenesis is an attractive target for treatment of MM as plasma cells grow and expand almost exclusively in the bone marrow (172), and both osteoblastic and vascular niches can support the proliferation of MM cells (173). Anti-angiogenic properties of thalidomide was first described in 1994 (174). Due to increased awareness of the importance of angiogenesis in the pathogenesis of cancer and especially in MM, Dr. Barlogie and colleagues at the University of Arkansas initiated a compassionate-use trial of anti-angiogenic therapy with thalidomide in MM with a response-rate of 32% (175). Lenalidomide has diverse mechanisms of action,

including angiogenesis (176), and maintenance therapy with lenalidomide after ASCT have been demonstrated to increase progression free survival (PFS) (177) and OS (178). International myeloma working group still doesn't recommend different treatments in different MM risk groups with the exception of bortezomib in induction to and maintenance after ASCT to high-risk MM patients (179). It would therefore be of interest to identify biologic or clinical risk-factors that could serve as a predictive marker of response to lenalidomide and other anti-angiogenic therapies in MM.

Human endothelial progenitor cells (EPC) with capability to incorporate in sites of active angiogenesis was first described more than a decade ago (180). A central part in the complex process of malignant angiogenesis is EPC, together with hematopoietic stem cells. This dual dependency in tumor progression has been demonstrated in angiogenic defective mice as transplantation of VEGF-mobilized wild-type EPC and hematopoietic stem cells were able to both reconstitute hematopoiesis and also restore tumor growth (181).

Primitive hematopoietic progenitor cells from bone marrow and umbilical cord blood express high levels of cytoplasmic aldehyde dehydrogenase (ALDH) as compared to lymphocytes and monocytes (182). The cytosolic enzyme ALDH is expressed at high levels in HSC and progenitors and seems to be responsible for the resistance to the alkylating agent cyclophosphamide (183, 184). It has been developed a fluorescent substrate for ALDH and demonstrated that $ALDH^{hi}$ cells were highly enriched for primitive hematopoietic progenitor cells (185). ALDH expression is a functional marker of both hematopoietic and non-hematopoietic bone marrow derived progenitor cells (186). A hematopoietic stem cell population from human umbilical cord blood isolated by depletion of cells with mature lineage markers and selected by high ALDH activity had enriched CD34 and CD133 expression (187), and isolation of $ALDH^{hi}CD133^{+}$ cells selected a population of stem cells with ability of multi-lineage reconstitution and possession of long-term repopulating ability in secondary murine recipients (188). Hence, a functional stem cell marker such as high intracellular ALDH activity in combination with conserved stem cell surface markers may both serve as a useful tool for the delineation of distinct hematopoietic stem and progenitor cell compartments and to isolate and functionally characterize non-hematopoietic progenitors (188). Thus, some recent studies identified EPC as solely

ALDH^{hi} cells (189, 190). Based on previous studies documenting the importance of angiogenesis in MM it is therefore of interest to characterize levels of EPC in B cell malignancies.

1.2.5 Defect signaling in tumor-infiltrating T cells in lymphoma

The immune microenvironment plays an essential role in clinical outcome. Defects in tumor-infiltrating T-cell function in patients with hematologic malignancies might influence their capacity to mount efficient anti-tumor immune responses. Malignant B-CLL cells have to ability to impair the T cell actin cytoskeleton that is essential for T cell activation and function (191). Both CD4$^+$ and CD8$^+$ tumor-infiltrating T cells in FL have defects in their ability to mobilize F-actin at the immune synapse and have decreased recruitment of the signaling proteins LFA-1, Lck, Itk, Filamin-A and Rab27A (192). T cells from CLL patients exhibits signs of exhaustion demonstrated by up-regulation of CD244, CD160 and PD1 and a subset of PD1$^+$ T cells have up-regulated Blimp-1 which is implicated in the mechanism of exhaustion (193). It is also demonstrated that autologous, activated CD4$^+$ T cells can induce proliferation of malignant B-CLL through CD40L and IL-21 production (194).
In FL, T-cell responses have been demonstrated to affect clinical outcome, as patients with impaired IL-7–induced phosphorylation of STAT5 in tumor-infiltrating T cells had adverse OS (195). Furthermore, tumor-infiltrating T cells in FL have impaired IL-4-, IL-10-, and IL-21-induced phosphorylation of STAT6 and STAT3, in contrast to the corresponding autologous peripheral blood subset. This can be explained by differential expression of the inhibitory receptor PD-1 in FL tumor-infiltrating T cells and peripheral blood T cells (196).

1.2.6 Risk adapted therapy in B cell malignancies

Risk adapted treatment is used in many hematological malignancies, such as acute lymphoblastic leukemia or acute myeloid leukemia (197). Although molecular profiling have capabilities to both identifying new subgroups of lymphoma entities and patients with adverse prognosis in CLL (99, 100) FL,(198), DLBCL (199), clinical prognostic tools like International Prognostic Index (IPI) in DLBCL (200), Follicular Lymphoma International Prognostic Index in FL (201), Mantel cell lymphoma international

prognostic index in MCL (202), ISS in MM (162) and Rai or Binet staging in CLL (94, 95) still remains cornerstones in clinical practice.

Molecular markers in hematological malignancies may have the property to reflect biology, measure tumor load or provide targets for new therapy. Combination of clinical, pathological and molecular markers has potential to provide more precise prognostic models for risk stratification aiming to improve clinical outcome (203). TP53 mutations and/or 17p deletions has been shown to be strongly associated with adverse clinical outcome in CLL (96, 204). In MM, translocations (4;14) or deletions 17p are associated with high risk stratification and adverse OS (159, 166, 167). However, several established molecular prognostic markers such as IGHV mutational status in CLL may not translate into clinical practice. Clinical decision making is still mostly based on symptomatic disease and not the molecular profile (205). This is demonstrated by the observation that even subgroups of CLL patients with adverse prognostic markers as deletions 17p and mutated IGHV may have an indolent course of disease (206).

2. Aims of thesis

It is of interest to further expanding our knowledge of B cell malignancies risk factors both to consult patients and to provide strategies for risk-adapted therapy and disease follow-up. The overall aim of the present thesis was to investigate potential biological prognostic markers for B-cell lymphoma and myeloma patients by use of flow cytometry.

More specifically the aims were to:

- Characterize BCR and co-receptor signaling profiles of tumor B cells and tumor-infiltrating T cells from patients with SLL/CLL and MZL and compare with signaling profiles in normal B and T cells.

- Explore the importance of tumor cell heterogeneity in SLL/CLL and MZL on the basis of the phosphorylation levels of signal transduction molecules.

- Identify signaling signatures in SLL/CLL that correlate with patient's clinical outcome.

- Examine the presence of EPC with high ALDH expression in autologous peripheral blood stem cell (PBSC) grafts from patients with DLBCL and MM.

- Compare the level of EPC in patients with DLBCL and MM and to examine if the level of EPC in patients could be associated with markers of clinical outcome.

3. Main results

Paper I

Knowledge about signaling pathways in malignant cells may provide prognostic and diagnostic information, in addition to identify potential molecular targets for therapy. BCR and co-receptor CD40 signaling is essential for normal B cells, and there is increasing evidence that signaling via BCR and CD40 plays an important role in the pathogenesis of B-cell lymphoma. The aim of the study was to investigate basal- and induced signaling in lymphoma B cells and in infiltrating T cells in single-cell suspensions of biopsies from SLL/CLL and MZL patients.

Cells were then left unstimulated or activated with anti-BCR, anti-BCR+H_2O_2, IL-2, IL-7, IL-15, soluble CD40L or with PMA and ionomycin for 4, 15 or 45 minutes. Samples were stained for 30 minutes with a unique combination of two different dyes, Pacific Blue and Pacific Orange-succinimidyl ester, each used in one of 3 different concentrations. This fluorescent cell barcoding made it possible to identify 9 different cell populations as previously described (195, 207). The barcoded cells were then aliquoted into six tubes for staining with different antibody panels. Each panel contained a backbone of the antibodies anti-BCL2, anti-CD20, and anti-CD5 in addition to two different phospho-antibodies. The cells were collected on a FACSAria flow cytometer and data were analyzed using Cytobank software. In summary, a panel of 9 stimulation conditions targeting B and T cells, including crosslinking of the BCR, CD40L and interleukins in combination with 12 matching phospho-protein readouts was used to study signaling, creating a total of 108 signaling nodes.

Malignant B cells from SLL/CLL patients showed significant higher basal levels of p-SFKs, p-PLCγ, p-ERK, p-p38, p-p65 (NF-κB), p-STAT5 and p-STAT6 when compared to healthy donor B cells, and the basal levels of these signaling proteins varied considerably between different patients. In contrast, anti-BCR induced signaling was highly impaired in SLL/CLL and MZL B cells as determined by low p-SFK, p-SYK and p-PLCγ levels. Impaired anti-BCR-induced p-PLCγ was associated with reduced surface expression of IgM and CD79b. Similarly, CD40L-induced signaling was also impaired in SLL/CLL and MZL lymphoma B cells compared to normal B cells with significant lower phosphorylation of p38, ERK and S6 in SLL/CLL

and lower p38 and ERK in MZL, whereas p-p65 (NF-κB) was equal to that of normal B cells. In contrast, IL-2, IL-7 and IL-15 induced p-STAT5 in tumor-infiltrating T cells were not different from normal T cells. In conclusion, this work provided a better overview of basal, BCR- and CD40L-induced signaling in malignant SLL/CLL and MZL cells as well as cytokine-induced signaling responses in tumor-infiltrating T cells. Although no unique signaling profiles that could distinguish SLL/CLL from MZL were identified, we identified contrasting signaling abnormalities in the lymphoma B cells when compared to normal B cells.

Paper II

In FL, a new lymphoma subset with abnormal and impaired B-cell antigen receptor signaling has been identified by phospho-flow cytometry (195). This new subset of lymphoma cells has clinical significance as the prevalence of this subset at the time of diagnosis was negatively associated with OS. The aim of the present study was to investigate whether similar correlations could be found in SLL/CLL and MZL. Therefore, we performed further analysis of data generated and presented in paper I.

We found that CD40 stimulation induced p-S6 and p-p65 signaling in both SLL/CLL and MZL cells, but at highly variable frequencies, with median fluorescence intensity (MFI) ranging 0.07 - 2.61. Survival analysis with log-rank test revealed that high expression of p-S6 and p-p65 in lymphoma B cells after CD40 stimulation were associated with improved OS (p=0.022). Similar to observation in FL, per-cell analysis revealed a subpopulation of lymphoma B cells with no apparent signaling after BCR-crosslinking in SLL/CLL and MZL patients. The frequency of this subpopulation was highly variable with percentage of BCR-insensitive cells ranging from 5% to 100%. Survival analysis showed that patients with more than 60% BCR-insensitive tumor-cells had significant shorter OS as compared to patients with fewer than 60% BCR-insensitive cells (p=0.032). Therefore, our results suggest that alterations in signaling through CD40 and BCR can influence patient survival also for SLL/CLL and MZL patients and represent interesting targets for therapy.

Paper III

MM is considered an incurable B-cell malignancy, although many patients can benefit from high-dose therapy with ASCT as first line treatment. In NHL, ASCT is usually

performed after relapse, with curative intent. Disease progression in MM is often associated with increased angiogenesis, in which EPC may have a central role. Here, we investigated the clinical impact of EPC levels in PBSC autografts for MM and NHL patients who received ASCT. Forty-one patients (MM; n = 24, NHL; n = 17) with available cryopreserved PBSC autograft samples collected in the period between 1995 and 2006 were included in this study. Cryopreserved PBSC were thawed, and incubated with Aldefluor, a fluorescent substrate which can be used to identify subsets of cells with increased ALDH activity. Cells were then co-stained with anti-VEGFR2, anti-CD34, and anti-CD133, and collected on a FACSCanto flow cytometer and analysed with FlowJo software.

EPC were identified by flow cytometry as $ALDH^{hi}CD34^{+}VEGFR2^{+}CD133^{+}$ cells in both MM and NHL autografts, ranging from 0.02% - 7.56% of $CD34^{+}$ cells. In MM, there was a positive correlation between percentage of EPC in PBSC grafts and s-β_2M levels (r^2 = 0.371, P = .002). Unlike for NHL patients, survival analysis with log-rank test showed that MM patients with higher than cohort median percentage of EPC in PBSC grafts had significant shorter time to next treatment (P = .023), but not PFS or OS. The actual amount of EPC per kg infused cells during ASCT, termed EPC (cells/kg), was calculated as a ratio of EPC (% of $CD34^{+}$ population), and divided by number of stem cells infused during ASCT ($CD34^{+}$ cells x 10^6 /kg). MM patients with high numbers of infused EPC (cells/kg) during ASCT had significant shorter PFS (P = 0.035), OS (P = 0.044) and time to next treatment (P = 0.009). In multivariate analysis, EPC (cells/kg) was a significant independent negative prognostic indicator of PFS (P = 0.033). In conclusion, this study indicates that presence of high numbers of EPC in PBSC grafts is associated with adverse prognosis after ASCT in MM.

4 General discussion

4.1 Methodological consideration

4.1.1 Patient population

The patient samples included in paper I and II were from tumor biopsies from previously untreated patients with SLL/CLL. Median follow-up of the study population was 72 months/6.0 years (range 9 - 257 months). Tumor biopsies from previously untreated patients with SLL/CLL were collected at the Norwegian Radium Hospital after informed consent from the patients between 1988 and 2006 and the clinical data were collected retrospectively. The study was approved by Regional Committee for Medical Research Ethics (REK 2.2007.2949). All patient biopsies included were subjected to review by a reference pathologist in 2010. A total of 27 SLL/CLL samples were initially analyzed by phospho-flow cytometry. Only patients' samples where at least 50% of the tumor cells responded to any stimulation condition with a phospho-protein readout were included and carried forward in the analysis. In this regard, 10/27 samples were excluded from further analysis. One sample was excluded because pathology review re-diagnosed SLL sample to mantle cell lymphoma. Of the remaining 16 samples, five were re-diagnosed as MZL after review by pathologist resulting in a sample cohort of SLL; n = 11 and MZL; n = 5.

In the EPC study (paper III), forty-one patients (MM; n=24, NHL; n=17) with cryopreserved PBSC autograft samples collected before ASCT in the period 1995-2006 at University Hospital of North Norway where included. The study was approved by Regional Committee for Medical Research Ethics (REK-Nord 2011/724). Follow-up in EPC study ended January 31, 2014. Median follow-up in NHL cohort was 6.2 years (range 0.3 – 13.2 years). Biopsies from NHL patients included were subjected to review by a reference pathologist in 2014. Median follow-up in MM patient cohort was 10.2 years (range 0.5 - 17.2) according to the reverse Kaplan-Meyer estimator (208). Clinical data were collected from University Hospital of North Norway.

4.1.2 Description of phospho-specific flow cytometry

Fluorescence-activated flow cytometry allows fast single-cell multi-parametric analysis and sorting of highly purified populations of viable cells. The technique was born in the Herzenberg laboratory at Stanford University in the late 1960s (209, 210). In the beginning, cytometers only measured one fluorescent signal and two scattered-light signals. However, in the mid-1980s four-colour machines that could distinguish six parameters for each cell allowed to discriminate functionally distinct subsets of leukocytes became available. Further technological advances in the 1990s with developments of hardware, software and chemistry made it possible to create machines capable of measuring more than ten colors (Figure 4) (210). For simplistic description regarding analyzing a few of the multiple subsets in the immune system, as few as two physical parameters (forward and side scatter) and two fluorescence parameters can be sufficient. Forward- and side-scatter characteristics can distinguish monocytes and granulocytes (high side scatter and high forward scatter) from platelets and erythrocytes (low side scatter and low forward scatter). Lymphocytes and circulating dendritic cells show intermediate forward and side scatter. Lymphocytes can be further segregated by lineage (B cells, T cells and NK cells) using one or more fluorescent antibodies. For example, B cells can be identified using fluorescent labeled antibodies specific for the CD19 or CD20 cell-surface marker.

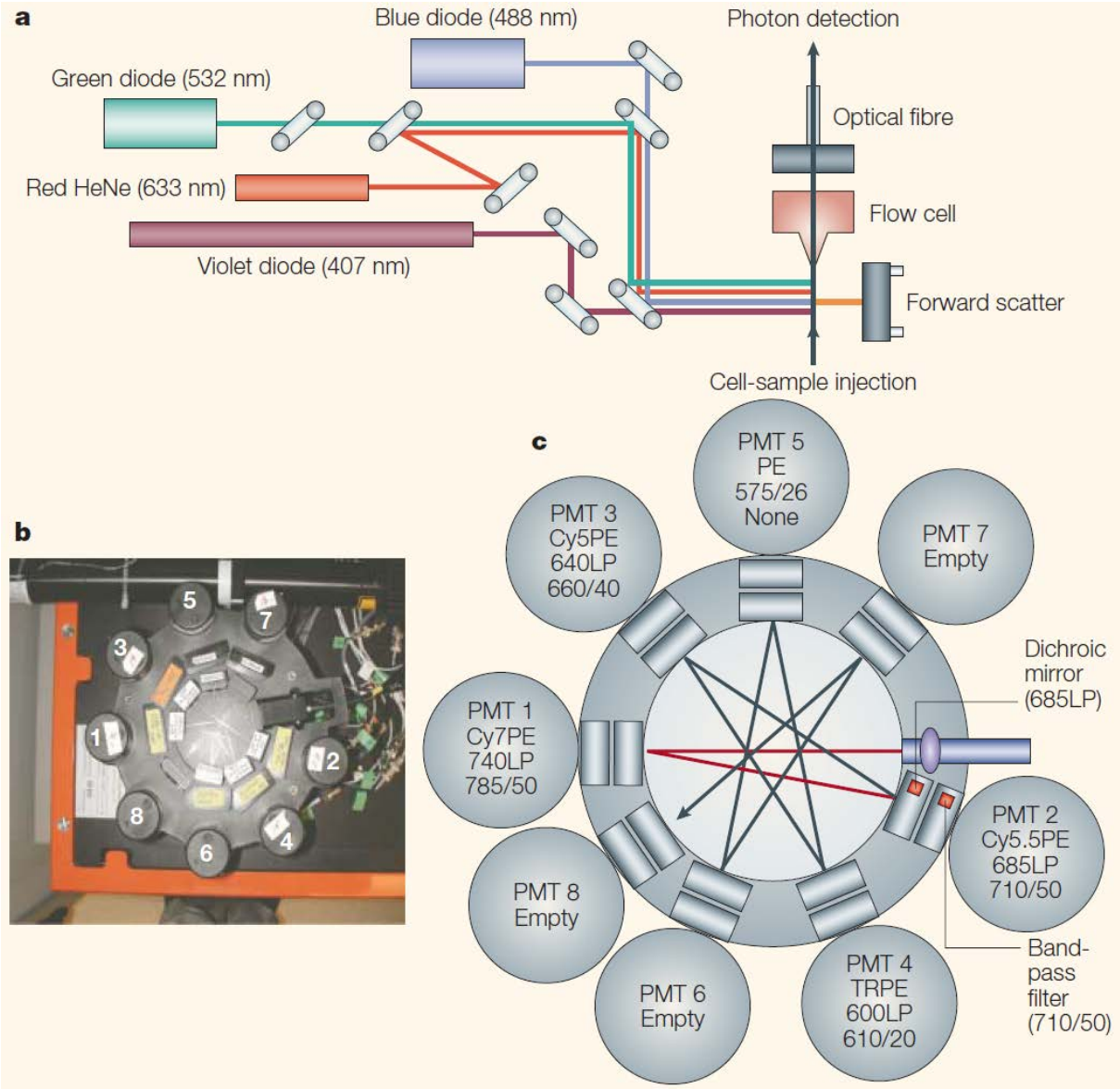


Figure 4. A model of laser geometry and fluorescence-emission detection system. The laser geometry and fluorescence-emission detection system of a flow cytometer. **(A)** A schematic representation of four lasers; blue (488 nm), green (534 nm), violet (407 nm) and a red helium-neon (HeNe) laser (633 nm). **(B-C)** Presentation of the octagon assembly for detection of laser-excited emissions, composed of a series of photomultiplier tubes (PMTs 1–8) and optics in an octagonal arrangement. Light enter this system and are systematically reflected to the next detector by a dichroic mirror or transmitted through a band-pass filter and counted in a PMT. As an example; for PMT 1, '740LP' (740 nm long-pass filter) indicates that all wavelengths longer than 740 nm are transmitted and that all wavelengths shorter than 740 nm are reflected (210). Reprinted by permission from Nature Publishing Group.

Flow cytometry is an important tool in both clinical diagnostics and in basic immunological research due to its capability to rapidly distinguish different subsets in heterogeneous cell populations. Advances in the technique are made through

improvement in flow cytometry instrumentation and applications, it is possible to perform simultaneously analysis of 17 colors and 19 parameters (210). Recently, the first 27-color fluorescence experiment was performed (unpublished data announced by Chattopadhyay, Perfetto and Roederer by mail 03.05.14). Traditionally, flow cytometry protocols have utilized staining of different surface antigens to characterize and define subsets in the hematopoietic system. However, surface staining provides limited functional information of the cell subset analyzed. Methods are available to characterize different intracellular epitopes like DNA (211), cytokines (212), enzymes (213), hormone receptors (214), cell cycle proteins (215) and phosphorylated signaling proteins. Technical advances the last decade with the development of phospho-specific antibodies and protocols for cell fixation and permeabilization have expanded the ability to gain additional functional information with the study of kinase cascades in single cells (207, 216-227).

Phospho-specific analyses as phospho-flow and Western blot are based on the premise that there exist a correlation between the phosphorylation status of a given protein and its biologic status (222, 228). In kinases, phosphorylation typically results in enhanced activity and propagation of a signal downstream, as in the mitogen-activated protein kinase cascade (229). However, phosphorylation can also lead to inactivation of enzymatic activity, as in the case of phosphorylated Y505 in LCK and Y508 in LYN downstream of the T and B cell receptor, respectively, due to recruitment of phosphatases (230).

4.1.3 Advantages and limitations of phospho-specific flow cytometry

The main advantages of utilizing a phospho-specific flow cytometry technique in signaling studies are abilities of performing single-cell analysis and to analyze cell subsets within heterogeneous cell populations. Compared to traditional Western blot, phospho-specific flow cytometry provides additional parameters like fold change of the single-cell phospho-protein level and the percentage of cells responding to a stimulus (224). Further, phospho-flow offers an ability to measure multiple parameters simultaneously from the same cell, and to do analyses in cases where there is only limited sample material available with small number of cells. The flow cytometry technique provides statistical properties as means, medians, standard

deviations and coefficients of variations. In addition, peak shapes as Gaussian versus slanted or skewed peaks may be informative of small changes during analysis (222).

However, several technical considerations must be done. Antibody titration is important to obtain optimal staining concentrations; lower antibody concentration can often reduce unwanted background which may not be present when staining intact cells (224). One concern is if the potential phospho-antibody is cross-reactive with another, but structurally similar phospho-epitope in another protein. For example, the phospho-antibody p-SYK also cross-reacts with ZAP-70 (Y319) due to homology of the phosphorylation site SYK (Y352).To verify that antibodies exhibiting specificity for the phosphorylated state of the kinase, antibodies can be tested by treating cells with appropriate kinase-inducing agents or selective inhibitors of kinase activity. In addition it is possible to do Western blot analysis and kinase activity assays (218). In addition, it is possible to test phospho-antibody specificity by phosphatase treatment or eventually by blocking with a phospho-specific peptide (231).

Different phospho-specific monoclonal antibodies have been tested for phosphospecificity and are demonstrated to correlate with kinase activity. Antibodies against phospho (p)-p44/p42 (ERK1/2) and p-p38 amongst others were tested with FACS, Western blot and kinase activity assays in protocols including specific stimulus to kinase activation or inhibitors of the upstream kinase (218). Further, MFI as measured by flow cytometry correlated well with densitometry values from Western blots, validating the different probes of being able to measure small changes in activated kinases, and also reflecting activated kinase levels as in Western blot analysis (218). Other publications have compared flow cytometry and Western blot with phospho-specific monoclonal antibodies directed against p-ERK, p-p38, p-JNK, p-STAT1, p-STAT5 and p-STAT6 (219), or p-S6 (232).

Intracellular signaling is transient in nature, and fixation must be rapid. Fixation of cells with formaldehyde before methanol permeabilization is a simple and fast technique and preserves the phospho-epitopes (219). The stability of phospho-epitopes during the protocol is also fundamental as de-phosphorylation would affect results. Experiments have shown that samples can be stored in cold methanol for up

to one month with only minor loss of phospho-epitope staining (219). An important aspect is that the antigen of interest may not be accessible for antibody binding after cellular fixation as fixatives like formaldehyde potentially can lead to the epitope of interest are buried into protein interfaces. However, experimental evidence shows that with proper permeabilization with methanol, intracellular phospho-epitopes can be measured (219). Surface antigens and light scatter properties potentially can be altered by fixation and permeabilization of cells (219, 224). Therefore, during protocol development and refinements, one must monitor potential effects off cell fixation and permeabilization on maintaining surface epitope recognition and how light scatter properties are affected. The signal-to-noise ratio when analyzing low-abundance signaling proteins may be too small. Another important question is if different cellular localization of antigens limits antibody binding. Experience with protocols using formaldehyde and methanol shows that both nuclear antigens like the STAT transcription factors and antigens in the cytosol like PLCγ can be assessed with phospho-specific flow (222). Of note, subcellular localization of a given antigen cannot be assessed with flow cytometry and microscopy techniques or Western blot after cellular fractionation must be considered.

Many antibodies that work well within Western blot protocols are not suitable for phospo-specific flow cytometry. In addition, experiments show that different antibody clones have heterogeneous staining levels and caution must be taken when considering a negative result. Positive controls with well-established stimulation conditions for instance PMA activation of p-ERK is requisite when new antibodies are tested in protocols (219). Until now, there are limitations in the diversity of commercially available phospho-flow antibodies. One recent study illustrates this challenge. A total of 247 possible PEG2 regulated phospho-epitopes in lymphoid cells were detected by a quantitative phosphoproteomic approach, but only five commercially available specific phospho-antibodies were identified (233). Western blotting has the potential of measuring small amounts of a given signaling protein due to better antigen accessibility and no concern of auto-fluorescence (222). When choosing a fluorochrome for flow cytometry it is important to consider that the fluorophore's absorbance spectrum must match the laser line in the flow cytometer and that emission fall within the available detection filter sets. Furthermore, the fluorophore must not interfere with binding of antibody to antigen or hamper

permeability through the fixed cell structure due to large size (PE or APC). Alexa Fluor 488 and 647 ore often chosen for phospho-antibodies as they are small-molecule fluorophores with spectral properties suitable for flow cytometry and have the advantages of being extremely bright and photostable (219). Phospho-specific flow cytometry protocols are relative fast and time saving when compared to immunohistochemistry. However, one important limitation compared to microscopy is lack of information concerning tissue morphology.

4.1.4 Rationale for choosing phospho-flow in exploring signaling in lymphoma samples

Altogether, a “high-throughput” strategy of obtaining a comprehensive picture of the signaling network in lymphoma cells upon stimulatory signals at the single cell level will potentially provide new information in relation to lymphoma pathogenesis. Detecting of the phosphorylation status of signaling proteins is essential for examination of intracellular signaling pathways and has traditionally been performed by “bulk” analysis like Western blot or ELISA techniques. However, these methods will only measure the average expression of the proteins of interest in the sample, and sample heterogeneity is masked.

Single cell phosphorylation studied by flow cytometry has provided us with new insight in the study of different tumor cell characteristics. Data can be used to generate signaling network maps and link specific signaling profiles with clinical data as biomarkers of diagnosis. As an example, in a study with patients with juvenile myelomonocytic leukemia (JMML) there were identified that the tumor cells had a unique signature with strong phosphorylation of STAT5 after stimulation with GM-CSF (234). Furthermore, single-cell phospho-flow has provided information about response to therapy. Earlier work in acute myelogenous leukemia (AML) has detected subsets of patients with impaired response to first line chemotherapy in AML patients (221). Recently, a FL cell subset with impaired BCR and co-receptor signaling together with aberrant signaling in tumor-infiltrating T cells with significant impact on OS has been identified and thoroughly characterized (195). Based on these prior studies, we decided to use phospho flow cytometry as the method of choice to study cell signaling in SLL/CLL and MZL patient samples (paper I and II).

4.1.5 Rare cell analysis by flow cytometry

EPC are rare events in normal peripheral blood, the frequency are somewhere between 0.01% and 0.0001% of peripheral mononuclear cells (235). A consensus immunophenotype have not been established, so different cell surface markers have been used to detect EPC. Reliable enumeration of EPCs remains a technical challenge. For accurate detection of rare events by flow cytometry the background noise have to be substantially less than the frequency of the sought events. It is recommended to consider several methodological aspects to reduce background noise when rare events flow cytometry protocols are developed (235). Therefore, our protocol included a viability stain (like propidium iodide (PI) 7-aminoactinomycin D (7-AAD), or in our case Aldefluor), pre-incubation with a Fc-receptor blocking serum and extensive washing procedure to remove carry-over of cells between different patient samples on the cytometer instrument. Furthermore, in antibody and fluorochrome selection it is important to select fluorochromes with high signal-to-noise ratio like PE or tandem conjugates of PE (PE-Cy5 or PE-Cy7) or APC and its conjugates (APCAlexafluor700 or APC-H7) (236). In the EPC study (paper III) we defined and characterized EPC with three phenotypic surface markers together with a functional marker for high ALDH activity. This method is quite specific, but since EPC are rare cells in peripheral blood, measurement error according to Poisson distribution is high (237). Of note, we characterized and identified EPC in stem cell grafts mobilized by G-CSF which are enriched for stem and progenitor cells. In the $ALDH^{hi}$ population, we identified the frequency of EPC with a mean of 1.83%, and EPC in the whole population analyzed was mean 0.03%. This results in CV in the range 1-5% for $5x10^6$ collected events (236). As both CD34 and VEGFR2 are found on mature endothelium, we believe the addition of CD133 and ALDH are necessary to define cells with progenitor or stem cell properties.

4.1.6 Limitations of patient cohort and outcome prediction

The association between clinical features and tumor cell signaling features were hampered by the low number of patients included (paper II). There were few patients with SLL/CLL and MZL included due to few available tumor biopsies with long clinical

follow-up and good enough quality of live tumor cells frozen in DMSO. Furthermore, due to the limited number of patients, we decided to pool SLL/CLL and MZL patients into one cohort of indolent B cell lymphoma. However, although SLL/CLL and MZL share many clinical characteristics as indolent lymphoma, they constitute two distinct different diseases with unique clinical and pathological characteristics.

The conclusion in the EPC study (paper III) is weakened by the relative small number of MM patients included. In addition, data regarding response after HMAS was hampered by missing immunofixation data. Complete response in MM after HMAS is defined as normal bone marrow examination and absence of monoclonal component on immunofixation (238). Therefore, the category of complete response after HMAS does not exist. Patients underwent two different induction regimen before the ASCT and this may have an impact of the survival. However, MM patients had no difference in OS, PFS or TNT according to the induction chemotherapy (VAD vs. Cy-Dex), and there were no significant difference in percentage of EPC within the MM cohort according to induction chemotherapy prior to ASCT (VAD vs. Cy-Dex). Last, there were not available data on cytogenetic abnormalities as t(4:14), t(14:16) and deletion 17p13. These genomic aberrations preferentially should have been included in the multivariate cox model.

4.2 Flow cytometry to study signaling pathways and predict outcome in B cell malignancies

4.2.1 Basal BCR signaling in lymphoma cells

When examining cellular signaling, the unstimulated state is referred to as the basal, tonic or constitutive state. Usually, in resting normal cells, the basal state is inactive. However, in resting cancer cells, the basal state might be elevated and suggests constitutive activation of signaling pathways (239). We therefore explored if there were differences in the basal phospho-protein expression levels between malignant B cells and healthy donor B cells (paper I). The fold change in MFI for phospho-proteins in unstimulated malignant B cells from SLL/CLL or MZL patients were normalized to the MFI in normal, peripheral blood $CD20^+$ B cells. We found significantly elevated basal levels of p-SFKs, p-PLCγ, p-ERK, p-p38, p-p65 (NF-κB),

p-STAT5 and p-STAT6 (paper I). Of note, the basal levels of phospho-proteins were heterogeneous and ranged from small elevations (i.e. fold change <0.5) in most cases to higher basal levels in a minority of patients samples.

Previously, SYK was found to be constitutive phosphorylated in primary CLL samples relative to cell lines (129, 240). Interestingly, we did not detect significant elevations in basal p-SYK (paper I). A possible explanation for not identifying higher constitutive levels of SYK in unstimulated lymphoma B cells compared to normal B cells could possibly be dephosphorylation during handling of tissue samples. However, only tumor samples where the majority of B cells induced signaling were included in the study and all samples were rested in tissue culture incubator before staining with phospho-specific antibodies. Of note, a small subset of SLL/CLL and MZL samples showed basal p-SYK levels above normal B cells.

Recently, it has been demonstrated that BCR signaling in CLL is not dependent on exogenous antigens. Instead, BCR activation involves the binding of one region of the BCR to self-epitopes on variable regions of the same receptor. Malignant B-CLL cells induce cell-autonomous Ca^{2+}-signaling independent of extrinsic antigens which is dependent on the heavy-chain complementary determining region 3 and an internal epitope on the BCR (241). The clinical significance of these BCR interactions in vivo, where cells are surrounded by high levels of serum Ig that potentially compete with these Ig/Ig interactions is unclear (242). Mutated CLL have higher basal Ca^{2+}-signaling as compared to unmutated-CLL (243), and the increased basal signaling in mutated-CLL are potentially due to a more efficient route to anergy for mutated than for unmutated-CLL (131).

It has been demonstrated that malignant B-CLL cells have elevated basal Lyn tyrosine kinase activity (244), ERK (245) and p-p38 (246, 247). Activation of PLCγ and PI3K initiate downstream p-ERK and p-p38. The Ras-Raf-ERK signaling pathway is important in B cells as decreased Ras activation in mice impairs B cell proliferation (248, 249). The p38 pathway was initially identified as a mediator of inflammation and stress responses, but it is demonstrated that it interacts with p53 and regulates cell cycle checkpoints (249-251). However, the exact role of p38 in the physiology of normal and malignant B cells is largely unknown (252). The p-p38

expression does not merely indicate proliferation status since GC B cells within reactive lymphoid tissue show negligible expression of p-p38 (252). Differential phosphorylation of ERK and p38 might direct B cells to diverse cellular outcomes, but it is unknown whether a B cell activates these two pathways simultaneously or favors one pathway depending on additional signaling context (57).

Furthermore, we also detected elevated basal p-p65 in SLL/CLL patient samples (paper I). This is in line with previous studies which report constitutively activated NF-κB in CLL patients (137, 253, 254). In CLL, there was heterogeneity in basal and inducible NF-κB subunit Rel A (p65) activity, but all CLL samples had higher basal NF-κB compared to normal B cells (254). The clinical importance of Rel A (p65) in CLL has been demonstrated as Rel A (p65) activity was predictive of CLL patients time to first treatment and survival in addition to the observation that Rel A (p65) clearly increased in serial samples tested during treatment (255). These data indicate that p65 is constitutively elevated in CLL patients with more aggressive disease and that p-p65 can be induced by chemotherapy.

4.2.2 Activated BCR signaling in lymphoma cells

Normal, mature B cell survival depends on an intact BCR (14), and BCR signaling most likely constitutes of both basal signaling as well as antigen-activated BCR signaling (14, 15). We found impaired BCR signaling in both SLL/CLL and MZL patient samples (paper I). Both proximal signaling molecules like p-SFK, p-SYK/p-ZAP-70 and p-PLCγ and the distal signaling molecule p-ERK had low expression in both SLL/CLL and MZL lymphoma B cells, when compared to normal B cells. Furthermore, we found reduced levels of surface IgM and CD79b in CLL/SLL lymphoma cells, and both IgM and CD79b expression correlated with anti-BCR activated p-PLCγ signaling (paper I). Of note, measuring B-cell signaling capability after BCR cross-linking *in vitro* will be influenced by the *in vivo* activation status at the time the sample was acquired. Hence, if the BCR recently had been activated *in vivo*, this might have induced internalization of the BCR, making the cells less responsive to the following *in vitro* BCR-induced signaling.

This is supported by our data, showing reduced surface IgM and CD79b expression in SLL/CLL cells (paper I). Others have also found surface IgM expression to vary considerably among primary CLL samples, and a subset of patients had markedly decreased IgM expression on the malignant cells (126-128). It has been reported an association between IgM expression and BCR signaling capacity in CLL (131). Low expression of CD79b in CLL cells has also been reported previously (256). CLL tumor cells which are unresponsive to anti-IgM, can respond to anti-CD79a treatment, indicating a deficit in signal transmission from the BCR to CD79a/b (129). However, since a subgroup of CLL samples was unresponsive to activation with anti-CD79a, a potential defect further downstream in the BCR signaling pathway is also possible (129). Furthermore, the cell-autonomous Ca^{2+}-signaling independent of extrinsic antigens shown in CLL cells (241) further suggests that impaired BCR-signaling response upon in vitro re-activation could be due to a recent in vivo activation.

There are also other possible explanations for the impaired BCR signaling response in SLL/CLL and MZL malignant B cells. Potential mechanisms of impaired BCR signaling in malignant B cells might include high constitutive signaling, increased negative regulation, or loss-of-function mutation of a proximal kinase, such as SYK or SFKs. If an increase in negative regulation was responsible for impaired BCR signaling in malignant B cells, this signaling defect might be reversed by inhibiting tyrosine phosphatases that fine-tune BCR signaling, such as CD45, CD22-associated SHP-1, or PTPROt (51, 57, 257-259). However, if BCR signaling were impaired through loss of kinase function, signaling in malignant B cells would not be restored by phosphatase inhibition. Of note, inhibition of BCR signaling in the malignant B cell was significantly reversed when BCR was engaged in the presence of H_2O_2. Adding H_2O_2 alone in FL triggered no significant signaling (257). In FL it has been demonstrated that anti-BCR+H_2O_2 induced ERK and p38 signaling was blocked by the SYK inhibitor R406, in contrast to no effect by SYK inhibitor on PMA induced p-ERK signaling (195). Therefore, the framework for BCR-dependent signaling remained intact in these cells. Taken together, these results indicate that BCR signaling was not permanently lost and that the impaired BCR signaling was reversible in the malignant B cells generally and especially in the BCR insensitive population (195).

The observation that BCR signaling in SLL/CLL and MZL was restored when H_2O_2 was added together with BCR cross-linking can partially be explained by high levels of negatively regulating protein tyrosine phosphatases (PTPs) in lymphoma samples (paper I). Normal BCR signaling is dependent on a balance between PTK and protein tyrosine phosphatases that attenuate signaling. Stimulation of the BCR results in generation of H_2O_2 that leads to an inhibition of PTPs (51). The addition of H_2O_2 as a stimulus by itself does not induce signaling (57). Overexpression of the BCR regulating phosphatase PTPROt inhibited proliferation of lymphoma cell lines and resulted in increased apoptosis (259). However, it has been demonstrated that reduction of reactive oxygen species (ROS) like H_2O_2 in ataxia telangiectasia (ATM) $^{-/-}$ mice prevented lymphomagenesis (260). So far, there are few available data about the magnitude and function of ROS in lymphoma B cells. We observed that malignant B cells in SLL/CLL and MZL patient samples had sustained BCR induced signaling. This has previously also been reported for FL (257), but the reason for this is currently unknown.

We did not detect significant differences in BCR-induced signaling between unmutated and mutated SLL/CLL, possibly due to small sample size (paper I). BCR signaling in CLL are known to be heterogeneous among patients. CLL patients with unmutated BCR have increased global tyrosine phosphorylation (129), increased progression into G1 (130), and increased intracellular Ca^{2+}-signaling after BCR stimulation (261). In addition, B-CLL cells expressing ZAP-70 had increased BCR signaling and also increased levels of phosphorylated CD79b following anti-BCR treatment compared to CLL cells with no ZAP-70 expression (107). Unmutated CLL cells have higher levels of p-ERK after BCR stimulation compared to mutated CLL (262). As p-ERK signaling was more prominent in CLL with adverse prognostic marker this points to a role for p-ERK in CLL pathophysiology (263).

The term anergy, defined as a state of reduced responsiveness to BCR activation, is used to describe the tumor cell status in B-CLL cells. It is characterized by reversibility and points to in vivo antigen-induced modulation of BCR. Different levels of anergy in unmutated and mutated B-CLL cells may influence clinical course (131). In a proportion of patients BCR signaling in malignant B-CLL cells is shown to be

down-modulated (129, 131). There is a strong tendency for the anergic profile with surface IgM downmodulation and impaired signaling to be in the CLL patients with a good prognosis, especially in mutated CLL and/or ZAP-70 negative samples (102, 129-131, 261, 263-265).

Increased BCR signaling have been demonstrated to be associated with shorter time to first treatment in a cohort of CLL patients (266). Recently, a study on malignant B cells from CLL patients demonstrated highly heterogeneous BCR signaling after combined anti-BCR and H_2O_2 stimulation. A model with phospho-response signature stratified CLL patient's cohort into clinically relevant subgroups where patients whose malignant B cells responded to BCR activation with high responsiveness required treatment after shorter periods of expectant monitoring (267).

The impaired BCR signaling in SLL/CLL discovered in paper I was based on MFI of the total malignant B cell population. However, this analysis could not distinguish between uniform low signaling response in all malignant cells, or high signaling in only a subset of cells (heterogeneous signaling response). A recent publication in FL using phospho-specific flow cytometry and per-cell analysis revealed that FL patients having a large fraction of their tumor cells being non-responsive to BCR crosslinking, had an inferior clinical outcome (195). Importantly, per-cell analysis in SLL/CLL and MZL also revealed a subpopulation of lymphoma B cells with no apparent signaling after BCR stimulation and the frequency of this specific BCR insensitive lymphoma B cell subpopulation in SLL/CLL and MZL patients were associated with a shortened OS (paper II). Of note, there is previously demonstrated evidence of intraclonal heterogeneity in CLL (268). Heterogeneous BCR signaling within individual samples from CLL patients have previously been reported. Ca^{2+} flux as a marker for BCR signaling were highly variable ranging from less than 5% responding cells to almost 100% across a population of CLL patients (131). Analysis of subgroups within B-CLL cells from patients have demonstrated that a subgroup with low p-PLCγ and p-ERK signaling after BCR activation had low IgM expression and contained cells with highest Ki-67 expression (269). High levels of Ki-67+ cells circulating in plasma are shown to be correlated shorter survival in CLL patients, and interestingly this finding was independent on IGHV mutation in multivariate analysis (270). CLL patients with unusual clinical regression after more than 10 years of follow-up showed that BCR

signaling pathway genes are overrepresented in the CLL clones with spontaneous regression (271). In CLL patients, a defined side population of leukemia cells with phenotypic and cytogenetic hallmarks of B-CLL has recently been demonstrated to be chemo-resistant even before therapy (272). However, this B-CLL side population is considerably smaller than the subpopulation of BCR insensitive lymphoma cells demonstrated here. It therefore remains unknown whether there is a functional relationship between these two subpopulations of lymphoma B cells. High BCR signaling capacity has been associated with an unmutated IGHV status in CLL (129, 273, 274), in contrast to SLL and MZL where there are reported no significant difference in OS between patients with unmutated and mutated IGHV (275, 276). IGHV mutational status did not predict OS in our SLL/MZL patient cohort, nor was it associated with the prevalence of the BCR insensitive lymphoma subset (paper II).

4.2.3 Activated CD40 signaling in lymphoma cells

Proliferation of B-CLL cells require more than antigen activation of BCR, and for normal B cells the most important co-stimulus comes from CD40 stimulation and additional cytokines, both from $CD4^+$ T helper cells in the lymph node microenvironment (277). CD40 signaling has potential to contribute to drive leukemic cell proliferation in the lymph node microenvironment, as autologous T cells can induce proliferation of B-CLL cells with combined CD40L and IL-21 stimulation (194, 278). In normal B cells, CD40L stimulation resulted in increase in p-p65 (NF-κB), p-S6, p-p38, and to a lesser extent p-ERK (paper I). Malignant B cells from SLL/CLL showed significantly impaired p-ERK, p-p38 and p-S6 after CD40L stimulation, whereas malignant B cells from MZL had significantly less p-ERK and p-p38 relative to normal B cells (paper I). Altogether, CD40L signaling was partly impaired in malignant B cells from SLL/CLL and MZL patients and was characterized by diminished p38 phosphorylation. The reason for impaired p-p38 expression in SLL/CLL and MZL lymphoma B cells is not known, but likely provides improved survival for the malignant cells. Activation of p38 has a pro-apoptotic function in CLL cells, and earlier work has shown that rituximab-induced apoptosis is dependent on phosphorylation of p38 (279). Furthermore, recent work in primary CLL cells illustrates that chemotherapy-induced up-regulation of the pro-apoptotic protein

NOXA is at least partly dependent on p38 (280). Thus, loss of p38 function is likely to give the tumor cells a survival advantage.

In contrast to the overall weak CD40L induced phosphorylation of p38 in malignant B cells, p-p65 (NF-κB) expression was markedly heterogeneous in SLL/CLL and MZL, but similar to the observed variation in healthy donor B cells. This finding is in accordance with earlier work where lymphoma B cells from CLL patients were heterogeneous in basal- as well as activation-induced NF-κB (254). This has potentially clinical implications as a correlation between the NF-κB subunit Rel-A (p65) DNA binding in CLL cells and lymphocyte doubling time was identified, and Rel-A DNA binding was positively correlated with in vitro resistance to fludarabine (254).

Recently, it was reported that high p-p65 in response to CD40L in FL patients samples correlated to better prognosis (195). In contrast, CD40L stimulation of B-CLL cells induced expression of micro-RNA 155, a critical regulator of post-transcriptional gene expression demonstrated to be an independent adverse prognostic marker in CLL (281). In this study CD40L stimulated malignant B-CLL cells had increased microRNA-155 expression, reduced expression of the phosphatase SHIP1, and enhanced BCR activated Ca^{2+}-signaling (281). We therefore examined CD40L induced signaling in lymphoma B cells, and correlated relative MFI to patient survival. We found that SLL/CLL and MZL patients with malignant B cells with higher than cohort median MFI of p-p65 (NF-κB) and p-S6 in response to CD40L had a better OS (paper II). This interesting observation about CD40 signaling and its importance for cell survival have previously been reported in CLL. Autologous B-CLL cells transfected with human or murine CD154 (CD40L) to achieve CD40 cross-linking showed enhanced susceptibility to death-receptor-mediated or drug-induced apoptosis (282), and induced anti-leukemic immune responses (283). Of note, Irish et al investigated CD40 signaling within the BCR-insensitive cell subset and found it to be intact. These data provided evidence that altered CD40 signaling might support tumor maintenance in patients wherein clinical outcome was not determined by levels of BCR insensitive population alone (195).

4.2.3 Bone marrow angiogenesis in multiple myeloma

Aberrant angiogenesis is one of the important hallmarks in the multistep pathogenesis of MM disease progression (153). Recruitment of VEGFR2$^+$ EPC and VEGFR1$^+$ hematopoietic precursor cells from bone marrow is central in complex process of malignant angiogenesis (181). However, the importance of EPC in MM or B cell lymphoma progression and clinical outcome is not clear. We found that high number of CD34$^+$VEGFR2$^+$CD133$^+$ EPC with high ALDH activity in PBSC grafts predicted adverse outcome in MM, but not in NHL patients (paper III). Interestingly, we did not detect significant differences in the amount of EPC within PBSC grafts from NHL and MM patients although PBSC grafts were mobilized with different protocols in the two cohorts. This observation is in line with previous work showing that there was no significant difference in EPC levels between MM and NHL after mobilization to peripheral blood by cyclophosphamide and G-CSF (284). Unlike for MM, we could not observe any trends towards worse outcome in NHL patients with high levels of EPC, although this result could be due to the small cohort of NHL Accordingly, the role of angiogenesis in DLBCL measured by microvessel density has shown different results in regard to clinical outcome (170, 171). In contrast to NHL, MM cells grow and expand almost exclusively in the bone marrow (172), and both osteoblastic and vascular niches can support the proliferation of MM cells (173).

EPC level in stem cell grafts was associated with increased pre-treatment s-β_2M in the MM cohort (paper III). Of importance, we found a correlation between EPC in PBSC grafts and s-β_2M in the MM cohort. S-β2M is a marker of tumor cell burden and an important prognostic factor in MM (285-288). The association between the levels of EPC in PBSC grafts and s- β_2M in peripheral blood at time of diagnosis are concordant with previous studies in MM, which have reported a correlation between s- β_2M and circulating endothelial cells (289), or circulating EPC (290). It is an unsolved question whether levels of EPC in stem cell grafts has a direct effect on relapse or purely acts as a surrogate marker. The correlation s- β_2M before treatment and EPC in the graft could indicate that MM patients with high tumor load at baseline mobilize more EPC together with PBSC.

We defined EPC as progenitor cells with high intracellular ALDH expression combined with the phenotypic surface markers CD34, CD133 and VEGFR2 (paper

III). Although the properties of EPC to differentiate into mature endothelial cells in vitro and to contribute to vessel formation after transplantation was described more than a decade ago (180), no consensus has been reached regarding a uniform definition of EPC. EPC characterized as $CD34^+CD133^+VEGFR2^+$ has previously been identified in NHL and MM (284), non-small cell lung cancer (291, 292), myelofibrosis with myeloid metaplasia (293), and glioma (294). However, there are controversies if $CD34^+VEGFR2^+CD133^+$ cells have angiogenic or hematopoietic capacities (295). These markers are also demonstrated to be expressed on hematopoietic stem- and progenitor cells, making it difficult to distinguish between endothelial and hematopoietic progenitors (296-298). Furthermore, $ALDH^{hi}CD133^+$ cells have ability of multi-lineage reconstitution and possessed long-term repopulating ability in secondary murine recipients (188). Therefore, high ALDH activity is a functional marker of both hematopoietic and non-hematopoietic bone marrow derived progenitor cells (186). Some recent studies characterized EPC solely as $ALDH^{hi}$ or as $CD34^+CD133^+$ cells (189, 190). Of note, the process of angiogenesis is still incompletely understood as both HSC and non-hematopoietic cells can be transplanted to augment vascularization in mice (299-301).

5. Conclusion

Papers I and II provides comprehensive information about differences in BCR signaling in samples from SLL/CLL and MZL patients compared to normal B and T cells. Although we did not identify unique signaling profiles that could distinguish SLL/CLL from MZL, we identified contrasting signaling abnormalities in the lymphoma B cells when compared to normal B cells. Further studies using single-cell phospho-specific flow cytometry to obtain patient specific signaling aberrations could provide an opportunity to personalize inhibitor treatment in B cell lymphoma patients. Although BCR signaling in CLL is well characterized and on-going studies are expected to expand our knowledge about new targets for therapy, it is less knowledge about BCR signaling in MZL. In addition, even if the field of BCR signaling in both normal B cells and malignant B-CLL cells has evolved over a decade, it is only limited knowledge regarding intraclonal heterogeneity of malignant B cells in lymphoma and leukemia, and how they potentially can expand over time following successive therapies and tumor cell progression. The work presented in paper II demonstrated that it can be especially valuable to combine phenotypic surface markers with an input stimulus and measure induced phosphorylation in order to dissect subset of malignant B cells from patient's samples rather than only measuring homeostatic states of such pathways in the whole tumor cell population.

Paper III is the first one to comprehensively study the impact of EPC in autografts from NHL and MM and to demonstrate that EPC have impact on MM patient's overall survival after ASCT. However, the conclusion of the study is weakened by the limited number of patients included. In addition, we did not have information of cytogenetic abnormalities in MM cells and how this might could influence EPC. Finally, in order to gain a better insight whether the increased numbers of EPCs in the autografts is a surrogate marker of poor prognosis and survival or facilitate relapse by direct action, future EPC protocols should consider to study the presence of contaminating MM cells checking the number of $CD138^{+}$ cells, stromal cells and regulatory T cells in the autograft together to the number of EPC, and to analyze whether the presence these factors influence EPC or have impact on the PFS and OS.

Papers I-III

6. References

1. Blander, J. M., and L. E. Sander. 2012. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nat Rev Immunol* 12:215-225.
2. Parham, P. 2009. *The Immune system*. Garland Science, Taylor & Francis Group, LLC, New York.
3. Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. 2011. Innate or Adaptive Immunity? The Example of Natural Killer Cells. *Science* 331:44-49.
4. Rothstein, T. L., D. O. Griffin, N. E. Holodick, T. D. Quach, and H. Kaku. 2013. Human B-1 cells take the stage. *Annals of the New York Academy of Sciences* 1285:97-114.
5. Wang, C. Y., R. A. Good, P. Ammirati, G. Dymbort, and R. L. Evans. 1980. Identification of a p69,71 complex expressed on human T cells sharing determinants with B-type chronic lymphatic leukemic cells. *The Journal of Experimental Medicine* 151:1539-1544.
6. Lanier, L. L., N. L. Warner, J. A. Ledbetter, and L. A. Herzenberg. 1981. Expression of Lyt-1 antigen on certain murine B cell lymphomas. *The Journal of Experimental Medicine* 153:998-1003.
7. Freedman, A., G. Freeman, J. Whitman, J. Segil, J. Daley, and L. Nadler. 1989. Studies of in vitro activated CD5+ B cells. *Blood* 73:202-208.
8. Sims, G. P., R. Ettinger, Y. Shirota, C. H. Yarboro, G. G. Illei, and P. E. Lipsky. 2005. Identification and characterization of circulating human transitional B cells. *Blood* 105:4390-4398.
9. Lee, J., S. Kuchen, R. Fischer, S. Chang, and P. E. Lipsky. 2009. Identification and Characterization of a Human CD5+ Pre-Naive B Cell Population. *The Journal of Immunology* 182:4116-4126.
10. Hardy, R. R., C. E. Carmack, S. A. Shinton, J. D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *The Journal of Experimental Medicine* 173:1213-1225.
11. Blom, B., and H. Spits. 2006. DEVELOPMENT OF HUMAN LYMPHOID CELLS. *Annual Review of Immunology* 24:287-320.
12. Takahashi, M., Y. Matsuoka, K. Sumide, R. Nakatsuka, T. Fujioka, H. Kohno, Y. Sasaki, K. Matsui, H. Asano, K. Kaneko, and Y. Sonoda. 2014. CD133 is a positive marker for a distinct class of primitive human cord blood-derived CD34-negative hematopoietic stem cells. *Leukemia* 28:1308-1315.
13. Gallacher, L., B. Murdoch, D. M. Wu, F. N. Karanu, M. Keeney, and M. Bhatia. 2000. Isolation and characterization of human CD34−Lin− and CD34+Lin− hematopoietic stem cells using cell surface markers AC133 and CD7. *Blood* 95:2813-2820.
14. Lam, K. P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 90:1073-1083.
15. Kraus, M., M. B. Alimzhanov, N. Rajewsky, and K. Rajewsky. 2004. Survival of Resting Mature B Lymphocytes Depends on BCR Signaling via the Ig[alpha]/[beta] Heterodimer. *Cell* 117:787-800.
16. Dorshkind, K., and E. Montecino-Rodriguez. 2007. Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential. *Nat Rev Immunol* 7:213-219.
17. Lichtman, M. A. 1981. The ultrastructure of the hemopoietic environment of the marrow: a review. *Exp Hematol* 9:391-410.
18. Scadden, D. T. 2006. The stem-cell niche as an entity of action. *Nature* 441:1075-1079.

19. Zhu J, Garrett R., Jung Y, Zhang Y, Kim N, Wang J, Joe GJ, Hexner E, Choi Y, Taichman RS and Emerson SG. 2007. Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood* 109:3706-3712.
20. Mansour, A., A. Anginot, S. J. Mancini, C. Schiff, G. F. Carle, A. Wakkach, and C. Blin-Wakkach. 2011. Osteoclast activity modulates B-cell development in the bone marrow. *Cell Res* 21:1102-1115.
21. Sipkins, D. A., X. Wei, J. W. Wu, J. M. Runnels, D. Cote, T. K. Means, A. D. Luster, D. T. Scadden, and C. P. Lin. 2005. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature* 435:969-973.
22. Rafii, S., R. Mohle, F. Shapiro, B. M. Frey, and M. A. S. Moore. 1997. Regulation of Hematopoiesis by Microvascular Endothelium. *Leukemia & Lymphoma* 27:375-386.
23. Sugiyama, T., H. Kohara, M. Noda, and T. Nagasawa. 2006. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity* 25:977-988.
24. Egawa, T., K. Kawabata, H. Kawamoto, K. Amada, R. Okamoto, N. Fujii, T. Kishimoto, Y. Katsura, and T. Nagasawa. 2001. The Earliest Stages of B Cell Development Require a Chemokine Stromal Cell-Derived Factor/Pre-B Cell Growth-Stimulating Factor. *Immunity* 15:323-334.
25. Tokoyoda, K., T. Egawa, T. Sugiyama, B.-I. Choi, and T. Nagasawa. 2004. Cellular Niches Controlling B Lymphocyte Behavior within Bone Marrow during Development. *Immunity* 20:707-718.
26. Hargreaves, D. C., P. L. Hyman, T. T. Lu, V. N. Ngo, A. Bidgol, G. Suzuki, Y.-R. Zou, D. R. Littman, and J. G. Cyster. 2001. A Coordinated Change in Chemokine Responsiveness Guides Plasma Cell Movements. *The Journal of Experimental Medicine* 194:45-56.
27. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer, and B. L. Davison. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *The Journal of Experimental Medicine* 180:1955-1960.
28. Corfe, S. A., and C. J. Paige. 2012. The many roles of IL-7 in B cell development; Mediator of survival, proliferation and differentiation. *Seminars in Immunology* 24:198-208.
29. Nutt, S. L., and D. M. Tarlinton. 2011. Germinal center B and follicular helper T cells: siblings, cousins or just good friends[quest]. *Nat Immunol* 12:472-477.
30. Klein, U., and R. Dalla-Favera. 2008. Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol* 8:22-33.
31. Kinoshita, K., and T. Honjo. 2001. Linking class-switch recombination with somatic hypermutation. *Nat Rev Mol Cell Biol* 2:493-503.
32. Tunyaplin, C., A. L. Shaffer, C. D. Angelin-Duclos, X. Yu, L. M. Staudt, and K. L. Calame. 2004. Direct Repression of prdm1 by Bcl-6 Inhibits Plasmacytic Differentiation. *The Journal of Immunology* 173:1158-1165.
33. Inamine, A., Y. Takahashi, N. Baba, K. Miyake, T. Tokuhisa, T. Takemori, and R. Abe. 2005. Two waves of memory B-cell generation in the primary immune response. *International Immunology* 17:581-589.
34. Kunkel, E. J., and E. C. Butcher. 2003. Plasma-cell homing. *Nat Rev Immunol* 3:822-829.
35. Dal Porto, J. M., S. B. Gauld, K. T. Merrell, D. Mills, A. E. Pugh-Bernard, and J. Cambier. 2004. B cell antigen receptor signaling 101. *Molecular Immunology* 41:599-613.

36. Takata, M., H. Sabe, A. Hata, T. Inazu, Y. Homma, T. Nukada, H. Yamamura, and T. Kurosaki. 1994. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways. *EMBO J* 13:1341-1349.
37. Campbell, M. A., and B. M. Sefton. 1992. Association between B-lymphocyte membrane immunoglobulin and multiple members of the Src family of protein tyrosine kinases. *Mol Cell Biol* 12:2315-2321.
38. Yamanashi, Y., T. Kakiuchi, J. Mizuguchi, T. Yamamoto, and K. Toyoshima. 1991. Association of B cell antigen receptor with protein tyrosine kinase Lyn. *Science* 251:192-194.
39. Burkhardt, A. L., M. Brunswick, J. B. Bolen, and J. J. Mond. 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. *Proc Natl Acad Sci U S A* 88:7410-7414.
40. Geahlen, R. L. 2009. Syk and pTyr'd: Signaling through the B cell antigen receptor. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1793:1115-1127.
41. Arita, A., D. C. McFarland, J. H. Myklebust, S. Parekh, B. Petersen, J. Gabrilove, and J. D. Brody. 2013. Signaling pathways in lymphoma: pathogenesis and therapeutic targets. *Future Oncology* 9:1549-1571.
42. Kurosaki, T., and M. Hikida. 2009. Tyrosine kinases and their substrates in B lymphocytes. *Immunological Reviews* 228:132-148.
43. Ishiai, M., H. Sugawara, M. Kurosaki, and T. Kurosaki. 1999. Cutting Edge: Association of Phospholipase C-γ2 Src Homology 2 Domains with BLNK Is Critical for B Cell Antigen Receptor Signaling. *The Journal of Immunology* 163:1746-1749.
44. Beitz, L. O., D. A. Fruman, T. Kurosaki, L. C. Cantley, and A. M. Scharenberg. 1999. SYK Is Upstream of Phosphoinositide 3-Kinase in B Cell Receptor Signaling. *Journal of Biological Chemistry* 274:32662-32666.
45. Gold, M. R., M. P. Scheid, L. Santos, M. Dang-Lawson, R. A. Roth, L. Matsuuchi, V. Duronio, and D. L. Krebs. 1999. The B Cell Antigen Receptor Activates the Akt (Protein Kinase B)/Glycogen Synthase Kinase-3 Signaling Pathway Via Phosphatidylinositol 3-Kinase. *The Journal of Immunology* 163:1894-1905.
46. Marshall, B. N., Yun, Clark. 2000. Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase Cγ pathways. *Immunological Reviews* 176:30-46.
47. Lin, X., and D. Wang. 2004. The roles of CARMA1, Bcl10, and MALT1 in antigen receptor signaling. *Seminars in Immunology* 16:429-435.
48. Su, T. T., B. Guo, Y. Kawakami, K. Sommer, K. Chae, L. A. Humphries, R. M. Kato, S. Kang, L. Patrone, R. Wall, M. Teitell, M. Leitges, T. Kawakami, and D. J. Rawlings. 2002. PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling. *Nat Immunol* 3:780-786.
49. Saijo, K., I. Mecklenbräuker, A. Santana, M. Leitger, C. Schmedt, and A. Tarakhovsky. 2002. Protein Kinase C β Controls Nuclear Factor κB Activation in B Cells Through Selective Regulation of the IκB Kinase α. *The Journal of Experimental Medicine* 195:1647-1652.
50. Hashimoto, A., H. Okada, A. Jiang, M. Kurosaki, S. Greenberg, E. A. Clark, and T. Kurosaki. 1998. Involvement of Guanosine Triphosphatases and Phospholipase C-γ2 in Extracellular Signal–regulated Kinase, c-Jun NH2-terminal Kinase, and p38 Mitogen-activated Protein Kinase Activation by the B Cell Antigen Receptor. *The Journal of Experimental Medicine* 188:1287-1295.
51. Reth, M. 2002. Hydrogen peroxide as second messenger in lymphocyte activation. *Nat Immunol* 3:1129-1134.

52. Meffre, E., and M. C. Nussenzweig. 2002. Deletion of immunoglobulin β in developing B cells leads to cell death. *Proceedings of the National Academy of Sciences* 99:11334-11339.
53. Shaffer, A. L., and M. S. Schlissel. 1997. A truncated heavy chain protein relieves the requirement for surrogate light chains in early B cell development. *The Journal of Immunology* 159:1265-1275.
54. Wienands, J., O. Larbolette, and M. Reth. 1996. Evidence for a preformed transducer complex organized by the B cell antigen receptor. *Proceedings of the National Academy of Sciences* 93:7865-7870.
55. Monroe, J. G. 2006. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat Rev Immunol* 6:283-294.
56. Rickert, R. C. 2013. New insights into pre-BCR and BCR signalling with relevance to B cell malignancies. *Nat Rev Immunol* 13:578-591.
57. Irish, J. M., D. K. Czerwinski, G. P. Nolan, and R. Levy. 2006. Kinetics of B cell receptor signaling in human B cell subsets mapped by phosphospecific flow cytometry. *J Immunol* 177:1581-1589.
58. Richards, J. D., S. H. Davé, C.-H. G. Chou, A. A. Mamchak, and A. L. DeFranco. 2001. Inhibition of the MEK/ERK Signaling Pathway Blocks a Subset of B Cell Responses to Antigen. *The Journal of Immunology* 166:3855-3864.
59. Piatelli, M. J., C. Doughty, and T. C. Chiles. 2002. Requirement for a hsp90 Chaperone-dependent MEK1/2-ERK Pathway for B Cell Antigen Receptor-induced Cyclin D2 Expression in Mature B Lymphocytes. *Journal of Biological Chemistry* 277:12144-12150.
60. Hauser, J., N. Sveshnikova, A. Wallenius, S. Baradaran, J. Saarikettu, and T. Grundström. 2008. B-cell receptor activation inhibits AID expression through calmodulin inhibition of E-proteins. *Proceedings of the National Academy of Sciences* 105:1267-1272.
61. Niiro, H., and E. A. Clark. 2002. Regulation of B-cell fate by antigen-receptor signals. *Nat Rev Immunol* 2:945-956.
62. Graham, J. P., K. M. Arcipowski, and G. A. Bishop. 2010. Differential B-lymphocyte regulation by CD40 and its viral mimic, latent membrane protein 1. *Immunological Reviews* 237:226-248.
63. Allen, R., R. Armitage, M. Conley, H. Rosenblatt, N. Jenkins, N. Copeland, M. Bedell, S. Edelhoff, C. Disteche, D. Simoneaux, and a. et. 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* 259:990-993.
64. Aruffo, A., M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L. S. Grosmaire, R. Stenkamp, M. Neubauer, R. L. Roberts, R. J. Noelle, J. A. Ledbetter, U. Francke, and H. D. Ochs. 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* 72:291-300.
65. DiSanto, J. P., J. Y. Bonnefoy, J. F. Gauchatt, A. Fischer, and G. d. Saint Basile. 1993. CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature* 361:541-543.
66. Fuleihan, R., N. Ramesh, R. Loh, H. Jabara, R. S. Rosen, T. Chatila, S. M. Fu, I. Stamenkovic, and R. S. Geha. 1993. Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc Natl Acad Sci U S A* 90:2170-2173.
67. Korthauer, U., D. Graf, H. W. Mages, F. Briere, M. Padayachee, S. Malcolm, A. G. Ugazio, L. D. Notarangelo, R. J. Levinsky, and R. A. Kroczek. 1993. Defective

expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* 361:539-541.

68. Saito, M., J. Gao, K. Basso, Y. Kitagawa, P. M. Smith, G. Bhagat, A. Pernis, L. Pasqualucci, and R. Dalla-Favera. 2007. A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. *Cancer Cell* 12:280-292.
69. Kuppers, R. 2005. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer* 5:251-262.
70. Sant, M., C. Allemani, C. Tereanu, R. De Angelis, R. Capocaccia, O. Visser, R. Marcos-Gragera, M. Maynadie, A. Simonetti, J. M. Lutz, and F. Berrino. 2010. Incidence of hematologic malignancies in Europe by morphologic subtype: results of the HAEMACARE project. *Blood* 116:3724-3734.
71. Campo, E., S. H. Swerdlow, N. L. Harris, S. Pileri, H. Stein, and E. S. Jaffe. 2011. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood* 117:5019-5032.
72. Morton, L. M., S. S. Wang, S. S. Devesa, P. Hartge, D. D. Weisenburger, and M. S. Linet. 2006. Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001. *Blood* 107:265-276.
73. Zenz, T., D. Mertens, R. Kuppers, H. Dohner, and S. Stilgenbauer. 2010. From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nat Rev Cancer* 10:37-50.
74. Klein, U., Y. Tu, G. A. Stolovitzky, M. Mattioli, G. Cattoretti, H. Husson, A. Freedman, G. Inghirami, L. Cro, L. Baldini, A. Neri, A. Califano, and R. Dalla-Favera. 2001. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med* 194:1625-1638.
75. Seifert, M., and R. Kuppers. 2009. Molecular footprints of a germinal center derivation of human IgM+(IgD+)CD27+ B cells and the dynamics of memory B cell generation. *J Exp Med* 206:2659-2669.
76. Stevenson, F. K., S. Krysov, A. J. Davies, A. J. Steele, and G. Packham. 2011. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood* 118:4313-4320.
77. Seifert, M., L. Sellmann, J. Bloehdorn, F. Wein, S. Stilgenbauer, J. Dürig, and R. Küppers. 2012. Cellular origin and pathophysiology of chronic lymphocytic leukemia. *The Journal of Experimental Medicine* 209:2183-2198.
78. Pasqualucci, L., A. Migliazza, N. Fracchiolla, C. William, A. Neri, L. Baldini, R. S. K. Chaganti, U. Klein, R. Küppers, K. Rajewsky, and R. Dalla-Favera. 1998. BCL-6 mutations in normal germinal center B cells: Evidence of somatic hypermutation acting outside Ig loci. *Proceedings of the National Academy of Sciences* 95:11816-11821.
79. Seifert, M., and R. Küppers. 2009. Molecular footprints of a germinal center derivation of human IgM+(IgD+)CD27+ B cells and the dynamics of memory B cell generation. *The Journal of Experimental Medicine* 206:2659-2669.
80. Novak, U., K. Basso, L. Pasqualucci, R. Dalla-Favera, and G. Bhagat. 2011. Genomic analysis of non-splenic marginal zone lymphomas (MZL) indicates similarities between nodal and extranodal MZL and supports their derivation from memory B-cells. *Br J Haematol* 155:362-365.
81. Bahler, D. W., J. A. Pindzola, and S. H. Swerdlow. 2002. Splenic marginal zone lymphomas appear to originate from different B cell types. *Am J Pathol* 161:81-88.
82. Anderson, K. C., and R. D. Carrasco. 2011. Pathogenesis of myeloma. *Annu Rev Pathol* 6:249-274.

83. Johnsen, H. E., K. S. Bergkvist, A. Schmitz, M. K. Kjeldsen, S. M. Hansen, M. Gaihede, M. A. Norgaard, J. Baech, M. L. Gronholdt, F. S. Jensen, P. Johansen, J. S. Bodker, M. Bogsted, and K. Dybkaer. 2013. Cell of origin associated classification of B-cell malignancies by gene signatures of the normal B-cell hierarchy. *Leuk Lymphoma* 55:1251-1260.
84. Bakkus, M. H., C. Heirman, I. Van Riet, B. Van Camp, and K. Thielemans. 1992. Evidence that multiple myeloma Ig heavy chain VDJ genes contain somatic mutations but show no intraclonal variation. *Blood* 80:2326-2335.
85. Sahota, S., T. Hamblin, D. G. Oscier, and F. K. Stevenson. 1994. Assessment of the role of clonogenic B lymphocytes in the pathogenesis of multiple myeloma. *Leukemia* 8:1285-1289.
86. Johnsen, H. E., K. S. Bergkvist, A. Schmitz, M. K. Kjeldsen, S. M. Hansen, M. Gaihede, M. A. Nørgaard, J. Bæch, M.-L. Grønholdt, F. S. Jensen, P. Johansen, J. S. Bødker, M. Bøgsted, and K. Dybkær. Cell of origin associated classification of B-cell malignancies by gene signatures of the normal B-cell hierarchy. *Leukemia & Lymphoma* 0:1-10.
87. Dillman, R. O. 2008. Immunophenotyping of chronic lymphoid leukemias. *J Clin Oncol* 26:1193-1194.
88. Dillman, R. O., J. C. Beauregard, J. W. Lea, M. R. Green, R. E. Sobol, and I. Royston. 1983. Chronic lymphocytic leukemia and other chronic lymphoid proliferations: surface marker phenotypes and clinical correlations. *J Clin Oncol* 1:190-197.
89. Hammerstrøm J, and e. al. 2012. Nasjonalt handlingsprogram med retningslinjer for diagnostikk, behandling og oppfølging av maligne blodsykdommer. *Helsedirektoratet*.
90. Tjonnfjord, G. E., B. E. Ly, T. B. Johannesen, A. Tierens, K. Beiske, S. Heim, and V. Jonsson. 2012. Chronic lymphocytic leukaemia in Norway--incidence and prognostic markers at diagnosis. *Tidsskr Nor Laegeforen* 132:2056-2059.
91. Chiorazzi, N., K. R. Rai, and M. Ferrarini. 2005. Chronic lymphocytic leukemia. *N Engl J Med* 352:804-815.
92. Dighiero, G. 2005. CLL Biology and Prognosis. *Hematology Am Soc Hematol Educ Program*:278-284.
93. Keating, M. J., N. Chiorazzi, B. Messmer, R. N. Damle, S. L. Allen, K. R. Rai, M. Ferrarini, and T. J. Kipps. 2003. Biology and treatment of chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program*:153-175.
94. Binet, J. L., A. Auquier, G. Dighiero, C. Chastang, H. Piguet, J. Goasguen, G. Vaugier, G. Potron, P. Colona, F. Oberling, M. Thomas, G. Tchernia, C. Jacquillat, P. Boivin, C. Lesty, M. T. Duault, M. Monconduit, S. Belabbes, and F. Gremy. 1981. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer* 48:198-206.
95. Rai, K. R., A. Sawitsky, E. P. Cronkite, A. D. Chanana, R. N. Levy, and B. S. Pasternack. 1975. Clinical staging of chronic lymphocytic leukemia. *Blood* 46:219-234.
96. Zenz, T., B. Eichhorst, R. Busch, T. Denzel, S. Habe, D. Winkler, A. Buhler, J. Edelmann, M. Bergmann, G. Hopfinger, M. Hensel, M. Hallek, H. Dohner, and S. Stilgenbauer. 2010. TP53 Mutation and Survival in Chronic Lymphocytic Leukemia. *J Clin Oncol* 28:4473-4479.
97. Dighiero, G., K. Maloum, B. Desablens, B. Cazin, M. Navarro, R. Leblay, M. Leporrier, J. Jaubert, G. Lepeu, B. Dreyfus, J.-L. Binet, P. Travade, F.-L. Turpin, G. Tertian, A. Bichoffe, and The French Cooperative Group on Chronic Lymphocytic Leukemia. 1998. Chlorambucil in Indolent Chronic Lymphocytic Leukemia. *N Engl J Med* 338:1506-1514.

98. Fais, F., F. Ghiotto, S. Hashimoto, B. Sellars, A. Valetto, S. L. Allen, P. Schulman, V. P. Vinciguerra, K. Rai, L. Z. Rassenti, T. J. Kipps, G. Dighiero, H. W. Schroeder, Jr., M. Ferrarini, and N. Chiorazzi. 1998. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest* 102:1515-1525.
99. Damle, R. N., T. Wasil, F. Fais, F. Ghiotto, A. Valetto, S. L. Allen, A. Buchbinder, D. Budman, K. Dittmar, J. Kolitz, S. M. Lichtman, P. Schulman, V. P. Vinciguerra, K. R. Rai, M. Ferrarini, and N. Chiorazzi. 1999. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 94:1840-1847.
100. Hamblin, T. J., Z. Davis, A. Gardiner, D. G. Oscier, and F. K. Stevenson. 1999. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 94:1848-1854.
101. Maloum, K., F. Davi, H. Merle-Beral, O. Pritsch, C. Magnac, F. Vuillier, G. Dighiero, X. Troussard, F. F. Mauro, and J. Benichou. 2000. Expression of unmutated VH genes is a detrimental prognostic factor in chronic lymphocytic leukemia. *Blood* 96:377-379.
102. Chen, L., G. Widhopf, L. Huynh, L. Rassenti, K. R. Rai, A. Weiss, and T. J. Kipps. 2002. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood* 100:4609-4614.
103. Crespo, M., F. Bosch, N. Villamor, B. Bellosillo, D. Colomer, M. Rozman, S. Marcé, A. López-Guillermo, E. Campo, and E. Montserrat. 2003. ZAP-70 Expression as a Surrogate for Immunoglobulin-Variable-Region Mutations in Chronic Lymphocytic Leukemia. *New England Journal of Medicine* 348:1764-1775.
104. Wiestner, A., A. Rosenwald, T. S. Barry, G. Wright, R. E. Davis, S. E. Henrickson, H. Zhao, R. E. Ibbotson, J. A. Orchard, Z. Davis, M. Stetler-Stevenson, M. Raffeld, D. C. Arthur, G. E. Marti, W. H. Wilson, T. J. Hamblin, D. G. Oscier, and L. M. Staudt. 2003. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood* 101:4944-4951.
105. Orchard, J. A., R. E. Ibbotson, Z. Davis, A. Wiestner, A. Rosenwald, P. W. Thomas, T. J. Hamblin, L. M. Staudt, and D. G. Oscier. 2004. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. *The Lancet* 363:105-111.
106. Rassenti, L. Z., L. Huynh, T. L. Toy, L. Chen, M. J. Keating, J. G. Gribben, D. S. Neuberg, I. W. Flinn, K. R. Rai, J. C. Byrd, N. E. Kay, A. Greaves, A. Weiss, and T. J. Kipps. 2004. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* 351:893-901.
107. Chen, L., L. Huynh, J. Apgar, L. Tang, L. Rassenti, A. Weiss, and T. J. Kipps. 2008. ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. *Blood* 111:2685-2692.
108. Brachtl, G., J. Piñón Hofbauer, R. Greil, and T. Hartmann. 2014. The pathogenic relevance of the prognostic markers CD38 and CD49d in chronic lymphocytic leukemia. *Annals of Hematology* 93:361-374.
109. Friedberg, J. W., J. Sharman, J. Sweetenham, P. B. Johnston, J. M. Vose, A. LaCasce, J. Schaefer-Cutillo, S. De Vos, R. Sinha, J. P. Leonard, L. D. Cripe, S. A. Gregory, M. P. Sterba, A. M. Lowe, R. Levy, and M. A. Shipp. 2010. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* 115:2578-2585.
110. Byrd, J. C., R. R. Furman, S. E. Coutre, I. W. Flinn, J. A. Burger, K. A. Blum, B. Grant, J. P. Sharman, M. Coleman, W. G. Wierda, J. A. Jones, W. Zhao, N. A.

Heerema, A. J. Johnson, J. Sukbuntherng, B. Y. Chang, F. Clow, E. Hedrick, J. J. Buggy, D. F. James, and S. O'Brien. 2013. Targeting BTK with Ibrutinib in Relapsed Chronic Lymphocytic Leukemia. *New England Journal of Medicine* 369:32-42.

111. Brown JR, B. J., Coutre SE, Benson DM, Flinn IW, Wagner-Johnston ND, Spurgeon SE, Kahl BS, Bello C, Webb HK, Johnson DM, Peterman S, Li D, Jahn TM, Lannutti BJ, Ulrich RG, Yu AS, Miller LL, Furman RR. 2014. Idelalisib, an inhibitor of phosphatidylinositol 3-kinase p110δ, for relapsed/refractory chronic lymphocytic leukemia. *Blood* 123:3390-3397.
112. de Jong, D., B. M. Voetdijk, G. J. Van Ommen, J. C. Kluin-Nelemans, G. C. Beverstock, and P. M. Kluin. 1989. Translocation t(14;18) in B cell lymphomas as a cause for defective immunoglobulin production. *The Journal of Experimental Medicine* 169:613-624.
113. Cleary, M. L., T. C. Meeker, S. Levy, E. Lee, M. Trela, J. Sklar, and R. Levy. 1986. Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. *Cell* 44:97-106.
114. Meeker, T., J. Lowder, M. L. Cleary, S. Stewart, R. Warnke, J. Sklar, and R. Levy. 1985. Emergence of Idiotype Variants during Treatment of B-Cell Lymphoma with Anti-Idiotype Antibodies. *New England Journal of Medicine* 312:1658-1665.
115. Gunven, P., G. Klein, E. Klein, T. Norin, and S. Singh. 1980. Surface immunoglobulins on Burkitt's lymphoma biopsy cells from 91 patients. *Int J Cancer* 25:711-719.
116. Segal, G. H., M. G. Edinger, M. Owen, M. McNealis, P. Lopez, A. Perkins, M. D. Linden, A. J. Fishleder, M. H. Stoler, and R. R. Tubbs. 1991. Concomitant delineation of surface Ig, B-cell differentiation antigens, and HLADR on lymphoid proliferations using three-color immunocytometry. *Cytometry* 12:350-359.
117. Yano T, v. K. J., Magrath IT, Longo DL, Jaffe ES, Raffeld M. 1992. Histogenetic correlations between subcategories of small noncleaved cell lymphomas. *Blood* 79:1282-1290.
118. Gururajan, M., C. D. Jennings, and S. Bondada. 2006. Cutting Edge: Constitutive B Cell Receptor Signaling Is Critical for Basal Growth of B Lymphoma. *J Immunol* 176:5715-5719.
119. Hivroz, C., B. Geny, J. C. Brouet, and C. Grillot-Courvalin. 1990. Altered signal transduction secondary to surface IgM cross-linking on B-chronic lymphocytic leukemia cells. Differential activation of the phosphatidylinositol-specific phospholipase C. *J Immunol* 144:2351-2358.
120. Lankester, A. C., G. M. van Schijndel, C. E. van der Schoot, M. H. van Oers, C. J. van Noesel, and R. A. van Lier. 1995. Antigen receptor nonresponsiveness in chronic lymphocytic leukemia B cells. *Blood* 86:1090-1097.
121. Michel, F., H. Merle-Beral, E. Legac, A. Michel, P. Debre, and G. Bismuth. 1993. Defective calcium response in B-chronic lymphocytic leukemia cells. Alteration of early protein tyrosine phosphorylation and of the mechanism responsible for cell calcium influx. *J Immunol* 150:3624-3633.
122. Semichon, M., H. Merle-Beral, V. Lang, and G. Bismuth. 1997. Normal Syk protein level but abnormal tyrosine phosphorylation in B-CLL cells. *Leukemia* 11:1921-1928.
123. D'Arena, G., N. Cascavilla, P. Musto, R. Colella Bisogno, G. Pistolese, and M. Carotenuto. 2000. CD79b expression in B-cell chronic lymphocytic leukemia. *Haematologica* 85:556-557.
124. Thompson, A. A., Talley JA, Do HN, Kagan HL, Kunkel L, Berenson J, Cooper MD, Saxon A, Wall R. 1997. Aberrations of the B-Cell Receptor B29 (CD79b) Gene in Chronic Lymphocytic Leukemia. *Blood* 90:1387-1394.

125. Alfarano A, I. S., Circosta P, Minuzzo S, Vallario A, Zamarchi R, Fregonese A, Calderazzo F, Faldella A, Aragno M, Camaschella C, Amadori A, Caligaris-Cappio F. 1999. An Alternatively Spliced Form of CD79b Gene May Account for Altered B-Cell Receptor Expression in B-Chronic Lymphocytic Leukemia. *Blood* 93:2327-2335.
126. Lewis, R. E., J. M. Cruse, S. Pierce, J. Lam, and Y. Tadros. 2005. Surface and cytoplasmic immunoglobulin expression in B-cell chronic lymphocytic leukemia (CLL). *Experimental and Molecular Pathology* 79:146-150.
127. Minuzzo, S., S. Indraccolo, V. Tosello, E. Piovan, A. Cabrelle, L. Trentin, G. Semenzato, and A. Amadori. 2005. Heterogeneous intracellular expression of B-cell receptor components in B-cell chronic lymphocytic leukaemia (B-CLL) cells and effects of CD79b gene transfer on surface immunoglobulin levels in a B-CLL-derived cell line. *British Journal of Haematology* 130:878-889.
128. Vuillier, F., G. Dumas, C. Magnac, M.-C. Prevost, A. I. Lalanne, P. Oppezzo, E. Melanitou, G. Dighiero, and B. Payelle-Brogard. 2005. Lower levels of surface B-cell-receptor expression in chronic lymphocytic leukemia are associated with glycosylation and folding defects of the {micro} and CD79a chains. *Blood* 105:2933-2940.
129. Lanham, S., T. Hamblin, D. Oscier, R. Ibbotson, F. Stevenson, and G. Packham. 2003. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. *Blood* 101:1087-1093.
130. Guarini, A., S. Chiaretti, S. Tavolaro, R. Maggio, N. Peragine, F. Citarella, M. R. Ricciardi, S. Santangelo, M. Marinelli, M. S. De Propris, M. Messina, F. R. Mauro, I. Del Giudice, and R. Foa. 2008. BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgV H unmutated chronic lymphocytic leukemia (CLL) cells. *Blood* 112:782-792.
131. Ian Mockridge, C., K. N. Potter, I. Wheatley, L. A. Neville, G. Packham, and F. K. Stevenson. 2007. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood* 109:4424-4431.
132. Chen, L., J. Apgar, L. Huynh, F. Dicker, T. Giago-McGahan, L. Rassenti, A. Weiss, and T. J. Kipps. 2005. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. *Blood* 105:2036-2041.
133. Ranheim, E. A., and T. J. Kipps. 1993. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J Exp Med* 177:925-935.
134. Berberich, I., G. Shu, and E. Clark. 1994. Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. *J Immunol* 153:4357-4366.
135. Lalmanach-Girard, A. C., T. C. Chiles, D. C. Parker, and T. L. Rothstein. 1993. T cell-dependent induction of NF-kappa B in B cells. *J Exp Med* 177:1215-1219.
136. Sutherland, C., A. Heath, S. Pelech, P. Young, and M. Gold. 1996. Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. *J Immunol* 157:3381-3390.
137. Cuni, S., P. Perez-Aciego, G. Perez-Chacon, J. A. Vargas, A. Sanchez, F. M. Martin-Saavedra, S. Ballester, J. Garcia-Marco, J. Jorda, and A. Durantez. 2004. A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. *Leukemia* 18:1391-1400.
138. Gricks, C. S., D. Zahrieh, A. J. Zauls, G. Gorgun, D. Drandi, K. Mauerer, D. Neuberg, and J. G. Gribben. 2004. Differential regulation of gene expression following CD40 activation of leukemic compared to healthy B cells. *Blood* 104:4002-4009.
139. Romano, M. F., A. Lamberti, P. Tassone, F. Alfinito, S. Costantini, F. Chiurazzi, T. Defrance, P. Bonelli, F. Tuccillo, M. C. Turco, and S. Venuta. 1998. Triggering of

CD40 Antigen Inhibits Fludarabine-Induced Apoptosis in B Chronic Lymphocytic Leukemia Cells. *Blood* 92:990-995.

140. Mazloom, A., L. J. Medeiros, P. W. McLaughlin, V. Reed, F. F. Cabanillas, L. E. Fayad, B. Pro, G. Gonzalez, P. Iyengar, D. L. Urbauer, and B. S. Dabaja. 2010. Marginal zone lymphomas. *Cancer* 116:4291-4298.
141. Swerdlow, S. H., and International Agency for Research on Cancer. 2008. *WHO classification of tumours of haematopoietic and lymphoid tissue*. IARC, Lyon.
142. Matutes, E., D. Oscier, C. Montalban, F. Berger, E. Callet-Bauchu, A. Dogan, P. Felman, V. Franco, E. Iannitto, M. Mollejo, T. Papadaki, E. D. Remstein, A. Salar, F. Sole, K. Stamatopoulos, C. Thieblemont, A. Traverse-Glehen, A. Wotherspoon, B. Coiffier, and M. A. Piris. 2007. Splenic marginal zone lymphoma proposals for a revision of diagnostic, staging and therapeutic criteria. *Leukemia* 22:487-495.
143. Ferreri, A. J. M., and E. Zucca. 2007. Marginal-zone lymphoma. *Critical Reviews in Oncology/Hematology* 63:245-256.
144. Heilgeist, A., F. McClanahan, A. D. Ho, and M. Witzens-Harig. 2013. Prognostic value of the Follicular Lymphoma International Prognostic Index score in marginal zone lymphoma: an analysis of clinical presentation and outcome in 144 patients. *Cancer* 119:99-106.
145. Montalban, C., V. Abraira, L. Arcaini, E. Domingo-Domenech, P. Guisado-Vasco, E. Iannitto, M. Mollejo, E. Matutes, A. Ferreri, A. Salar, S. Rattotti, A. Carpaneto, R. Perez Fernandez, J. L. Bello, M. Hernandez, D. Caballero, F. Carbonell, and M. A. Piris. 2012. Risk stratification for Splenic Marginal Zone Lymphoma based on haemoglobin concentration, platelet count, high lactate dehydrogenase level and extrahilar lymphadenopathy: development and validation on 593 cases. *Br J Haematol* 159:164-171.
146. Salido, M., C. Baro, D. Oscier, K. Stamatopoulos, J. Dierlamm, E. Matutes, A. Traverse-Glehen, F. Berger, P. Felman, C. Thieblemont, S. Gesk, A. Athanasiadou, Z. Davis, A. Gardiner, F. Milla, A. Ferrer, M. Mollejo, M. J. Calasanz, L. Florensa, B. Espinet, E. Luno, I. Wlodarska, G. Verhoef, M. Garcia-Granero, A. Salar, T. Papadaki, S. Serrano, M. A. Piris, and F. Sole. 2010. Cytogenetic aberrations and their prognostic value in a series of 330 splenic marginal zone B-cell lymphomas: a multicenter study of the Splenic B-Cell Lymphoma Group. *Blood* 116:1479-1488.
147. Sutton, L. A., A. Agathangelidis, C. Belessi, N. Darzentas, F. Davi, P. Ghia, R. Rosenquist, and K. Stamatopoulos. 2013. Antigen selection in B-cell lymphomas--tracing the evidence. *Semin Cancer Biol* 23:399-409.
148. Niemann, C. U., and A. Wiestner. 2013. B-cell receptor signaling as a driver of lymphoma development and evolution. *Semin Cancer Biol* 23:410-421.
149. Yan, Q., Y. Huang, A. J. Watkins, S. Kocialkowski, N. Zeng, R. A. Hamoudi, P. G. Isaacson, L. de Leval, A. Wotherspoon, and M. Q. Du. 2012. BCR and TLR signaling pathways are recurrently targeted by genetic changes in splenic marginal zone lymphomas. *Haematologica* 97:595-598.
150. Carreras, J., N. Villamor, L. Colomo, C. Moreno, S. R. y. Cajal, M. Crespo, F. Tort, F. Bosch, A. López-Guillermo, D. Colomer, E. Montserrat, and E. Campo. 2005. Immunohistochemical analysis of ZAP-70 expression in B-cell lymphoid neoplasms. *The Journal of Pathology* 205:507-513.
151. Rinaldi, A., F. Forconi, L. Arcaini, M. Mian, E. Sozzi, S. Zibellini, L. Baldini, S. Franceschetti, G. Gaidano, R. Marasca, M. Mollejo, M. A. Piris, A. Tucci, F. Facchetti, G. Bhagat, R. D. Favera, P. M. Rancoita, E. Zucca, I. Kwee, and F. Bertoni. 2010. Immunogenetics features and genomic lesions in splenic marginal zone lymphoma. *Br J Haematol* 151:435-439.

152. Gopal, A. K., B. S. Kahl, S. de Vos, N. D. Wagner-Johnston, S. J. Schuster, W. J. Jurczak, I. W. Flinn, C. R. Flowers, P. Martin, A. Viardot, K. A. Blum, A. H. Goy, A. J. Davies, P. L. Zinzani, M. Dreyling, D. Johnson, L. L. Miller, L. Holes, D. Li, R. D. Dansey, W. R. Godfrey, and G. A. Salles. 2014. PI3Kdelta inhibition by idelalisib in patients with relapsed indolent lymphoma. *N Engl J Med* 370:1008-1018.
153. Palumbo, A., and K. Anderson. 2011. Multiple Myeloma. *New England Journal of Medicine* 364:1046-1060.
154. Mahindra, A., T. Hideshima, and K. C. Anderson. 2010. Multiple myeloma: biology of the disease. *Blood Reviews* 24, Supplement 1:S5-S11.
155. Solly, S. 1844. Remarks on the pathology of mollities ossium; with cases. *Med Chir Trans* 27:435-498 438.
156. Kyle, R. A., and S. V. Rajkumar. 2008. Multiple myeloma. *Blood* 111:2962-2972.
157. Cancer Registry of Norway. Cancer Registry of Norway. Cancer in Norway 2011 - Cancer incidence, mortality, survival and prevalence in Norway.
Oslo: .
158. Cavo, M., S. V. Rajkumar, A. Palumbo, P. Moreau, R. Orlowski, J. Bladé, O. Sezer, H. Ludwig, M. A. Dimopoulos, M. Attal, P. Sonneveld, M. Boccadoro, K. C. Anderson, P. G. Richardson, W. Bensinger, H. E. Johnsen, N. Kroeger, G. Gahrton, P. L. Bergsagel, D. H. Vesole, H. Einsele, S. Jagannath, R. Niesvizky, B. G. M. Durie, J. San Miguel, and S. Lonial. 2011. International Myeloma Working Group consensus approach to the treatment of multiple myeloma patients who are candidates for autologous stem cell transplantation. *Blood* 117:6063-6073.
159. Neben, K., A. Jauch, U. Bertsch, C. Heiss, T. Hielscher, A. Seckinger, T. Mors, N. Z. Muller, J. Hillengass, M. S. Raab, A. D. Ho, D. Hose, and H. Goldschmidt. 2010. Combining information regarding chromosomal aberrations t(4;14) and del(17p13) with the International Staging System classification allows stratification of myeloma patients undergoing autologous stem cell transplantation. *Haematologica* 95:1150-1157.
160. Ludwig, H., V. Bolejack, J. Crowley, J. Blade, J. S. Miguel, R. A. Kyle, S. V. Rajkumar, K. Shimizu, I. Turesson, J. Westin, P. Sonneveld, M. Cavo, M. Boccadoro, A. Palumbo, P. Tosi, J. L. Harousseau, M. Attal, B. Barlogie, A. K. Stewart, and B. Durie. 2010. Survival and years of life lost in different age cohorts of patients with multiple myeloma. *J Clin Oncol* 28:1599-1605.
161. Avet-Loiseau, H., M. Attal, L. Campion, D. Caillot, C. Hulin, G. Marit, A. M. Stoppa, L. Voillat, M. Wetterwald, B. Pegourie, E. Voog, M. Tiab, A. Banos, J. Jaubert, D. Bouscary, M. Macro, B. Kolb, C. Traulle, C. Mathiot, F. Magrangeas, S. Minvielle, T. Facon, and P. Moreau. 2012. Long-term analysis of the IFM 99 trials for myeloma: cytogenetic abnormalities [t(4;14), del(17p), 1q gains] play a major role in defining long-term survival. *J Clin Oncol* 30:1949-1952.
162. Greipp, P. R., J. S. Miguel, B. G. M. Durie, J. J. Crowley, B. Barlogie, J. Bladé, M. Boccadoro, J. A. Child, H. Avet-Loiseau, R. A. Kyle, J. J. Lahuerta, H. Ludwig, G. Morgan, R. Powles, K. Shimizu, C. Shustik, P. Sonneveld, P. Tosi, I. Turesson, and J. Westin. 2005. International Staging System for Multiple Myeloma. *Journal of Clinical Oncology* 23:3412-3420.
163. Avet-Loiseau, H. 2007. Role of genetics in prognostication in myeloma. *Best Practice & Research Clinical Haematology* 20:625-635.
164. Fonseca, R., P. L. Bergsagel, J. Drach, J. Shaughnessy, N. Gutierrez, A. K. Stewart, G. Morgan, B. Van Ness, M. Chesi, S. Minvielle, A. Neri, B. Barlogie, W. M. Kuehl, P. Liebisch, F. Davies, S. Chen-Kiang, B. G. Durie, R. Carrasco, O. Sezer, T. Reiman, L. Pilarski, and H. Avet-Loiseau. 2009. International Myeloma Working Group

molecular classification of multiple myeloma: spotlight review. *Leukemia* 23:2210-2221.

165. Munshi, N. C., K. C. Anderson, P. L. Bergsagel, J. Shaughnessy, A. Palumbo, B. Durie, R. Fonseca, A. K. Stewart, J. L. Harousseau, M. Dimopoulos, S. Jagannath, R. Hajek, O. Sezer, R. Kyle, P. Sonneveld, M. Cavo, S. V. Rajkumar, J. San Miguel, J. Crowley, and H. Avet-Loiseau. 2011. Consensus recommendations for risk stratification in multiple myeloma: report of the International Myeloma Workshop Consensus Panel 2. *Blood* 117:4696-4700.
166. Boyd, K. D., F. M. Ross, L. Chiecchio, G. P. Dagrada, Z. J. Konn, W. J. Tapper, B. A. Walker, C. P. Wardell, W. M. Gregory, A. J. Szubert, S. E. Bell, J. A. Child, G. H. Jackson, F. E. Davies, and G. J. Morgan. 2012. A novel prognostic model in myeloma based on co-segregating adverse FISH lesions and the ISS: analysis of patients treated in the MRC Myeloma IX trial. *Leukemia* 26:349-355.
167. Avet-Loiseau, H., B. G. Durie, M. Cavo, M. Attal, N. Gutierrez, J. Haessler, H. Goldschmidt, R. Hajek, J. H. Lee, O. Sezer, B. Barlogie, J. Crowley, R. Fonseca, N. Testoni, F. Ross, S. V. Rajkumar, P. Sonneveld, J. Lahuerta, P. Moreau, and G. Morgan. 2013. Combining fluorescent in situ hybridization data with ISS staging improves risk assessment in myeloma: an International Myeloma Working Group collaborative project. *Leukemia* 27:711-717.
168. Vacca, A., D. Ribatti, L. Roncali, G. Ranieri, G. Serio, F. Silvestris, and F. Dammacco. 1994. Bone marrow angiogenesis and progression in multiple myeloma. *Br J Haematol* 87:503-508.
169. Vacca, A., D. Ribatti, M. Presta, M. Minischetti, M. Iurlaro, R. Ria, A. Albini, F. Bussolino, and F. Dammacco. 1999. Bone Marrow Neovascularization, Plasma Cell Angiogenic Potential, and Matrix Metalloproteinase-2 Secretion Parallel Progression of Human Multiple Myeloma. *Blood* 93:3064-3073.
170. Cardesa-Salzmann, T. M., L. Colomo, G. Gutierrez, W. C. Chan, D. Weisenburger, F. Climent, E. Gonzalez-Barca, S. Mercadal, L. Arenillas, S. Serrano, R. Tubbs, J. Delabie, R. D. Gascoyne, J. M. Connors, J. L. Mate, L. Rimsza, R. Braziel, A. Rosenwald, G. Lenz, G. Wright, E. S. Jaffe, L. Staudt, P. Jares, A. Lopez-Guillermo, and E. Campo. 2011. High microvessel density determines a poor outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus chemotherapy. *Haematologica* 96:996-1001.
171. Jorgensen, J. M., F. B. Sorensen, K. Bendix, J. L. Nielsen, M. L. Olsen, A. M. Funder, and F. d'Amore. 2007. Angiogenesis in non-Hodgkin's lymphoma: clinico-pathological correlations and prognostic significance in specific subtypes. *Leuk Lymphoma* 48:584-595.
172. Abe, M. 2011. Targeting the interplay between myeloma cells and the bone marrow microenvironment in myeloma. *Int J Hematol* 94:334-343.
173. Chen, Z., R. Z. Orlowski, M. Wang, L. Kwak, and N. McCarty. 2014. Osteoblastic niche supports the growth of quiescent multiple myeloma cells. *Blood* 123:2204-2208.
174. D'Amato, R. J., M. S. Loughnan, E. Flynn, and J. Folkman. 1994. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci U S A* 91:4082-4085.
175. Singhal, S., J. Mehta, R. Desikan, D. Ayers, P. Roberson, P. Eddlemon, N. Munshi, E. Anaissie, C. Wilson, M. Dhodapkar, J. Zeddis, and B. Barlogie. 1999. Antitumor activity of thalidomide in refractory multiple myeloma. *N Engl J Med* 341:1565-1571.
176. Quach, H., D. Ritchie, A. K. Stewart, P. Neeson, S. Harrison, M. J. Smyth, and H. M. Prince. 2010. Mechanism of action of immunomodulatory drugs (IMiDS) in multiple myeloma. *Leukemia* 24:22-32.

177. Attal, M., V. Lauwers-Cances, G. Marit, D. Caillot, P. Moreau, T. Facon, A. M. Stoppa, C. Hulin, L. Benboubker, L. Garderet, O. Decaux, S. Leyvraz, M. C. Vekemans, L. Voillat, M. Michallet, B. Pegourie, C. Dumontet, M. Roussel, X. Leleu, C. Mathiot, C. Payen, H. Avet-Loiseau, and J. L. Harousseau. 2012. Lenalidomide maintenance after stem-cell transplantation for multiple myeloma. *N Engl J Med* 366:1782-1791.
178. McCarthy, P. L., K. Owzar, C. C. Hofmeister, D. D. Hurd, H. Hassoun, P. G. Richardson, S. Giralt, E. A. Stadtmauer, D. J. Weisdorf, R. Vij, J. S. Moreb, N. S. Callander, K. Van Besien, T. Gentile, L. Isola, R. T. Maziarz, D. A. Gabriel, A. Bashey, H. Landau, T. Martin, M. H. Qazilbash, D. Levitan, B. McClune, R. Schlossman, V. Hars, J. Postiglione, C. Jiang, E. Bennett, S. Barry, L. Bressler, M. Kelly, M. Seiler, C. Rosenbaum, P. Hari, M. C. Pasquini, M. M. Horowitz, T. C. Shea, S. M. Devine, K. C. Anderson, and C. Linker. 2012. Lenalidomide after stem-cell transplantation for multiple myeloma. *N Engl J Med* 366:1770-1781.
179. Chng, W. J., A. Dispenzieri, C. S. Chim, R. Fonseca, H. Goldschmidt, S. Lentzsch, N. Munshi, A. Palumbo, J. S. Miguel, P. Sonneveld, M. Cavo, S. Usmani, B. G. Durie, and H. Avet-Loiseau. 2014. IMWG consensus on risk stratification in multiple myeloma. *Leukemia* 28:269-277.
180. Asahara, T., T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, and J. M. Isner. 1997. Isolation of Putative Progenitor Endothelial Cells for Angiogenesis. *Science* 275:964-966.
181. Lyden, D., K. Hattori, S. Dias, C. Costa, P. Blaikie, L. Butros, A. Chadburn, B. Heissig, W. Marks, L. Witte, Y. Wu, D. Hicklin, Z. Zhu, N. R. Hackett, R. G. Crystal, M. A. Moore, K. A. Hajjar, K. Manova, R. Benezra, and S. Rafii. 2001. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 7:1194-1201.
182. Kastan, M., E. Schlaffer, J. Russo, O. Colvin, C. Civin, and J. Hilton. 1990. Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood* 75:1947-1950.
183. Hilton, J. 1984. Role of Aldehyde Dehydrogenase in Cyclophosphamide-resistant L1210 Leukemia. *Cancer Research* 44:5156-5160.
184. Sladek, N. E., and G. J. Landkamer. 1985. Restoration of Sensitivity to Oxazaphosphorines by Inhibitors of Aldehyde Dehydrogenase Activity in Cultured Oxazaphosphorine-resistant L1210 and Cross-Linking Agent-resistant P388 Cell Lines. *Cancer Research* 45:1549-1555.
185. Storms, R. W., A. P. Trujillo, J. B. Springer, L. Shah, O. M. Colvin, S. M. Ludeman, and C. Smith. 1999. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci U S A* 96:9118-9123.
186. Capoccia, B. J., D. L. Robson, K. D. Levac, D. J. Maxwell, S. A. Hohm, M. J. Neelamkavil, G. I. Bell, A. Xenocostas, D. C. Link, D. Piwnica-Worms, J. A. Nolta, and D. A. Hess. 2009. Revascularization of ischemic limbs after transplantation of human bone marrow cells with high aldehyde dehydrogenase activity. *Blood* 113:5340-5351.
187. Hess, D. A., T. E. Meyerrose, L. Wirthlin, T. P. Craft, P. E. Herrbrich, M. H. Creer, and J. A. Nolta. 2004. Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. *Blood* 104:1648-1655.
188. Hess, D. A., L. Wirthlin, T. P. Craft, P. E. Herrbrich, S. A. Hohm, R. Lahey, W. C. Eades, M. H. Creer, and J. A. Nolta. 2006. Selection based on CD133 and high

aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. *Blood* 107:2162-2169.

189. Povsic, T. J., K. L. Zavodni, F. L. Kelly, S. Zhu, P. J. Goldschmidt-Clermont, C. Dong, and E. D. Peterson. 2007. Circulating Progenitor Cells Can Be Reliably Identified on the Basis of Aldehyde Dehydrogenase Activity. *Journal of the American College of Cardiology* 50:2243-2248.
190. Povsic, T., S. Najjar, K. Prather, J. Zhou, S. Adams, K. Zavodni, F. Kelly, L. Melton, V. Hasselblad, J. Heitner, S. Raman, G. Barsness, M. Patel, R. Kim, E. Lakatta, R. Harrington, and S. Rao. 2013. EPC mobilization after erythropoietin treatment in acute ST-elevation myocardial infarction: the REVEAL EPC substudy. *J Thromb Thrombolysis* 36:1-9.
191. Ramsay, A. G., A. J. Johnson, A. M. Lee, G. Gorgun, R. Le Dieu, W. Blum, J. C. Byrd, and J. G. Gribben. 2008. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. *J Clin Invest* 118:2427-2437.
192. Ramsay, A. G., A. J. Clear, G. Kelly, R. Fatah, J. Matthews, F. MacDougall, T. A. Lister, A. M. Lee, M. Calaminici, and J. G. Gribben. 2009. Follicular lymphoma cells induce T-cell immunologic synapse dysfunction that can be repaired with lenalidomide: implications for the tumor microenvironment and immunotherapy. *Blood* 114:4713-4720.
193. Riches, J. C., J. K. Davies, F. McClanahan, R. Fatah, S. Iqbal, S. Agrawal, A. G. Ramsay, and J. G. Gribben. 2013. T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. *Blood* 121:1612-1621.
194. Pascutti, M. F., M. Jak, J. M. Tromp, I. A. M. Derks, E. B. M. Remmerswaal, R. Thijssen, M. H. A. van Attekum, G. G. van Bochove, D. M. Luijks, S. T. Pals, R. A. W. van Lier, A. P. Kater, M. H. J. van Oers, and E. Eldering. 2013. IL-21 and CD40L signals from autologous T cells can induce antigen-independent proliferation of CLL cells. *Blood* 122:3010-3019.
195. Irish, J. M., J. H. Myklebust, A. A. Alizadeh, R. Houot, J. P. Sharman, D. K. Czerwinski, G. P. Nolan, and R. Levy. 2010. B-cell signaling networks reveal a negative prognostic human lymphoma cell subset that emerges during tumor progression. *Proc Natl Acad Sci U S A* 107:12747-12754.
196. Myklebust, J. H., J. M. Irish, J. Brody, D. K. Czerwinski, R. Houot, H. E. Kohrt, J. Timmerman, J. Said, M. R. Green, J. Delabie, A. Kolstad, A. A. Alizadeh, and R. Levy. 2013. High PD-1 expression and suppressed cytokine signaling distinguish T cells infiltrating follicular lymphoma tumors from peripheral T cells. *Blood* 121:1367-1376.
197. Gleissner, B., R. Küppers, R. Siebert, B. Glass, L. Trümper, W. Hiddemann, and M. Dreyling. 2008. Report of a workshop on malignant lymphoma: a review of molecular and clinical risk profiling. *British Journal of Haematology* 142:166-178.
198. O'Shea, D., C. O'Riain, C. Taylor, R. Waters, E. Carlotti, F. MacDougall, J. Gribben, A. Rosenwald, G. Ott, L. M. Rimsza, E. B. Smeland, N. Johnson, E. Campo, T. C. Greiner, W. C. Chan, R. D. Gascoyne, G. Wright, L. M. Staudt, T. A. Lister, and J. Fitzgibbon. 2008. The presence of TP53 mutation at diagnosis of follicular lymphoma identifies a high-risk group of patients with shortened time to disease progression and poorer overall survival. *Blood* 112:3126-3129.
199. Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, R. Levy, W. Wilson, M. R. Grever, J. C.

Byrd, D. Botstein, P. O. Brown, and L. M. Staudt. 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403:503-511.

200. Shipp, M. A. 1993. A Predictive Model for Aggressive Non-Hodgkin's Lymphoma. *New England Journal of Medicine* 329:987-994.
201. Solal-Céligny, P., P. Roy, P. Colombat, J. White, J. O. Armitage, R. Arranz-Saez, W. Y. Au, M. Bellei, P. Brice, D. Caballero, B. Coiffier, E. Conde-Garcia, C. Doyen, M. Federico, R. I. Fisher, J. F. Garcia-Conde, C. Guglielmi, A. Hagenbeek, C. Haïoun, M. LeBlanc, A. T. Lister, A. Lopez-Guillermo, P. McLaughlin, N. Milpied, P. Morel, N. Mounier, S. J. Proctor, A. Rohatiner, P. Smith, P. Soubeyran, H. Tilly, U. Vitolo, P.-L. Zinzani, E. Zucca, and E. Montserrat. 2004. Follicular Lymphoma International Prognostic Index. *Blood* 104:1258-1265.
202. Hoster, E., M. Dreyling, W. Klapper, C. Gisselbrecht, A. van Hoof, H. C. Kluin-Nelemans, M. Pfreundschuh, M. Reiser, B. Metzner, H. Einsele, N. Peter, W. Jung, B. Wormann, W. D. Ludwig, U. Duhrsen, H. Eimermacher, H. Wandt, J. Hasford, W. Hiddemann, and M. Unterhalt. 2008. A new prognostic index (MIPI) for patients with advanced-stage mantle cell lymphoma. *Blood* 111:558-565.
203. Kwong, Y.-L. 2007. Predicting the outcome in non-Hodgkin lymphoma with molecular markers. *British Journal of Haematology* 137:273-287.
204. Fink, A. M., S. Bottcher, M. Ritgen, K. Fischer, N. Pflug, B. Eichhorst, C. M. Wendtner, D. Winkler, A. Buhler, T. Zenz, P. Staib, J. Mayer, M. Hensel, G. Hopfinger, M. Wenger, G. Fingerle-Rowson, H. Dohner, M. Kneba, S. Stilgenbauer, R. Busch, and M. Hallek. 2013. Prediction of poor outcome in CLL patients following first-line treatment with fludarabine, cyclophosphamide and rituximab. *Leukemia* 27:1949-1952.
205. Zenz, T., D. Mertens, and S. Stilgenbauer. 2010. Biological diversity and risk-adapted treatment of chronic lymphocytic leukemia. *Haematologica* 95:1441-1443.
206. Best, O. G., A. C. Gardiner, Z. A. Davis, I. Tracy, R. E. Ibbotson, A. Majid, M. J. Dyer, and D. G. Oscier. 2009. A subset of Binet stage A CLL patients with TP53 abnormalities and mutated IGHV genes have stable disease. *Leukemia* 23:212-214.
207. Krutzik, P. O., and G. P. Nolan. 2006. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat Methods* 3:361-368.
208. Schemper, M., and T. L. Smith. 1996. A note on quantifying follow-up in studies of failure time. *Controlled Clinical Trials* 17:343-346.
209. Herzenberg, L. A., D. Parks, B. Sahaf, O. Perez, M. Roederer, and L. A. Herzenberg. 2002. The History and Future of the Fluorescence Activated Cell Sorter and Flow Cytometry: A View from Stanford. *Clinical Chemistry* 48:1819-1827.
210. Perfetto, S. P., P. K. Chattopadhyay, and M. Roederer. 2004. Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* 4:648-655.
211. Ormerod, M. G. 2002. Investigating the relationship between the cell cycle and apoptosis using flow cytometry. *J Immunol Methods* 265:73-80.
212. Pala, P., T. Hussell, and P. J. Openshaw. 2000. Flow cytometric measurement of intracellular cytokines. *J Immunol Methods* 243:107-124.
213. Ruitenberg, J. J., and C. A. Waters. 2003. A rapid flow cytometric method for the detection of intracellular cyclooxygenases in human whole blood monocytes and a COX-2 inducible human cell line. *J Immunol Methods* 274:93-104.
214. Cao, S., S. D. Hudnall, F. Kohen, and L. J. Lu. 2000. Measurement of estrogen receptors in intact cells by flow cytometry. *Cytometry* 41:109-114.
215. Zamai, L., B. Canonico, A. Gritzapis, F. Luchetti, C. Felici, M. Della Felice, L. Margomenou, M. Papamichail, J. W. Gratama, and S. Papa. 2002. Intracellular

detection of Bcl-2 and p53 proteins by flow cytometry: comparison of monoclonal antibodies and sample preparation protocols. *J Biol Regul Homeost Agents* 16:289-302.

216. Fleisher, T. A., S. E. Dorman, J. A. Anderson, M. Vail, M. R. Brown, and S. M. Holland. 1999. Detection of Intracellular Phosphorylated STAT-1 by Flow Cytometry. *Clinical Immunology* 90:425-430.
217. Chow, S., H. Patel, and D. W. Hedley. 2001. Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: Potential for pharmacodynamic monitoring of signal transduction inhibitors. *Cytometry* 46:72-78.
218. Perez, O. D., and G. P. Nolan. 2002. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. *Nat Biotechnol* 20:155-162.
219. Krutzik, P. O., and G. P. Nolan. 2003. Intracellular phospho-protein staining techniques for flow cytometry: Monitoring single cell signaling events. *Cytometry Part A* 55A:61-70.
220. Perez, O. D., D. Mitchell, G. C. Jager, S. South, C. Murriel, J. McBride, L. A. Herzenberg, S. Kinoshita, and G. P. Nolan. 2003. Leukocyte functional antigen 1 lowers T cell activation thresholds and signaling through cytohesin-1 and Jun-activating binding protein 1. *Nat Immunol* 4:1083-1092.
221. Irish, J. M., R. Hovland, P. O. Krutzik, O. D. Perez, O. Bruserud, B. T. Gjertsen, and G. P. Nolan. 2004. Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell* 118:217-228.
222. Krutzik, P. O., J. M. Irish, G. P. Nolan, and O. D. Perez. 2004. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. *Clinical Immunology* 110:206-221.
223. Perez, O. D., P. O. Krutzik, and G. P. Nolan. 2004. Flow cytometric analysis of kinase signaling cascades. *Methods Mol Biol* 263:67-94.
224. Krutzik, P. O., M. R. Clutter, and G. P. Nolan. 2005. Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry. *J Immunol* 175:2357-2365.
225. Perez, O. D., D. Mitchell, R. Campos, G. J. Gao, L. Li, and G. P. Nolan. 2005. Multiparameter analysis of intracellular phosphoepitopes in immunophenotyped cell populations by flow cytometry. *Curr Protoc Cytom* Chapter 6:Unit 6 20.
226. Sachs, K., O. Perez, D. Pe'er, D. A. Lauffenburger, and G. P. Nolan. 2005. Causal protein-signaling networks derived from multiparameter single-cell data. *Science* 308:523-529.
227. Perez, O. D., and G. P. Nolan. 2006. Phospho-proteomic immune analysis by flow cytometry: from mechanism to translational medicine at the single-cell level. *Immunol Rev* 210:208-228.
228. Schlessinger, J. 2000. Cell Signaling by Receptor Tyrosine Kinases. *Cell* 103:211-225.
229. Harnett, M. M., E. Katz, and C. A. Ford. 2005. Differential signalling during B-cell maturation. *Immunology Letters* 98:33-44.
230. Saunders, A. E., and P. Johnson. 2010. Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45. *Cellular Signalling* 22:339-348.
231. Bordeaux, J., A. Welsh, S. Agarwal, E. Killiam, M. Baquero, J. Hanna, V. Anagnostou, and D. Rimm. 2010. Antibody validation. *Biotechniques* 48:197-209.
232. Donahue, A. C., and D. A. Fruman. 2007. Distinct signaling mechanisms activate the target of rapamycin in response to different B-cell stimuli. *Eur J Immunol* 37:2923-2936.

233. Oberprieler, N. G., S. Lemeer, M. E. Kalland, K. M. Torgersen, A. J. R. Heck, and K. Tasken. 2010. High-resolution mapping of prostaglandin E2-dependent signaling networks identifies a constitutively active PKA signaling node in CD8+CD45RO+ T cells. *Blood* 116:2253-2265.
234. Kotecha, N., N. J. Flores, J. M. Irish, E. F. Simonds, D. S. Sakai, S. Archambeault, E. Diaz-Flores, M. Coram, K. M. Shannon, G. P. Nolan, and M. L. Loh. 2008. Single-cell profiling identifies aberrant STAT5 activation in myeloid malignancies with specific clinical and biologic correlates. *Cancer Cell* 14:335-343.
235. Khan, S. S., M. A. Solomon, and J. P. McCoy, Jr. 2005. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. *Cytometry B Clin Cytom* 64:1-8.
236. Hedley, B. D., and M. Keeney. 2013. Technical issues: flow cytometry and rare event analysis. *International Journal of Laboratory Hematology* 35:344-350.
237. Fadini, G. P., A. Avogaro, and C. Agostini. 2007. Critical Assessment of Putative Endothelial Progenitor Phenotypes. *Experimental Hematology* 35:1479-1480.
238. Harousseau, J.-L., H. Avet-Loiseau, M. Attal, C. Charbonnel, F. Garban, C. Hulin, M. Michallet, T. Facon, L. Garderet, G. Marit, N. Ketterer, T. Lamy, L. Voillat, F. Guilhot, C. Doyen, C. Mathiot, and P. Moreau. 2009. Achievement of at Least Very Good Partial Response Is a Simple and Robust Prognostic Factor in Patients With Multiple Myeloma Treated With High-Dose Therapy: Long-Term Analysis of the IFM 99-02 and 99-04 Trials. *Journal of Clinical Oncology* 27:5720-5726.
239. Irish, J. M., N. Kotecha, and G. P. Nolan. 2006. Mapping normal and cancer cell signalling networks: towards single-cell proteomics. *Nat Rev Cancer* 6:146-155.
240. Gobessi, S., L. Laurenti, P. G. Longo, L. Carsetti, V. Berno, S. Sica, G. Leone, and D. G. Efremov. 2009. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia* 23:686-697.
241. Minden, M. D.-v., R. Ubelhart, D. Schneider, T. Wossning, M. P. Bach, M. Buchner, D. Hofmann, E. Surova, M. Follo, F. Kohler, H. Wardemann, K. Zirlik, H. Veelken, and H. Jumaa. 2012. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* 489:309-312.
242. Packham, G., S. Krysov, A. Allen, N. Savelyeva, A. J. Steele, F. Forconi, and F. K. Stevenson. 2014. The outcome of B-cell receptor signaling in chronic lymphocytic leukemia: proliferation or anergy. *Haematologica* 99:1138-1148.
243. Muggen, A. F., S. Y. Pillai, L. P. Kil, M. C. van Zelm, J. J. M. van Dongen, R. W. Hendriks, and A. W. Langerak. 2014. Basal Ca2+ signaling is particularly increased in mutated chronic lymphocytic leukemia. *Leukemia*. advance online publication, 4 July. doi: 10.1038/leu.2014.188.
244. Contri, A., A. M. Brunati, L. Trentin, A. Cabrelle, M. Miorin, L. Cesaro, L. A. Pinna, R. Zambello, G. Semenzato, and A. Donella-Deana. 2005. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J Clin Invest* 115:369-378.
245. Muzio, M., B. Apollonio, C. Scielzo, M. Frenquelli, I. Vandoni, V. Boussiotis, F. Caligaris-Cappio, and P. Ghia. 2008. Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood* 112:188-195.
246. Ringshausen, I., T. Dechow, F. Schneller, K. Weick, M. Oelsner, C. Peschel, and T. Decker. 2004. Constitutive activation of the MAPkinase p38 is critical for MMP-9 production and survival of B-CLL cells on bone marrow stromal cells. *Leukemia* 18:1964-1970.

247. Poggi, A., S. Catellani, A. Bruzzone, F. Caligaris-Cappio, M. Gobbi, and M. R. Zocchi. 2008. Lack of the leukocyte-associated Ig-like receptor-1 expression in high-risk chronic lymphocytic leukaemia results in the absence of a negative signal regulating kinase activation and cell division. *Leukemia* 22:980-988.
248. Coughlin, J. J., S. L. Stang, N. A. Dower, and J. C. Stone. 2005. RasGRP1 and RasGRP3 Regulate B Cell Proliferation by Facilitating B Cell Receptor-Ras Signaling. *The Journal of Immunology* 175:7179-7184.
249. Johnson, G. L., and R. Lapadat. 2002. Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. *Science* 298:1911-1912.
250. Harris, S. L., and A. J. Levine. 2005. The p53 pathway: positive and negative feedback loops. *Oncogene* 24:2899-2908.
251. Wu, G. S. 2004. The functional Interactions Between the MAPK and p53 Signaling Pathways. *Cancer Biology & Therapy* 3:156-161.
252. Ding, H., A. M. Gabali, S. D. Jenson, M. S. Lim, and K. S. Elenitoba-Johnson. 2009. P38 mitogen activated protein kinase expression and regulation by interleukin-4 in human B cell non-Hodgkin lymphomas. *J Hematop* 2:195-204.
253. Furman, R. R., Z. Asgary, J. O. Mascarenhas, H. C. Liou, and E. J. Schattner. 2000. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. *J Immunol* 164:2200-2206.
254. Hewamana, S., S. Alghazal, T. T. Lin, M. Clement, C. Jenkins, M. L. Guzman, C. T. Jordan, S. Neelakantan, P. A. Crooks, A. K. Burnett, G. Pratt, C. Fegan, C. Rowntree, P. Brennan, and C. Pepper. 2008. The NF-kappaB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. *Blood* 111:4681-4689.
255. Hewamana, S., T. T. Lin, C. Rowntree, K. Karunanithi, G. Pratt, R. Hills, C. Fegan, P. Brennan, and C. Pepper. 2009. Rel A Is an Independent Biomarker of Clinical Outcome in Chronic Lymphocytic Leukemia. *Journal of Clinical Oncology* 27:763-769.
256. Zomas, A. P., E. Matutes, R. Morilla, K. Owusu-Ankomah, B. K. Seon, and D. Catovsky. 1996. Expression of the immunoglobulin-associated protein B29 in B cell disorders with the monoclonal antibody SN8 (CD79b). *Leukemia* 10:1966-1970.
257. Irish, J. M., D. K. Czerwinski, G. P. Nolan, and R. Levy. 2006. Altered B-cell receptor signaling kinetics distinguish human follicular lymphoma B cells from tumor-infiltrating nonmalignant B cells. *Blood* 108:3135-3142.
258. Singh, D. K., D. Kumar, Z. Siddiqui, S. K. Basu, V. Kumar, and K. V. S. Rao. 2005. The Strength of Receptor Signaling Is Centrally Controlled through a Cooperative Loop between Ca2+ and an Oxidant Signal. *Cell* 121:281-293.
259. Chen, L., P. Juszczynski, K. Takeyama, R. C. T. Aguiar, and M. A. Shipp. 2006. Protein tyrosine phosphatase receptor-type O truncated (PTPROt) regulates SYK phosphorylation, proximal B-cell-receptor signaling, and cellular proliferation. *Blood* 108:3428-3433.
260. Ito, K., K. Takubo, F. Arai, H. Satoh, S. Matsuoka, M. Ohmura, K. Naka, M. Azuma, K. Miyamoto, K. Hosokawa, Y. Ikeda, T. W. Mak, T. Suda, and A. Hirao. 2007. Regulation of reactive oxygen species by Atm is essential for proper response to DNA double-strand breaks in lymphocytes. *J Immunol* 178:103-110.
261. Nedellec, S., Y. Renaudineau, A. Bordron, C. Berthou, N. Porakishvili, P. M. Lydyard, J. O. Pers, and P. Youinou. 2005. B cell response to surface IgM cross-linking identifies different prognostic groups of B-chronic lymphocytic leukemia patients. *J Immunol* 174:3749-3756.

262. Chatzouli, M., S. Ntoufa, N. Papakonstantinou, E. Chartomatsidou, A. Anagnostopoulos, P. Kollia, P. Ghia, M. Muzio, K. Stamatopoulos, and C. Belessi. 2014. Heterogeneous Functional Effects of Concomitant B Cell Receptor and TLR Stimulation in Chronic Lymphocytic Leukemia with Mutated versus Unmutated Ig Genes. *The Journal of Immunology* 192:4518-4524.
263. Allsup, D. J., A. S. Kamiguti, K. Lin, P. D. Sherrington, Z. Matrai, J. R. Slupsky, J. C. Cawley, and M. Zuzel. 2005. B-cell receptor translocation to lipid rafts and associated signaling differ between prognostically important subgroups of chronic lymphocytic leukemia. *Cancer Res* 65:7328-7337.
264. Petlickovski, A., L. Laurenti, X. Li, S. Marietti, P. Chiusolo, S. Sica, G. Leone, and D. G. Efremov. 2005. Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. *Blood* 105:4820-4827.
265. Deglesne, P. A., N. Chevallier, R. Letestu, F. Baran-Marszak, T. Beitar, C. Salanoubat, L. Sanhes, J. Nataf, C. Roger, N. Varin-Blank, and F. Ajchenbaum-Cymbalista. 2006. Survival response to B-cell receptor ligation is restricted to progressive chronic lymphocytic leukemia cells irrespective of Zap70 expression. *Cancer Res* 66:7158-7166.
266. Cesano, A., O. Perbellini, E. Evensen, C. C. Chu, F. Cioffi, J. Ptacek, R. N. Damle, R. Chignola, J. Cordeiro, X.-j. Yan, R. E. Hawtin, I. Nichele, J. R. Ware, C. Cavallini, O. Lovato, R. Zanotti, K. R. Rai, N. Chiorazzi, G. Pizzolo, and M. T. Scupoli. 2013. Association between B-cell receptor responsiveness and disease progression in B-cell chronic lymphocytic leukemia: results from single cell network profiling studies. *Haematologica* 98:626-634.
267. Palomba, M. L., K. Piersanti, C. G. Ziegler, H. Decker, J. W. Cotari, K. Bantilan, I. Rijo, J. R. Gardner, M. Heaney, D. Bemis, R. Balderas, S. N. Malek, E. Seymour, A. D. Zelenetz, M. R. van den Brink, and G. Altan-Bonnet. 2014. Multidimensional single-cell analysis of BCR signaling reveals proximal activation defect as a hallmark of chronic lymphocytic leukemia B cells. *PLoS One* 9:e79987.
268. Calissano, C., R. N. Damle, S. Marsilio, X. J. Yan, S. Yancopoulos, G. Hayes, C. Emson, E. J. Murphy, M. K. Hellerstein, C. Sison, M. S. Kaufman, J. E. Kolitz, S. L. Allen, K. R. Rai, I. Ivanovic, I. M. Dozmorov, S. Roa, M. D. Scharff, W. Li, and N. Chiorazzi. 2011. Intraclonal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Mol Med* 17:1374-1382.
269. Coelho, V., S. Krysov, A. Steele, M. Sanchez Hidalgo, P. W. Johnson, P. S. Chana, G. Packham, F. K. Stevenson, and F. Forconi. 2013. Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function. *Blood* 122:2664-2672.
270. Bruey, J.-M., H. Kantarjian, W. Ma, Z. Estrov, C. Yeh, A. Donahue, H. Sanders, S. O'Brien, M. Keating, and M. Albitar. 2010. Circulating Ki-67 index in plasma as a biomarker and prognostic indicator in chronic lymphocytic leukemia. *Leukemia Research* 34:1320-1324.
271. Del Giudice, I., S. Chiaretti, S. Tavolaro, M. S. De Propris, R. Maggio, F. Mancini, N. Peragine, S. Santangelo, M. Marinelli, F. R. Mauro, A. Guarini, and R. Foa. 2009. Spontaneous regression of chronic lymphocytic leukemia: clinical and biologic features of 9 cases. *Blood* 114:638-646.
272. Gross, E., F. E. L'Faqihi-Olive, L. Ysebaert, M. Brassac, S. Struski, S. Kheirallah, J. J. Fournie, G. Laurent, and A. Quillet-Mary. 2010. B-chronic lymphocytic leukemia chemoresistance involves innate and acquired leukemic side population cells. *Leukemia* 24:1885-1892.

273. Guarini, A., S. Chiaretti, S. Tavolaro, R. Maggio, N. Peragine, F. Citarella, M. R. Ricciardi, S. Santangelo, M. Marinelli, M. S. De Propris, M. Messina, F. R. Mauro, I. Del Giudice, and R. Foà. 2008. BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgVH unmutated chronic lymphocytic leukemia (CLL) cells. *Blood* 112:782-792.
274. Song, Z., P. Lu, R. R. Furman, J. P. Leonard, P. Martin, L. Tyrell, F. Y. Lee, D. M. Knowles, M. Coleman, and Y. L. Wang. 2010. Activities of SYK and PLCgamma2 predict apoptotic response of CLL cells to SRC tyrosine kinase inhibitor dasatinib. *Clin Cancer Res* 16:587-599.
275. Daudignon, A., S. Poulain, P. Morel, D. Penther, F. Parmentier, B. Bouchindhomme, J. Fernandes, P. Duthilleul, and C. Bastard. 2010. Increased trisomy 12 frequency and a biased IgVH 3–21 gene usage characterize small lymphocytic lymphoma. *Leukemia Research* 34:580-584.
276. Traverse-Glehen, A., F. Davi, E. Ben Simon, E. Callet-Bauchu, P. Felman, L. Baseggio, S. Gazzo, C. Thieblemont, C. Charlot, B. Coiffier, F. Berger, and G. Salles. 2005. Analysis of VH genes in marginal zone lymphoma reveals marked heterogeneity between splenic and nodal tumors and suggests the existence of clonal selection. *Haematologica* 90:470-478.
277. Packham, G., S. Krysov, A. Allen, N. Savelyeva, A. J. Steele, F. Forconi, and F. K. Stevenson. 2014. The outcome of B-cell receptor signaling in chronic lymphocytic leukemia: proliferation or anergy. *Haematologica* 99:1138-1148.
278. Ahearne, M. J., S. Willimott, L. Piñon, D. B. Kennedy, F. Miall, M. J. S. Dyer, and S. D. Wagner. 2013. Enhancement of CD154/IL4 proliferation by the T follicular helper (Tfh) cytokine, IL21 and increased numbers of circulating cells resembling Tfh cells in chronic lymphocytic leukaemia. *British Journal of Haematology* 162:360-370.
279. Pedersen, I. M., A. M. Buhl, P. Klausen, C. H. Geisler, and J. Jurlander. 2002. The chimeric anti-CD20 antibody rituximab induces apoptosis in B-cell chronic lymphocytic leukemia cells through a p38 mitogen activated protein-kinase-dependent mechanism. *Blood* 99:1314-1319.
280. Tonino, S. H., J. van Laar, M. H. van Oers, J. Y. Wang, E. Eldering, and A. P. Kater. 2011. ROS-mediated upregulation of Noxa overcomes chemoresistance in chronic lymphocytic leukemia. *Oncogene* 30:701-713.
281. Cui, B., L. Chen, S. Zhang, M. Mraz, J.-F. Fecteau, J. Yu, E. M. Ghia, L. Zhang, L. Bao, L. Z. Rassenti, K. Messer, G. A. Calin, C. M. Croce, and T. J. Kipps. 2014. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood* 124:546-554.
282. Wierda, W. G., J. E. Castro, R. Aguillon, D. Sampath, A. Jalayer, J. McMannis, C. E. Prussak, M. Keating, and T. J. Kipps. 2010. A phase I study of immune gene therapy for patients with CLL using a membrane-stable, humanized CD154. *Leukemia* 24:1893-1900.
283. Kato, K., M. J. Cantwell, S. Sharma, and T. J. Kipps. 1998. Gene transfer of CD40-ligand induces autologous immune recognition of chronic lymphocytic leukemia B cells. *The Journal of Clinical Investigation* 101:1133-1141.
284. Mauro, E., G. M. Rigolin, C. Fraulini, O. Sofritti, M. Ciccone, C. D. Angeli, G. Castoldi, and A. Cuneo. 2007. Mobilization of endothelial progenitor cells in patients with hematological malignancies after treatment with filgrastim and chemotherapy for autologous transplantation. *European Journal of Haematology* 78:374-380.
285. Cassuto, J. P., B. P. Krebs, G. Viot, P. Dujardin, and R. Masseyeff. 1978. β2-MICROGLOBULIN, A TUMOUR MARKER OF LYMPHOPROLIFERATIVE DISORDERS. *The Lancet* 312:108-109.

286. Norfolk, D., J. A. Child, E. H. Cooper, S. Kerruish, and A. M. Ward. 1980. Serum beta 2-microglobulin in myelomatosis: potential value in stratification and monitoring. *Br J Cancer* 42:510-515.
287. Bataille, R., B. G. Durie, and J. Grenier. 1983. Serum beta2 microglobulin and survival duration in multiple myeloma: a simple reliable marker for staging. *Br J Haematol* 55:439-447.
288. Durie, B. G., D. Stock-Novack, S. E. Salmon, P. Finley, J. Beckord, J. Crowley, and C. A. Coltman. 1990. Prognostic value of pretreatment serum beta 2 microglobulin in myeloma: a Southwest Oncology Group Study. *Blood* 75:823-830.
289. Zhang, H., V. Vakil, M. Braunstein, E. L. P. Smith, J. Maroney, L. Chen, K. Dai, J. R. Berenson, M. M. Hussain, U. Klueppelberg, A. J. Norin, H. O. Akman, T. Ozcelik, and O. A. Batuman. 2005. Circulating endothelial progenitor cells in multiple myeloma: implications and significance. *Blood* 105:3286-3294.
290. Bhaskar, A., R. Gupta, L. Kumar, A. Sharma, M. C. Sharma, M. Kalaivani, and S. C. Thakur. 2012. Circulating endothelial progenitor cells as potential prognostic biomarker in multiple myeloma. *Leukemia & Lymphoma* 53:635-640.
291. Dome, B., J. Timar, J. Dobos, L. Meszaros, E. Raso, S. Paku, I. Kenessey, G. Ostoros, M. Magyar, A. Ladanyi, K. Bogos, and J. Tovari. 2006. Identification and clinical significance of circulating endothelial progenitor cells in human non-small cell lung cancer. *Cancer Res* 66:7341-7347.
292. Pircher, A., C. M. Kahler, S. Skvortsov, M. Dlaska, G. Kawaguchi, T. Schmid, E. Gunsilius, and W. Hilbe. 2008. Increased numbers of endothelial progenitor cells in peripheral blood and tumor specimens in non-small cell lung cancer: a methodological challenge and an ongoing debate on the clinical relevance. *Oncol Rep* 19:345-352.
293. Massa, M., V. Rosti, I. Ramajoli, R. Campanelli, A. Pecci, G. Viarengo, V. Meli, M. Marchetti, R. Hoffman, and G. Barosi. 2005. Circulating CD34+, CD133+, and Vascular Endothelial Growth Factor Receptor 2–Positive Endothelial Progenitor Cells in Myelofibrosis With Myeloid Metaplasia. *Journal of Clinical Oncology* 23:5688-5695.
294. Zheng, P. P., W. C. Hop, T. M. Luider, P. A. Sillevis Smitt, and J. M. Kros. 2007. Increased levels of circulating endothelial progenitor cells and circulating endothelial nitric oxide synthase in patients with gliomas. *Ann Neurol* 62:40-48.
295. Dome, B., J. Timar, A. Ladanyi, S. Paku, F. Renyi-Vamos, W. Klepetko, G. Lang, P. Dome, K. Bogos, and J. Tovari. 2009. Circulating endothelial cells, bone marrow-derived endothelial progenitor cells and proangiogenic hematopoietic cells in cancer: From biology to therapy. *Critical Reviews in Oncology/Hematology* 69:108-124.
296. Yin, A. H., S. Miraglia, E. D. Zanjani, G. Almeida-Porada, M. Ogawa, A. G. Leary, J. Olweus, J. Kearney, and D. W. Buck. 1997. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 90:5002-5012.
297. de Wynter, E. A., D. Buck, C. Hart, R. Heywood, L. H. Coutinho, A. Clayton, J. A. Rafferty, D. Burt, G. Guenechea, J. A. Bueren, D. Gagen, L. J. Fairbairn, B. I. Lord, and N. G. Testa. 1998. CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. *STEM CELLS* 16:387-396.
298. Ziegler, B. L., M. Valtieri, G. A. Porada, R. De Maria, R. Muller, B. Masella, M. Gabbianelli, I. Casella, E. Pelosi, T. Bock, E. D. Zanjani, and C. Peschle. 1999. KDR receptor: a key marker defining hematopoietic stem cells. *Science* 285:1553-1558.
299. Takakura, N., T. Watanabe, S. Suenobu, Y. Yamada, T. Noda, Y. Ito, M. Satake, and T. Suda. 2000. A role for hematopoietic stem cells in promoting angiogenesis. *Cell* 102:199-209.

300. De Palma, M., M. A. Venneri, C. Roca, and L. Naldini. 2003. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med* 9:789-795.
301. Urbich, C., C. Heeschen, A. Aicher, E. Dernbach, A. M. Zeiher, and S. Dimmeler. 2003. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation* 108:2511-2516.