UNIVERSITY OF AMSTERDAM

UvA-DARE (Digital Academic Repository)

Targeting the BCR signalosome in B cell malignancies

de Rooij, M.F.M.

Publication date
2017

Document Version
Final published version

License
Other

Link to publication

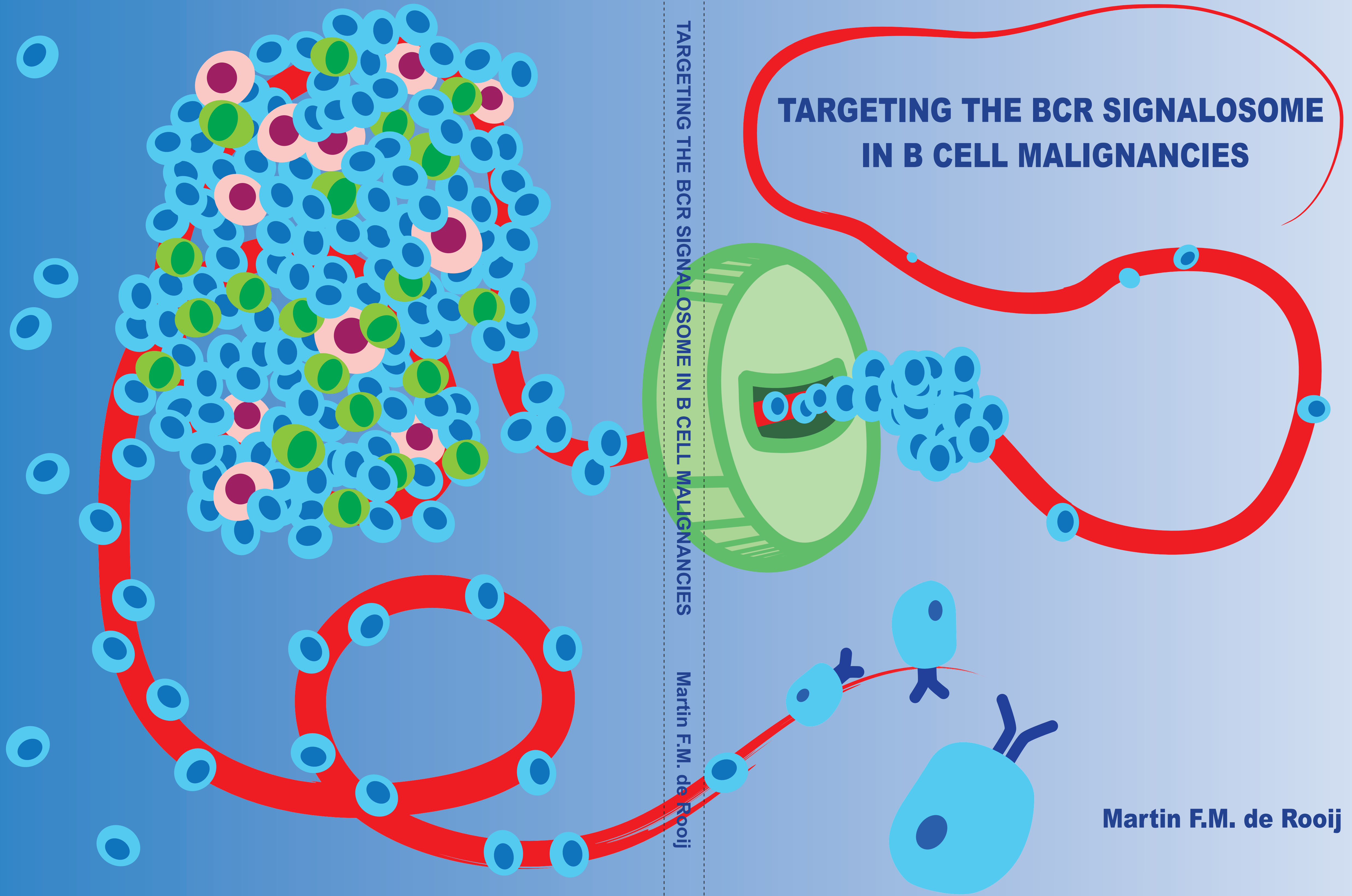
Citation for published version (APA):
de Rooij, M. F. M. (2017). *Targeting the BCR signalosome in B cell malignancies*.

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (https://dare.uva.nl)

Download date:11 Nov 2022


TARGETING THE BCR SIGNALOSOME IN B CELL MALIGNANCIES
Martin F.M. de Rooij
TARGETING THE BCR SIGNALOSOME
IN B CELL MALIGNANCIES
Martin F.M. de Rooij

TARGETING THE BCR SIGNALOSOME IN B CELL MALIGNANCIES

Martin F.M. de Rooij

Targeting the BCR signalosome in B cell malignancies

Academic thesis, University of Amsterdam, Amsterdam, The Netherlands

ISBN 978-94-6299-746-2

Author Martin F.M. de Rooij

Cover design Wim J.V. van Est

Layout Eelco Roos

Print Ridderprint BV, Ridderkerk

The research in this thesis was financially supported by the Dutch Cancer Society, Lymph&Co, Fonds Stimulans, and Pharmacyclics Inc.

Printing of this thesis was financially supported by Department of Pathology, Academic Medical Center, Amsterdam

Copyright ©2017, Martin F.M. de Rooij, Amsterdam, The Netherlands

All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means, without express written permission from the author

TARGETING THE BCR SIGNALOSOME IN B CELL MALIGNANCIES

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. K.I.J. Maex

ten overstaan van een door het college voor promoties ingestelde commissie,

in het openbaar te verdedigen in de Agnietenkapel

op woensdag 20 december 2017, te 14.00 uur

door

Martin Franciscus Maria de Rooij

geboren te Eindhoven

Promotiecommissie:

Promotor: Prof. dr. S.T. Pals, AMC-UvA

Co-promotor: Dr. M. Spaargaren, AMC-UvA

Overige leden: Dr. J.E.J. Guikema, AMC-UvA
Prof. dr. M.H.J. van Oers, AMC-UvA
Prof. dr. R.A.W. van Lier, AMC-UvA
Prof. dr. J.H. Veelken, Universiteit Leiden
Prof. dr. C.G. Figdor, Radboud Universiteit Nijmegen

Faculteit der Geneeskunde

Contents

Chapter 1	General introduction	9
Chapter 2	The clinically active BTK inhibitor ibrutinib targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia *Blood (2012) 119:2590-4*	57
Chapter 3	Egress of $CD19^{+}CD5^{+}$ cells into peripheral blood following treatment with the Bruton tyrosine kinase inhibitor ibrutinib in mantle cell lymphoma patients *Blood (2013) 122:2412-24*	79
Chapter 4	BTK inhibitors in chronic lymphocytic leukemia: a glimpse to the future *Oncogene (2014) 34:2426-36*	107
Chapter 5	Ibrutinib and idelalisib synergistically target BCR-controlled adhesion in MCL and CLL: a rationale for combination therapy *Blood (2015) 125:2306-9*	139
Chapter 6	Ibrutinib and idelalisib target B cell receptor- but not CXCL12/CXCR4-controlled integrin-mediated adhesion in Waldenström macroglobulinemia *Haematologica (2016) 101:e111-5*	151
Chapter 7	General discussion	173
Chapter 8	Summary	197
	PhD portfolio	201
	Nederlandse samenvatting	203
	Curriculum vitae	205
	Dankwoord	206

Chapter 1

General introduction

1

Immune system

Our immune system protects us against pathogens. The first line of defense consists of mechanical barriers, like impenetrable epithelia, the acidic environment of the stomach and antibacterial substances in body fluids. When a pathogen evades these barriers, the immune system is activated. All leukocytes (white blood cells) participate in the immune system, which mechanistically can be divided in two parts, the innate and the adaptive immune system.

Evolutionary, the innate immune system is the oldest of the two parts. It consists of cellular components (*e.g.* macrophages, granulocytes, natural killer cells and platelets) and liver-derived humoral components (*e.g.* complement, kinin, and coagulation systems). Upon infection, these components orchestrate the inflammatory reaction, resulting in access for immune cells and components to the inflamed tissue, killing of the pathogens, prevention of pathogen dissemination, clearance of pathogens and damaged cells, and repair of damaged tissues. Cells of the innate immune system express receptors that recognize structural patterns of damaged cells (damage-associated molecular pattern receptors) and pathogens (pathogen-associated molecular pattern receptors). In addition, the complement system targets damaged cells and pathogens in a nonspecific manner. During evolution, many pathogens acquired means to evade these recognition mechanisms. Higher organisms (vertebrates) therefore developed an adaptive immune system to beat these pathogens.

The adaptive immune system is an extension of the innate immune system, rather than a separate system. In most cases, it is activated by innate cells (antigen presenting cells). The adaptive immune system consists of cellular components (T cells) and humoral components (B cell-derived antibodies). When a T cell recognizes an antigen, it can kill the (infected) cell directly (cluster of differentiation 8 positive ($CD8^+$) T cells) or activate other cells of the immune system ($CD4^+$ T cells). When an antibody recognizes an antigen, it can use components of the innate immune system (*e.g.* complement or phagocytes) to kill or clear its target. Furthermore, an antibody can neutralize a virus or toxin directly by blocking its interaction with the targeted cell. In contrast to the innate recognition receptors, T cell receptors and antibodies recognize one specific epitope. They develop via random gene segment recombination and mutation, and by negative (against self-antigens) and positive (against pathogenic antigens) selection. In addition, a characteristic property of the adaptive immune system is the development of memory, enabling quick clearance upon re-exposure to known pathogens and rapid adaption to mutated pathogens.

Normal B cells

B cells are lymphocytes that express and produce antibodies. Characteristic of B cells is their B-cell antigen receptor (BCR), the membrane bound form of an antibody. At the final differentiated, plasma cell stage, the intracellular part of the BCR is removed by alternative splicing, enabling the BCR to be secreted as soluble antibody.

B cell development

B cell development is a complex and unique process, which includes rearrangements and mutations in DNA, enabling production of antibodies against virtually every existing (bio)molecule. In mammals, early B cell development occurs in the bone marrow (Fig. 1). The hematopoietic stem cell is the ancestor of all blood cells, including lymphocytes. Via induction of the transcription factor Ikaros, hematopoietic stem cells differentiate into the lymphoid lineage,[1] and via induction of E2A and PAX5, the B cell lineage is determined.[2]

A key event in early B cell differentiation is the formation of the BCR, a process called V(D)J-recombination.[3] The immunoglobulin gene of the heavy chain consists of variable (V), diverse (D) and joining (J) gene segments, and the light chain of multiple V and J gene segments. Recombination activating genes (RAG) 1 and 2 make double stranded breaks in recombination signal sequences (RSS) between the gene segments. Subsequently, the gene segments are ligated via the error prone non-homologous end joining mechanism. In addition, terminal deoxynucleotidyl transferase can add additional nucleotides between the gene segments. Via these mechanisms highly diverse antibodies can be generated.

At first, the pro-B cell starts to recombine their heavy chain locus. One D and one J gene segment (pre-pro-B cells), and subsequently one V gene segment are recombined (in pro-B cell).[3] The chance of a correct reading frame is one out of three. When the reading frame is not correct, and therefore a full length heavy chain molecule cannot be made, the recombination is edited. Alternatively, the other allele is used. If this results in an expressible protein, the heavy chain will be expressed together with the surrogate light chain.[4]

The surrogate light chain has the property to bind to a constant region of the immunoglobulin M (IgM) heavy chain.[5] This results in (subtle) pre-B cell receptor signaling, that suppresses further recombination and induces proliferation (large pre-B cell). Subsequently the recombination of the light chain is initiated (small pre-B cell).

For the light chains, there are 2 genes, κ and λ, and thus 4 loci. First the κ loci are rearranged, and if these two fail, the λ locus is rearranged.[6] Since there

is a 1/3 chance for a good reading frame, about 2/3 of B cells are κ and 1/3 is λ.[7] Together, the light chain and the heavy chain form a unique BCR. The BCR is expressed on the membrane and signals to stop the light chain rearrangement. When BCR-mediated signaling is too strong, in case of (pre)BCR recognition of an

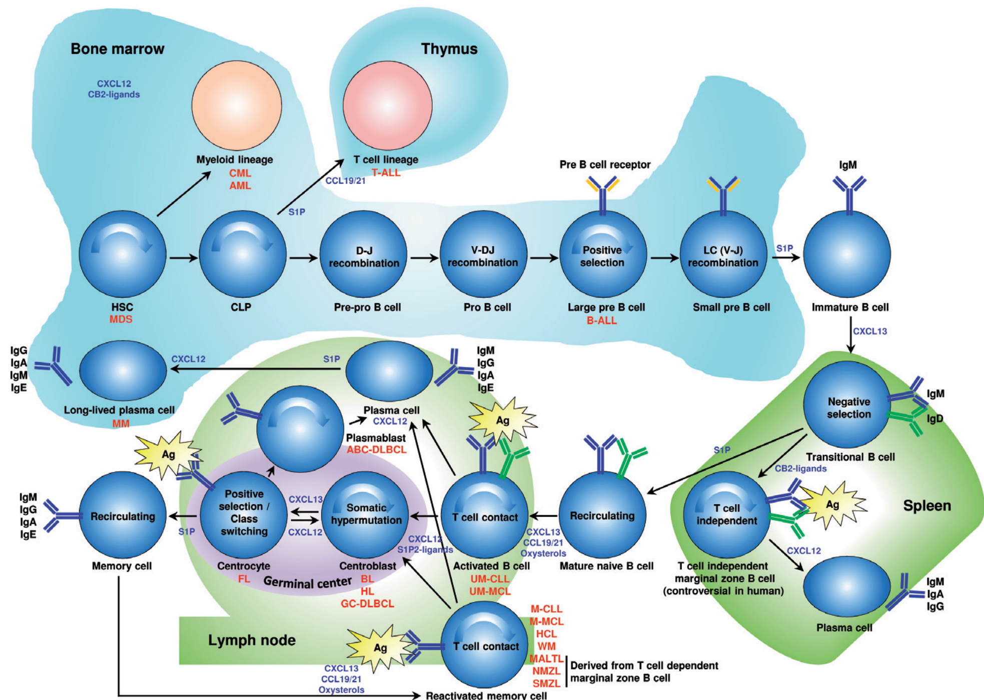


Figure 1. B cell differentiation, homing, and related lymphomas/leukemias
B cells originate from hematopoietic stem cells in the bone marrow. First they develop their BCRs by V(D)J recombination, and subsequently move to the spleen. In the spleen they differentiate into T-cell dependent or independent B cells. Upon antigen encounter, both types will produce antibody secreting plasma cells. T-cell dependent B cells form germinal centers. The result of a germinal center reaction is the production of memory and plasma cells with high affinity BCRs/antibodies. The germinal center is also the major place where class switch recombination takes place, however, T-cell independent B cells can do it too. A fraction of the plasma cells move back to the bone marrow as long-lived plasma cells. These journeys are regulated by different kind of chemokines (shown in blue). B cell stages which are able to proliferate are indicated with an intracellular arrow. Every stage of differentiation has their own characteristic malignant counterpart (shown in red). Some counterparts are a matter of debate. See text for details. Lymphoma/leukemia abbreviations: MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia (T: T cell-derived, B: B cell-derived); CLL, chronic lymphocytic leukemia (UM: unmutated BCR, M: mutated BCR); MCL, mantle cell lymphoma; BL, Burkitt lymphoma; HL, Hodgkin lymphoma; DLBCL diffuse large B cell lymphoma (GC: germinal center-type, ABC: activated B cell-type); FL, follicular lymphoma; HCL, hairy cell lymphoma; WM, Waldenström macroglobulinemia; MALTL, mucosa-associated lymphoid tissue lymphoma; NMZL, nodal marginal zone lymphoma; SMZL, splenic marginal zone lymphoma; MM, multiple myeloma.

auto-antigen, the receptor is either edited, or the B cell is eliminated, preventing autoimmune disease.[6;8]

When the BCR is expressed successfully, immature B cells leave the bone marrow as transitional B cells and migrate to the spleen. Here, these cells are again negatively selected for autoreactivity before they become mature B cells.[9] Via B-cell activating factor (BAFF) signaling the B cells are protected against FAS (CD95)-mediated apoptosis.[9;10] Most transitional B cells become T cell-dependent follicular B cells (in mice called B2 cells).[11] Mature naive B cells express BCRs of the IgM and IgD isotype. These naive B cells recirculate between B cell follicles in secondary lymphoid organs (lymph nodes, spleen, etc.). Another subset of B cells differentiate into T cell independent B cells via Notch2 signaling.[12] These are marginal zone B cells, located in the marginal zone of the spleen. In humans, they can also recirculate to mucosa-associated lymphoid tissues, while in mice, they only stay in the spleen. The marginal zone B cells are responsible for the natural antibody repertoire.[13] These B cells quickly become activated upon bacterial infections, due to massive crosslinking of their BCRs and induction of Toll-like receptor (TLR) signaling.[14] They differentiate into plasma cells, and produce low affinity IgM antibodies as a quick response to the infection. These plasma cells are long-lived and are also located in the spleen.[15] Human marginal zone B cells can form germinal centers, and undergo somatic hypermutation, and can also switch their immunoglobin isotype to IgA or IgG, and are therefore unlikely to be T cell independent.[16] In mice, also other T cell independent B cells have been identified, the B1 cells.[11] They are abundantly present in the peritoneal and pleural cavities, but their presence in humans remains controversial.

B cell lymphopoiesis takes place throughout life, however, the incidence of B-cell derived acute lymphocytic lymphoma (B-ALL) in childhood suggests that most B cell development takes place early in life. In adults, 0.46% of the naive B cells are newly synthesized every day,[17] suggesting that B-cells have an average lifespan of 200 days. This indicates that the B cell repertoire is continuously refreshed.

Germinal center reaction

When conventional naive B cells recognize an encountered antigen, they become activated, eventually leading to maturation into specific antibody secreting plasma cells and non-secreting memory cells (Fig. 1). The germinal center, where the BCRs are mutated and selected, plays a crucial role in the development of high affinity antibodies against antigens.

Antigens enter lymph nodes via the lymph fluid, which drains from the tissues via afferent lymph vessels into the subcapsular sinus.[18;19] In the lymph

node, the lymph fluid drains from the subcapsular sinus via the B cell follicles to the lymph node medulla, and subsequently via efferent lymph vessels to the blood.[19] Small antigens opsonized by complement factors are trapped by follicular dendritic cells in the B cell follicles. Large antigens are presented to B cells by subcapsular sinus macrophages, which are lining the border of the subcapsular sinus and B cell follicles.[20] Antigens that are not trapped, are cleared by macrophages in the medulla, ensuring that the lymph fluid flowing back into blood is clean. In the B cell follicles, the B cells recognize the antigens via their BCRs. The complement receptor CD21 together with CD19 and CD81 on the B cells act as a co-receptor for the BCR. Hereby, opsonization of antigens by complement facilitates BCR signaling.[21;22]

When the B cell binds a specific antigen, the BCR-antigen complex is internalized by endocytosis and transported to lysosomes. The antigen is degraded, and the components of the pathogen are presented to TLRs. Human B cells have high expression of intracellular TLR7 and -9, which recognizes ssRNA and CpG (DNA), respectively.[23] B cells also express TLR10, but the ligand remains elusive.[24] In this way, the B cell identifies the nature of the antigen. TLR signaling induces nuclear factor kappa B (NFκB) activation, which facilitates BCR signaling. From the lysosomes, peptides of the degraded antigen are presented to T cells at the plasma membrane on major histocompatibility complex (MHC) class II molecules.[25] This can be a totally different epitope or molecule than recognized by the BCR. Subsequently, cognate T cells, which are already activated by dendritic cells, stimulate the B cells via co-stimulatory molecules, CD40L (CD154), which is also a strong inducer of NFκB activation, and interleukin 4 (IL4) and IL21, which induce B cell proliferation and survival, respectively. Without co-stimulation, activated B cells die or become anergic to prevent auto immune disease. Inactivating mutations of CD40 or CD40L cause hyper IgM syndrome due to impaired costimulation resulting in absence of germinal centers and class switch recombination.[26;27]

When B cells are activated, they start proliferating. Initially, part of the progeny differentiates into plasma cells, which move to the medullary cords of the lymph node[28] and provide the lymph fluid IgM antibodies, that quickly flow into the blood stream and are recruited to the sites of inflammation.

Other progeny of the activated B cells form a germinal center. This is a specialized niche within the B cell follicles where plasma and memory cells with high affinity antibodies/BCRs are developed. Germinal centers are specific for the T cell-dependent B cells. Germinal centers starts with tens to hundreds of different B cell clones.[29] The differentiation of activated B cell into germinal center B cells is induced by the master regulator B-cell lymphoma 6 (BCL6).[30] BCL6 promotes proliferation and inhibits plasma cell differentiation. In addition, it inhibits apoptosis, in order to

allow the occurrence of DNA mutations (somatic hypermutation).

Histological, the germinal center is divided in a dark and a light zone.[31] The dark zone has a much higher density of B cells than the light zone. The dark zone consists of dividing B cells, called centroblasts. Here, the B cells mutate their immunoglobulin genes in a process called somatic hypermutation, which is dependent on activation-induced (cytidine) deaminase (AID).[32] AID deaminates a cytidine to an uracil. Via error prone base excision repair, this leads to mutation in G:C base pairs (~40%), and via error prone mismatch repair, in A:T base pairs (~60%).[33] AID-mediated mutations are localized in hotspots. Somatic hypermutation appears at high frequencies at the V(D)J region,[33] the domains of the BCR which recognizes its antigen. AID is active in the G_1 phase and is quickly degraded upon G_1-S transition.[34] After some rounds of cell division and somatic hypermutation, the cells move to the light zone and stay there for several hours.[35] The B cells in the light zone are smaller and are called centrocytes.[31] Centrocytes stop dividing and upregulate proteins involved in antigen presentation. Inhibition of forkhead box O transcription factors (FOXOs) by phosphoinositide-3-kinase (PI3K) activity is associated with centrocytic differentiation.[36] A recent study shows that centroblast-centrocyte transition is regulated by B cell intrinsic mechanisms, rather than by extracellular cues.[37]

In the light zone, apart from B cells, follicular dendritic cells, T cells, and macrophages are abundantly present. Via complement- and Fc-receptors, follicular dendritic cells present the antigen on their surface to the centrocytes. The cells with improved affinity BCRs are better antigen presenting cells that centrocytes with low affinity BCRs. Follicular T helper cells recognize loaded MHC class II molecules and prevent these centrocytes from apoptosis via costimulatory molecules, like CD40L and IL21.[38] Centrocytes with poorly loaded MHC class II get FAS-mediated apoptosis signals.[39;40] A histological study showed that FASL (CD95L) is expressed by follicular dendritic cells, rather than by T cells.[41] Most mutations in the BCR will result in low affinity BCRs, therefore most centrocytes are intended to die. Interestingly, patients and mice with hyper IgM syndrome due to AID mutations have giant germinal centers.[42] This is explained by the lack of somatic hypermutation and, as a consequence, lack of incorrectly mutated BCRs. The apoptotic B cells in the light zone are cleared by tingible body macrophages.[43]

The surviving centrocytes have the opportunity to change the isotype of the BCR, a process called class switch recombination,[32] which is also mediated by AID. AID makes nicks in Switch (S) regions, resulting in double stranded breaks. The free ends of the DNA are ligated by non-homologous end joining, by which the intervening DNA between the S regions is cut out of the chromosome. This makes

class switch recombination irreversible. The two ends of the remaining small DNA fragment are religated, thereby forming circular DNA, which is not replicated and is therefore diluted during each cell division. A comparable mechanism also occurs during RAG-mediated V(D)J-recombination. Quantification of these circular DNA fragments in B cell populations reveals the number of cell divisions.[44] After light chain recombination (small pre-B cell stage), the post-germinal center memory and plasma cells of children have passed on average 8 cell divisions, and memory cells of adults on average 11 cell divisions, while naive B cells have passed only 2 division in both children and adults.[45] This suggests that most B cell proliferation takes place during a germinal center reaction.

Class switch recombination is regulated cytokines produced by follicular T helper cells.[46;47] The type of T helper cell, and therefore the cytokines they produce, is previously determined by dendritic cells.[48] Human IgM cells can switch to IgG1, -2a, -2b, -3 or -4, IgA or IgE.[49] The different isotypes have different half-lives, ranging from 2 days (IgE) to 20 days (IgG).[50] They vary in multimerization, ranging from monomeric (IgG) to pentameric (IgM). They also have different immunological properties: some are better in recruiting the complement system (complement-dependent cytotoxicity; CDC), whereas others are recognized by isotype specific Fc-receptors on phagocytes and natural killer cells (antibody-dependent cell-mediated cytotoxicity; ADCC). Certain isotypes have a specialized function. IgA is suitable for secretion in mucosa and body fluids, like in intestinal fluids or tear fluids, and thereby preventing infections. IgG can pass the placental barrier, to protect the fetus against pathogens. IgE is involved in mast cell activation which leads to defense against parasites, but also to allergic reactions. The most common switched isotype is IgG1 (70%), which is involved in the defense against bacteria, viruses and yeasts. It can neutralize antigens and is involved in CDC and ADCC.[49]

After class switch recombination, centrocytes differentiate into plasma cells or memory cells. Cells with the highest affinity BCRs differentiate into plasma blasts/plasma cells, and cells with lower affinity BCRs differentiate into memory cells.[51;52] Plasma blasts move via the outer T zone to the medullary cords of the lymph node.[53] Here, after some cell divisions, they become antibody-secreting terminally differentiated plasma cells. In the medullary cord, they are supported by macrophages which produces the survival factors IL6 and a proliferation-inducing ligand (APRIL).[53] Some of these plasma cells migrate to the bone marrow and reside there as long-lived plasma cells.[54] These plasma cells can survive for more than 10 years, and constantly produce antibodies, whereby a second exposure to the same antigen is quickly restrained. To keep the antibody repertoire up to date throughout life, old plasma cells in the bone marrow niche are out competed by new plasma cells.[55]

Memory cells recirculate, and upon second exposure of the same antigen, quickly differentiate into plasma cells under the control of memory helper T cells.[56] Memory cells (especially IgM memory cells)[57] can also form new germinal centers to optimize the BCR affinity, enabling them to adapt to evolving pathogens. For this reason, memory cells add value to the memory facilitated by long-lived plasma cells. In secondary lymphoid tissues, memory cells are localized at strategic locations, such as the mucosal epithelium of the tonsils.[58] Hereby, the antigens first passage the memory cells and then drain trough the primary follicles containing naive B cells.

To improve BCR affinity, centrocytes can also dedifferentiate into centroblasts and regain somatic hypermutation.[59] This process is called affinity maturation. Centrocytes with high affinity BCRs can transiently express c-MYC, which reprograms the centrocytes to become centroblasts.[60;61] Abrogation of c-MYC expression induces germinal centers collapse, so the reentry of a germinal center cycle (from centrocytes to centroblasts) maintains the germinal center. On average, a germinal center reaction takes about one month, but the duration varies strongly, dependent on the type of antigen. Once the antigen is defeated, antigen presentation in the germinal center stops, therefore T cells cease their survival signals and the germinal center reaction ends.[62]

B cell traffic

As described in the previous paragraph, B cells develop in the bone marrow, then migrate to the blood and recirculate between secondary lymphoid tissues. After antigen encounter, long-lived plasma cells return to the bone marrow. The trafficking and relocalization of B cells throughout the various developmental and differentiation stages is mainly regulated by chemokines (Fig. 1).

Chemokines are divided in chemo-attractants and chemo-repellents. In B cell traffic, only chemo-attractive chemokines are known. The chemokine family involved in regulation of migration of B cells and other hematopoietic cells consists of small secreted proteins with a characteristic cysteine motif. They are divided in four subfamilies, the CXC-, CC-, C- and CX_3C-chemokines.[63] In most cases, the CXC-chemokine members are homeostatic chemokines, which are responsible for normal leukocyte recirculation. CC-chemokine members are inflammatory chemokines, which attract leukocytes to inflammatory sites.[64] In tissues, chemokines can be either soluble or bound to heparan sulphate proteoglycans (HSPG) or other proteoglycans located in the extracellular matrix or on cell membranes.[65] The interaction of chemokines with HSPGs is important for retaining the chemokines in tissues with flowing fluids, such as in the interstitial fluid flow or in the blood and lymph flow. Chemokines can also bind to HSPGs expressed on the targeted cells,

and are thereby presented to their cognate receptors on the membranes of these cells.

The chemokine receptors are seven transmembrane G protein-coupled receptors. They are also divided in CXC-, CC-, C- and CX_3C-receptors, dependent on the chemokines they bind. Most chemokines and chemokine receptors are promiscuous, meaning that they can interact with different partners.[66] Most chemokine pathways act in chemokine networks,[64] which are used for proper guiding of the target cells in three dimensions.

The main chemokine in bone marrow is CXCL12, also known as stroma-derived factor 1 (SDF1). It is produced by bone marrow stromal cells.[67;68] The main receptor for CXCL12 is CXCR4, which is expressed by all B cell subsets. An alternative receptor is CXCR7, but the significance of this receptor on hematopoietic cells is unknown.[69] In B cells, CXCL12 induces activation of integrin $\alpha_4\beta_1$, which is a receptor for fibronectin and vascular cell adhesion molecule 1 (VCAM1).[70] Bone marrow stromal cells have high VCAM1 expression, which keeps the developing B cell in close contact with the stromal cell. Remarkably, in chimeric CXCR4 knockout mice, in which CXCR4 is knocked out early during B cell development, the B cells still differentiate and are not outcompeted by wild type cells.[37] It is possible that blocking a chemokine receptor does not necessarily cause immobilization, but only halts migration. Other chemokines involved in bone marrow organization are the cannabinoid receptor 2 (CB2) agonists. A recent study showed that immature B cells require CB2 for localization to the sinusoidal niche of the bone marrow.[71] In this model, CXCR4 was dispensable for localization to the sinusoidal niche, but it was required for localization to the bone marrow parenchyma. Once the B cell is ready to leave the bone marrow, the sphingosine-1-phosphate receptor 1 (S1P1) is expressed, which induces bone marrow egress.[72]

During B cell maturation, CXCR5 and CCR7 are expressed,[73] which are the receptors for CXCL13 and CCL19/21 respectively. When B cells egress from the bone marrow as transitional B cells, they first move to the spleen. They home to the spleen through open arterioles, controlled by CXCR5, which is an integrin independent process.[74] After maturation in the spleen, the mature B cells upregulate L-selectin.[75] Selectins are low affinity adhesion molecules, that bind sugar moieties in a calcium dependent manner. Ligands of L-selectin are highly expressed in high endothelial venules. These are blood vessels of specialized cuboidal endothelial cells, present in all secondary lymphoid tissues, except the spleen. In lymph nodes, high endothelial venules are localized in the paracortex. At these sites, B and T lymphocytes migrate from blood into the lymph node.[76] During their rolling on the endothelium, which is mediated by L-selectin, the B cells are slowed down. CXCL13

and CCL19/21, presented by heparan sulfates on the surface of the high endothelial cells,[77;78] stimulate passing B cells. This leads to activation of integrin $\alpha_L\beta_2$ (aka lymphocyte function-associated antigen 1; LFA1) and $\alpha_4\beta_1$ (aka very late antigen 4; VLA4), which bind to intercellular cell adhesion molecule 1 (ICAM1) and VCAM1 respectively, resulting in firm adhesion. Integrins are adhesion molecules, which bind to extracellular matrix (ECM) components or cell surface proteins in a calcium and magnesium dependent manner. Via these strong interactions, the lymphocyte undergoes trans- or paracellular migration from the luminal side to the basal side of the endothelial cell.

The role of CXCR4 in the circulation mature B cells is somewhat controversial. Some studies show that CXCR4 is involved in homing to lymph nodes and Peyer patches, [79] and even in egress from Peyer patches,[80] whereas other studies show that CXCR4 signaling is inactivated in mature B cells via an unknown intrinsic mechanism.[81;82] The latter could explain why circulating mature B cells (with the exception of plasma cells) do not home to the bone marrow, and why they are not affected by CXCL12 expression in blood vessels, when it is executing its role in blood vessel formation.[83;84]

Upon extravasation, B cells must migrate through tissue structures. First they have to pass the basal lamina of the endothelium. For this purpose, B cells remodel the basal lamina and other ECM components using the proteolytic enzyme matrix metalloproteinase MMP9.[85] For trafficking through the tissues, lymphocytes follow chemokine gradients. B cells home to the B cell follicles via CXCL13 and oxysterols (EBI2 ligands) gradients and by using follicular dendritic cell networks.[86;87] T cells home to T zones via CCL19/CCL21 gradients and by using the conduit system (reticular fiber network).[88]

Upon chemokine binding, the chemokine receptor is internalized, whereby the lymphocytes become insensitive to lymph node resident chemokines. Subsequently, the B and T cells move, via the efferent lymph vessel, to the blood. This process is independent of integrins,[89;90] and is controlled by the chemokine sphingosine-1-phosphate (S1P), which is present In blood and lymph fluid.[90] During this process, the S1P1 receptor is internalized, and CXCR4, CXCR5 and CCR7 are re-expressed, enabling the lymphocytes to recirculate to other lymph nodes.

When a B or T cell recognize an antigen in the lymph node, they keep the integrins in an activated state via the BCR/TCR controlled signaling. Additionally, they express CD69, which inhibits S1P1,[91] preventing egress from the lymph node. Furthermore, activated T cells upregulate CXCR5, enabling them to move to the border of the B cell follicle, and activated B cells upregulate Epstein-Barr virus-induced G protein-coupled receptor 2 (EBI2) and CCR7 allowing them to move to

the border with the T cell zone.

During germinal center formation, CCR7 and EBI2 expression on B cells is downregulated,[92] whereas S1P2 expression is upregulated,[93] resulting in movement of the B cells into the center of the follicle, initiating the germinal center reaction. Due to increased membrane expression of CXCR4, the germinal center cells (centroblasts) will retain in the dark zone.[94] In addition, hepatocyte growth factor (HGF) plays a role in dark zone organization.[95] Via down regulation of CXCR4 and upregulation of CXCR5, the germinal center cells (centrocytes) move to the light zone.[94] In the dark zone of the germinal center, CXCL12 is produced by reticular cells and, in the light zone, CXCL13 by follicular dendritic cells.[37;96] Both are highly branched cell types contacting all B cells. The two cell types probably have a common origin, since only chemokines and some receptors (like complement and Fc receptors) are differentially expressed. These cells are already present in primary follicles as a germinal center primordium.

At the end of the germinal center reaction, the produced plasma cells gain CXCR4 expression and lose expression of CXCR5 and CCR7,[97] resulting in migration to the medulla of the lymph node and to the bone marrow. Some plasma cell may home to other functional localizations, such as IgA-producing plasma cells that home to mucosal sites. These plasma cells express integrin $\alpha_4\beta_7$,[98] that binds to MadCAM1, which is expressed on the endothelium of mucosal sites. The memory cells, also derived from the germinal center reaction, retain CXCR4, CXCR5 and CCR7 expression, allowing recirculation between the lymphoid organs.[97]

Other chemokine receptors involved in B cell and plasma cell homing are: CCR9 (intestines), CCR10 (salivary glands, trachea, mammary glands, intestines) CCR6 (Peyer patches), CXCR3 (inflamed tissues), and CB2 (marginal zone of the spleen).[97]

The malignant counterparts of B cells, B cell leukemias and lymphomas imitate the homing and migration behavior of their non-malignant cells of origin, which is orchestrated by their differential expression of chemokine receptors and integrins. Most malignant B cells are very motile, making dissemination of the malignancy the rule rather than the exception.

B cell malignancies

85% of hematopoietic malignancies are from of lymphoid origin. Of these lymphoid malignancies, 95% is of B cell origin and only 5% of T cell origin.[99] The explanation for this overrepresentation of B-cell malignancies is that B cells undergo additional somatic hypermutation and class switch recombination of the immunoglobulin genes,

which can lead to DNA damage. Although T cells also undergo V(D)J recombination, most T-cell development takes place during childhood, while B cell development takes place throughout the entire lifespan.

Apart from the fact that leukemia is characterized by high lymphocyte counts in the blood, there is no clear difference between the biology of leukemia and lymphoma. Lymphomas can be divided into Hodgkin and non-Hodgkin lymphoma (NHL). Hodgkin lymphoma is characterized by the presence of the large multinucleated Reed-Sternberg cells. There are 4 subtypes of Hodgkin lymphoma and more than 60 of NHL.[100] As mentioned, B cell differentiation involves multiple different stages. Each stage can give rise to specific malignancies (Fig. 1).[99]

In most of these malignancies, the B cells do not lose their BCR expression, because BCR signaling is an important survival pathway for B cells. Most BCRs of lymphomas are IgM isotype, even post germinal center lymphomas. However, MM cells, which do not express a BCR, produce mainly IgG or IgA antibodies. It has been suggested that IgM BCRs strongly induce proliferation, whereas IgG BCRs mainly promote plasma cell differentiation.[101]

Many lymphomas have characteristic translocations, especially involving the immunoglobulin loci, resulting from aberrant V(D)J-recombination or class switch recombination.[99] For example, *BCL2* translocation is characteristic for follicular lymphoma (FL) (BCL2 prevents FAS-mediated apoptosis in the germinal center), *MYC* for Burkitt lymphoma (BL) (c-MYC induces reentry of the germinal center reaction) and *BCL6* for diffuse large B cell lymphoma (DLBCL) (BCL6 prevents differentiation of germinal center B cells). The differentiation stage of the B cells at which the translocation occurs decides which malignancy will develop: when *CCND1* (cyclin D1) is translocated during V(D)J-recombination, it will result in mantle cell lymphoma (MCL), but when translocated during class switch recombination, it will result in multiple myeloma (MM), keeping the resting naive B cells or plasma cells in cell cycle.

Another B cell specific process involved in lymphomagenesis is aberrant somatic hypermutation, which is executed by the enzyme AID. Besides the immunoglobulin genes, AID can also target other genes, such as *BCL6*, *FAS*, *MB1* (CD79α), and *B29* (CD79β).[102] Furthermore, mutations enhancing activity of the NFκB pathway are especially oncogenic, including mutations in *B29*, *CARD11* and *A20* mutations in DLBCL, and myeloid differentiation primary response gene 88 (*MYD88)*-mutations in DLBCL, Waldenström macroglobulinemia (WM) and chronic lymphocytic leukemia (CLL). This aberrant NFκB activity keeps the B cells in an activated state.[103;104] The B cell malignancies studied in this thesis will be described in more detail below.

Chronic lymphocytic leukemia

CLL is characterized by an accumulation of malignant, small, mature B cells. CLL cells are $CD19^+CD5^+CD23^+$, which is used for diagnosing the disease.[105] The percentage of $CD38^+$ CLL cells is a marker for progression.[106] CLL cells express an unmutated (UM) or a mutated (M) BCR, reflecting a pre- and post-germinal center B cell origin, respectively.[107;108] Expression of zeta-chain-associated protein kinase 70 (ZAP70) is used as a biomarker for the unmutated form of CLL.[109] The exact B cell counterpart is still unknown, but gene expression profiling and B cell differentiation markers points towards memory cells or B1 cells.[110;111] However, CLL cells do require T cell help for proliferation, suggesting that CLL cells are not derived from the T cell independent B1 cells. The expression of IgD on CLL cells also suggests that they may be derived from naive B cells or marginal zone B cells.[112] The tumors are located in lymph nodes, bone marrow, spleen and blood. Symptoms are pancytopenia (causing anemia, recurrent infections, and hemorrhage), enlarged lymph nodes and abdominal pain due to spleen involvement. CLL can transform to an aggressive DLBCL (Richter transformation), which worsens prognosis. The incidence of CLL in the Netherlands is 700 cases per year (Nederlandse Vereniging voor Hematologie), and the median age is >60 years. The current treatment of CLL consists of rituximab, cyclophosphamide, chlorambucil, fludarabine, or lenalidomide, and/or allogeneic stem cell transplantation, but the disease is still incurable. The survival rate depends on the BCR mutation status. The patients with an unmutated BCR ($ZAP70^+$) have a worse prognosis (median survival is now 8 years) than those with mutated BCR ($ZAP70^-$) (median survival is now 25 years). The indolent subgroup does not always require treatment.

CLL develops from a premalignant phase: monoclonal B cell lymphocytosis (MBL), which is frequently observed in elderly people.[113] MBL is usually asymptomatic and does not easily transform to CLL. This premalignant condition is therefore not treated. Translocations involving immunoglobulin loci are not present in CLL. Common mutations are 17q deletions and 11q deletions involving *TP53* and *ATM*, respectively. Other recurrent mutations are found in *NOTCH1*, *MYD88*, *BIRC3,* and *SF3B1*.[114;115] Some of these mutations are driver mutations, which are present in all the malignant cells (such as *MYD88*), whereas others mediate treatment resistance and will grow out upon treatment with chemotherapy (such as *TP53*).[115] In a mouse study employing xenotransplanted hematopoietic stem cells from CLL patients, the mice developed MBL and CLL with different V(D)J-recombined BCR, suggesting that these genetic predispositions were already present in the stem cell compartment.[116]

CLL cells proliferate in proliferation centers,[117] located in lymph nodes, bone marrow and spleen, migrate to blood where they become quiescent, and

subsequently die by apoptosis. Heavy water studies have shown that CLL cells have a high turnover rate,[118] and telomere length analysis demonstrated that blood-derived CLL cells have undergone multiple cell division; UM-CLL more than M-CLL.[119] However, the clinical course is relatively indolent. Many drugs rather efficiently kill blood derived CLL cells, but hardly affect the lymphoid tissue resident cells. This can be explained by the fact that the lymphoid tissues form protective niches, where the CLL cells are provided various survival and proliferation signals (Fig. 2).

Homing and retention in these microenvironments is mainly regulated by chemokines, integrins and the BCR.[120;121] CLL cells home from blood into lymph nodes via the chemokines CCL19/21 and CXCL12/13 and the integrins $\alpha_4\beta_1$ and $\alpha_L\beta_2$.

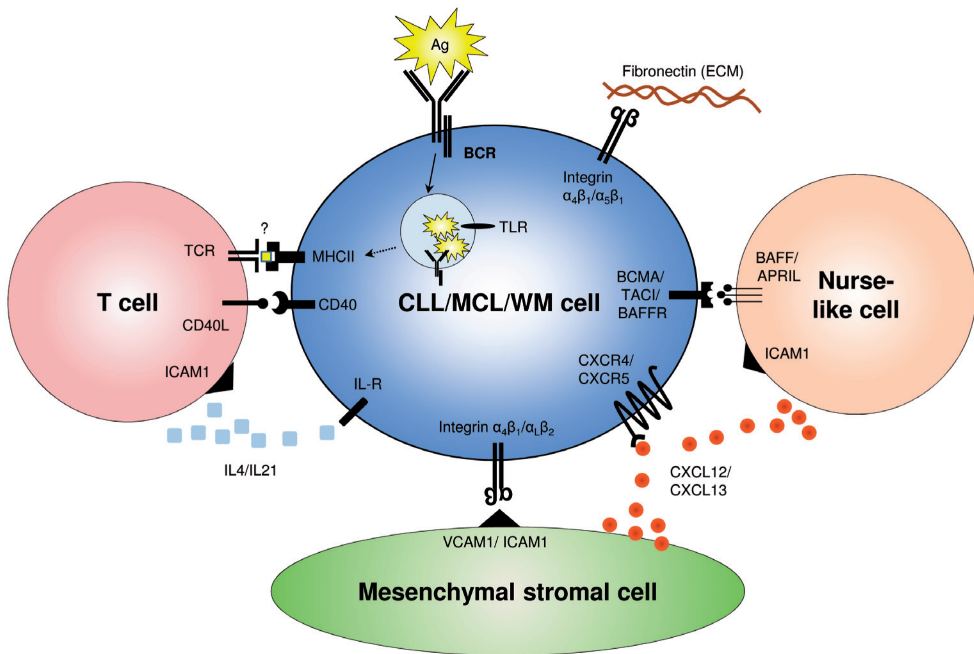


Figure 2. Lymphoma microenvironment in lymphoid tissues
CLL, MCL, and WM cells interact via integrins $\alpha_4\beta_1$ and $\alpha_L\beta_2$ to VCAM1 and ICAM1, respectively, which are expressed on mesenchymal stromal cells, nurse-like cells (tumor-associated macrophages) and T cells, and via integrin $\alpha_4\beta_1/\alpha_5\beta_1$ to ECM components, for example, fibronectin. The activation of these integrins is controlled by chemokines (CXCL12/13 and CCL19/21 (not shown)) and BCR signaling. Antigen (Ag)-stimulated BCR signaling is an important microenvironmental signal for CLL, MCL, and WM, as it provides strong retention signals. Internalized antigens might activate intracellular TLR, and might be presented to the T cells via MHC class II molecules. Subsequently, the malignant cells are stimulated by CD40L and IL4/21 provided by T cells. Furthermore, the malignant cells are stimulated by BAFF and APRIL, which are provided by nurse-like cells. Combined, these microenvironmental interactions have critical roles in the regulation of proliferation and survival of the malignant cells in specialized niches within the lymphoid tissues. See text for details. This figure was adapted from chapter 4.

CCL19/21 and CXCR13 are expressed on high endothelial venules, and CXCL12 is produced by mesenchymal stromal cells and nurse-like cells (tumor-associated macrophages). Expression of CCR7 and integrin $\alpha_4\beta_1$ is strongly correlated with lymphadenopathy.[77] Upon chemokine binding, the chemokine receptor will be internalized to prevent overstimulation. Due to this mechanism, CXCR4 membrane expression can be used to discriminate between blood and lymph node/bone marrow derived CLL cells.[122;123]

Important signals from the microenvironment for the CLL cells are antigens. Half of CLL patients have a biased immunoglobulin gene segment usage and a stereotypic BCR repertoire,[107] strongly indicating that antigens, and thus BCR signaling, play a role in CLL development. In many cases, the UM-BCRs are auto- or polyreactive, thus recognizing self-antigens.[124] Interestingly, some CLL BCRs can even recognize an intrinsic epitope of the BCR itself.[125] The epitopes of M-BCRs were yet unknown, but recently a mutated stereotypic subgroup was found to recognize yeast cell wall components.[126] Probably other mutated subgroups recognize other pathogens. In a microarray study, CLL cells located in lymphoid tissues (lymph nodes and bone marrow) were found to have a more active BCR signaling as compared to blood derived cells.[123] This was irrespective of mutational status or stereotypic B cell repertoire, indicating that BCR signaling plays a role in all CLL patients. *In vitro*, blood derived CLL cells with mutated BCRs are generally anergic to BCR activation, meaning that the lymph node resident CLL cells differ from blood derived cells with respect to BCR signaling. Many CLL cells express also T cell receptor signaling molecules, such as ZAP70 and LCK, which enhances BCR signaling.[109;127]

BCR signaling is of critical importance for the CLL cells because it induces strong retention signals via integrin activation,[128] it attracts T cells and nurse-like cells via CCL3 and CCL4 production,[129] and it promotes proliferation and survival via NFκB, protein kinase B/RAC-alpha serine/threonine-protein kinase (PKB/AKT) and mitogen-activated protein kinase (MAPK) activation.[130] Among these pathways, the NFκB presumably is the most important pathway, since many recurrent mutations (in *MYD88* and *BIRC3*), and signals from the microenvironment (CD40L, TLR, tumor necrosis factor alpha (TNFα), BAFF, and APRIL) are involved in NFκB activation.[103;104]

Upon BCR ligation by antigen, antigens are internalized for presentation to follicular helper T cells. For growth of primary human CLL cells in mice autologous T cells are required,[131] suggesting that antigen presentation to T cells takes place by MHC class II on CLL cells.[132] Internalization of BCR-antigen complexes also feeds into TLR signaling via activation of the endosomal receptors TLR7/9. This possibly plays a role in CLL, because CLL cells express functional TLR7/9,[133;134] and 10% of CLL patients have a mutation in *MYD88* (L265P), a key adapter protein in TLR signaling.[114]

The recruited T cells and nurse-like cells provide the CLL cells with various survival and proliferation stimuli, most of which are also required for normal B cell activation.[135] The T cells support the CLL cells with CD40L and cytokines (TNFα, IL4, and IL21), which induce survival and proliferation pathways in CLL cells, such as NFκB-, JAK/STAT-, MAPK- and PI3K-signaling. Nurse-like cells provide the CLL cells with BAFF and APRIL,[136] TNF family members which activate B-cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), and the BAFF receptor (BAFFR), and strongly induce survival signals via NFκB activation. The presence of all these survival and proliferation factors within the microenvironment provides the CLL cells strong protection, hampering the treatment of these tissue resident CLL cells by conventional drugs.

Mantle cell lymphoma

MCL is characterized by an accumulation of malignant small naive B cells. MCL cells are $CD19^+CD5^+$ and cyclin $D1^+$.[105] Most MCL patients have an unmutated BCR. However, there is also a subgroup of MCLs with a mutated BCR, which are likely derived from post-germinal center B cells. The mutated subgroup has an indolent phenotype and can be distinguished from the unmutated aggressive form by the lack of SOX11 expression.[137] The tumors are located in the mantle zones of reactive follicles of lymph nodes, but during progression also in bone marrow, spleen, gastrointestinal tract, and in blood. Symptoms are pancytopenia, fever, unexplained weight loss, lymphadenopathy, splenomegaly, and gastrointestinal, pulmonary, or central nervous system complications. The incidence of MCL in the Netherlands is 150 cases per year (Nederlandse Vereniging voor Hematologie), and the median age is >60 years. MCL will be treated with rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone, bortezomib, and/or autologous stem cell transplantation, but is still incurable (median survival is now 6 years).

MCL develops from a premalignant phase: *in situ* MCL.[138;139] *In situ* MCL was accidentally found by examination of reactive lymph nodes, and the prevalence in healthy individuals was 7%.[138;139] The initiating mutation is a translocation of the *CCND1* gene with the immunoglobulin heavy chain locus t(11;14), which is present in >95% of all MCL tumors, including *in situ* MCL. This translocation occurs during V(D)J-recombination at the pre-B cell stage. Cyclin D1 is an import regulator of G_1-S transition of the cell cycle, the most important switch between cell division or no cell division. Normal naive B cells are in cell cycle arrest due to expression of the Krüppel family genes. Upon BCR triggering, these factors are downregulated and the cell cycle inducer cyclin D1 is expressed,[140] resulting in escape from cell cycle arrest. Progression of MCL involves aberrations in genes controlling the DNA

damage response, cell cycle control, apoptosis, deregulating the PI3K-, JAK/STAT-, NOTCH-, WNT-, and NFκB-signaling pathways.[141] Apart from genomic alterations, the microenvironment plays a central role in the regulation of proliferation and survival of MCL cells (Fig. 2).[142] Similar to CLL, half of MCL patients have a biased immunoglobulin gene segment usage and a stereotypic BCR repertoire,[143] and lymph node-derived MCL cells show active BCR signaling,[144] thus antigen-induced BCR signaling may play a role in MCL development. In MCL, T cells, tumor-associated macrophages and mesenchymal stromal cells are present. Although not studied to the same extend as in CLL, they are believed to be equally important for the survival and proliferation of MCL cells.[142]

Waldenström macroglobulinemia

WM is characterized by an accumulation of malignant $CD19^+CD5^-$ mature B cells and $CD138^+CD38^+CD45^+$ plasma cells in the bone marrow.[145] Frequently, the BCR contains somatic hypermutation, suggesting that they are derived from post-germinal center B cells. In almost all patients, the BCR is of the IgM isotype. Typically, high levels of monoclonal pentameric IgM are produced (M protein), which can result in a hyperviscosity syndrome. Symptoms of hyperviscosity are loss of vision, heart and kidney problems, polyneuropathy and cryoglubulinemia. Other symptoms of WM are pancytopenia, unexplained weight loss, lymphadenopathy, and splenomegaly. The incidence of WM in the Netherlands is 75 cases per year (Nederlandse Vereniging voor Hematologie), and the median age is >60 years. The disease is quite indolent, and the first policy is watchful waiting. When the disease progresses, it is treated with chlorambucil, fludarabine, rituximab, cyclophosphamide, doxorubicin, vincristine, and/or prednisolone. The disease is still incurable (median survival is now 6.5 year).

WM develops from the premalignant stadium termed IgM monoclonal gammapathy of undetermined significance (IgM-MGUS).[146] Only about 5% of IgM-MGUS actually develops into WM. Gene expression analysis indicates that WM is closer to CLL than to MM.[147] Unlike MM, WM tumors do not only consists of malignant plasma cells, but also of clonal post-germinal center B cells.[145] Another difference between WM and MM is that osteolytic bone disease is frequently observed in MM, but not in WM.

WM cells express a biased, but not stereotypic BCR.[148] This suggests that antigens play a role in the pathogenesis, but that there is no stringent selection. The occurrence of WM is correlated to conditions with chronic immune stimulation (autoimmune disease or chronic infections),[149] again suggesting that antigens play a role in WM pathogenesis.

In WM, translocations with the immunoglobulin loci are rare.[145] In contrast,

almost all (95%) WM patients have a hotspot mutation in MYD88 (95%), resulting in constitutive activation of NFκB. In addition, 30-40% of WMs have a mutation in CXCR4,[150] which is involved in homing of the tumor cells. The CXCR4 mutations found in WM patients are WHIM-like mutations (explained below, under "CXCL12/CXCR4 signaling"), and prevent desensitization of the receptor. Apart from of homing, the mutant receptor acts as a retention factor. CXCR4 is important for bone marrow homing and retention.

As described for the former B-cell malignancies, the microenvironment also plays an important role in WM (Fig. 2). However, it has been less extensively studied as in CLL. Characteristic for WM tumors is the invasion of mast cells, which provide the WM cells with CD40L, APRIL, and BAFF.[151;152] T cells provides the WM cells with IL21,[153] and macrophages together with mast cells induce angiogenesis.[154]

Signal transduction in (malignant) B cells

BCR and chemokine signaling play crucial roles in cell growth, homing, and retention of (malignant) B cells. The molecular details of these pathways will be described here, together with promising drugable targets of these pathways, which can be used in the clinic to treat B cell malignancies. For chemokine signaling, CXCL12/CXCR4 is used as a model chemokine.

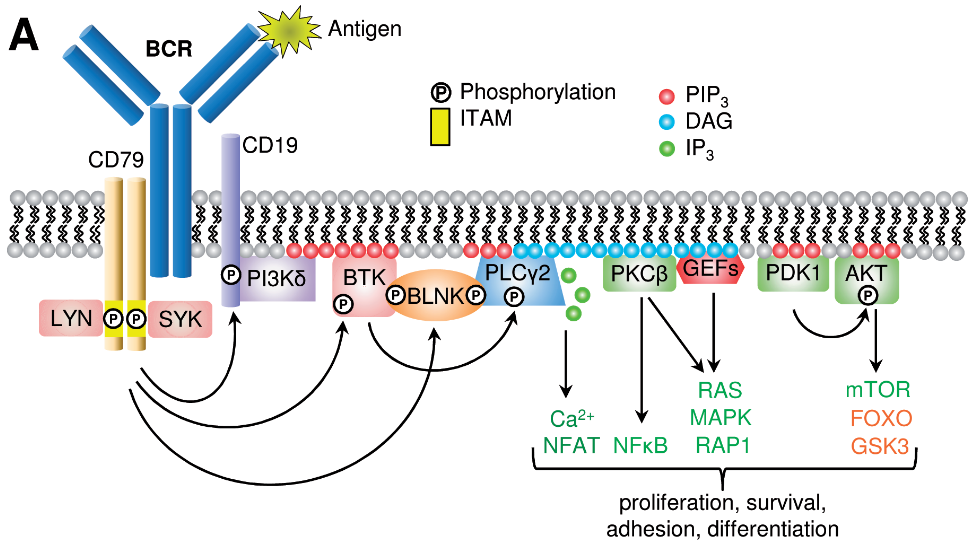
B-cell antigen receptor signaling

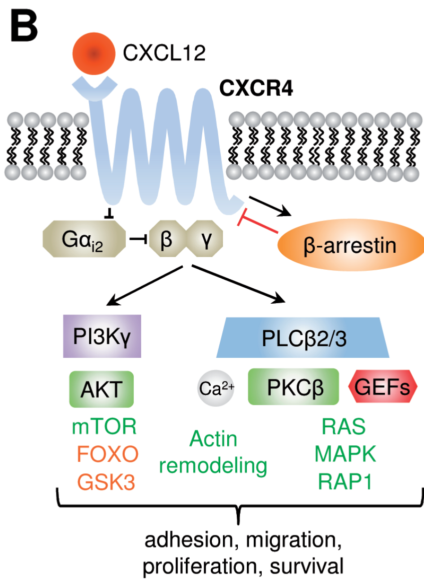
The BCR is exclusively expressed on B cells. The BCR can signal in three flavors: tonic, chronic, and antigen-driven.[155;156] In the absence of antigen, the BCR activates the PI3K pathway in an autonomous manner (tonic signaling), which is of critical importance for B cell survival.[157] The BCR is linked to the co-receptors CD79α and -β, which are involved in cytoplasmic membrane localization, turnover, and signal transduction. Some mutations in CD79, observed in DLBCL, prevent internalization of the BCR, which enhances tonic signaling, resulting in chronic signaling.[155] Whereas tonic signaling does not activate NFκB, chronic signaling does.[157;158] Thus, the strength of BCR-signaling sets the threshold for activating downstream pathways.

Upon antigen binding, the BCR-antigen complex is internalized, which is required for antigen processing, TLR activation, and antigen presentation to T cells. Internalization of BCR-antigen complexes enhances BCR signaling.[159] At a molecular level, the activated BCR activates different pathways involved in B cell activation, adhesion, survival, proliferation, and differentiation, including the MAPK-, PI3K-, NFκB-, and nuclear factor of activated T-cells (NFAT)-pathways.[130]

In the currently accepted model, BCRs are crosslinked upon antigen binding. However, a recent study showed that resting BCRs are present as oligomers and that antigen binding actually results in dissociation of the BCR,[160] offering a novel dissociation activation model. In any case, upon antigen binding, LCK/YES novel tyrosine kinase (LYN) phosphorylates the immunoreceptor tyrosine-based activation motif (ITAM)-containing BCR-subunit CD79, which then recruits spleen tyrosine kinase (SYK). LYN (and/or SYK) phosphorylates CD19, resulting in the binding and activation of PI3K class IA (α, β, and δ, in which the δ isoform is the major class I PI3K isoform involved in antigen-controlled BCR-signaling),[161] which in turn generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 production results in the recruitment of pleckstrin homology (PH)-domain containing proteins, including Bruton tyrosine kinase (BTK) and phospholipase-γ2 (PLCγ2), to the membrane (Fig. 3A).[162] In parallel, SYK phosphorylates B-cell linker protein (BLNK), a scaffold protein which upon phosphorylation binds both BTK and PLCγ2. Subsequently, LYN and/or SYK phosphorylate BTK on Y551, and hereby activate this molecule.[163;164] BTK also autophosphorylates on Y223, however the exact function of this autophosphorylation is not known; an Y223F mutation which cannot be phosphorylated, does not prevent (pre)BCR-controlled BTK activity,[165] but rather enhances BTK-activity,[166] suggesting phosphorylation of Y223 is an autoinhibitory signal. Another important regulatory phosphorylation site on BTK is S180, which is phosphorylated by protein kinase C (PKC), and acts as a negative feedback loop.[167] PKC seems to be an important negatively regulator for many pathways, since almost all PKC mutations in cancer are loss of function mutations.[168]

Direct substrates of BTK include PLCγ2 and general transcription factor II-I (TFII-I). Upon SYK-mediated phosphorylation of the docking protein BLNK, both BTK and PLCγ2 are recruited to BLNK. BTK subsequently activates PLCγ2 by phosphorylation on Y753/Y759.[169;170] Activated PLCγ2 produces diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG then activates RAS-GRP/RAS/MAPK-signaling (involved in proliferation), PKC/NFκB-signaling (involved in proliferation and differentiation), and CalDAG-GEF1/RAS-related protein 1 (RAP1)-signaling (involved in integrin activation). In addition, IP_3-mediated calcium release from the ER induces NFAT and NFκB signaling (involved in proliferation and differentiation), and cytoskeleton rearrangements (involved in cell-cell interactions such as synapse formation with T cells, motility and morphology).[171-178] Furthermore, upon BCR crosslinking, BTK recruits PIP5K, which is involved in the production of PIP_2, the substrate for BTK's upstream regulator PI3K, as well as its downstream effector PLCγ2.[179] A second pathway induced by BTK is the TFII-I pathway.[180] Upon BCR crosslinking, TFII-I is phosphorylated by BTK and subsequently translocates to the





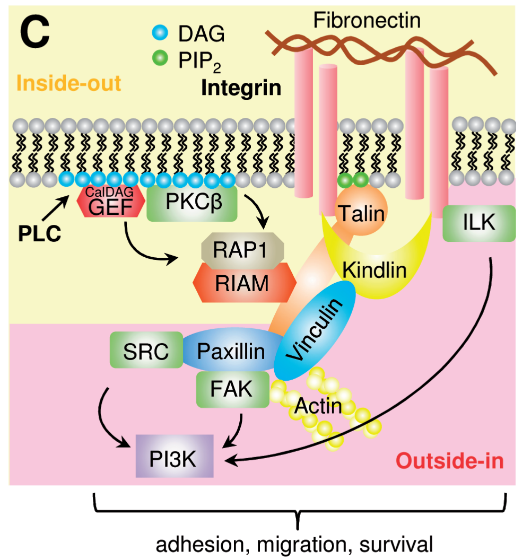


Figure 3. BCR, CXCR4, and integrin signaling
BCR- (A) and chemokine signaling (B) induce via PLC integrin activation (C). See text for details. Ca^{2+}, calcium ions; GEF, guanine nucleotide exchange factor. Figures A and B were adapted from chapter 4.

1

nucleus where it acts as a transcription factor.[181] In B cells, TFII-I is, together with ARID3A, involved in regulation of immunoglobulin heavy chain expression,[182-184] and in BCR-controlled cell cycle arrest.[185]

Another important BCR-controlled pathway is the PI3K/AKT-pathway. This pathway is activated independent of BTK activity.[128] The PH-domain containing serine/threonine kinases phosphoinositide-dependent kinase 1 (PDK1) and AKT are recruited to PIP_3, by which PDK1 phosphorylates and activates AKT. AKT controls mammalian target of rapamycin (mTOR)- (involved in regulations of protein synthesis), FOXO- (involved in regulation of apoptosis), and glycogen synthase kinase 3 beta (GSK3β)-signaling (involved in cell metabolism, NFκB, NFAT, and WNT signaling).[186-188]

BCR signaling is strictly regulated by different phosphatases. Phosphorylated ITAM motifs are dephosphorylated by SRC homology region 2 domain-containing phosphatase 1 and 2 (SHP1/2). Also, other SYK and BTK mediated phosphorylations can be reversed by these phosphatases. In addition, phosphatase and tensin homolog (PTEN) reverses PI3K activity (dephosphorylates PIP_3 to PIP_2), which results in inhibition of BTK/PLCγ2 and AKT activity. Another inositol phosphatase, SRC homology region 2 containing inositol phosphatase 1 (SHIP1), dephosphorylates PIP_3 to another isoform of PIP_2 which is still capable to activate AKT but not BTK/PLCγ2.

The phosphatases SHIP1 and SHP1/2 are recruited to immunoreceptor tyrosine-based inhibitory motifs (ITIM). An important ITIM containing protein is CD22. The ITIM motifs of CD22 is phosphorylated by LYN, which then recruits and activates SHIP1 and SHP1/2.[189] Interestingly, inhibition of LYN prevents BCR internalization,[155] and loss of LYN causes Lupus-like autoimmune disease due to overactivation of BCR signaling.[189;190] Furthermore, the low affinity IgG Fc-receptor (FcγRIIB1, CD32) contains ITIM motifs, which inhibit B cell and plasma cell activity in order to regulate antibody titers. Finally, another tyrosine phosphatase involved in BCR signaling is the common leukocyte marker CD45. In contrast to the other phosphatases, its activity is associated with positive regulation of BCR signaling, probably by dephosphorylation of inhibitory phosphorylation sites on LYN (as described, LYN is initially involved in BCR activation, and subsequently in inhibition) and other SRC family kinases.[191]

BCR signalosome inhibitors

The BCR and CD79 are exclusively expressed in B cells.[192] Proximal signaling molecules (SYK, PI3Kδ, and BTK) are (almost) exclusively expressed in hematopoietic cells. More distal signaling molecules are ubiquitously expressed. This makes the proximal kinases promising therapeutic targets in B cell malignancies and other B

cell related diseases (*e.g.* autoimmune disease). Here, inhibitors against SYK, BTK, and PI3Kδ, will be discussed. Since the proximal kinase LYN also controls inhibitory signals, this might not be a good clinical target.

Spleen tyrosine kinase

SYK is a non-receptor tyrosine kinase of the ZAP70 family.[193] By means of its two SRC homology region 2 (SH2) domains, it is recruited to phosphorylated ITAM motifs of CD79. These SH2 domains are linked to a kinase domain. SYK has at least 37 direct targets,[194] of which BTK and PLCγ2 are the most important in B cells. It plays a role in immune cell activation, cell morphology, proliferation and survival, and regulation of gene expression.

Apart from B cells, SYK is also expressed in platelets, mast cells, and macrophages, and outside the hematopoietic system in fibroblasts, endothelial cells, some epithelial cells and neurons.[195] SYK deficient mice are not viable due to aberrant blood and lymph vessel formation.[196] Irradiated mice reconstituted with SYK-deficient liver-derived hematopoietic stem cells show a B cell development arrest at the pro- to pre-B cell transition.[197]

The pharmaceutical company Rigel Therapeutics Inc. developed the selective SYK inhibitor fostamatinib (R788). Fostamatinib is a prodrug, which has to be metabolized to the active drug R406. R406 is an ATP-competitive SYK inhibitor and has a 50% inhibitory concentration (IC_{50}) of 41nM.[198] The first clinical trial in CLL was very promising, with an overall response rate (ORR; >50% reduction in tumor size) of 55%.[199] Fostamatinib was also active in other NHL subtypes, like DLBCL (22%), MCL (11%), and FL (10%).[199] This was the first study applying a BCR signalosome inhibitor in B cell lymphoma patients. A remarkable clinical observation in CLL was the occurrence of transient lymphocytosis upon fostamatinib treatment.

Because BTK and PI3K inhibitors have better clinical activities, the clinical trials with NHL patients were ended, and subsequently fostamatinib was studied in autoimmune disease. The clinical trials in rheumatoid arthritis failed, and currently it is studied in immune thrombocytopenic purpura, autoimmune hemolytic anemia and IgA nephropathy. Fostamatinib is well tolerated, but it is still not approved by the US Food and Drug Administration (FDA). Apart from SYK, fostamatinib also has affinity for FLT3, KIT, LCK, JAK1, and JAK3 kinases.[200] Novel specific SYK-inhibitors are under development, including entospletinib (GS-9973)[201] and P505-15 (PRT-062607).[202]

Bruton tyrosine kinase

BTK is named after Dr. Ogden Bruton, the pediatrician who described X-linked

agammaglobulinemia (XLA) in 1952.[203] This is a hereditary disease in which B cells and immunoglobulins are absent, resulting in recurrent infections early in childhood. In 1993 the genetic defect was mapped in the *BTK* locus.[204;205] Meanwhile more than 1250 mutations in the BTK gene have been found, which are either missense, nonsense, frame shift, or splice site mutations.[206] Most of these mutations alter the structure of the protein.

BTK is a non-receptor tyrosine kinase of the TEC family. The protein uses a zinc ion as cofactor, which is bound to its TH (TEC homology)-domain. With its PH-domain, it interacts with phospholipids (*e.g.* PIP_3), and with its SH2-domain to phosphorylated tyrosines on BLNK. The known substrates of BTK are PLCγ2 and TFII-I, which are phosphorylated by the tyrosine kinase (TK) domain.[207] Furthermore, BTK contains a SH3-domain. This domain is not essential for its function, since no mutations are found in this domain resulting in XLA (with the exception of nonsense or frame shift mutations which alter the rest of the protein).[206]

BTK plays a crucial role in B cell development and function. When BTK is nonfunctional, B cells are completely absent in humans (XLA). In mice, the numbers of B cells are only decreased (X-linked immunodeficiency; *Xid*), and the present B cells can still form germinal centers,[208] so BTK is not as essential in mice B cells as it is in human B cells. Most likely this is due to the prominent expression of the related tyrosine kinase Tec in mouse B cells.[209] Apart from B cells, no other cells are absent or non-functional in XLA and *Xid*. This makes BTK a promising drug target in B cell malignancies and autoimmune diseases.

The pharmaceutical company Celera Genomics and later Pharmacyclics Inc. developed ibrutinib (formerly known as PCI-32765), a selective inhibitor against BTK which is orally available.[210] Ibrutinib binds covalently and irreversibly to cysteine-481 in the active site of BTK, and has an IC_{50} of 0.5nM.[211] Only 8 other kinases in the human kinome have a homologous cysteine in their active site, but most of them have much lower affinity for ibrutinib, with the exception of BLK and BMX.[211]

The first clinical trials with ibrutinib in NHL were very promising, with high ORRs in CLL (71%), MCL (72%), WM (100%), and activated B cell-type DLBCL (40%).[212-215] Note that the tumor regression in WM was measured indirectly by analyzing IgM antibody levels, while the other diseases by tumor mass (PET scan). In CLL and MCL, the mechanism of action was different from DLBCL. Ibrutinib kills the malignant cells directly in a subset of DLBCL patients with chronic active BCR signaling caused by mutations in CD79.[155] However, in DLBCL patients with CARD11 mutations, which act downstream from BTK, the malignant cells are insensitive to ibrutinib. In CLL and MCL, ibrutinib causes a decrease in lymphadenopathy, which was accompanied by a transient lymphocytosis, similar to fostamatinib.[216] Apparently,

ibrutinib mobilizes the malignant cells and does not direct kill them. Lymphocytosis is a marker for progression;[217] however this lymphocytosis turned out to be asymptomatic and resolved after a few months to a year.[218] It was concluded that the malignant CLL and MCL cells are not directly killed by ibrutinib, but are mobilized from the lymphoid microenvironment into the circulation, resulting in indirect cell death. Meanwhile, ibrutinib is FDA and European Medicines Agency (EMA) approved for CLL, MCL, and WM. Other BTK inhibitors are in development, in which spebrutinib (CC-292)[219] and acalabrutinib (ACP-196)[220] are interesting competitors.

Phosphatidylinositon-3-kinase p110δ (PI3Kδ)

PI3Kδ is a class IA type in the family of PI3K. PI3Kδ consists of two subunits, a p85α regulatory subunit and a catalytic p110δ subunit. The p85α subunit contains SH2 domains which interact with phosphorylated tyrosines. PI3Kδ is expressed by B and T cells and acts downstream of the B and T cell receptor.[221] It is also active in other leukocytes, such as in neutrophils, macrophages, natural killer cells and mast cells.[222] p110δ mutant mice still develop B and T cells, however the B and T cells cannot be activated. p110α mediates pre-B cell signaling and tonic BCR signaling, but cannot mediate antigen-dependent BCR signaling.[161;223] As a result, germinal centers are absent. Furthermore, these mice lack B1 and marginal zone B cells. Mice that are double deficient for p110α and -δ,[161] as well as p85α deficient mice and patients show a complete absence of B cells.[224;225]

The pharmaceutical company Gilead Sciences Inc. developed a PI3K inhibitor selective for the p110δ isoform, called idelalisib (formerly known as GS-1101 and CAL-101). Idelalisib is an ATP-competitive inhibitor and is highly selective for p110δ with an IC_{50} of 2.5nM.[226] Idelalisib has much lower affinity for other members of the PI3K family, in which the p110γ is the closest with an IC_{50} of 89nM.

The first clinical trial in CLL was very promising, with an ORR of 72%.[227] Idelalisib is also active in small lymphocytic lymphoma (61%), MCL (40%), WM (55-80%), FL (54%), and marginal zone B cell lymphoma (47%).[228-230] Again like fostamatinib and ibrutinib, idelalisib induced a lymphocytosis in CLL and MCL. By now, idelalisib has been FDA and EMA approved for FL, and in combination with rituximab, for CLL. Other PI3K inhibitors (sometimes dual inhibitors for other p110 isoforms or mTOR) are in development, in which duvelisib (IPI-145)[231] and buparlisib (BKM-120)[232] are interesting competitors.

CXCL12/CXCR4 signaling

The best studied chemokine receptor in immunology is CXCR4, because of its role in HIV entry in T cells. CXCL12 is the ligand of CXCR4. There are 6 different splice

variants of CXCL12 with different affinities for CXCR4.[233] The different CXCL12 isoforms also have different affinities for HSPGs. Most likely this is important to make the perfect gradient for efficient guiding of the target cells. The CXCL12-CXCR4 axis is involved in the traffic of many leukocytes and hematopoietic stem cells, angiogenesis, and organ development. CXCR4 or CXCL12 knockout mice are not viable, due to impaired development of several organs.[234;235]

CXCR4 and other chemokine receptors are G protein-coupled receptors, which are coupled to $G\alpha i_2$. B cells lacking $G\alpha i_2$ poorly enter lymph nodes.[236] Gαi pathways can be targeted by pertussis toxin. Gαi proteins inhibit adenylyl cyclase, but this does not seem to be involved in chemotaxis.[237;238] $G\alpha i_2$ is associated to Gβγ, en thereby inhibits it. Upon binding of CXCL12 to CXCR4, $G\alpha i_2$ dissociates from Gβγ, in which Gβγ activates PLCβ2/3 and PI3Kγ (Fig. 3B).[239] PLCβ produces DAG and IP_3, that activate RAP1 and calcium pathways respectively.[240] Consequently, integrins are activated and the cytoskeleton is rearranged, resulting in adhesion and migration, respectively. Cell migration requires a chemokine gradient. The difference in chemokine concentration on different cell sides causes a polarized intracellular calcium flux. This results in polarized cytoskeletal rearrangements, by which an uropod and a leading edge are formed, resulting in cell movement towards the gradient.[241] Furthermore, DAG also activates PKC and RAS-MAPK pathways, and PI3Kγ activates AKT, which can induce proliferation and survival signals.[242;243]

Chemokine signaling is tightly regulated by negative feedback loops.[244] CXCR4 is phosphorylated on its C-terminal by G protein-coupled receptor kinases (GRKs) and PKC. β-arrestin is recruited to the phosphorylated C-terminus, thereby inhibiting CXCR4's activity, and inducing clathrin-mediated internalization. Truncating mutations of the C-terminal part of CXCR4 results in warts, hypogammaglobulinemia, recurrent bacterial infections, and myelokathexis (WHIM) syndrome.[245;246] These CXCR4 molecules cannot be desensitized, therefore the mutant CXCR4 act as a retention factor, and the leukocytes do not move out the bone marrow. This results in low numbers of circulating leukocytes and increased incidence of infections.[247] As discussed earlier, these mutations are also frequently found in WM.

CXCR4 can be pharmacologically targeted. A highly specific inhibitor of CXCR4 is plerixafor (AMD3100),[248] which inhibits CXCL12 mediated adhesion and migration of MM cells.[249] Plerixafor is used in the clinic to enhance granulocyte colony-stimulating factor (G-CSF) mediated hematopoietic stem cell mobilization (for transplantation).[250] Probably, G-CSF causes hematopoietic stem cell egress, and plerixafor prevents back homing. Thus far, clinical trials with plerixafor in CLL and MM did not yield any breakthrough publications. Likely, this could be due to redundancy to other chemokines and unknown retention mechanisms.

Integrin activation

Integrins are transmembrane molecules which connect the actin cytoskeleton to the extracellular matrix. The integrin family consists of 18 α- and 8 β-chains.[251] Each integrin consist of a combination of one of the α- and one of the β-chains. 24 unique integrins have been discovered in humans. As discussed previously, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_L\beta_2$ are the most important integrins on B cells. Integrins form calcium and magnesium dependent adhesion interactions, mainly with arginine-glycine-aspartate (RGD)-motifs on many extracellular matrix components such as fibronectin, collagens and laminins, but can also bind to cell surface proteins of the immunoglobulin superfamily such as VCAM1 and ICAM1.[252] Integrins are expressed in an inactive state and can rapidly be activated (within a second upon chemokine signaling) and inactivated (within minutes).[253]

A common event in BCR and chemokine signaling is RAP1 activation (Fig. 3C).[254;255] RAP1 is a small G protein, and is a molecular switch involved in integrin activation. RAP1-GTP binds to RAP1-GTP-interacting adapter molecule (RIAM) and subsequently, RIAM activates talin.[256] Talin is normally inactive via an auto-inhibitory mechanism.[257] The talin head domain interacts with the rod domain, and therefore the head domain cannot bind to integrins. Upon engagement with RIAM, talin associates to the phospholipid PIP_2 via positively charged amino acids.[258] Then, the talin head domain is able to bind to the β-chain of integrins.[259] This results in a conformational change of the integrin molecule, enabling it to bind its extracellular ligands. In addition, talin also bind to the actin cytoskeleton. Due to adhesion forces, the talin molecule is stretched out which exposes the cryptic vinculin binding sites.[260] Vinculin subsequently binds to talin and to the actin cytoskeleton, and thereby strengthens the adhesion complex. Furthermore, multiple activated integrin molecules are cross linked by kindlins, resulting in enhanced avidity.[261] In this way, a strong adhesion complex can be formed, in which the cytoskeleton is strongly connected to the extracellular substrate via the integrin adhesion complex, the focal adhesion complex. Mutations in kindlin-3, the main leukocyte and platelet kindlin, is associated with leukocyte adhesion deficiency type III, a disease characterized by recurrent infection and bleeding disorders.[262] Remarkably, the B and T cells are only mildly affected in these patients.[263] The activation of integrins by these signaling pathways is called inside-out signaling.

Upon adhesion, the integrin adhesion complex can also exert signals into the cell; outside-in signaling. Paxillin is recruited to the focal adhesion complex, which interacts with vinculin, and is an adaptor protein for different kinases, such as focal adhesion kinase (FAK) and SRC.[264;265] In addition, integrin-linked kinase

(ILK), which binds to the integrins β-chain, is recruited.[266] These kinases induce actin remodeling which is involved in cell spreading, contraction, motility, and migration. They also induce cell survival mainly via PI3K activation.[267;268] Especially in adherent cells (like fibroblasts and epithelial cells), apoptosis will be induced when adhesion is lost, due to the absence of these survival signals.[269] This type of cell death is called anoikis. However, lymphocytes do not immediately die upon loss of integrin signaling, because they have to recirculate between the lymph nodes via blood and lymph. They are more dependent on tonic BCR signaling (especially via the PI3K-pathway).[157] Nevertheless, germinal center B cells obtain survival signals by follicular dendritic cells via integrin $\alpha_4\beta_1$/VCAM1- and $\alpha_L\beta_2$/ICAM1-mediated outside-in signaling.[270] This facilitates the selection of centrocytes with high affinity BCRs, which are capable to activate their integrins via BCR-controlled inside-out signaling. In addition, integrin-mediated adhesion can also protect lymphoma cells against drug-induced apoptosis. This is called cell adhesion-mediated drug resistance (CAM-DR).[271]

Pharmacologically, integrins can be targeted by blocking antibodies. Natalizumab, an α_4-integrin blocking antibody, is approved for Crohn disease and multiple sclerosis. In these diseases, it blocks migration of leukocytes to inflamed intestinal and neural tissues.[272] A major drawback of this treatment is the development of progressive multifocal leukoencephalopathy, a viral infection, which is often seen in immunocompromised patients. In preclinical studies of B cell malignancies, natalizumab prevents stromal protection and CAM-DR of B-ALL cells and several NHL cells.[273;274] Future studies must establish if natalizumab or other integrin blocking antibodies are also effective in CLL and other NHL subtypes.

Aim and outline of this thesis

During the first clinical trials of ibrutinib in CLL patients, a rapid and sustained reduction of lymphadenopathy was observed, which was accompanied by transient lymphocytosis.[216] The treatment regimen of 4 weeks ibrutinib, followed by a drug holiday of 1 week, resulted in a saw-tooth pattern of the absolute peripheral blood lymphocyte count (ALC) (Fig. 4A). In subsequent clinical trials, a continuous treatment regimen resulted in a transient) lymphocytosis that could last for several months (Fig. 4B). Together, these clinical observations suggested that ibrutinib may mobilize CLL cells in a reversible manner (Fig. 4C).

In **chapter 2** we studied the underlying mechanism of action of ibrutinib at the molecular and cellular level in CLL. Since BTK is involved in BCR- as well as in

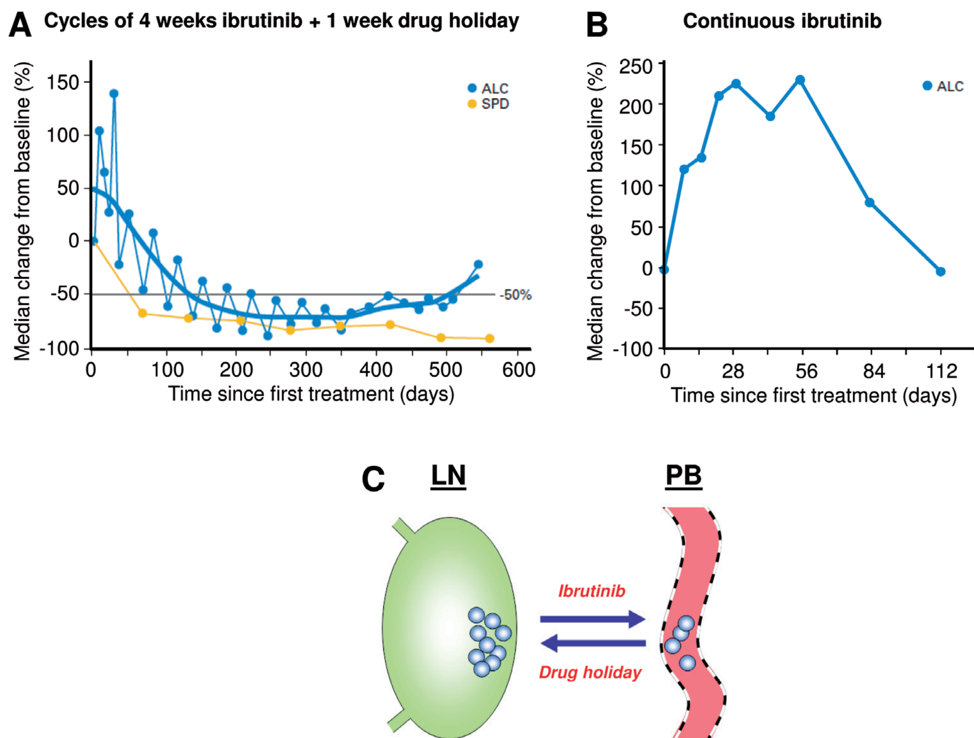


Figure 4. Ibrutinib reversibly mobilizes CLL cells from lymphoid tissues into the circulation
CLL patients were treated with ibrutinib in cycles of 4 weeks treatment and 1 week holiday (A), or continuously (B). A decrease in lymphadenopathy accompanied by a transient lymphocytosis was observed. The ibrutinib-induced lymphocytosis was reversible upon drug deprivation. We hypothesized that ibrutinib mobilizes CLL cells from lymphoid tissues into the circulation (C). The molecular mechanism of this mobilization is studied in this thesis. ALC, absolute lymphocyte count (in peripheral blood); SPD, sum of product diameter (lymph node size); LN, lymph node; PB, peripheral blood. Figure A was adapted from ref [216], figure B from ref [275], and figure C from chapter 2.

1

chemokine-controlled pathways,[70;128] we studied the effect of ibrutinib on adhesion and migration of CLL cells. We show that ibrutinib partially inhibits chemokine-controlled signaling, adhesion and migration, whereas it strongly inhibits BCR-controlled signaling and adhesion. In **chapter 3** we studied the mechanism of action of ibrutinib in mantle cell lymphoma, including a more detailed analysis of the clinical effects. Next, in **chapter 4**, we present a review on BTK inhibitors in CLL, focusing on the biology and clinical applications of ibrutinib and other BTK inhibitors in CLL.

Apart from ibrutinib, the PI3Kδ inhibitor idelalisib also resulted in very good response rates in CLL and MCL patients, including mobilization of the malignant cells into the circulation of these patients. In **chapter 5** we show that idelalisib also inhibits BCR-controlled integrin-mediated adhesion. In combination, ibrutinib and idelalisib

inhibit BCR-controlled adhesion in a strong synergistic manner. We propose that combined treatment of CLL and MCL patients with both ibrutinib and idelalisib could mobilize the malignant cells from their protective niche more efficiently, which may reduce the chance of therapy-resistance. Lastly, in **chapter 6,** we studied the effects of ibrutinib and idelalisib on growth, signaling, adhesion and migration in WM. We show that the major effect of ibrutinib and idelalisib is the inhibition of BCR-controlled adhesion of WM cells.

Taken together, our studies indicate that inhibition of the BCR signalosome targets BCR-controlled integrin-mediated adhesion; the abrogation of this retention signal causes mobilization of CLL, MCL and WM cells from their protective lymphoid organ niche, resulting in tumor regression.

References

1. Wang JH, Nichogiannopoulou A, Wu L et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity.* 1996;5(6):537-549.
2. Baker SJ, Reddy EP. B cell differentiation: role of E2A and Pax5/BSAP transcription factors. *Oncogene* 1995;11(3):413-426.
3. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V(D)J recombination. *Cell* 2002;109 Suppl:S45-S55.
4. Karasuyama H, Rolink A, Shinkai Y et al. The expression of Vpre-B/lambda 5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. *Cell* 1994;77(1):133-143.
5. Ubelhart R, Bach MP, Eschbach C et al. N-linked glycosylation selectively regulates autonomous precursor BCR function. *Nat.Immunol.* 2010;11(8):759-765.
6. Retter MW, Nemazee D. Receptor editing occurs frequently during normal B cell development. *J.Exp.Med.* 1998;188(7):1231-1238.
7. Mole CM, Bene MC, Montagne PM, Seilles E, Faure GC. Light chains of immunoglobulins in human secretions. *Clin.Chim.Acta* 1994;224(2):191-197.
8. Hartley SB, Cooke MP, Fulcher DA et al. Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. *Cell* 1993;72(3):325-335.
9. Carsetti R, Kohler G, Lamers MC. Transitional B cells are the target of negative selection in the B cell compartment. *J.Exp.Med.* 1995;181(6):2129-2140.
10. Schiemann B, Gommerman JL, Vora K et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 2001;293(5537):2111-2114.
11. Allman D, Pillai S. Peripheral B cell subsets. *Curr.Opin.Immunol.* 2008;20(2):149-157.
12. Saito T, Chiba S, Ichikawa M et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity.* 2003;18(5):675-685.
13. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity.* 2001;14(5):617-629.
14. Rubtsov AV, Swanson CL, Troy S et al. TLR agonists promote marginal zone B cell activation and facilitate T-dependent IgM responses. *J.Immunol.* 2008;180(6):3882-3888.
15. Bohannon C, Powers R, Satyabhama L et al. Long-lived antigen-induced IgM plasma cells demonstrate somatic mutations and contribute to long-term protection. *Nat.Commun.* 2016;7:11826.
16. Weill JC, Weller S, Reynaud CA. Human marginal zone B cells. *Annu.Rev.Immunol.* 2009;27:267-285.
17. Macallan DC, Wallace DL, Zhang Y et al. B-cell kinetics in humans: rapid turnover of peripheral blood memory cells. *Blood* 2005;105(9):3633-3640.
18. Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity.* 2007;27(1):160-171.

19. Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. *Nat.Rev. Immunol.* 2009;9(1):15-27.
20. Gonzalez SF, Lukacs-Kornek V, Kuligowski MP et al. Complement-dependent transport of antigen into B cell follicles. *J.Immunol.* 2010;185(5):2659-2664.
21. Cherukuri A, Cheng PC, Pierce SK. The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. *J.Immunol.* 2001;167(1):163-172.
22. Cherukuri A, Cheng PC, Sohn HW, Pierce SK. The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts. *Immunity.* 2001;14(2):169-179.
23. Hornung V, Rothenfusser S, Britsch S et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J.Immunol.* 2002;168(9):4531-4537.
24. Hasan U, Chaffois C, Gaillard C et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J.Immunol.* 2005;174(5):2942-2950.
25. Vascotto F, Le RD, Lankar D et al. Antigen presentation by B lymphocytes: how receptor signaling directs membrane trafficking. *Curr.Opin.Immunol.* 2007;19(1):93-98.
26. Foy TM, Laman JD, Ledbetter JA et al. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *J.Exp.Med.* 1994;180(1):157-163.
27. Kawabe T, Naka T, Yoshida K et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity.* 1994;1(3):167-178.
28. Hargreaves DC, Hyman PL, Lu TT et al. A coordinated change in chemokine responsiveness guides plasma cell movements. *J.Exp.Med.* 2001;194(1):45-56.
29. Tas JM, Mesin L, Pasqual G et al. Visualizing antibody affinity maturation in germinal centers. *Science* 2016;351(6277):1048-1054.
30. Basso K, Dalla-Favera R. BCL6: master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. *Adv.Immunol.* 2010;105:193-210.
31. Stein H, Bonk A, Tolksdorf G et al. Immunohistologic analysis of the organization of normal lymphoid tissue and non-Hodgkin's lymphomas. *J.Histochem.Cytochem.* 1980;28(8):746-760.
32. Honjo T, Kinoshita K, Muramatsu M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu.Rev.Immunol.* 2002;20:165-196.
33. Peled JU, Kuang FL, Iglesias-Ussel MD et al. The biochemistry of somatic hypermutation. *Annu.Rev. Immunol.* 2008;26:481-511.
34. Le Q, Maizels N. Cell cycle regulates nuclear stability of AID and determines the cellular response to AID. *PLoS.Genet.* 2015;11(9):e1005411.
35. Allen CD, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. *Science* 2007;315(5811):528-531.
36. Sander S, Chu VT, Yasuda T et al. PI3 Kinase and FOXO1 transcription factor activity differentially control B cells in the germinal center light and dark z ones. *Immunity.* 2015;43(6):1075-1086.

37. Bannard O, Horton RM, Allen CD et al. Germinal center centroblasts transition to a centrocyte phenotype according to a timed program and depend on the dark zone for effective selection. *Immunity.* 2013;39(5):912-924.
38. Zotos D, Coquet JM, Zhang Y et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J.Exp.Med.* 2010;207(2):365-378.
39. Allen CD. Germinal center quality control: death by Fas. *Immunity.* 2015;42(5):783-785.
40. Butt D, Chan TD, Bourne K et al. FAS inactivation releases unconventional germinal center B cells that escape antigen control and drive IgE and autoantibody production. *Immunity.* 2015;42(5):890-902.
41. Verbeke CS, Wenthe U, Zentgraf H. Fas ligand expression in the germinal centre. *J.Pathol.* 1999;189(2):155-160.
42. Revy P, Muto T, Levy Y et al. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 2000;102(5):565-575.
43. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity.* 2007;27(2):190-202.
44. van Zelm MC, Berkowska MA, van Dongen JJ. Studying the replication history of human B lymphocytes by real-time quantitative (RQ)-PCR. *Methods Mol.Biol.* 2013;971:113-122.
45. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *J.Exp.Med.* 2007;204(3):645-655.
46. Coffman RL, Savelkoul HF, Lebman DA. Cytokine regulation of immunoglobulin isotype switching and expression. *Semin.Immunol.* 1989;1(1):55-63.
47. Coffman RL, Seymour BW, Lebman DA et al. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol.Rev.* 1988;102:5-28.
48. Ballesteros-Tato A, Randall TD. Priming of T follicular helper cells by dendritic cells. *Immunol.Cell Biol.* 2014;92(1):22-27.
49. Spiegelberg HL. Biological role of different antibody classes. *Int.Arch.Allergy Appl.Immunol.* 1989;90 Suppl 1:22-27.
50. Brekke OH, Sandlie I. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat.Rev.Drug Discov.* 2003;2(1):52-62.
51. Smith KG, Light A, O'Reilly LA et al. bcl-2 transgene expression inhibits apoptosis in the germinal center and reveals differences in the selection of memory B cells and bone marrow antibody-forming cells. *J.Exp.Med.* 2000;191(3):475-484.
52. Shinnakasu R, Inoue T, Kometani K et al. Regulated selection of germinal-center cells into the memory B cell compartment. *Nat.Immunol.* 2016;17(7):861-869.
53. Mohr E, Serre K, Manz RA et al. Dendritic cells and monocyte/macrophages that create the IL-6/APRIL-rich lymph node microenvironments where plasmablasts mature. *J.Immunol.* 2009;182(4):2113-2123.

54. Fooksman DR, Schwickert TA, Victora GD et al. Development and migration of plasma cells in the mouse lymph node. *Immunity.* 2010;33(1):118-127.
55. Radbruch A, Muehlinghaus G, Luger EO et al. Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat.Rev.Immunol.* 2006;6(10):741-750.
56. McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigen-specific memory B cell development. *Annu.Rev.Immunol.* 2005;23:487-513.
57. Dogan I, Bertocci B, Vilmont V et al. Multiple layers of B cell memory with different effector functions. *Nat.Immunol.* 2009;10(12):1292-1299.
58. Liu YJ, Barthelemy C, de BO et al. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity.* 1995;2(3):239-248.
59. Schwickert TA, Lindquist RL, Shakhar G et al. In vivo imaging of germinal centres reveals a dynamic open structure. *Nature* 2007;446(7131):83-87.
60. Calado DP, Sasaki Y, Godinho SA et al. The cell-cycle regulator c-Myc is essential for the formation and maintenance of germinal centers. *Nat.Immunol.* 2012;13(11):1092-1100.
61. Dominguez-Sola D, Victora GD, Ying CY et al. The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry. *Nat.Immunol.* 2012;13(11):1083-1091.
62. Kesmir C, De Boer RJ. A mathematical model on germinal center kinetics and termination. *J.Immunol.* 1999;163(5):2463-2469.
63. Murphy PM, Baggiolini M, Charo IF et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol.Rev.* 2000;52(1):145-176.
64. Moser B, Wolf M, Walz A, Loetscher P. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol.* 2004;25(2):75-84.
65. Lortat-Jacob H. The molecular basis and functional implications of chemokine interactions with heparan sulphate. *Curr.Opin.Struct.Biol.* 2009;19(5):543-548.
66. Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu.Rev.Immunol.* 2000;18:217-242.
67. D'Apuzzo M, Rolink A, Loetscher M et al. The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *Eur.J.Immunol.* 1997;27(7):1788-1793.
68. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity.* 2006;25(6):977-988.
69. Sierro F, Biben C, Martinez-Munoz L et al. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc.Natl.Acad.Sci.U.S.A.* .2007;104(37):14759-14764.
70. de Gorter DJ, Beuling EA, Kersseboom R et al. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity.* 2007;26(1):93-104.

71. Pereira JP, An J, Xu Y, Huang Y, Cyster JG. Cannabinoid receptor 2 mediates the retention of immature B cells in bone marrow sinusoids. *Nat.Immunol.* 2009;10(4):403-411.
72. Allende ML, Tuymetova G, Lee BG et al. S1P1 receptor directs the release of immature B cells from bone marrow into blood. *J.Exp.Med.* 2010;207(5):1113-1124.
73. Bowman EP, Campbell JJ, Soler D et al. Developmental switches in chemokine response profiles during B cell differentiation and maturation. *J.Exp.Med.* 2000;191(8):1303-1318.
74. Manevich-Mendelson E, Grabovsky V, Feigelson SW et al. Talin1 is required for integrin-dependent B lymphocyte homing to lymph nodes and the bone marrow but not for follicular B-cell maturation in the spleen. *Blood* 2010;116(26):5907-5918.
75. Loder F, Mutschler B, Ray RJ et al. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J.Exp.Med.* 1999;190(1):75-89.
76. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. *Nat.Immunol.* 2001;2(2):123-128.
77. Till KJ, Lin K, Zuzel M, Cawley JC. The chemokine receptor CCR7 and alpha4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes. *Blood* 2002;99(8):2977-2984.
78. de Paz JL, Moseman EA, Noti C et al. Profiling heparin-chemokine interactions using synthetic tools. *ACS Chem.Biol.* 2007;2(11):735-744.
79. Okada T, Ngo VN, Ekland EH et al. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *J.Exp.Med.* 2002;196(1):65-75.
80. Schmidt TH, Bannard O, Gray EE, Cyster JG. CXCR4 promotes B cell egress from Peyer's patches. *J.Exp.Med.* 2013;210(6):1099-1107.
81. Glodek AM, Honczarenko M, Le Y, Campbell JJ, Silberstein LE. Sustained activation of cell adhesion is a differentially regulated process in B lymphopoiesis. *J.Exp.Med.* 2003;197(4):461-473.
82. Palmesino E, Moepps B, Gierschik P, Thelen M. Differences in CXCR4-mediated signaling in B cells. *Immunobiology* 2006;211(5):377-389.
83. Ara T, Tokoyoda K, Okamoto R, Koni PA, Nagasawa T. The role of CXCL12 in the organ-specific process of artery formation. *Blood* 2005;105(8):3155-3161.
84. Takabatake Y, Sugiyama T, Kohara H et al. The CXCL12 (SDF-1)/CXCR4 axis is essential for the development of renal vasculature. *J.Am.Soc.Nephrol.* 2009;20(8):1714-1723.
85. Trocme C, Gaudin P, Berthier S et al. Human B lymphocytes synthesize the 92-kDa gelatinase, matrix metalloproteinase-9. *J.Biol.Chem.* 1998;273(32):20677-20684.
86. Bajenoff M, Egen JG, Koo LY et al. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity.* 2006;25(6):989-1001.
87. Liu C, Yang XV, Wu J et al. Oxysterols direct B-cell migration through EBI2. *Nature* 2011;475(7357):519-523.
88. Bajenoff M, Egen JG, Koo LY et al. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity.* 2006;25(6):989-1001.
89. Matloubian M, Lo CG, Cinamon G et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 2004;427(6972):355-360.

90. Lo CG, Xu Y, Proia RL, Cyster JG. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. *J.Exp. Med.* 2005;201(2):291-301.
91. Shiow LR, Rosen DB, Brdickova N et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 2006;440(7083):540-544.
92. Pereira JP, Kelly LM, Xu Y, Cyster JG. EBI2 mediates B cell segregation between the outer and centre follicle. *Nature* 2009;460(7259):1122-1126.
93. Green JA, Suzuki K, Cho B et al. The sphingosine 1-phosphate receptor S1P(2) maintains the homeostasis of germinal center B cells and promotes niche confinement. *Nat.Immunol.* 2011;12(7):672-680.
94. Allen CD, Ansel KM, Low C et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat.Immunol.* 2004;5(9):943-952.
95. Tjin EP, Bende RJ, Derksen PW et al. Follicular dendritic cells catalyze hepatocyte growth factor (HGF) activation in the germinal center microenvironment by secreting the serine protease HGF activator. *J.Immunol.* 2005;175(5):2807-2813.
96. Rodda LB, Bannard O, Ludewig B, Nagasawa T, Cyster JG. Phenotypic and morphological properties of germinal center dark zone Cxcl12-expressing reticular cells. *J.Immunol.* 2015;195(10):4781-4791.
97. Kunkel EJ, Butcher EC. Plasma-cell homing. *Nat.Rev.Immunol.* 2003;3(10):822-829.
98. Berlin C, Berg EL, Briskin MJ et al. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* 1993;74(1):185-195.
99. Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat.Rev.Cancer* 2005;5(4):251-262.
100. Morton LM, Wang SS, Devesa SS et al. Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001. *Blood* 2006;107(1):265-276.
101. Niemann CU, Wiestner A. B-cell receptor signaling as a driver of lymphoma development and evolution. *Semin.Cancer Biol.* 2013;23(6):410-421.
102. de Yebenes VG, Ramiro AR. Activation-induced deaminase: light and dark sides. *Trends Mol.Med.* 2006;12(9):432-439.
103. Krappmann D, Vincendeau M. Mechanisms of NF-kappaB deregulation in lymphoid malignancies. *Semin.Cancer Biol.* 2016;29:3-14.
104. Lim KH, Yang Y, Staudt LM. Pathogenetic importance and therapeutic implications of NF-kappaB in lymphoid malignancies. *Immunol.Rev.* 2012;246(1):359-378.
105. Barna G, Reiniger L, Tatrai P, Kopper L, Matolcsy A. The cut-off levels of CD23 expression in the differential diagnosis of MCL and CLL. *Hematol.Oncol.* 2008;26(3):167-170.
106. Damle RN, Wasil T, Fais F et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 1999;94(6):1840-1847.
107. Fais F, Ghiotto F, Hashimoto S et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J.Clin.Invest* 1998;102(8):1515-1525.
108. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999;94(6):1848-1854.

109. Wiestner A, Rosenwald A, Barry TS et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood* 2003;101(12):4944-4951.
110. Klein U, Tu Y, Stolovitzky GA et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J.Exp.Med.* 2001;194(11):1625-1638.
111. Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. *Curr.Opin. Immunol.* 2001;13(2):195-201.
112. Oppezzo P, Magnac C, Bianchi S et al. Do CLL B cells correspond to naive or memory B-lymphocytes? Evidence for an active Ig switch unrelated to phenotype expression and Ig mutational pattern in B-CLL cells. *Leukemia* 2002;16(12):2438-2446.
113. Rawstron AC. Monoclonal B cell lymphocytosis--what does it really mean? *Curr.Hematol.Malig.Rep.* 2013;8(1):52-59.
114. Wang L, Lawrence MS, Wan Y et al. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *N.Engl.J.Med.* 2011;365(26):2497-2506.
115. Landau DA, Carter SL, Stojanov P et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* 2013;152(4):714-726.
116. Kikushige Y, Ishikawa F, Miyamoto T et al. Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia. *Cancer Cell* 2011;20(2):246-259.
117. Soma LA, Craig FE, Swerdlow SH. The proliferation center microenvironment and prognostic markers in chronic lymphocytic leukemia/small lymphocytic lymphoma. *Hum.Pathol.* 2006;37(2):152-159.
118. Messmer BT, Messmer D, Allen SL et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J.Clin.Invest* 2005;115(3):755-764.
119. Damle RN, Batliwalla FM, Ghiotto F et al. Telomere length and telomerase activity delineate distinctive replicative features of the B-CLL subgroups defined by immunoglobulin V gene mutations. *Blood* 2004;103(2):375-382.
120. Burger JA. Chemokines and chemokine receptors in chronic lymphocytic leukemia (CLL): from understanding the basics towards therapeutic targeting. *Semin.Cancer Biol.* 2010;20(6):424-430.
121. Davids MS, Burger JA. Cell Trafficking in Chronic Lymphocytic Leukemia. *Open.J.Hematol.* 2012;3(S1).
122. Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood* 1999;94(11):3658-3667.
123. Herishanu Y, Perez-Galan P, Liu D et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* 2011;117(2):563-574.
124. Herve M, Xu K, Ng YS et al. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J.Clin.Invest* 2005;115(6):1636-1643.

125. Duhren-von MM, Ubelhart R, Schneider D et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* 2012;489(7415):309-312.
126. Hoogeboom R, van Kessel KP, Hochstenbach F et al. A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *J.Exp.Med.* 2013;210(1):59-70.
127. Talab F, Allen JC, Thompson V, Lin K, Slupsky JR. LCK is an important mediator of B-cell receptor signaling in chronic lymphocytic leukemia cells. *Mol.Cancer Res.* 2013;11(5):541-554.
128. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J.Exp.Med.* 2003;198(10):1539-1550.
129. Burger JA, Quiroga MP, Hartmann E et al. High-level expression of the T-cell chemokines CCL3 and CCL4 by chronic lymphocytic leukemia B cells in nurselike cell cocultures and after BCR stimulation. *Blood* 2009;113(13):3050-3058.
130. Dal Porto JM, Gauld SB, Merrell KT et al. B cell antigen receptor signaling 101. *Mol.Immunol.* 2004;41(6-7):599-613.
131. Bagnara D, Kaufman MS, Calissano C et al. A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease. *Blood* 2011;117(20):5463-5472.
132. Kremer AN, van der Meijden ED, Honders MW et al. Human leukocyte antigen-DO regulates surface presentation of human leukocyte antigen class II-restricted antigens on B cell malignancies. *Biol. Blood Marrow Transplant.* 2014;20(5):742-747.
133. Chen M, Bittencourt MC, Feugier P, Faure GC, Bene MC. Active Toll-like receptor-7 triggering of chronic lymphocytic leukemia B-cells by imiquimod. *Leuk.Lymphoma* 2012;53(4):739-742.
134. Grandjenette C, Kennel A, Faure GC, Bene MC, Feugier P. Expression of functional toll-like receptors by B-chronic lymphocytic leukemia cells. *Haematologica* 2007;92(9):1279-1281.
135. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood* 2009;114(16):3367-3375.
136. Nishio M, Endo T, Tsukada N et al. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. *Blood* 2005;106(3):1012-1020.
137. Navarro A, Clot G, Royo C et al. Molecular subsets of mantle cell lymphoma defined by the IGHV mutational status and SOX11 expression have distinct biologic and clinical features. *Cancer Res.* 2012;72(20):5307-5316.
138. Carvajal-Cuenca A, Sua LF, Silva NM et al. In situ mantle cell lymphoma: clinical implications of an incidental finding with indolent clinical behavior. *Haematologica* 2012;97(2):270-278.
139. Lecluse Y, Lebailly P, Roulland S et al. t(11;14)-positive clones can persist over a long period of time in the peripheral blood of healthy individuals. *Leukemia* 2009;23(6):1190-1193.
140. Glynne R, Ghandour G, Rayner J, Mack DH, Goodnow CC. B-lymphocyte quiescence, tolerance and activation as viewed by global gene expression profiling on microarrays. *Immunol.Rev.* 2000;176:216-246.
141. Jares P, Colomer D, Campo E. Molecular pathogenesis of mantle cell lymphoma. *J.Clin.Invest* 2012;122(10):3416-3423.

142.Burger JA, Ford RJ. The microenvironment in mantle cell lymphoma: cellular and molecular pathways and emerging targeted therapies. *Semin.Cancer Biol.* 2011;21(5):308-312.

143.Hadzidimitriou A, Agathangelidis A, Darzentas N et al. Is there a role for antigen selection in mantle cell lymphoma? Immunogenetic support from a series of 807 cases. *Blood* 2011;118(11):3088-3095.

144.Saba NS, Liu D, Herman SE et al. Pathogenic role of B-cell receptor signaling and canonical NF-kappaB activation in mantle cell lymphoma. *Blood* 2016;128(1):82-92.

145.Shaheen SP, Talwalkar SS, Lin P, Medeiros LJ. Waldenstrom macroglobulinemia: a review of the entity and its differential diagnosis. *Adv.Anat.Pathol.* 2012;19(1):11-27.

146.Alexanian R, Weber D, Delasalle K, Cabanillas F, Dimopoulos M. Asymptomatic Waldenstrom's macroglobulinemia. *Semin.Oncol.* 2003;30(2):206-210.

147.Chng WJ, Schop RF, Price-Troska T et al. Gene-expression profiling of Waldenstrom macroglobulinemia reveals a phenotype more similar to chronic lymphocytic leukemia than multiple myeloma. *Blood* 2006;108(8):2755-2763.

148.Martin-Jimenez P, Garcia-Sanz R, Balanzategui A et al. Molecular characterization of heavy chain immunoglobulin gene rearrangements in Waldenstrom's macroglobulinemia and IgM monoclonal gammopathy of undetermined significance. *Haematologica* 2007;92(5):635-642.

149.Koshiol J, Gridley G, Engels EA, McMaster ML, Landgren O. Chronic immune stimulation and subsequent Waldenstrom macroglobulinemia. *Arch.Intern.Med.* 2008;168(17):1903-1909.

150.Hunter ZR, Xu L, Yang G et al. The genomic landscape of Waldenstrom macroglobulinemia is characterized by highly recurring MYD88 and WHIM-like CXCR4 mutations, and small somatic deletions associated with B-cell lymphomagenesis. *Blood* 2014;123(11):1637-1646.

151.Ho AW, Hatjiharissi E, Ciccarelli BT et al. CD27-CD70 interactions in the pathogenesis of Waldenstrom macroglobulinemia. *Blood* 2008;112(12):4683-4689.

152.Tournilhac O, Santos DD, Xu L et al. Mast cells in Waldenstrom's macroglobulinemia support lymphoplasmacytic cell growth through CD154/CD40 signaling. *Ann.Oncol.* 2006;17(8):1275-1282.

153.Hodge LS, Ziesmer SC, Yang ZZ et al. IL-21 in the bone marrow microenvironment contributes to IgM secretion and proliferation of malignant cells in Waldenstrom macroglobulinemia. *Blood* 2012;120(18):3774-3782.

154.Terpos E, Tasidou A, Kastritis E et al. Angiogenesis in Waldenstrom's macroglobulinemia. *Clin. Lymphoma Myeloma.* 2009;9(1):46-49.

155.Davis RE, Ngo VN, Lenz G et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. *Nature* 2010;463(7277):88-92.

156.Monroe JG. Ligand-independent tonic signaling in B-cell receptor function. *Curr.Opin.Immunol.* 2004;16(3):288-295.

157.Srinivasan L, Sasaki Y, Calado DP et al. PI3 kinase signals BCR-dependent mature B cell survival. *Cell* 2009;139(3):573-586.

158.Wilson WH, Young RM, Schmitz R et al. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. *Nat.Med.* 2015;21(8):922-926.

159. Chaturvedi A, Martz R, Dorward D, Waisberg M, Pierce SK. Endocytosed BCRs sequentially regulate MAPK and Akt signaling pathways from intracellular compartments. *Nat.Immunol.* 2011;12(11):1119-1126.
160. Yang J, Reth M. Oligomeric organization of the B-cell antigen receptor on resting cells. *Nature* 2010;467(7314):465-469.
161. Ramadani F, Bolland DJ, Garcon F et al. The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development. *Sci.Signal.* 2010;3(134):ra60.
162. Saito K, Scharenberg AM, Kinet JP. Interaction between the Btk PH domain and phosphatidylinositol-3,4,5-trisphosphate directly regulates Btk. *J.Biol.Chem.* 2001;276(19):16201-16206.
163. Kurosaki T, Kurosaki M. Transphosphorylation of Bruton's tyrosine kinase on tyrosine 551 is critical for B cell antigen receptor function. *J.Biol.Chem.* 1997;272(25):15595-15598.
164. Rawlings DJ, Scharenberg AM, Park H et al. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* 1996;271(5250):822-825.
165. Middendorp S, Dingjan GM, Maas A, Dahlenborg K, Hendriks RW. Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity. *J.Immunol.* 2003;171(11):5988-5996.
166. Park H, Wahl MI, Afar DE et al. Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity.* 1996;4(5):515-525.
167. Kang SW, Wahl MI, Chu J et al. PKCbeta modulates antigen receptor signaling via regulation of Btk membrane localization. *EMBO J.* 2001;20(20):5692-5702.
168. Antal CE, Hudson AM, Kang E et al. Cancer-associated protein kinase C mutations reveal kinase's role as tumor suppressor. *Cell* 2015;160(3):489-502.
169. Kim YJ, Sekiya F, Poulin B, Bae YS, Rhee SG. Mechanism of B-cell receptor-induced phosphorylation and activation of phospholipase C-gamma2. *Mol.Cell Biol.* 2004;24(22):9986-9999.
170. Takata M, Kurosaki T. A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-gamma 2. *J.Exp.Med.* 1996;184(1):31-40.
171. de Gorter DJ, Vos JC, Pals ST, Spaargaren M. The B cell antigen receptor controls AP-1 and NFAT activity through Ras-mediated activation of Ral. *J.Immunol.* 2007;178(3):1405-1414.
172. Rawlings DJ. Bruton's tyrosine kinase controls a sustained calcium signal essential for B lineage development and function. *Clin.Immunol.* 1999;91(3):243-253.
173. Genevier HC, Callard RE. Impaired Ca2+ mobilization by X-linked agammaglobulinaemia (XLA) B cells in response to ligation of the B cell receptor (BCR). *Clin.Exp.Immunol.* 1997;110(3):386-391.
174. Petro JB, Rahman SM, Ballard DW, Khan WN. Bruton's tyrosine kinase is required for activation of IkappaB kinase and nuclear factor kappaB in response to B cell receptor engagement. *J.Exp.Med.* 2000;191(10):1745-1754.
175. Antony P, Petro JB, Carlesso G et al. B-cell antigen receptor activates transcription factors NFAT (nuclear factor of activated T-cells) and NF-kappaB (nuclear factor kappaB) via a mechanism that involves diacylglycerol. *Biochem.Soc.Trans.* 2004;32(Pt 1):113-115.

176.McLeod SJ, Ingham RJ, Bos JL, Kurosaki T, Gold MR. Activation of the Rap1 GTPase by the B cell antigen receptor. *J.Biol.Chem.* 1998;273(44):29218-29223.
177.Petro JB, Khan WN. Phospholipase C-gamma 2 couples Bruton's tyrosine kinase to the NF-kappaB signaling pathway in B lymphocytes. *J.Biol.Chem.* 2001;276(3):1715-1719.
178.Scharenberg AM, Humphries LA, Rawlings DJ. Calcium signalling and cell-fate choice in B cells. *Nat. Rev.Immunol.* 2007;7(10):778-789.
179.Saito K, Tolias KF, Saci A et al. BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity.* 2003;19(5):669-678.
180.Yang W, Desiderio S. BAP-135, a target for Bruton's tyrosine kinase in response to B cell receptor engagement. *Proc.Natl.Acad.Sci.U.S.A.* 1997;94(2):604-609.
181.Novina CD, Kumar S, Bajpai U et al. Regulation of nuclear localization and transcriptional activity of TFII-I by Bruton's tyrosine kinase. *Mol.Cell Biol.* 1999;19(7):5014-5024.
182.Webb CF, Yamashita Y, Ayers N et al. The transcription factor Bright associates with Bruton's tyrosine kinase, the defective protein in immunodeficiency disease. *J.Immunol.* 2000;165(12):6956-6965.
183.Rajaiya J, Hatfield M, Nixon JC, Rawlings DJ, Webb CF. Bruton's tyrosine kinase regulates immunoglobulin promoter activation in association with the transcription factor Bright. *Mol.Cell Biol.* 2005;25(6):2073-2084.

184.Rajaiya J, Nixon JC, Ayers N et al. Induction of immunoglobulin heavy-chain transcription through the transcription factor Bright requires TFII-I. *Mol.Cell Biol.* 2006;26(12):4758-4768.
185.Ashworth T, Roy AL. Cutting Edge: TFII-I controls B cell proliferation via regulating NF-kappaB. *J.Immunol.* 2007;178(5):2631-2635.
186.Rokutanda S, Fujita T, Kanatani N et al. Akt regulates skeletal development through GSK3, mTOR, and FoxOs. *Dev.Biol.* 2009;328(1):78-93.
187.Gold MR, Ingham RJ, McLeod SJ et al. Targets of B-cell antigen receptor signaling: the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase-3 signaling pathway and the Rap1 GTPase. *Immunol.Rev.* 2000;176:47-68.
188.Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle* 2003;2(4):339-345.
189.Nishizumi H, Horikawa K, Mlinaric-Rascan I, Yamamoto T. A double-edged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. *J.Exp.Med.* 1998;187(8):1343-1348.
190.Hibbs ML, Tarlinton DM, Armes J et al. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell* 1995;83(2):301-311.
191.Yanagi S, Sugawara H, Kurosaki M et al. CD45 modulates phosphorylation of both autophosphorylation and negative regulatory tyrosines of Lyn in B cells. *J.Biol.Chem.* 1996;271(48):30487-30492.
192.Chu PG, Arber DA. CD79: a review. *Appl.Immunohistochem.Mol.Morphol.* 2001;9(2):97-106.
193.van Oers NS, Weiss A. The Syk/ZAP-70 protein tyrosine kinase connection to antigen receptor signalling processes. *Semin.Immunol.* 1995;7(4):227-236.
194.Xue L, Geahlen RL, Tao WA. Identification of direct tyrosine kinase substrates based on protein kinase assay-linked phosphoproteomics. *Mol.Cell Proteomics.* 2013;12(10):2969-2980.

195.Yanagi S, Inatome R, Takano T, Yamamura H. Syk expression and novel function in a wide variety of tissues. *Biochem.Biophys.Res.Commun.* 2001;288(3):495-498.
196.Bohmer R, Neuhaus B, Buhren S et al. Regulation of developmental lymphangiogenesis by Syk(+) leukocytes. *Dev.Cell* 2010;18(3):437-449.
197.Turner M, Mee PJ, Costello PS et al. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 1995;378(6554):298-302.
198.Braselmann S, Taylor V, Zhao H et al. R406, an orally available spleen tyrosine kinase inhibitor blocks fc receptor signaling and reduces immune complex-mediated inflammation. *J.Pharmacol.Exp.Ther.* 2006;319(3):998-1008.
199.Friedberg JW, Sharman J, Sweetenham J et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* 2010;115(13):2578-2585.
200.Mocsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat.Rev.Immunol.* 2010;10(6):387-402.
201.Sharman J, Hawkins M, Kolibaba K et al. An open-label phase 2 trial of entospletinib (GS-9973), a selective spleen tyrosine kinase inhibitor, in chronic lymphocytic leukemia. *Blood* 2015;125(15):2336-2343.
202.Spurgeon SE, Coffey G, Fletcher LB et al. The selective SYK inhibitor P505-15 (PRT062607) inhibits B cell signaling and function in vitro and in vivo and augments the activity of fludarabine in chronic lymphocytic leukemia. *J.Pharmacol.Exp.Ther.* 2013;344(2):378-387.
203.Bruton OC. Agammaglobulinemia. *Pediatrics* 1952;9(6):722-728.
204.Vetrie D, Vorechovsky I, Sideras P et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature* 1993;361(6409):226-233.
205.Tsukada S, Saffran DC, Rawlings DJ et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 1993;72(2):279-290.
206.Valiaho J, Smith CI, Vihinen M. BTKbase: the mutation database for X-linked agammaglobulinemia. *Hum.Mutat.* 2006;27(12):1209-1217.
207.Mohamed AJ, Yu L, Backesjo CM et al. Bruton’s tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. *Immunol.Rev.* 2009;228(1):58-73.
208.Ridderstad A, Nossal GJ, Tarlinton DM. The xid mutation diminishes memory B cell generation but does not affect somatic hypermutation and selection. *J.Immunol.* 1996;157(8):3357-3365.
209.Ellmeier W, Jung S, Sunshine MJ et al. Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. *J.Exp.Med.* 2000;192(11):1611-1624.
210.Pan Z, Scheerens H, Li SJ et al. Discovery of selective irreversible inhibitors for Bruton’s tyrosine kinase. *ChemMedChem.* 2007;2(1):58-61.
211.Honigberg LA, Smith AM, Sirisawad M et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc. Natl.Acad.Sci.U.S.A.* 2010;107(29):13075-13080.

212.Byrd JC, Furman RR, Coutre SE et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N.Engl.J.Med.* 2013;369(1):32-42.

213.Wang ML, Rule S, Martin P et al. Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. *N.Engl.J.Med.* 2013;369(6):507-516.

214.Wilson WH, Young RM, Schmitz R et al. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. *Nat.Med.* 2015;21(8):922-926.

215.Treon SP, Tripsas CK, Meid K et al. Ibrutinib in previously treated Waldenstrom's macroglobulinemia. *N.Engl.J.Med.* 2015;372(15):1430-1440.

216.Advani RH, Buggy JJ, Sharman JP et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J.Clin.Oncol.* 2013;31(1):88-94.

217.Cheson BD, Byrd JC, Rai KR et al. Novel targeted agents and the need to refine clinical end points in chronic lymphocytic leukemia. *J.Clin Oncol.* 2012;30(23):2820-2822.

218.Woyach JA, Smucker K, Smith LL et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood* 2014;123(12):1810-1817.

219.Robak P, Smolewski P, Robak T. Emerging immunological drugs for chronic lymphocytic leukemia. *Expert.Opin.Emerg.Drugs* 2015;20(3):423-447.

220.Byrd JC, Harrington B, O'Brien S et al. Acalabrutinib (ACP-196) in Relapsed Chronic Lymphocytic Leukemia. *N.Engl.J.Med.* 2016;374(4):323-332.

221.Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat.Rev.Immunol.* 2003;3(4):317-330.

222.Fung-Leung WP. Phosphoinositide 3-kinase delta (PI3Kdelta) in leukocyte signaling and function. *Cell Signal.* 2011;23(4):603-608.

223.Okkenhaug K, Bilancio A, Farjot G et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* 2002;297(5583):1031-1034.

224.Conley ME, Dobbs AK, Quintana AM et al. Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K. *J.Exp.Med.* 2012;209(3):463-470.

225.Fruman DA, Snapper SB, Yballe CM, Alt FW, Cantley LC. Phosphoinositide 3-kinase knockout mice: role of p85alpha in B cell development and proliferation. *Biochem.Soc.Trans.* 1999;27(4):624-629.

226.Lannutti BJ, Meadows SA, Herman SE et al. CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. *Blood* 2011;117(2):591-594.

227.Brown JR, Byrd JC, Coutre SE et al. Idelalisib, an inhibitor of phosphatidylinositol 3 kinase p110delta, for relapsed/refractory chronic lymphocytic leukemia. Blood 2014;123(22):3390-3397.

228.Kahl BS, Spurgeon SE, Furman RR et al. A phase 1 study of the PI3Kd inhibitor idelalisib in patients with relapsed/refractory mantle cell lymphoma (MCL). Blood 2014;123(22):3398-3405.

229.Gopal AK, Kahl BS, De VS et al. PI3Kdelta Inhibition by Idelalisib in Patients with Relapsed Indolent Lymphoma. N.Engl.J.Med. 2014;370(11):1008-1018.

230.Flinn IW, Kahl BS, Leonard JP et al. Idelalisib, a selective inhibitor of phosphatidylinositol 3-kinase-delta, as therapy for previously treated indolent non-Hodgkin lymphoma. *Blood* 2014;123(22):3406-3413.

231.Dong S, Guinn D, Dubovsky JA et al. IPI-145 antagonizes intrinsic and extrinsic survival signals in chronic lymphocytic leukemia cells. *Blood* 2014;124(24):3583-3586.

232.Amrein L, Shawi M, Grenier J, Aloyz R, Panasci L. The phosphatidylinositol-3 kinase I inhibitor BKM120 induces cell death in B-chronic lymphocytic leukemia cells in vitro. *Int.J.Cancer* 2013;133(1):247-252.

233.Altenburg JD, Broxmeyer HE, Jin Q et al. A naturally occurring splice variant of CXCL12/stromal cell-derived factor 1 is a potent human immunodeficiency virus type 1 inhibitor with weak chemotaxis and cell survival activities. *J.Virol.* 2007;81(15):8140-8148.

234.Tachibana K, Hirota S, Iizasa H et al. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 1998;393(6685):591-594.

235.Nagasawa T, Hirota S, Tachibana K et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 1996;382(6592):635-638.

236.Han SB, Moratz C, Huang NN et al. Rgs1 and Gnai2 regulate the entrance of B lymphocytes into lymph nodes and B cell motility within lymph node follicles. *Immunity.* 2005;22(3):343-354.

237.Neptune ER, Bourne HR. Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Gs. *Proc.Natl.Acad.Sci.U.S.A.* 1997;94(26):14489-14494.

238.Neptune ER, Iiri T, Bourne HR. Galphai is not required for chemotaxis mediated by Gi-coupled receptors. *J.Biol.Chem.* 1999;274(5):2824-2828.

239.Kamp ME, Liu Y, Kortholt A. Function and regulation of heterotrimeric G proteins during c hemotaxis. *Int.J.Mol.Sci.* 2016;17(1).

240.Abram CL, Lowell CA. The ins and outs of leukocyte integrin signaling. *Annu.Rev.Immunol.* 2009;27:339-362.

241.Vicente-Manzanares M, Montoya MC, Mellado M et al. The chemokine SDF-1alpha triggers a chemotactic response and induces cell polarization in human B lymphocytes. *Eur.J.Immunol.* 1998;28(7):2197-2207.

242.Nagasawa T, Kikutani H, Kishimoto T. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc.Natl.Acad.Sci.U.S.A.* 1994;91(6):2305-2309.

243.Nagasawa T, Nakajima T, Tachibana K et al. Molecular cloning and characterization of a murine pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 receptor, a murine homolog of the human immunodeficiency virus 1 entry coreceptor fusin. *Proc.Natl.Acad.Sci.U.S.A.* 1996;93(25):14726-14729.

244.Busillo JM, Benovic JL. Regulation of CXCR4 signaling. *Biochim.Biophys.Acta* 2007;1768(4):952-963.

245.McCormick PJ, Segarra M, Gasperini P, Gulino AV, Tosato G. Impaired recruitment of Grk6 and beta-Arrestin 2 causes delayed internalization and desensitization of a WHIM syndrome-associated CXCR4 mutant receptor. *PLoS.One.* 2009;4(12):e8102.

246.Balabanian K, Lagane B, Pablos JL et al. WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood* 2005;105(6):2449-2457.

247.Gulino AV. WHIM syndrome: a genetic disorder of leukocyte trafficking. *Curr.Opin.Allergy Clin. Immunol.* 2003;3(6):443-450.

248.Hatse S, Princen K, Bridger G, De CE, Schols D. Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. *FEBS Lett.* 2002;527(1-3):255-262.

249.Azab AK, Runnels JM, Pitsillides C et al. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. *Blood* 2009;113(18):4341-4351.

250.Flomenberg N, Devine SM, Dipersio JF et al. The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood* 2005;106(5):1867-1874.

251.Johnson MS, Lu N, Denessiouk K, Heino J, Gullberg D. Integrins during evolution: evolutionary trees and model organisms. *Biochim.Biophys.Acta* 2009;1788(4):779-789.

252.Ruoslahti E. RGD and other recognition sequences for integrins. *Annu.Rev.Cell Dev.Biol.* 1996;12:697-715.

253.Laudanna C, Kim JY, Constantin G, Butcher E. Rapid leukocyte integrin activation by chemokines. *Immunol.Rev.* 2002;186:37-46.

254.Gold MR, Ingham RJ, McLeod SJ et al. Targets of B-cell antigen receptor signaling: the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase-3 signaling pathway and the Rap1 GTPase. *Immunol.Rev.* 2000;176:47-68.

255.McLeod SJ, Li AH, Lee RL, Burgess AE, Gold MR. The Rap GTPases regulate B cell migration toward the chemokine stromal cell-derived factor-1 (CXCL12): potential role for Rap2 in promoting B cell migration. *J.Immunol.* 2002;169(3):1365-1371.

256.Lagarrigue F, Kim C, Ginsberg MH. The Rap1-RIAM-talin axis of integrin activation and blood cell function. *Blood* 2016;128(4):479-487.

257.Goksoy E, Ma YQ, Wang X et al. Structural basis for the autoinhibition of talin in regulating integrin activation. *Mol.Cell* 2008;31(1):124-133.

258.Yang J, Zhu L, Zhang H et al. Conformational activation of talin by RIAM triggers integrin-mediated cell adhesion. *Nat.Commun.* 2014;5:5880.

259.Calderwood DA, Zent R, Grant R et al. The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. *J.Biol.Chem.* 1999;274(40):28071-28074.

260.del RA, Perez-Jimenez R, Liu R et al. Stretching single talin rod molecules activates vinculin binding. *Science* 2009;323(5914):638-641.

261.Ye F, Petrich BG, Anekal P et al. The mechanism of kindlin-mediated activation of integrin alphaIIbbeta3. *Curr.Biol.* 2013;23(22):2288-2295.

262. Svensson L, Howarth K, McDowall A et al. Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. *Nat.Med.* 2009;15(3):306-312.
263. Morrison VL, Uotila LM, Llort AM, Savinko T, Fagerholm SC. Optimal T Cell Activation and B Cell Antibody Responses In Vivo Require the Interaction between Leukocyte Function-Associated Antigen-1 and Kindlin-3. *J.Immunol.* 2015;195(1):105-115.
264. Turner CE, Glenney JR, Jr., Burridge K. Paxillin: a new vinculin-binding protein present in focal adhesions. *J.Cell Biol.* 1990;111(3):1059-1068.
265. Turner CE. Paxillin and focal adhesion signalling. *Nat.Cell Biol.* 2000;2(12):E231-E236.
266. Wu C, Dedhar S. Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. *J.Cell Biol.* 2001;155(4):505-510.
267. Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. *Nat.Rev.Cancer* 2002;2(2):91-100.
268. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002;110(6):673-687.
269. Zouq NK, Keeble JA, Lindsay J et al. FAK engages multiple pathways to maintain survival of fibroblasts and epithelia: differential roles for paxillin and p130Cas. *J.Cell Sci.* 2009;122(Pt 3):357-367.
270. Koopman G, Keehnen RM, Lindhout E et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. *J.Immunol.* 1994;152(8):3760-3767.
271. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* 1999;93(5):1658-1667.
272. Polman CH, O'Connor PW, Havrdova E et al. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N.Engl.J.Med.* 2006;354(9):899-910.
273. Hsieh YT, Gang EJ, Geng H et al. Integrin alpha4 blockade sensitizes drug resistant pre-B acute lymphoblastic leukemia to chemotherapy. *Blood* 2013;121(10):1814-1818.
274. Mraz M, Zent CS, Church AK et al. Bone marrow stromal cells protect lymphoma B-cells from rituximab-induced apoptosis and targeting integrin alpha-4-beta-1 (VLA-4) with natalizumab can overcome this resistance. *Br.J.Haematol.* 2011;155(1):53-64.
275. Burger JA, O'Brien S, Fowler N et al. The Bruton's tyrosine kinase inhibitor, PCI-32765, is well tolerated and demonstrates promising clinical activity in chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL): An Update on Ongoing Phase 1 Studies. *Blood* 2010;116(21):32.

Chapter 2

The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia

Martin F.M. de Rooij[1], Annemieke Kuil[1], Christian R. Geest[2], Eric Eldering[2], Betty Y. Chang[3], Joseph J. Buggy[3], Steven T. Pals[1*], and Marcel Spaargaren[1*]

Departments of [1]Pathology and [2]Experimental Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and [3]Pharmacyclics Inc, Sunnyvale, CA 94085-4521. *S.T.P. and M.S. share senior authorship.

Blood (2012) 119:2590-4

Abstract

Small-molecule drugs that target the B-cell antigen receptor (BCR) signalosome show clinical efficacy in the treatment of B-cell non-Hodgkin lymphoma. These agents, including the Bruton tyrosine kinase (BTK) inhibitor PCI-32765, display an unexpected response in patients with chronic lymphocytic leukemia (CLL): a rapid and sustained reduction of lymphadenopathy accompanied by transient lymphocytosis, which is reversible upon temporary drug deprivation. We hypothesized that this clinical response reflects impaired integrin-mediated adhesion and/or migration. Here, we show that PCI-32765 strongly inhibits BCR-controlled signaling and integrin $\alpha_4\beta_1$-mediated adhesion to fibronectin and VCAM-1 of lymphoma cell lines and primary CLL cells. Furthermore, PCI-32765 also inhibits CXCL12-, CXCL13-, and CCL19-induced signaling, adhesion, and migration of primary CLL cells. Our data indicate that inhibition of BTK by PCI-32765 overcomes BCR- and chemokine-controlled integrin-mediated retention and homing of malignant B cells in their growth- and survival-supporting lymph node and bone marrow microenvironment, which results in clinically evident CLL regression.

Introduction

Chronic lymphocytic leukemia (CLL), the most common adult leukemia, is an incurable malignancy of mature B lymphocytes characterized by the accumulation of resting malignant B cells in peripheral blood and the presence of proliferating malignant B cells in the lymph nodes (LN), spleen, and bone marrow (BM).[1-3] It is well established that the tumor microenvironment plays a major role in the pathogenesis of CLL: various cytokines, chemokines, and adhesion molecules provided within the LN, spleen, and BM microenvironment, as well as signaling by the B-cell antigen receptor (BCR), play a critical role in the localization, growth, survival, and drug resistance of CLL cells.[2-9]

Because either tonic, chronic, or antigen-driven BCR signaling is involved in the pathogenesis of most types of B-cell malignancies, the BCR signalosome provides a rational therapeutic target, including for CLL.[9] Regarding selectivity and clinical safety, Bruton tyrosine kinase (BTK) is a particularly promising target: it is a key component of the BCR signaling pathway, is only critical for B cells, and loss of BTK function is not lethal (*e.g.*, X-linked agammaglobulinemia patients and Btk-deficient mice).[10] Indeed, the selective, potent, orally administered, and irreversible small-molecule BTK-inhibitor PCI-32765[11] shows promising clinical activity in phase 1 and 2 studies in B-cell non-Hodgkin lymphoma, including complete or partial remission in a significant proportion of enrolled patients with diffuse large B-cell lymphoma, mantle cell lymphoma, and CLL.[12-14]

Interestingly, the CLL patients display an unexpected clinical response on treatment with PCI-32765: a rapid (within days) and sustained reduction of lymphadenopathy is accompanied by transient lymphocytosis, which is reversible upon temporary deprivation of the drug[13] (R. Advani, J.J.B., and N. Fowler, unpublished observations, 2010). Notably, some of the other efficacious small-molecule drugs that target the BCR signaling pathway, that is, inhibitors of SYK (R788/R406) and PI3K (CAL-101), show a similar response in clinical trials with CLL.[15;16] On the basis of our previous studies on B-cell adhesion and migration,[17-19] we hypothesized that this clinical response reflects attenuated microenvironment retention and homing of the CLL cells because of impaired BCR- or chemokine-controlled integrin-mediated adhesion or migration.

Materials and methods

Namalwa, Daudi, L363, or primary patient CLL cells, pretreated for 1 hour with 1μM PCI-32765, were allowed to adhere to either fibronectin- or VCAM-1–coated 96-well plates in the presence of anti-IgM, phorbol-12-myristate-13-acetate (PMA), or chemokines (CXCL12, CXCL13, or CCL19), or were allowed to migrate toward these chemokines in VCAM-1–coated transwells, essentially as described previously.[18;20] For further details and other methods, see supplemental Methods.

This study was conducted and approved by the Academic Medical Center Medical Committee on Human Experimentation. Informed consent was obtained in accordance with the Declaration of Helsinki.

Results and discussion

In line with the reported BTK specificity of PCI-32765[11;21] in the Burkitt lymphoma cell line Namalwa, we observed concentration-dependent inhibition by PCI-32765 of anti-IgM–induced phosphorylation of protein kinase B (PKB/AKT), ERK, and the BTK substrate site Y759 of phospholipase C-γ2 (PLCγ2), whereas phosphorylation of the activating LYN/SYK substrate site Y551 of BTK itself was actually augmented, which suggests inhibition of BTK-mediated negative feedback (Figs. 1A-B and S1A). Moreover, anti-IgM–induced adhesion to the extracellular matrix component fibronectin and the cellular adhesion molecule VCAM-1, which is mediated by integrin $\alpha_4\beta_1$,[18] was almost completely abolished by PCI-32765 treatment (Figs. 1C and S1B). In contrast, integrin-mediated adhesion induced by PMA, which activates protein kinase C downstream of BTK, was not affected (Fig. 1C). This demonstrates that the observed inhibitory effect of PCI-32765 on BCR-controlled integrin activation was specific and not caused by general cellular insensitivity or toxicity. Targeting of PI3K (wortmannin) or SYK (R406), but not MEK (PD-98059), also abolished BCR-controlled integrin-mediated adhesion (Fig. 1D).

Screening a panel of CLL patients revealed that CLL cells obtained from the majority (ie, 77%) of patients with a germline unmutated BCR, which have a worse prognosis,[2;3;9] could be further stimulated to adhere to either VCAM-1 or fibronectin by anti-IgM treatment, in contrast to the mutated BCR subgroup (Table S1). This most likely reflects the relatively anergic state of the mutated CLL cells with regard to BCR signaling,[22] as supported by their prominent adhesion in response to PMA and chemokines (Table S1). Importantly, in all unmutated CLL anti-IgM responders, apart from inhibition of BCR signaling (Fig. 1E), PCI-32765 strongly inhibited the

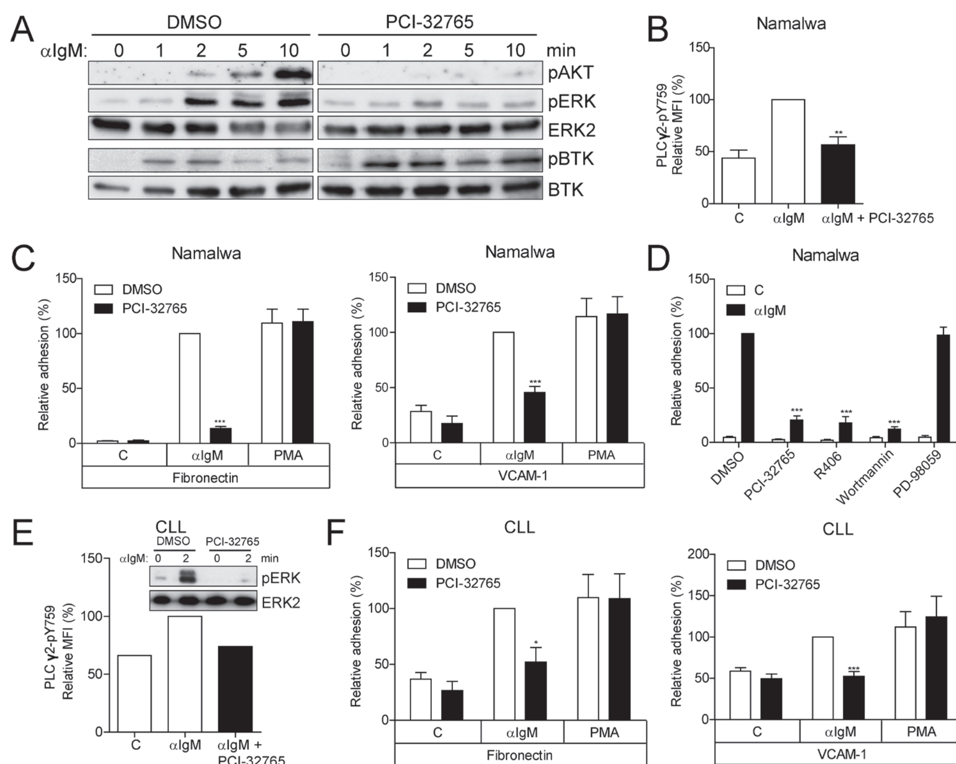


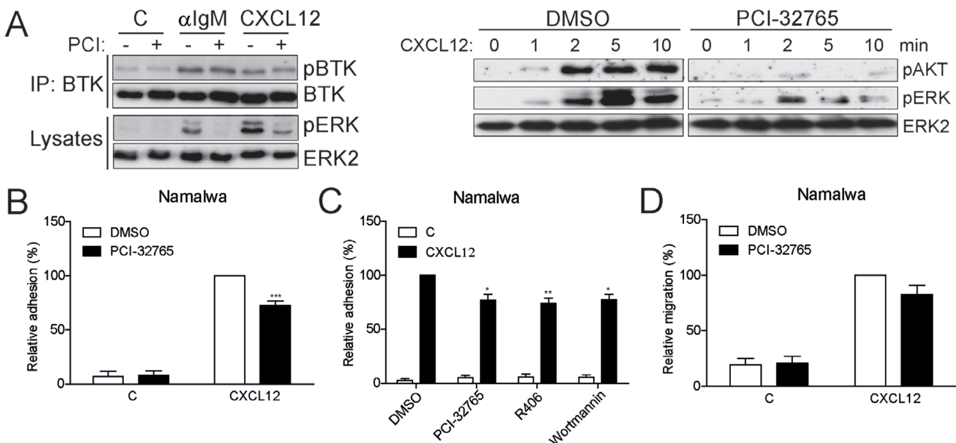
Figure 1. PCI-32765 abrogates BCR-controlled signaling and adhesion.
(A) Namalwa cells pretreated with 1μM PCI-32765 were stimulated with αIgM and immunoblotted for phosphorylated (p) AKT, ERK, and BTK (pY551). Total ERK2 and BTK were used as loading controls. The blots are representative of 4 independent experiments. (B) Namalwa cells pretreated with PCI-32765 were stimulated with αIgM, and phosphorylated phospholipase C-γ2 (PLCγ2-pY759) was measured by flow cytometry (n = 6). (C) Namalwa cells pretreated with PCI-32765 were stimulated with αIgM or PMA and allowed to adhere to fibronectin-coated (n = 13) or VCAM-1–coated (n = 8) surfaces. (D) Namalwa cells pretreated with PCI-32765 (BTK inhibitor), R406 (SYK inhibitor), wortmannin (PI3K inhibitor), or PD-98059 (MEK inhibitor) were stimulated with αIgM and allowed to adhere to fibronectin-coated surfaces (n = 7). (E) Primary CLL cells (patient 898) pretreated with 1μM PCI-32765 were stimulated with αIgM and immunoblotted for pERK. Total ERK2 was used as loading control. Phosphorylated PLCγ2 (pY759) was measured by flow cytometry from the same patient sample. (F) CLL cells pretreated with PCI-32765 were stimulated with αIgM or PMA and allowed to adhere to fibronectin-coated (n = 5 patients) or VCAM-1–coated (n = 6 patients) surfaces. Graphs are presented as normalized mean + SEM (100% = stimulated cells without inhibitors). C indicates control (unstimulated); and MFI, mean fluorescence intensity. **P* < .05; ***P* < .01; ****P* < .001.

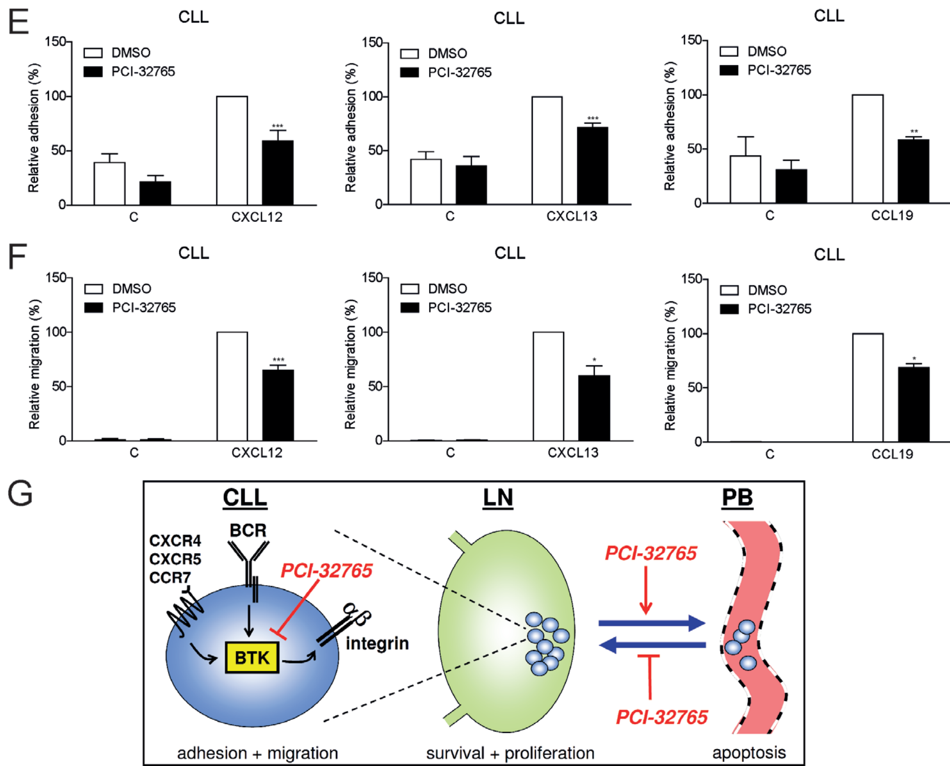
anti-IgM–stimulated integrin-mediated adhesion to fibronectin (mean inhibition ~75%) and VCAM-1 (mean inhibition ~100%; Fig. 1F). PMA-stimulated adhesion was unaffected (Fig. 1F), which again excluded cellular toxicity and demonstrated the specificity of PCI-32765, also in CLL cells.

Next, we addressed the possible effect of PCI-32765 on chemokine responses. The chemokine CXCL12 also induced phosphorylation of the activating LYN/SYK substrate site Y551 of BTK (Fig. 2A). Apart from concentration-dependent inhibition of CXCL12-induced phosphorylation of AKT and ERK, but not BTK (Figs. 2A and S1A), CXCL12-induced adhesion to VCAM-1 was inhibited in part by treatment of the Namalwa cells with PCI-32765 (Fig. 2B). A similar reduction was observed with the SYK inhibitor R406 and the PI3K inhibitor wortmannin (Fig. 2C). CXCL12-induced signaling and adhesion of BTK-negative L363 myeloma cells was not affected, which demonstrates the BTK specificity of the PCI-32765 effect (Figs. S1A and S2A). Furthermore, migration of Namalwa cells toward CXCL12 was consistently but not significantly inhibited by PCI-32765 (Fig. 2D), whereas prominent inhibition was observed in Daudi B cells (Figs. S2B-C).

Finally, we studied the effect of PCI-32765 on the response of primary CLL cells to CXCL12, CXCL13, and CCL19, the major chemokines involved in homing and retention of CLL cells in the LN and BM microenvironment.[5] As far as determined, the CLL cells of all patients expressed the corresponding receptors, that is, CXCR4, CXCR5, and CCR7 (Fig. S3A), and the CLL cells of most patients showed enhanced adhesion or migration in response to these chemokines, irrespective of the IgH_V mutation status (Table S1). PCI-32765 inhibited chemokine-induced signaling (Fig. S3B), and without exception, strongly inhibited CXCL12-, CXCL13-, and CCL19-induced adhesion of CLL cells (Fig. 2E). Furthermore, without compromising cell viability (Fig. S4), PCI-32765 partially inhibited (30%-40%) migration of the CLL cells toward these chemokines (Fig. 2F).

Taken together, the present data demonstrate that inhibition of BTK by PCI-32765 impairs BCR-controlled adhesion and chemokine-controlled adhesion and





2

Figure 2. PCI-32765 inhibits chemokine-induced signaling, adhesion and migration. (A) Namalwa cells pretreated with 1μM PCI-32765 were stimulated with αIgM or CXCL12, and total BTK was immunoprecipitated and subsequently immunoblotted for phosphorylated (p) BTK. Total BTK was used as loading control. Cell lysates were immunoblotted for pAKT and pERK. Total ERK2 was used as loading control. The blots are representative of 4 independent experiments. (B) Namalwa cells pretreated with PCI-32765 were allowed to adhere to surfaces coated with both VCAM-1 and CXCL12 (n = 10). (C) Namalwa cells pretreated with PCI-32765 (BTK inhibitor), R406 (SYK inhibitor), or wortmannin (PI3K inhibitor) were allowed to adhere to surfaces coated with both VCAM-1 and CXCL12 (n = 5). (D) Namalwa cells pretreated with PCI-32765 were allowed to migrate toward CXCL12 on VCAM-1–coated transwells (n = 7). (E) CLL cells pretreated with 1μM PCI-32765 were allowed to adhere to surfaces coated with both VCAM-1 and either CXCL12 (n = 7 patients), CXCL13 (n = 5 patients), or CCL19 (n = 3 patients). (F) CLL cells pretreated with 1μM PCI-32765 were allowed to migrate toward CXCL12 (n = 6 patients), CXCL13 (n = 4 patients), or CCL19 (n = 3 patients) on VCAM-1–coated transwells. (G) Inhibition of BTK by PCI-32765 impairs BCR-controlled integrin-mediated adhesion and chemokine (CXCL12, CXCL13, and CCL19)–induced adhesion and migration of CLL cells. Consequently, PCI-32765 overcomes BCR- and chemokine-controlled integrin-mediated retention of CLL cells in their growth- and survival-supporting LN and BM microenvironment, which results in their egress from these protective niches into the circulation (peripheral blood), and will prevent chemokine-driven homing into these niches, resulting in CLL regression. Graphs are presented as normalized mean + SEM (100% = stimulated cells without inhibitors). IP indicates immunoprecipitation; C, control (absence of chemokines); and PB, peripheral blood. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

migration of CLL cells. These findings in human primary CLL cells are corroborated by previous studies in which we observed impaired BCR-controlled adhesion and reduced CXCL12-/CXCL13-controlled adhesion, migration, and LN homing of Btk-deficient chicken or murine B cells.[18,19] The present results nicely explain the observed clinical response of CLL patients treated with PCI-32765:[12-14] impaired BCR- and chemokine-controlled retention of malignant cells in the BM and LN will cause the observed reduced lymphadenopathy and (transient) lymphocytosis, which deprives the cells of critical microenvironmental growth and survival signals, resulting in tumor regression, whereas temporary drug deprivation restores chemokine-driven LN homing and retention of CLL cells (Fig. 2G). In accordance, shortly after PCI-32765 treatment of the patients, most circulating CLL cells displayed low CXCR4 expression (Fig. S5), characteristic of LN- and BM-derived CLL cells.[5;8] Likewise, because targeting SYK and PI3K also inhibits BCR- and CXCL12-controlled adhesion (Figs. 1D and 2C), as well as CXCL12-induced migration,[6;23;24] this may also explain the observed lymphocytosis in clinical trials of CLL patients with the SYK inhibitor R788/fostamatinib (the R406 prodrug) and the PI3Kδ inhibitor CAL-101.[15;16]

These novel insights regarding drugs that target the BCR (and chemokine receptor) signalosome may not only provide support for their further use as monotherapy, exploiting the microenvironment dependence as the Achilles' heel of CLL, but also for their exploration as rational combination therapy. Once the malignant B cells egress from their protective niches into circulation, they may become more accessible and vulnerable to chemotherapy and antibody therapy (*e.g.* rituximab). This would make a promising and highly efficacious combination therapy, possibly resulting in greater benefit for CLL patients.

Acknowledgment
The authors thank Robbert Hoogeboom for providing some of the CLL patient samples. This work was supported by Pharmacyclics Inc.

Authorship contributions
M.F.M.d.R. designed the research, performed experiments, analyzed the data, designed the figures, and wrote the manuscript; A.K. performed experiments; C.R.G. and E.E. provided CLL patient samples and data and reviewed the manuscript; B.Y.C. performed experiments and analyzed data; J.J.B. designed the research and reviewed the manuscript; S.T.P cosupervised the study and reviewed the manuscript; and M.S. designed the research, supervised the study, analyzed the data, and wrote and revised the manuscript.

Disclosure of conflicts of interest

B.Y.C. and J.J.B. are employees of Pharmacyclics Inc and have a financial interest in PCI-32765. M.S. has received research support from Pharmacyclics Inc. The remaining authors declare no competing financial interests.

References

1. Messmer BT, Messmer D, Allen SL et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J.Clin.Invest.* 2005;115(3):755-764.
2. Pleyer L, Egle A, Hartmann TN, Greil R. Molecular and cellular mechanisms of CLL: novel therapeutic approaches. *Nat.Rev.Clin.Oncol.* 2009;6(7):405-418.
3. Zenz T, Mertens D, Kuppers R, Dohner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nat.Rev.Cancer* 2010;10(1):37-50.
4. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood* 2009;114(16):3367-3375.
5. Burger JA. Chemokines and chemokine receptors in chronic lymphocytic leukemia (CLL): from understanding the basics towards therapeutic targeting. *Semin.Cancer Biol.* 2010;20(6):424-430.
6. Buchner M, Baer C, Prinz G et al. Spleen tyrosine kinase inhibition prevents chemokine- and integrin-mediated stromal protective effects in chronic lymphocytic leukemia. *Blood* 2010;115(22):4497-4506.
7. Smit LA, Hallaert DY, Spijker R et al. Differential Noxa/Mcl-1 balance in peripheral versus lymph node chronic lymphocytic leukemia cells correlates with survival capacity. *Blood* 2007;109(4):1660-1668.
8. Herishanu Y, Perez-Galan P, Liu D et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* 2011;117(2):563-574.
9. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood* 2011;118(16):4313-4320.
10. Conley ME, Dobbs AK, Farmer DM et al. Primary B cell immunodeficiencies: comparisons and contrasts. *Annu.Rev.Immunol.* 2009;27:199-227.
11. Honigberg LA, Smith AM, Sirisawad M et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc. Natl.Acad.Sci.U.S.A.* 2010;107(29):13075-13080.
12. Burger JA, O'Brien S, Fowler N et al. The Bruton's tyrosine kinase inhibitor, PCI-32765, is well tolerated and demonstrates promising clinical activity in chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL): an update on ongoing phase 1 studies. *Blood* 2010;116(21):32.
13. Byrd JC, Blum KA, Burger JA et al. Activity and tolerability of the Bruton's tyrosine kinase (Btk) inhibitor PCI-32765 in patients with chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL): Interim results of a phase Ib/II study. *J.Clin.Oncol.* 2011;29:6508.
14. Fowler N, Sharman JP, Smith SM et al. The Btk inhibitor, PCI-32765, induces durable responses with minimal toxicity in patients with relapsed/refractory B-cell malignancies: results from a phase I study. *Blood* 2010;116(21):425.
15. Friedberg JW, Sharman J, Sweetenham J et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* 2010;115(13):2578-2585.

16. Furman RR, Byrd JC, Brown JR et al. CAL-101, an isoform-selective inhibitor of phosphatidylinositol 3-kinase p110 delta, demonstrates clinical activity and pharmacodynamic effects in patients with relapsed or refractory chronic lymphocytic leukemia. *Blood* 2010;116(21):31.
17. Koopman G, Keehnen RM, Lindhout E et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM1 (CD106) pathways prevents apoptosis of germinal center B cells. *J.Immunol.* 1994;152(8):3760-3767.
18. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J.Exp.Med.* 2003;198(10):1539-1550.
19. de Gorter DJ, Beuling EA, Kersseboom R et al. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity.* 2007;26(1):93-104.
20. de Gorter DJ, Reijmers RM, Beuling EA et al. The small GTPase Ral mediates SDF-1-induced migration of B cells and multiple myeloma cells. *Blood* 2008;111(7):3364-3372.
21. Herman SE, Gordon AL, Hertlein E et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* 2011;117(23):6287-6296.
22. Guarini A, Chiaretti S, Tavolaro S et al. BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgV H unmutated chronic lymphocytic leukemia (CLL) cells. *Blood* 2008;112(3):782-792.
23. Hoellenriegel J, Meadows SA, Sivina M et al. The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood* 2011;118(13):3603-3612.
24. Niedermeier M, Hennessy BT, Knight ZA et al. Isoform-selective phosphoinositide 3'-kinase inhibitors inhibit CXCR4 signaling and overcome stromal cell-mediated drug resistance in chronic lymphocytic leukemia: a novel therapeutic approach. *Blood* 2009;113(22):5549-5557.

Supplemental appendix

Supplementary materials and methods

Materials

The following reagents were used in this study: the phosphorylation state-specific antibodies phospho-p44/42 MAP kinase [T202/Y204] against ERK1 and -2, phospho-AKT [Ser473] against PKB/AKT (Cell Signaling Technology), phospho-BTK [Y551] against BTK (BD Biosciences) and phospho-PLCγ2 [Y759] against PLCγ2; anti-ERK2 (C-14; Santa Cruz Biotechnology), anti-BTK (Clone 53; BD Bioscience), goat F(ab)'2 anti-human IgM (LE/AF; Southern Biotech), horseradish peroxidase (HRP)-conjungated rabbit anti-mouse and HRP-conjungated goat anti-rabbit (DAKO), phycoerythrin-conjungated anti-CXCR4 (clone 12G5), fluorescein-conjungated anti-CXCR5 and fluorescein-conjungated anti-CCR7 (R&D Systems); protein A-Sepharose beads (Pharmacia); the pharmacological inhibitors PCI-32765 (Pharmacyclics, Sunnyvale, CA 94085-4521), R406 (Axon Medchem), PD-98059, wortmannin and phorbol-12-myristate-13-acetate (Sigma-Aldrich); recombinant human sVCAM-1 (R&D Systems), human plasma fibronectin, BSA (fraction V), recombinant human CXCL12 and recombinant human CXCL13 (R&D Systems), recombinant human CCL19 (MT-diagnostics), poly-l-Lysine (PLL) (Sigma-Aldrich), FITC-conjungated annexin V (IQ products) and TO-PRO-3-'iodide (Invitrogen).

Cell lines and primary material

The Burkitt's lymphoma cell lines Namalwa clone V3M[1] and Daudi,[2] and multiple myeloma cell line L363[3] were cultured as described. Peripheral blood derived CLL cells were obtained after routine diagnostic or follow-up procedures at the departments of Hematology or Pathology of the Academic Medical Center (AMC) Amsterdam, and were isolated and subsequently frozen and stored as previously described.[4] CLL samples included in this study contained 81-99% $CD5^+/CD19^+$ cells. This study was conducted and approved by the AMC Medical Committee on Human Experimentation. Informed consent was obtained in accordance with the Declaration of Helsinki.

Adhesion assay

The cell adhesion assays were performed essentially as described.[5] In detail, adhesion assays were done in triplicate on EIA/RIA 96-well plates (Costar) coated overnight at 4°C with PBS containing, 10μg/ml fibronectin or 500ng/ml VCAM-1, 4% BSA, or for 15 min at 37°C with 1mg/ml poly-l-lysine (PLL), and blocked for 2h at

37°C with 4% BSA in RPMI 1640. Cells were pretreated with 1μM PCI-32765, 200nM wortmannin, 1μM R406 or 50μM PD-98059 at 37°C for 1h in RPMI with 1% BSA. Subsequently, cells were stimulated with either 200ng/ml goat $F(ab')_2$ anti-human IgM, or 50ng/ml PMA, and $1.5.10^5$ Namalwa or 3.10^5 CLL-cells were immediately plated in 100μl/well and incubated at 37°C for 30 min. After extensive washing of the plate with RPMI containing 1% BSA to remove non-adhered cells, the adherent cells were fixed for 10 min with 10% glutaraldehyde in PBS and subsequently stained for 45 min with 0.5% crystal violet in 20% methanol. After extensive washing with water, the dye was eluted in methanol and absorbance was measured after 40 min at 570nm on a spectrophotometer (Multiskan RC spectrophotometer, Thermo labsystems). Background absorbance (no cells added) was subtracted. Absorbance due to nonspecific adhesion, as determined in wells coated with 4% BSA, was always less than 10% of the absorbance of anti-IgM-stimulated cells. Maximal adhesion (100%) was determined by applying the cells to wells coated with PLL, without washing the wells before fixation. Adhesion of the nonpretreated anti-IgM-stimulated cells was normalized to 100% and the bars represent the means + SEM of at least five independent experiments (Namalwa) or primary CLL cells obtained from at least three different patients, each assayed in triplicate.

Chemokine-mediated adhesion was assayed as described above, except that the chemokines 100ng/ml CXCL12, 500ng/ml CXCL13 or 100ng/ml CCL19 were coimmobilized with 500ng/ml VCAM-1. The plates were spun directly after applying the cells to the plate, and the cells were allowed to adhere for 2 min.

Migration assay

The cell migration assays were performed essentially as described.[6] In detail, migration assays were performed in triplicate with transwells (pore size 5μm (CLL) or 8μm (Namalwa and Daudi), Costar) coated with 500ng/ml VCAM-1 or uncoated. The lower compartment contained 100 ng/ml CXCL12, 500ng/ml CXCL13, or 100ng/ml CCL19. The cells, pretreated with 1μM PCI-32765 at 37°C for 1h in RPMI with 0.5% BSA, were applied to the upper compartment and allowed to migrate for 5h (Namalwa) or 2h (CLL cells) at 37°C. The amount of viable migrated cells was determined by FACS and expressed as a percentage of the input. The migration of nonpretreated cells on VCAM-1–coated transwells in the presence of CXCL12, CXCL13 or CCL19 was normalized to 100%, and the bars represent the means + SEM of seven independent experiments (Namalwa) or primary CLL cells from at least three different patients, each assayed in triplicate.

Immunoblotting

Immunoblotting was performed essentially as described.[5] In detail, 10^7 cells/ml RPMI were pretreated with 1µM PCI-32765 at 37°C for 1h. After stimulation with 200ng/ml goat anti-human IgM, $F(ab')_2$ or 100ng/ml CXCL12, 100ng/ml CXCL13 or 100ng/ml CCL19 for 2 min (or as indicated), cells were directly lysed in SDS-PAGE sample buffer. 2.10^5 cells (Namalwa) or 3.10^6 cells (CLL cells) were applied on a 10% SDS-PAGE gel and blotted with rabbit anti-phospho-ERK1/2 (Cell signaling), rabbit anti-phospho-AKT, mouse anti-phospho-BTK or mouse anti-β-actin followed by HRP-conjugated goat anti-rabbit or rabbit anti-mouse and developed by enhanced chemiluminescence (Amersham Pharmacia). To confirm equal expression and loading, the blots were stripped or occasionally blots were generated in a parallel fashion, and incubated with the antibodies rabbit anti-ERK2 and mouse anti-BTK.

Immunoprecipitation

Immunoprecipitation was performed essentially as described.[1] In detail, 10^7 Namalwa cells/ml RPMI were pretreated with 1µM PCI-32765 at 37°C for 1h. After stimulation with 200ng/ml goat anti-human IgM, $F(ab')_2$ or 100ng/ml CXCL12 for 2 min, the cells were directly lysed by addition of an equal volume ice-cold 2x lysis buffer (20mM Tris-HCl pH 8.0, 300mM NaCl, Nonidet P-40, 20% glycerol, 10mM EDTA, 4mM Na_3VO_4, 10mM NaF and 2 EDTA-free protease inhibitor cocktail tablets (Roche) per 50ml). After preclearance with protein A-Sepharose beads, the lysates were incubated with 1µg mouse anti-BTK at 4°C o/n, and immunoprecipitation was carried out by adding protein A-Sepharose beads for 1h. After extensive washing of the beads with 1x lysis buffer, immunoprecipitates were analyzed by immunoblotting, using mouse anti-phospho-BTK and reprobed with mouse anti-BTK.

PLCγ2 phosflow analysis

10^6 cells/ml RPMI (Namalwa or CLL cells) were pretreated with 1µM PCI-32765 at 37°C for 1h. The cells were incubated in suspension for 5 min with 200ng/ml goat $F(ab')_2$ anti-human IgM. Then, cells were fixed, washed, permeabilized (Perm buffer III) and incubated with phycoerythrin conjungated mouse anti-PLCγ2-pY759 (BD Biosciences Technology) for 30 min on ice and washed. Stainings were measured on a FACScanto II flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v6.0), and analyzed with Flow Jo (v7.2.1). Geometric means of the nonpretreated anti-IgM-stimulated cells was normalized to 100% and the bars represent the means + SEM of at least three independent experiments.

Flow cytometry

10^5 Namalwa or CLL cells were stained with phycoerythrin-conjungated anti-CXCR4, fluorescein-conjungated anti-CXCR5, fluorescein-conjungated anti-CCR7 or isotype control for 30 min on ice and washed. Stainings were measured on a FACScanto II flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v6.0), and analyzed with Flow Jo (v7.2.1).

Apoptosis analysis

10^6 cells/ml RPMI (Namalwa, L363 or CLL cells) were pretreated with 1µM PCI-32765 at 37°C for 3h. The cells were stained with FITC-conjungated annexin V for 30 min on ice and washed. Immediately before measuring, TO-PRO-3-‘iodide was added. Stainings were measured on a FACScantoII flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v6.0), and analyzed with Flow Jo (v7.2.1).

Statistical analysis

The unpaired two-tailed Student’s *t*-test was used to determine the significance of differences between two means. The one sample *t*-test was used to determine the significance of differences between means and normalized values (100%). $*P < .05$; $**P < .01$; $***P < .001$.

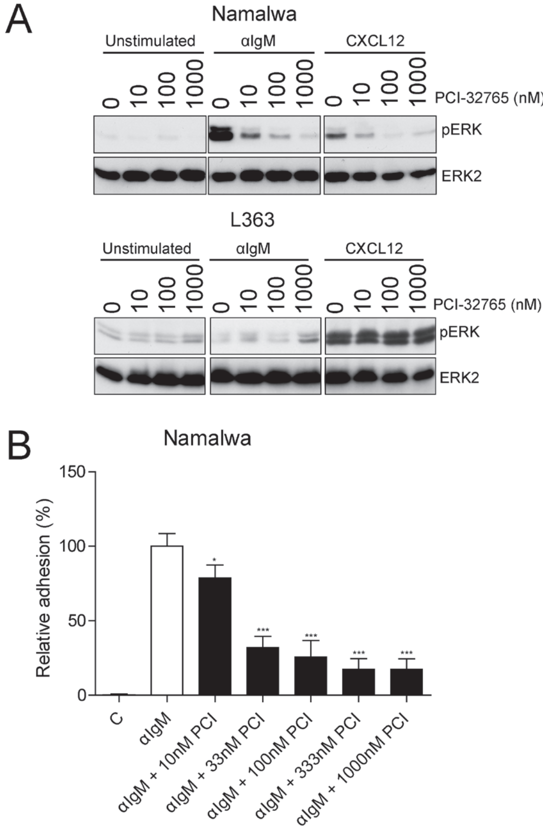
References

1. van der Voort R, Taher TE, Wielenga VJ et al. Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. *J.Biol.Chem.* 1999;274 (10) :6499-6506.
2. van der Voort R, Taher TE, Keehnen RM et al. Paracrine regulation of germinal center B cell adhesion through the c-met-hepatocyte growth factor/scatter factor pathway. *J.Exp.Med.* 1997;185 (12) :2121-2131.
3. Rozemuller H, van der Spek E, Bogers-Boer LH et al. A bioluminescence imaging based in vivo model for preclinical testing of novel cellular immunotherapy strategies to improve the graft-versus-myeloma effect. *Haematologica* 2008;93 (7) :1049-1057.
4. Hallaert DY, Jaspers A, van Noesel CJ et al. c-Abl kinase inhibitors overcome CD40-mediated drug resistance in CLL: implications for therapeutic targeting of chemoresistant niches. *Blood* 2008;112 (13) :5141-5149.
5. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J.Exp.Med.* 2003;198 (10) :1539-1550.
6. de Gorter DJ, Reijmers RM, Beuling EA et al. The small GTPase Ral mediates SDF-1-induced migration of B cells and multiple myeloma cells. *Blood* 2008;111 (7) :3364-3372.

Supplementary tables and figures

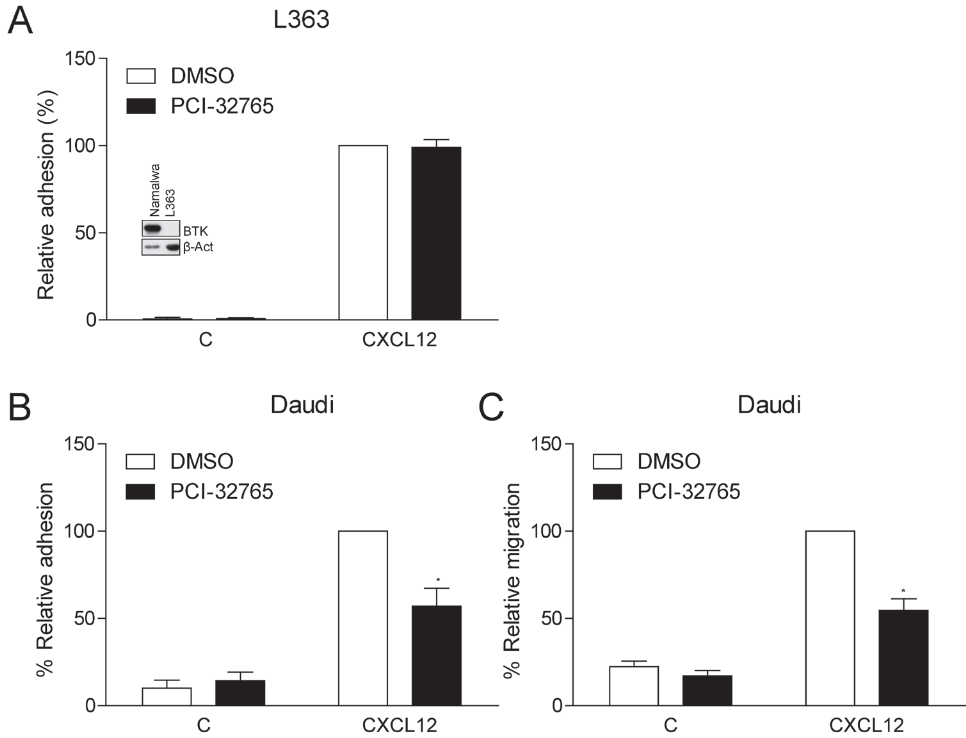
CLL patient no.	IgV_H gene status	αIgM FN/VCAM1	PMA FN/VCAM1	CXCL12 Adh./Mig.	CXCL13 Adh./Mig.	CCL19 Adh./Mig.	WBC count (10^9/l)	$CD3^+$ (%)	$CD19^+$ (%)	$CD5^+CD19^+$ (%)
81	U	+/+	o/o	+/o	o/o	o/o	122	5	92	82
233	U	+/+	+/+	+/o	o/+	o/o	80	1	98	98
479	U	+/+	+/+	+/+	o/o	o/o	232	1	99	99
560	U	-/-	+/+	o/o	o/o	o/o	69	13	80	79
745	U	-/-	+/+	o/+	+/+	o/+	48	7	84	84
801	U	o/+	o/o	o/o	o/o	o/o	94	7	87	87
804	U	o/+	o/+	o/+	-/-	o/-	167	8	85	84
898	U	+/o	+/o	+/+	+/o	o/o	127	4	95	93
947	U	+/+	+/o	-/o	o/o	o/o	234	3	94	94
409	M	-/-	+/+	+/o	-/o	o/o	47	9	81	81
1030	M	-/-	+/+	o/o	o/o	o/o	237	9	89	88
1044	M	-/o	+/o	+/o	+/o	o/o	153	3	96	96
2101	M	-/-	o/+	+/+	+/+	+/o	35	6	90	86
2103	M	-/-	+/+	o/+	+/+	+/+	183	2	96	96
2106	M	o/o	o/o	o/o	o/o	+/+	26	4	83	83
U: unmutated (<2%) M: mutated (>2%)		+: positive -: negative o: not determined						T-cells	B-cells	CLL-cells

Table S1. Patient characteristics.
The characteristics of individual CLL-patients are summarized in this table. FN, adhesion to fibronectin; VCAM1, adhesion to VCAM-1; Adh., adhesion; Mig., migration; WBC, white blood cell count.



2

Figure S1. Dose responsiveness of PCI-32765.
(A) Namalwa and L363 pretreated for 1h with different concentrations of PCI-32765 were stimulated with αIgM or CXCL12, and immunoblotted for p-ERK. Total ERK2 was used as loading control. Notice that L363 (a BTK negative multiple myeloma cell line) does not express membrane bound BCR. (B) Namalwa cells pretreated with different concentrations of PCI-32765 were stimulated with αIgM and allowed to adhere to fibronectin-coated surfaces. Graph is presented as normalized mean + SD from triplicates (100% = stimulated cells without inhibitor), C = control (unstimulated). *P < .05; **P < .01; ***P < .001.



2

Figure S2. CXCL12-induced adhesion and migration of Daudi is inhibited by PCI-32765.
(A) L363 (BTK negative cell line, see inset) pretreated for 1h with 1μM PCI-32765 were allowed to adhere to VCAM-1– and CXCL12-cocoated surfaces (n = 4 independent experiments. (B) Daudi cells pretreated with PCI-32765 were allowed to adhere to VCAM-1– and CXCL12-cocoated surfaces (n = 4 independent experiments). (C) Daudi cells pretreated with PCI-32765 were allowed to migrate towards CXCL12 (n = 3 independent experiments). Graphs are presented as the normalized mean + SEM of independent experiments (100% = stimulated cells without inhibitor). C = control (absence of CXCL12). $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

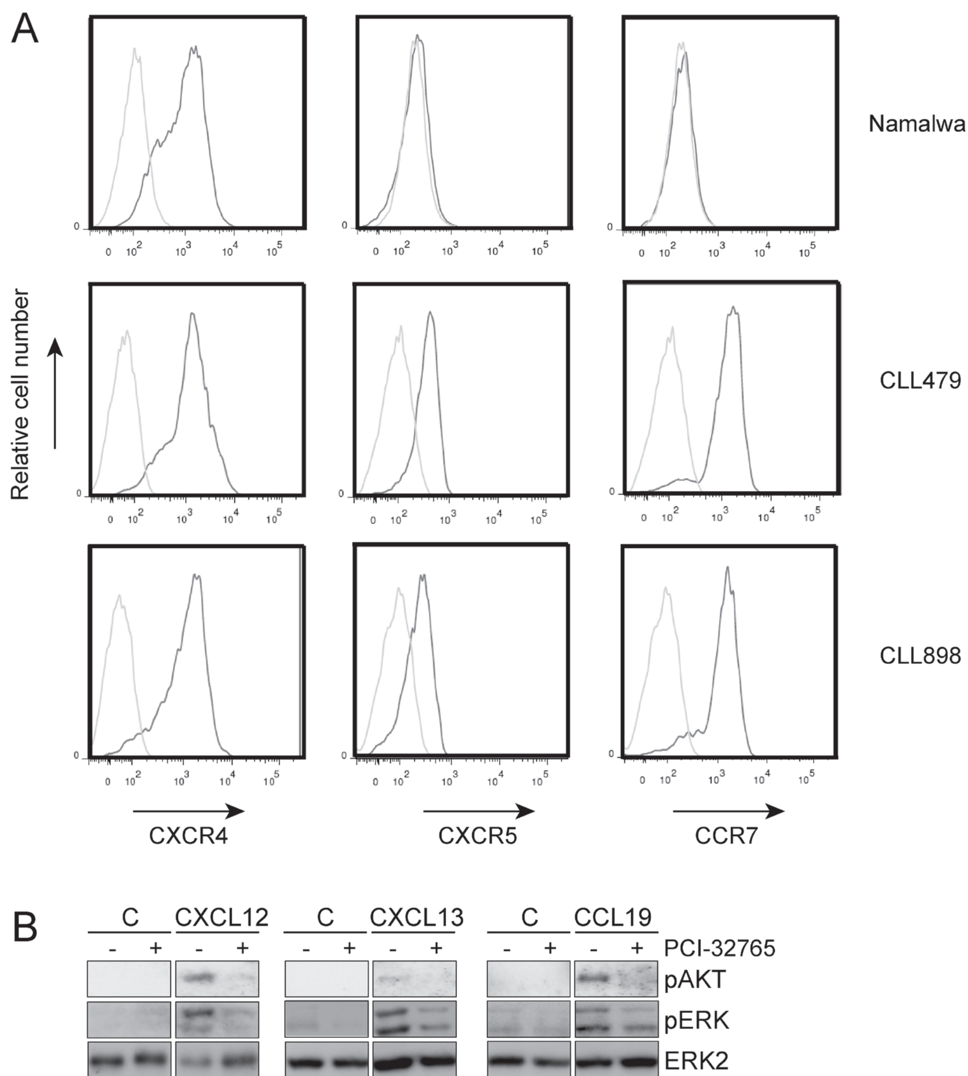


Figure S3. Chemokine receptor expression and signaling in CLL cells.
(A) Namalwa and CLL cells (representative for 5 patients) were stained for CXCR4, CXCR5 or CCR7 (red) or unstained (blue) and analyzed by flow cytometry. All patients analyzed (# 479, 560, 745, 898, and 2103) were positive for all three chemokine receptors. (B) Primary CLL cells (patient 898) pretreated with PCI-32765 were stimulated with CXCL12, CXCL13 or CCL19, and immunoblotted for p-ERK and p-AKT. Total ERK2 was used as loading control.

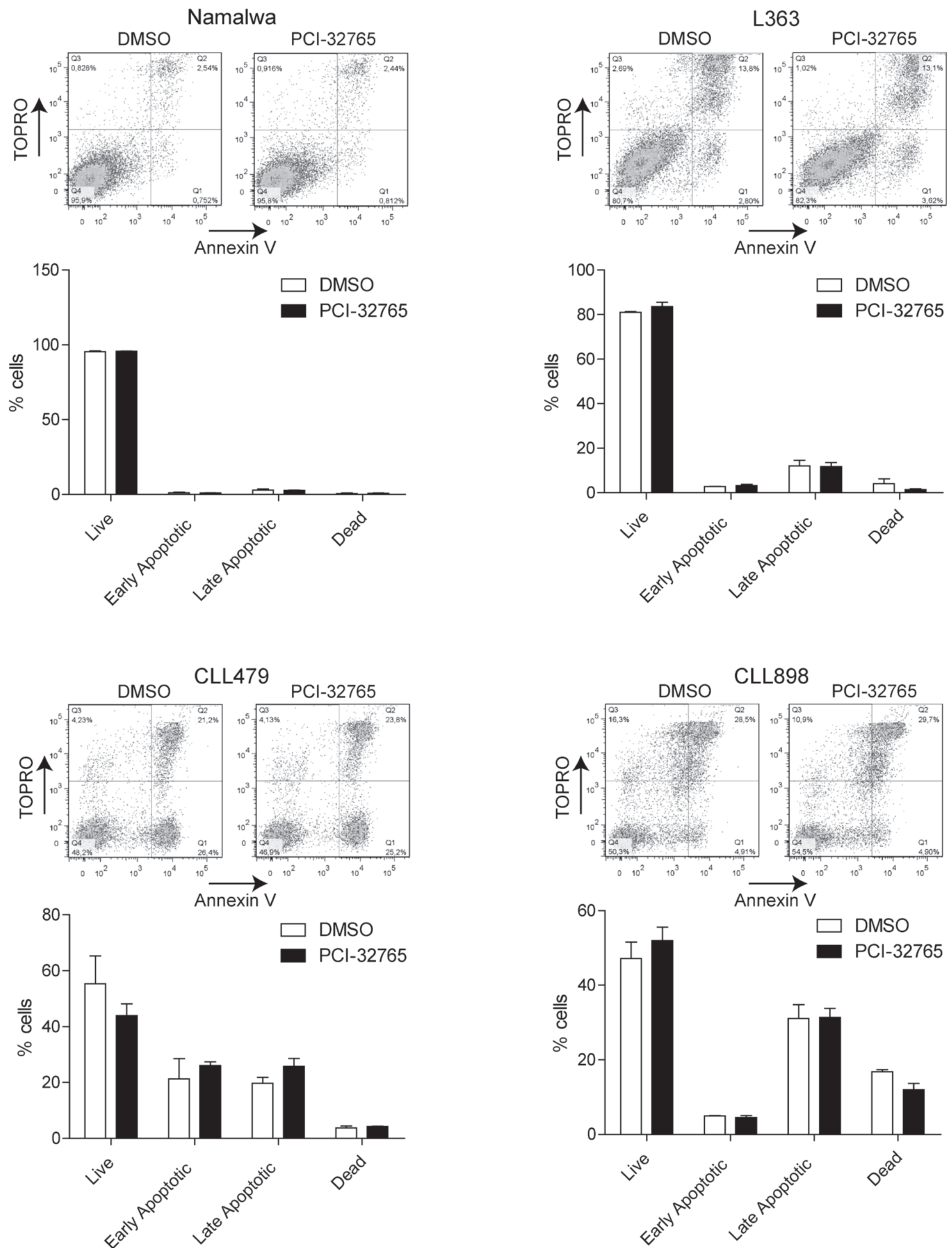


Figure S4. Cell viability is not affected by (short term) PCI-32765 treatment.
Namalwa, L363, and CLL cells treated with PCI-32765 for 3 hours were stained for annexin V and TO-PRO-3‘-iodide and analyzed by flow cytometry. Presented as percentage of live (annexin V and TOPRO double negative), early apoptotic: (annexin V positive and TOPRO negative), late apoptotic (annexin V and TOPRO double positive), or dead cells (annexin V negative and TOPRO positive). Representative FACS plots are shown and the graphs are presented as mean + SD (duplo measurement). *P < .05; **P < .01; ***P < .001.

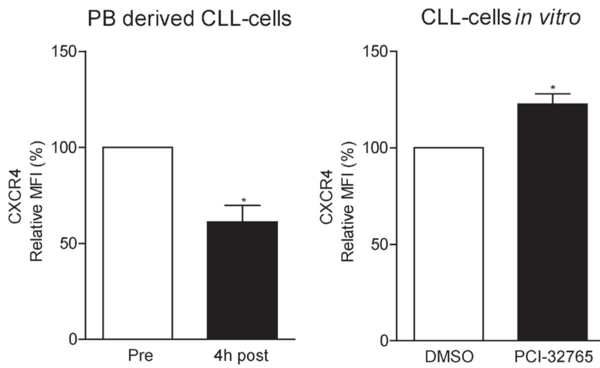


Figure S5. Reduced CXCR4 expression in circulating CLL cells in PCI-32765-treated patients. Peripheral blood (PB) derived CLL cells obtained either pre-administration (pre) or 4h post-administration (4h post) of PCI-32765 (n = 3 patients) and CLL cells (in vitro) treated with PCI-32765 (for 4 hours) (n = 2 patients) were stained for CXCR4 and analyzed by flow cytometry. Graphs are presented as normalized mean + SEM. *$P < .05$; **$P < .01$; ***$P < .001$.

Chapter 3

Egress of CD19$^+$CD5$^+$ cells into peripheral blood following treatment with the Bruton tyrosine kinase inhibitor ibrutinib in mantle cell lymphoma patients

Betty Y. Chang[1], Michelle Francesco[1*], Martin F.M. de Rooij[2*], Padmaja Magadala[1], Susanne M. Steggerda[1], Min Mei Huang[1], Annemieke Kuil[2], Sarah E. M. Herman[3], Stella Chang[1], Steven T. Pals[2], Wyndham Wilson[3], Adrian Wiestner[3], Marcel Spaargaren[2], Joseph J. Buggy[1], and Laurence Elias[1]

[1]Research Department, Pharmacyclics, Inc., Sunnyvale, CA; [2]Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and [3]Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD. [*]M.F. and M.F.M.d.R contributed equally to the manuscript.

Blood (2013) 122:2412-24

Abstract

Ibrutinib (PCI-32765) is a highly potent oral Bruton tyrosine kinase (BTK) inhibitor in clinical development for treating B-cell lymphoproliferative diseases. Patients with chronic lymphocytic leukemia (CLL) often show marked, transient increases of circulating CLL cells following ibrutinib treatments, as seen with other inhibitors of the B-cell receptor (BCR) pathway. In a phase 1 study of ibrutinib, we noted similar effects in patients with mantle cell lymphoma (MCL). Here, we characterize the patterns and phenotypes of cells mobilized among patients with MCL and further investigate the mechanism of this effect. Peripheral blood $CD19^+CD5^+$ cells from MCL patients were found to have significant reduction in the expression of CXCR4, CD38, and Ki67 after 7 days of treatment. In addition, plasma chemokines such as CCL22, CCL4, and CXCL13 were reduced 40% to 60% after treatment. Mechanistically, ibrutinib inhibited BCR- and chemokine-mediated adhesion and chemotaxis of MCL cell lines and dose-dependently inhibited BCR, stromal cell, and CXCL12/CXCL13 stimulations of pBTK, pPLCγ2, pERK, or pAKT. Importantly, ibrutinib inhibited migration of MCL cells beneath stromal cells in coculture. We propose that BTK is essential for the homing of MCL cells into lymphoid tissues, and its inhibition results in an egress of malignant cells into peripheral blood. This trial was registered at www.clinicaltrials.gov as #NCT00114738.

Introduction

Mantle cell lymphoma (MCL) is an aggressive type of B-cell malignancy, constituting 8% of non-Hodgkin lymphomas.[1-3] MCL is typically characterized by the t(11;14)(q13;q32) translocation, which drives cyclin D1 overexpression. Constitutive activation of the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and nuclear factor κB pathways contribute to the pathogenesis of MCL.[3] The majority of MCL patients present with advanced disease at diagnosis, and more than 90% of patients have extranodal manifestations with circulating MCL cells, bone marrow, and gastrointestinal involvement. In general, MCL patients have a poor prognosis, with a median overall survival time of 30 to 43 months and fewer than 15% of them are long-term survivors.[1;2] This demonstrates a clear need for new therapeutics for the treatment of this disease.

The interaction of neoplastic B cells with stromal cells in the lymph node (LN) or bone marrow microenvironment plays a critical role in the survival, progression, and drug resistance of various B-cell malignancies,[4-7] including MCL.[6;8;9] Importantly, the homing and trafficking of B cells into the microenvironment is tightly controlled and regulated by the interaction of chemokine receptors and adhesion molecules.[10-15] The contact between MCL cells and mesenchymal stromal cells (MSCs) is established and maintained by chemokine receptors and adhesion molecules. Stromal cells in lymphoid tissues constitutively express chemokines such as CXCL12 and CXCL13, forming gradients that allow the homing of B lymphocytes from the periphery into tissue compartments. MCL cells express G protein-coupled chemokine receptors such as CXCR4 and CXCR5 that bind CXCL12 and CXCL13, respectively.[6] Adhesion is facilitated by binding of integrins such as $\alpha_4\beta_1$ on B cells to VCAM-1 on stromal cells and fibronectin in the extracellular matrix.[16] In addition to the chemokine receptor and integrin engagement, it has been shown that B-cell receptor (BCR) activation is involved in integrin (such as $\alpha_4\beta_1$)-mediated adhesion[5;12;16-18] and is thought to contribute to the growth and survival of most types of B-cell malignancies.[18-21] BCR signaling pathway phosphoproteins are represented abundantly in MCL cell lines,[3;22;23] and genomic lesions or constitutive activation of signaling proteins downstream of the BCR pathways such as SYK and PI3Kα have been reported in MCL.[23-25]

Since BCR signaling is important for integrin-mediated adhesion, growth, and survival of B lymphocytes, Bruton tyrosine kinase (BTK), a key component of the BCR pathway, is thus significant in B lymphocyte adhesion and survival.[5;12;16;17] More recently, it has been shown that BTK plays a role in chemokine (such as CXCL12) -controlled B-cell chemotaxis and homing.[12] The BTK inhibitor ibrutinib (PCI-32765)

is an irreversible covalent inhibitor with a 50% inhibitory concentration of 0.5nM against BTK in biochemical assays, has been found to have broad antitumor activity in B-cell malignancies including MCL,[26;27] and is currently being evaluated in phase 3 clinical studies. In the initial phase 1 study, we noted that many MCL patients had rapid increases of $CD5^+$ B lymphocytes in the peripheral blood (PB) following ibrutinib treatment. This often occurred concomitantly with rapid reductions of lymphadenopathy, suggesting an egress of malignant cells from tissues into PB. The schedule of drug administration for most patients in the phase 1 study was in cycles of daily administration for 28 days, followed by 7 days without drug administration. We observed that the lymphoid flux was rapidly reversed during the 7-days-off portion of the treatment, with prompt reappearance at the beginning of the next cycle, resulting in a sawtooth pattern in PB. These patterns, along with immunophenotypic characterization of the cells involved, are presented here. We further investigated the mechanism of this effect by using MCL cell lines and primary cells and also in an MCL-stromal coculture system. We demonstrated that ibrutinib suppressed BCR- and CXCL12-/CXCL13-mediated adhesion and chemotaxis, suppressed migration of cells beneath the stromal cells (pseudo-emperipoiesis), and inhibited the phosphorylation of BTK, PLCγ2, and ERK in MCL cells. These observations effectively highlight the importance of BTK catalytic activity in the homing of MCL cells into lymphoid tissues and provide insight into the relevant mechanisms.

Materials and methods

Primary human MCL specimens from drug-treated patients

Blood was drawn from MCL patients (Table S1) enrolled in PCYC-04753[26] or PCYC-1104[27] (before April 1, 2012) in accordance with International Conference on Harmonisation Good Clinical Practice guidelines and principles of the Declaration of Helsinki, with informed consent from each patient and in compliance with the protocols approved by the relevant institutional review board. The blood samples were drawn into sodium heparin cell preparation tubes (BD) and shipped overnight to Pharmacyclics, Inc., within 24 hours. In a laminar flow hood, the peripheral blood mononuclear cells (PBMCs) were collected, washed with phosphate-buffered saline (PBS), and frozen in 90% fetal bovine serum and 10% dimethylsulfoxide in liquid nitrogen until use.

Cell lines and primary material for *ex vivo* studies

Adhesion studies were performed with PBMCs from MCL patients of the Academic

Medical Center (Amsterdam, The Netherlands). For *ex vivo* CXCR4 and CD38 staining studies, PB and LN biopsies were collected from treatment-naive MCL patients enrolled in National Cancer Institute Study 05-C-0170 (www.clinicaltrials.gov #NCT00114738) with approval from the National Institutes of Health Institutional Review Board and informed consent. Matched PB and LN samples were obtained on the same day and were processed and analyzed in parallel.

MCL phenotyping

PBMCs were washed, pelleted, and resuspended in PBS and 2% fetal bovine serum containing phenotyping surface antibodies. All staining cocktails were run in duplicate tubes. Cells were stained for 30 minutes, washed, pelleted, and fixed in PBS and 1.6% paraformaldehyde. Cells to be analyzed for proliferation with Ki67 were permeabilized with 70% ethanol at −20°C overnight, rehydrated with PBS, and stained with Ki67 antibody.

Flow cytometry

BD FACS (fluorescence-activated cell sorter) Canto II was used for all flow cytometry collections. Phosphoflow assays were stained and performed as described.[28] At least 10,000 CD19$^+$ cells were collected from each staining sample. The data were analyzed and quantified by using Flow Jo (v7.6), and the geometric mean was derived and presented for the mean fluorescence intensities (MFIs) of histograms for the specifically stained cell populations.

Coculture assays

Cocultures of M2-10B4 stromal cells and the MCL cell line Mino were established according to Burger et al.[10] Mino cells were treated with vehicle, pertussis toxin, or ibrutinib for 1 hour at 37°C and then added to confluent monolayers of stromal cells. The cocultures were incubated at 37°C for 5 hours to overnight to allow migration of Mino cells beneath the stromal cell layer, after which they were washed extensively to remove unmigrated cells. For cocultures using live-cell tracer dyes, M2-10B4 and Mino cells were first loaded with CellTracker Green 5-chloromethylfluorescein-diacetate (CMFDA) and CellTracker Orange 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethyl-rhodamine (CMTMR), respectively. For microscopy, cells were fixed with paraformaldehyde and mounted on slides with 4,6-diamidino-2-phenylindole mounting medium (Vectashield). For quantification of migration of Mino cells in cocultures by flow cytometry, cells were trypsinized and stained with APC-Cy7–labeled anti-CD19 antibody. Cells were counted by using CountBright absolute counting beads (Life Technologies).

Actin polymerization in Mino cells

Mino cells were adhered to coverslips in serum-free media for 30 minutes at 37°C and treated with dimethylsulfoxide, pertussis toxin, or ibrutinib for 1 hour. Cells were fixed with paraformaldehyde, permeabilized with Triton X-100, and stained with Alexa Fluor 594–labeled phalloidin (Molecular Probes). Coverslips were mounted on glass slides by using Vectashield mounting medium containing 4,6-diamidino-2-phenylindole. Microscopy was performed on a Zeiss Axioplan 2 microscope using a 63×/1.40 oil immersion Plan-Apochromat objective, and images were acquired with a Zeiss AxioCam MRm charge coupled device camera and AxioVision (v4.8) software. For densitometry, at least 30 cells were imaged for each condition.

Adhesion and migration assays

Cell adhesion[5;12] and migration[12;29] assays were performed as described.

Immunoblotting

Western blots were performed as previously described.[5;29]

Statistical analysis

Analyses were performed by using GraphPad Prism (v4.0). Statistically significant differences were determined by using either analysis of variance with Bonferroni's post hoc comparison, or unpaired two-tailed Student *t*-test was used to determine the significance of differences between two means.

Results

Transient increase in absolute lymphocyte count following ibrutinib administration to MCL patients

In a phase 1 study that enrolled patients with various non-Hodgkin lymphomas, MCL patients were treated with ibrutinib in 35-day cycles during which the drug was administered once a day for 28 days with a 7-day drug holiday between cycles.[26] Under these conditions, a cyclical pattern of increasing and decreasing absolute lymphocyte count (ALC) was observed. This was demonstrated by an increase in ALC following the first few weeks of treatment followed by a return to baseline after the 7-day drug holiday (Fig. 1A). This cyclic ALC pattern continued for the duration of the treatment (data not shown). During the course of ibrutinib treatments, tumor volumes (quantified by sum of perpendicular diameters measurements from

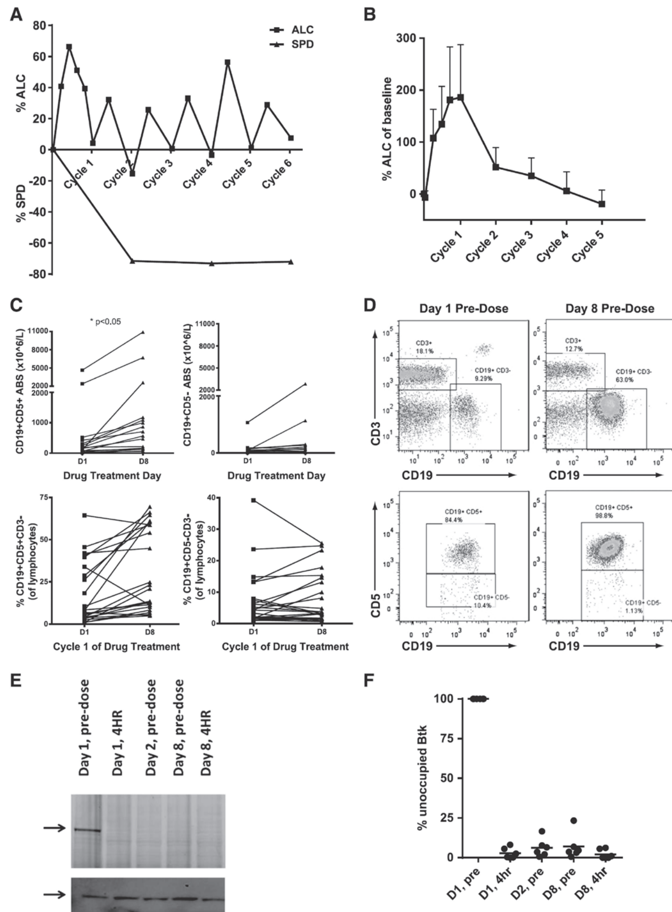


Figure 1. Transient mobilization of lymphocytes in MCL patients treated with ibrutinib.
The mean percentage change of ALC (over baseline) is graphed against treatment time. (A) Ibrutinib treatment was in 35-day cycles of 28 days on and 7 days off. Mean percentage ALC change and percentage of the sum of perpendicular diameters (SPD) change are plotted to treatment cycles (n = 9). (B) Ibrutinib treatment was continuous with no gap in treatment. Mean percentage ALC change compared with baseline (plotted as mean + standard error [SE]; n = 17) is plotted against treatment time. Time points plotted are D0 (day zero), D1, D8, D15, D22, and end of cycles 1, 2, 3, 4, and 5. (C) The absolute count (ABS) and percentage of $CD19^+CD5^+$ vs $CD19^+CD5^-$ cells following 1 week of ibrutinib treatment. Note the statistically significant change in the absolute count of $CD19^+CD5^+$ cells of MCL patients following treatment. *$P < .05$ (paired *t*-test; n = 16). (D) Flow plot of gated lymphocytes of PBMC samples from a representative MCL patient before and after ibrutinib treatment (560mg per day) for 7 days. PBMCs were stained with CD3, CD19, and CD5. Note increase of $CD19^+CD3^-$ and $CD19^+CD5^+$ population after 7 days of drug treatment. (E) Fluorescent probe occupancy assay of BTK in PBMCs isolated from a representative MCL patient before treatment (predose), and after 4 hours, 24 hours after first dose, before treatment on the eighth day (Day 8, predose), and 4 hours after the eighth day dose (Day 8, 4HR). The arrows point to the 75kDa BTK band on a scanned fluorescent gel (top) and a western blot (bottom). (F) An average of >90% occupancy of BTK by drug is achieved in MCL patients who were administered ibrutinib in the first week, determined by fluorescent probe assays (n = 6).

scans) decreased by 75% on average (Fig. 1A) during 2 to 6 treatment cycles. Thus, during the first 6 cycles of treatment, the cyclic changes of PB ALC occurred concomitantly with nodal responses in these patients. The increase in ALC was observed in a subsequent (phase 2) study in which MCL patients were treated with a fixed continuous daily dose of 560mg without interruption. In this trial, the ALC increased by 100% to 200% following the first 2 to 4 weeks of treatment, followed by notable gradual reductions in ALC commencing by the eighth week of treatment and continuing over the following months (Fig. 1B).

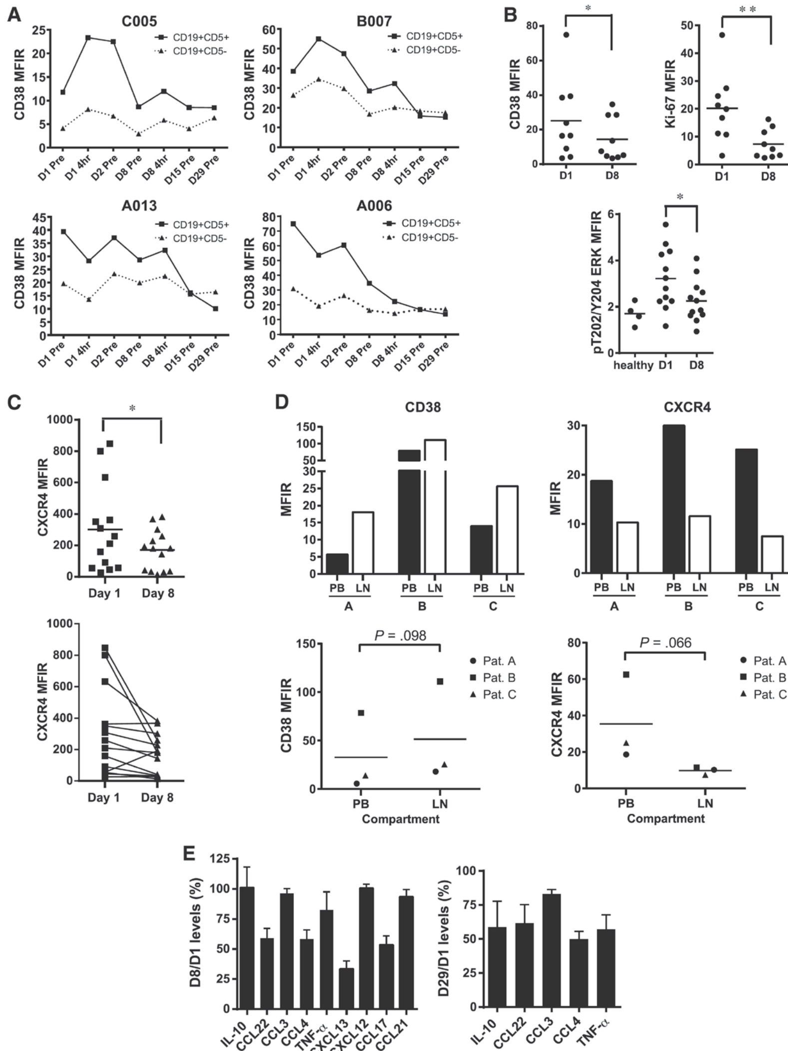
Elevated ALC is due to an increase of light chain-restricted $CD19^+CD5^+$ cells

In order to define the population of lymphocytes increased by ibrutinib, the PBMCs of patients isolated before (day 1 [D1]) and after 1 week of treatment (day 8 [D8]) were stained with CD19, CD3, and CD5 and analyzed by flow cytometry. The increased lymphocytes were characterized as $CD19^+CD3^-CD5^+$; both the absolute count and the percentage of $CD19^+CD5^+$ cells in the lymphocyte population were significantly increased after 1 week of ibrutinib treatment ($P < .05$), whereas the absolute count and the percentage of $CD19^+CD5^-$ cells was not (Fig. 1C). An illustrative patient is shown in Fig. 1D, in whom the $CD19^+CD3^-$ and $CD19^+CD5^+$ populations before drug treatments were 9.29% and 84.4%, respectively, and they increased to 63% and 98.8% after 1 week of treatment. The $CD19^+CD3^-CD5^+$ cells were light chain-restricted (data not shown), likely reflecting increased circulating MCL cells in the periphery following 1 week of drug treatment. In some cases, the mobilized cells made up a distinct subset of $CD45^{dim}$ small cells, which is also consistent with MCL (data not shown).

To confirm that full inhibition of the target BTK was achieved in these patients, occupancy of the BTK active site by ibrutinib was assessed in PBMCs from MCL patients by using a competitive binding fluorescent probe assay.[30] On average, more than 90% target occupancy was observed in patients following 1 week of treatment (Figs. 1E-F).

The peripheral $CD19^+CD5^+$ population has decreased Ki67, pERK, and surface CD38 and CXCR4 expression following drug treatment

Peripheral $CD19^+CD5^+$ cells were analyzed for markers commonly associated with cell proliferation/activation states such as CD38,[31;32] Ki67,[33] and pERK. CD38 surface expression was higher in the $CD19^+CD5^+$ cells than in the normal $CD19^+CD5^-$ cells prior to drug treatment and became even higher in some patients immediately after treatment (Fig. 2A), likely because of the higher CD38 expression in tissues (Fig. 2D), but it decreased significantly following 1 week of treatment ($P < .05$) (Fig. 2B)



3

Figure 2. CD19⁺CD5⁺ cells have decreased CXCR4, CD38, and Ki67 expression following ibrutinib treatment.

(A) Reduction of CD38 expression (mean fluorescence intensity ratio [MFIR]) in $CD19^+CD5^+$ cells but not $CD19^+CD5^-$ cells during 4 weeks of treatment in 4 patients treated with ibrutinib. (B) Surface CD38 expression (left panel; *P < .05) and intracellular Ki67 (right panel; **P < .01) is significantly reduced following 1 week of treatment. MFIR of intracellular phospho-ERK (pT202/Y204/ERK1/2) of $CD20^+CD5^+$ cells from healthy participants or MCL patients treated with ibrutinib before treatment (day 1 [D1]) and after 1 week of treatment (D8) (lower panel; *P < .05). (C) Significant reduction of surface CXCR4 expression (MFIR) in $CD19^+CD5^+$ cells following 1 week of ibrutinib treatment (n = 14; *P < .05). The line between the dot plots shows the mean. (D) CXCR4 and CD38 expression from LN biopsies and PBMCs (PB) of three MCL patients (patients A, B, and C) not treated with drug. (E) Plasma chemokine and cytokine concentrations on day 8 (D8; left) or day 29 (D29; right) of ibrutinib-treated MCL patients compared with pretreatment times 100% (n = 9).

and decreased even further with longer treatment, while the CD38 expression in the normal B cells did not change (Fig. 2A). *In vitro* ibrutinib treatment did not affect CD38 expression in MCL cells (Fig. S1). Furthermore, Ki67 expression, commonly used as a marker of proliferation, was significantly reduced after treatment ($P < .01$) (Figs. 2B and S7). pERK expression was generally higher in the $CD20^+CD5^+$ cells of MCL patients compared with healthy volunteers and was significantly reduced by ibrutinib treatment (Fig. 2B, lower panel; $P < .05$).

Since the chemokine receptor CXCR4 is important for B-cell homing to lymphoid tissues,[34;35] we determined the surface CXCR4 expression in the MCL cells and found that CXCR4 was significantly reduced ($P < .05$) in the $CD19^+CD5^+$ population following 1 week of treatment (Fig. 2C). We then analyzed CXCR4 expression on patient-matched LN- and PB-resident MCL cells from 3 untreated patients and found that CXCR4 expression was lower in MCL cells isolated from LNs compared with PB in all 3 patients examined (Fig. 2D), similar in trend to those noted in chronic lymphocytic leukemia (CLL) patients in whom CXCR4 surface expression is lower in LN compared with PB,[36;37] because of receptor endocytosis from high tissue concentrations of CXCL12.[4;11;37;38] Therefore, the newly circulating $CXCR4^{lo}$ MCL cell population is consistent with the mobilized cells originating from tissues such as LNs. This interpretation is further supported by the notable reduction in lymphadenopathy observed during the same period (Fig. 1A). Importantly, ibrutinib treatment *in vitro* did not have a direct effect on CXCR4 surface expression in primary MCL cells at concentrations of 1 to 1000nM (Fig. S1).

We also examined changes to plasma chemokines in the treated patients and found that chemokines important in B-cell trafficking/homing (CXCL13) and T-cell/accessory-cell attractions (CCL22, CCL4, CCL17)[6;39;40] were reduced on average by more than 50% following 1 week of treatment. By the end of the first cycle of treatment, in addition to the decrease of CCL4 and CCL22; cytokines important for MCL proliferation interleukin-10 (IL-10) and tumor necrosis factor α (TNF-α);[41;42] were also reduced by 50% (Fig. 2E).

Ibrutinib inhibits pseudo-emperipoiesis in MCL-stromal coculture

The transient increase of ALC in MCL patients treated with ibrutinib may be due to a disruption in cellular adhesion and migration within the LN or tissue compartment. To investigate this, we established the *in vitro* effect of ibrutinib on the pseudo-emperipoiesis of MCL in stromal cell cocultures. Primary MCL cells or the Mino cell line were grown in coculture with M2-10B4 murine bone marrow stromal cells. We found that primary MCL cells or Mino cells both adhered to and migrated beneath the M2-

10B4 cells. Significant inhibition of pseudo-emperipoiesis by ibrutinib was observed, as demonstrated by light microscopy (Figs. 3A-B), and the number of Mino cells or primary MCL cells remaining in the coculture were quantified by flow cytometry of $hCD19^{+}$ cells harvested by gentle washing following 4 hours of coculture (Figs. 3D-E, left panels). Ibrutinib dose-dependently inhibited migration of Mino cells beneath the stromal cells, and the inhibition was significant at 100nM ($P < .01$) and 1000nM ($P < .001$). Pertussin toxin, a well-studied G protein-coupled receptor inhibitor used as a positive control for inhibition of Mino cell migration significantly inhibited migration at 200ng/ml ($P < .001$). In addition, CXCL12, an important chemokine for B-cell homing produced by stromal cells, increased cortical actin of Mino cells, as assessed by phalloidin fluorescence microscopy, and this response was also dose-dependently and significantly inhibited by ibrutinib treatments at 10 and 100nM ($P < .001$) (Figs. 3C-D, right panel). Ibrutinib also suppressed actin polymerization of primary MCL in coculture at 100nM ($P < .001$) (Fig. 3E, right panel).

Ibrutinib inhibits BTK activity in MCL/stromal cell coculture and suppresses stromal cell-induced chemokine and cytokine secretion

To further understand the drug effect on MCL cells in coculture with stromal cells, Mino cells were treated with drug and cocultured with M2-10B4 or stimulated with anti-immunoglobulin M (anti-IgM). Ibrutinib dose-dependently inhibited pBTK, pPLCγ2, pAKT, and pERK in Mino cells in coculture with M2-10B4 cells and in Mino cells with BCR stimulation (Fig. 4A). Chemokine and cytokine concentrations of conditioned media were determined from ibrutinib-treated Mino cells alone or stimulated with anti-IgM or in coculture (Fig. 4B). Mino cells increased chemokine and cytokine secretions following BCR stimulation or coculture with M2 cells. Similar results were observed with the JeKo1 cell line (Fig. S2). Ibrutinib dose-dependently and potently suppressed production of human IL-10, CCL22, CCL3, CCL4, TNF-α, and CCL17 following BCR activation or in coculture, whereas the murine stromal cells alone did not produce human chemokines or cytokines (Fig. 4B). Similarly, ibrutinib suppressed the production of IL-10, CCL22, CCL3, CCL4, and TNF-α of JeKo1 cells in coculture with M2-10B4 (Fig. S2) or human stromal cell line HS-5 (data not shown). Interestingly, these chemokines/cytokines were also reduced in ibrutinib-treated patients, but the degree of reduction in plasma CCL3 was far less than that *in vitro* (Fig. 2E).

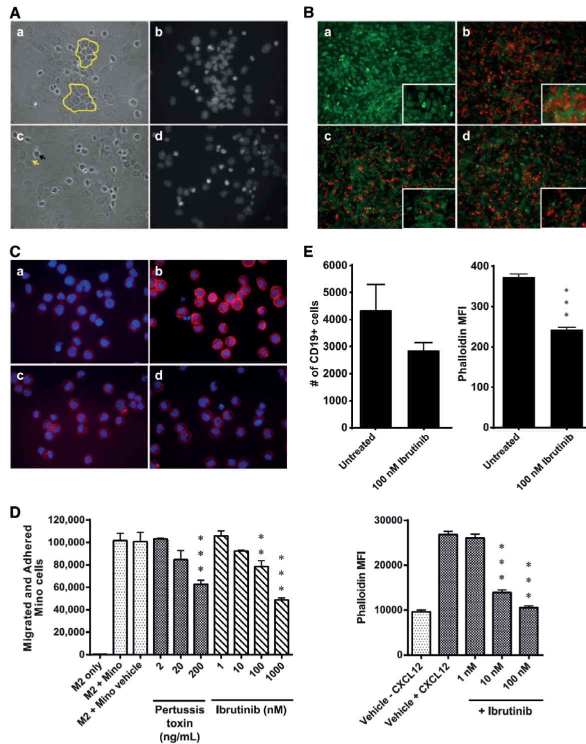


Figure 3. Ibrutinib inhibits migration of MCL cells beneath stromal cells (pseudo-emperipoiesis) and the formation of CXCL12-stimulated cortical actin.

(A) Phase contrast (left panel) and 4,6-diamidino-2-phenylindole (DAPI) staining (right panel) of Mino and BM stromal cell M2-10B4 coculture 24 hours after Mino cells were pretreated with vehicle (dimethylsulfoxide [DMSO]) (panels a and b) or ibrutinib (1000nM) (panels c and d). Highlighted yellow outlines show typical cobblestone appearance of migrated Mino cells beneath stromal cells. Black arrow points to a cell that is adhered on top of stromal cells but not migrated underneath. Yellow arrow points between two migrated Mino cells. (B) Mino cell and stromal cell coculture of Mino cells loaded with 5-(and-6)-(((4-chloromethyl) benzoyl)amino)-tetramethylrhodamine (red), and M2 cells loaded with 5-chloromethylfluorescein diacetate (green). (a) Stromal cells alone and (b) in coculture with Mino cells. (c) Mino cells pretreated with G protein-coupled receptor inhibitor pertussis toxin at 200ng/ml or (d) ibrutinib at 1000nM. (C) Mino cells were stimulated with CXCL12 at 100ng/ml and stained with rhodamine-phalloidin to determine actin polymerization and were counterstained with DAPI to identify nuclei of cells. Phalloidin staining of Mino cells (a) before and (b) after CXCL12 stimulation and after treatment with (c) pertussis toxin at 200ng/ml or (d) ibrutinib at 100nM. Magnification, ×200. (D) Mino cells were pretreated with ibrutinib, pertussis toxin, or vehicle for 30 minutes and then placed on a stromal cell-populated plate. After 4 hours, coculture was washed several times and migrated, and adhered Mino cells were counted in a flow cytometer with calibrated beads after staining with hCD19. Both pertussis toxin and ibrutinib dose-dependently inhibited migration and adhesion of Mino cells (left panel). Mino cells stimulated with CXCL12 and treated with vehicle or drug were stained with phalloidin, and its intensity was determined by using flow cytometry (right panel). (E) Ibrutinib (100nM) inhibited pseudo-emperipoiesis of primary MCL ($hCD19^+$ cells) in coculture with M2-10B4 stromal cells (left panel). Actin polymerization as assessed by phalloidin staining was significantly reduced by ibrutinib treatment in primary MCL cells. $^{**}P < .01$; $^{***}P < .001$. One-way analysis of variance (ANOVA) compared with vehicle control.

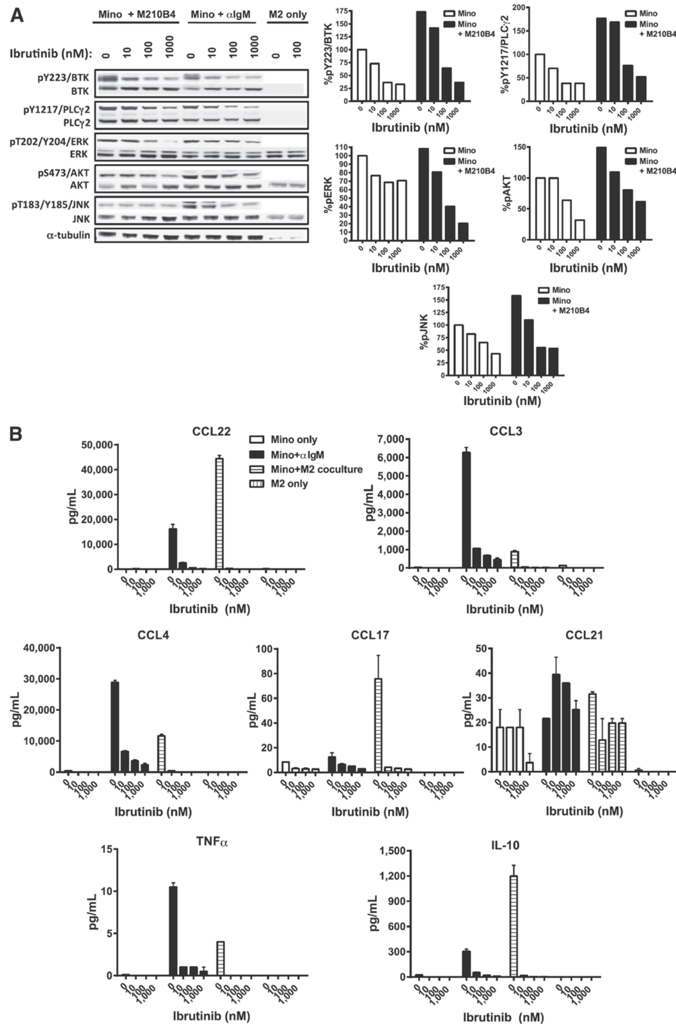


Figure 4. Ibrutinib suppresses BCR- and coculture-stimulated signaling and cytokine and chemokine production of MCL cells.

(A) Mino cells pretreated with vehicle or ibrutinib (10, 100, or 1000nM) were cultured alone (with anti-IgM stimulation) or in coculture with murine M2-10B4 stromal cells. M2-10B4 cells alone were pretreated with either vehicle or 100nM ibrutinib (M2 only). Mino cells in coculture, Mino cells alone, or M2-10B4 cells alone were collected after 48 hours, lysed, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analysis of pY223 BTK, pY1217 PLCγ2, pT202/Y204 ERK, pS473 AKT, and pT183/Y185 JNK. The phosphoblot was scanned, and signals were quantified (right panel); pBTK, pPLCγ2, pERK, pAKT, and pJNK signals were normalized to BTK, PLCγ2, ERK, AKT, and JNK total protein, respectively. Signals from each treatment were compared with vehicle-treated Mino cells. (B) Conditioned media from Mino cells only without stimulation, Mino cells stimulated with anti-IgM, or Mino cells in coculture with M2-10B4 or M2-10B4 alone were collected after 48 hours and analyzed for human cytokines and chemokines (IL-10, CCL22, CCL3, CCL4, TNF-α, CCL17, and CCL21). Note media from murine M2 cells alone do not react with human cytokines or chemokines.

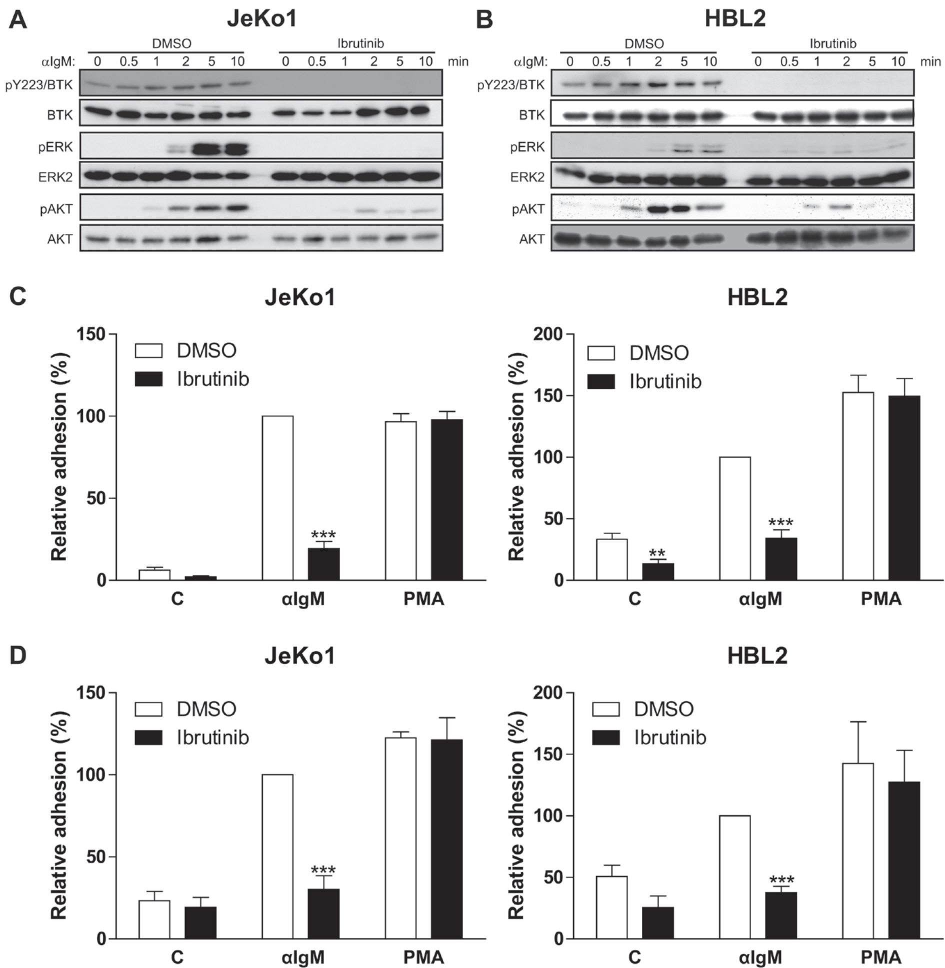


Figure 5. Ibrutinib inhibits BCR-activated adhesion of MCL cells.
(A-B) JeKo1 or HBL2 cells pretreated with vehicle or 100nM ibrutinib were stimulated with anti-IgM for 0, 0.5, 1, 2, 5, or 10 minutes and then immunoblotted for pBTK (Y223), pERK, and pAKT. Total BTK, ERK2, and AKT were used as loading controls. (C) JeKo1 (n = 8) or HBL2 (n = 8) cells pretreated with vehicle or 100nM ibrutinib were stimulated with anti-IgM or PMA and allowed to adhere to fibronectin-coated surfaces. (D) JeKo1 (n = 6) or HBL2 (n = 5) cells pretreated with vehicle or 100nM ibrutinib were stimulated with anti-IgM or PMA and allowed to adhere to VCAM-1–coated surfaces. PMA stimulations were used as a positive control for the assay and to show specificity of drug response. Graphs are presented as normalized means + SEM (100% = stimulated cells without inhibitors), C = control (unstimulated), PMA = phorbol-12-myristate-13-acetate. $^{**}P < .01$; $^{***}P < .001$. One-way ANOVA compared with vehicle control.

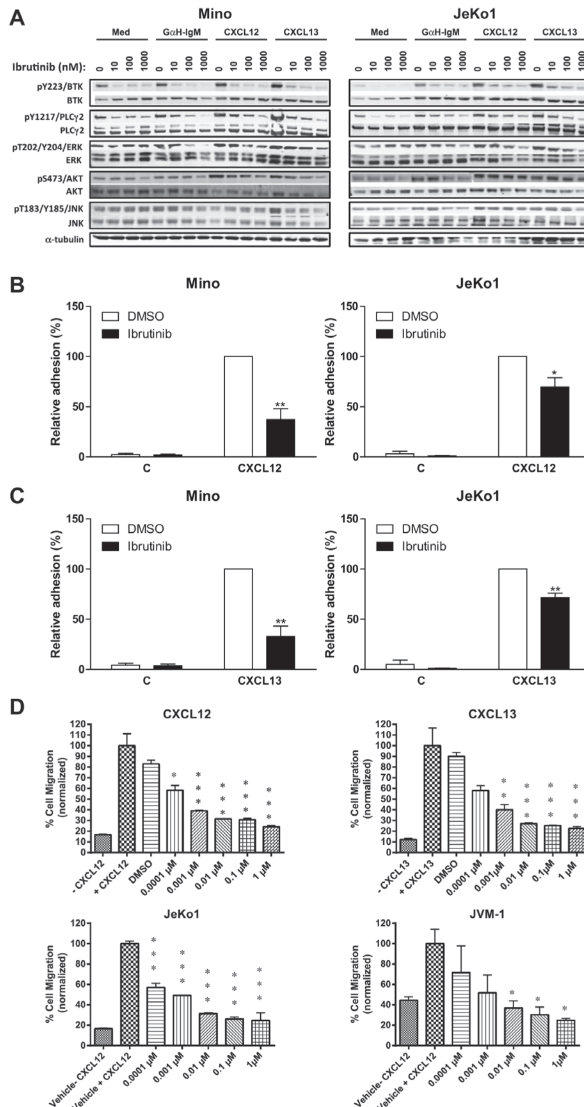


Figure 6. Ibrutinib inhibits CXCL12-/CXCL13-activated adhesion and migration of MCL cells. (A) Mino (left panel) or JeKo1 (right panel) cells pretreated with vehicle or ibrutinib 10, 100, or 1000nM were either stimulated with anti-IgM, CXCL12, or CXCL13 or treated with medium (Med) for 15 minutes and then immunoblotted for pBTK, pPLCγ2, pAKT, pERK, and pJNK. (B) Mino (n = 6) or JeKo1 (n = 4) cells pretreated with vehicle or 100nM ibrutinib were allowed to adhere to VCAM-1– and CXCL12-cocoated surfaces. (C) Mino (n = 5) or JeKo1 (n = 5) cells pretreated with vehicle or 100nM ibrutinib were allowed to adhere to VCAM-1– and CXCL13-cocoated surfaces. Graphs are presented as normalized means + SEM (100% = stimulated cells without inhibitors), C = control (unstimulated). (D) Cells from MCL cell lines Mino, JeKo1, and JVM-1 were treated with increasing concentration of ibrutinib and subjected to a chemotaxis migration assay in transwell plates with filters coated with VCAM-1, and CXCL12 or CXCL13 was added into the lower chamber as a chemoattractant. Ibrutinib dose-dependently inhibited CXCL12- and CXCL13-mediated migration of Mino cells, and CXCL12-mediated migration of JeKo1 and JVM-1 cells. *P < .05; **P < .01; ***P < .001. One-way ANOVA compared with vehicle control.

Ibrutinib inhibits BCR- and chemokine-mediated adhesion and migration *in vitro*

We measured the direct effect of ibrutinib on adhesion and migration of the MCL cell lines JeKo1, HBL2, Mino, and JVM-1. First, the effect of ibrutinib on BTK signaling was determined. As expected, ibrutinib inhibited phosphorylation of BTK and downstream signaling proteins PLCγ2, MAP kinases ERK, JNK, and AKT following stimulation by anti-IgM and chemokines CXCL12 and CXCL13 (Figs. 5A-B, 6A, S5, and S6). Cell surface expression of CXCR4, CXCR5, CCR7, surface IgM, and $\alpha_4\beta_1$ integrin was confirmed by flow cytometry (Fig. S3), and subsequent *in vitro* adhesion and chemotaxis assays were performed with the drug. Ibrutinib significantly inhibited anti-IgM–stimulated adhesion of JeKo1 and HBL2 cells onto fibronectin or VCAM-1 at 100nM (a clinically relevant concentration of ibrutinib) with more than 50% to 70% inhibition. The inhibition of adhesion by ibrutinib was also dose-dependent (Fig. S4). Similarly, the adhesion of both Mino and JeKo1 cells to VCAM-1 was inhibited by ibrutinib at 100nM following CXCL12- or CXCL13-activation. The extent of inhibition was greater in Mino cells (50% to 70%) than in JeKo1 cells (20% to 30%) (Figs. 6B-C). We found that, in addition to changes in adhesion, ibrutinib dose-dependently inhibited CXCL12-induced migration of Mino, JeKo1, and JVM-1 cells, with Mino and JeKo1 cells being more sensitive to drug than JVM-1 cells (Fig. 6D). Ibrutinib also significantly inhibited CXCL13-stimulated migration of Mino cells dose-dependently from 1nM to 1μM (Fig. 6D).

Next, we examined the effect of ibrutinib on signaling and adhesion in primary MCL cells. In primary MCL, pY223/BTK was increased compared with that in normal B lymphocytes, consistent with elevated BCR signaling in malignant B cells. Ibrutinib inhibited pBTK in both primary MCL and normal B cells on Y223, the autophosphorylation site of BTK, and Y551 (phosphorylated by SRC family kinases) and reduced pPLCγ2 on Y759 and Y1217 (Fig. 7A) at concentrations of 10nM and above. These results demonstrate that ibrutinib directly inhibits BTK activity in MCL primary cells. Importantly, ibrutinib also inhibited CXCL12- or CXCL13-activated adhesion to VCAM-1 as well as BCR-stimulated adhesion to fibronectin at 100nM in primary MCL cells. The degree of inhibition in these primary cells was about 10% to 20%, and the magnitude was less impressive compared with that in the MCL cell lines but the inhibition was statistically significant (Fig. 7B).

These studies collectively demonstrate that ibrutinib inhibits BCR-, CXCL12- and CXCL13-activated adhesion and migration in MCL cell lines as well as in primary MCL cells, which is associated with the BTK inhibition in these cells.

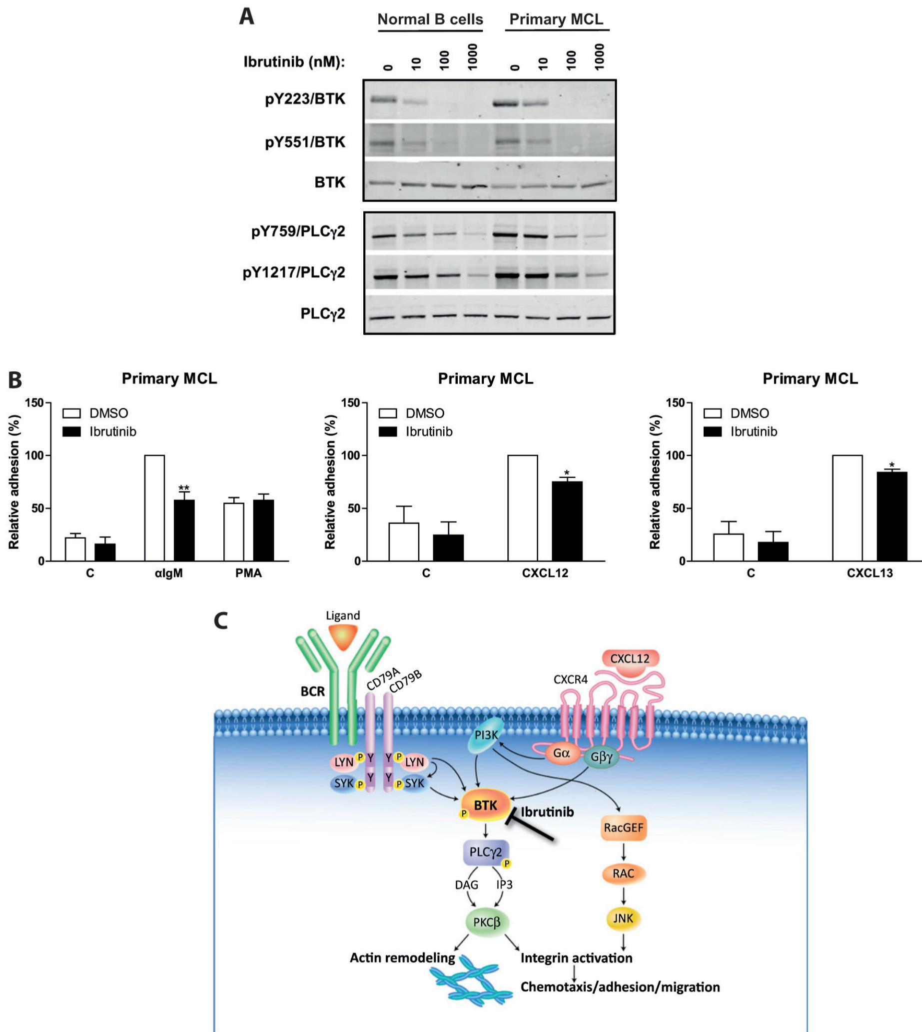


Figure 7. Ibrutinib inhibits BCR- and chemokine-induced adhesion of primary MCL cells. (A) CD19⁺ cells isolated from PBMCs of healthy volunteers or MCL patients were treated with ibrutinib at 100nM for 10 minutes prior to cell harvest, lysate generation, and western blotting. Primary MCL cells have increased BTK activity compared with B lymphocytes from healthy volunteers, which is further inhibited by ibrutinib in a dose-dependent manner. Tyrosine phosphorylation sites of PLCγ2 are also inhibited dose-dependently by drug. (B) Ibrutinib inhibited anti-IgM-controlled adhesion of primary MCL cells to fibronectin (n = 5 patients), or CXCL12- and CXCL13-controlled adhesion to VCAM-1 (n = 3 patients). Graphs are presented as normalized means + SEM (100% = stimulated cells without inhibitors), C = control (unstimulated). *$P < .05$; **$P < .01$. One-way ANOVA compared with vehicle control. (C) Schematic of mechanism of action of ibrutinib. Since BTK is downstream to both BCR and CXCR4 signaling, ibrutinib inhibits chemokine and BCR-mediated cell adhesion/migration of malignant cells thereby disrupting the microenvironment in the tissues, LNs, and BM, which results in the malignant cells egressing and eventually entering the peripheral circulation where they are presumably cleared.

Discussion

Dramatic early increases of PB lymphocytes occur consistently among CLL patients treated with small-molecule antagonists of the BCR signaling pathway (SYK, PI3Kδ, and BTK inhibitors).[26;43;44] Here we report and characterize similar early transient elevations of lymphocytes among MCL patients treated with the irreversible BTK inhibitor ibrutinib. The increase in ALC commenced as early as the first week, was maximal after 2 to 3 weeks of treatment, and then gradually subsided over several cycles of treatment. As with CLL patients,[26] MCL patients dosed on a 28-days-on and 7-days-off schedule exhibited decreases of ALC following the 7-day-off period at the end of each cycle, and subsequent increases of ALC were observed periodically at the beginning of each subsequent cycle. Despite these increases in circulating cells, lymphatic masses were noted to decrease concomitantly, consistent with cellular mobilization from tissues as opposed to disease progression.

We found that the newly appearing cells were light chain-restricted $CD19^+CD5^+$ cells, consistent with their identification as MCL cells. The identity of these cells as lymphomatous is not surprising since circulating PB tumor cells are often detectable among patients with MCL. Increase of circulating MCL cells following treatment has not been previously reported and, moreover, it was highly specific, not involving $CD19^+CD3^-CD5^-$ cells. Importantly, the $CD19^+CD5^+$ cells exhibited significantly lower CD38, Ki67, and pERK (downstream to BTK)[30] markers of cell activity. In addition, surface expression of CXCR4 was decreased. Consistent with studies on CLL cells, we found that MCL cells have lower expression of CXCR4 in LNs compared with PB. The shift in CXCR4 expression may thus largely reflect release from LNs or other tissues rather than a direct reduction of CXCR4 expression on circulating MCL cells because *in vitro* studies showed no effect of drug on surface CXCR4 on primary MCL cells (Fig. S1). $CXCR4^{hi}$ CLL cells have been shown to be able to reenter tissues and LNs, whereas $CXCR4^{lo}$ CLL cells likely have just exited tissues and can no longer reenter lymphoid organs.[18;36;37] Thus, the increase in MCL cells is likely to be derived from newly egressed MCLs from tissues and LNs. However, if these egressed MCLs just exited from tissues, why are they $CD38^{lo}$ since LN cells are $CD38^{hi}$. A closer look at CD38 expression in the first few days following drug treatment showed an initial increase of CD38 followed by a gradual decrease of expression over time (Fig. 2A). In CLL, CD38 expression serves as a real-time indicator for cell proliferation and activity,[31] and we speculate this may be true for MCL. The newly circulating MCL cells, having been exposed to target-saturating concentrations of ibrutinib, are likely to be less proliferative, as indicated by reduced Ki67, a prognostic marker for outcome of advanced MCL survival[2;33] and pERK.

3

Malignant CLL cells have been shown to be in a resting stage in the circulation but may be activated in proliferation centers of tissues and LNs.[45-47] MCL cells[6;8] may also exhibit properties similar to CLL cells which, after exiting the LNs, are poised for apoptosis and/or systemic clearance. In CLL, CD38-CD31 interactions are part of a network of accessory signals that modify the microenvironment leading to the proliferation and migration of CLL cells.[48-50] CD38 expression in MCL, unlike in CLL, has not been associated with disease prognosis; however, CD38 is associated with BCR signaling and lymphocyte activation and proliferation since B lymphocytes from Xid mice do not respond to anti-IgM or anti-CD38 while cells from wild-type mice do.[51] Recent reports indicate that positive CD38 expression in $CD20^{+}CD23^{-}$ cells with platelet counts predict the presence of t(11;14) translocation of MCL.[52] In addition, bortezomib-resistant MCL cells have higher CD38 expression.[3] To understand the precise fate of MCL cells after their exit from the tissues and LNs will require radiolabeling of patient cells such as that performed by heavy-water labeling of CLL cells and the subsequent calculation of the birth and death rate of CLL cells.[36]

We further probed the mechanisms of the increase in MCL cells by measuring cellular trafficking and PB immunophenotyping in a series of studies of cells treated with ibrutinib *in vitro.* We demonstrated the inhibition of BTK activity following BCR and chemokine stimulation on MCL cells alone and in coculture. Under these conditions, the drug inhibited BTK and PLCγ2 activities and reduced pERK, pJNK, and pAKT similar to the results with primary CLL cells.[5] In addition, the secretion of CCL3, CCL4, CCL22, and IL-10 was substantially reduced in Mino cell cultures and the plasma of patients treated with ibrutinib. These results substantiate our finding on the mechanism of action of ibrutinib in MCL. Presumably, ibrutinib inhibits chemokine and BCR signaling in MCL cells in tissues and lymphoid organs, which leads to loss of adhesion and migratory ability in the microenvironment where the MCL cells are well protected and nurtured by the stromal and accessory cells. As the cells lose their adhesion to the accessory cells in the microenvironment, they exit the tissues and LNs and eventually reenter peripheral circulation. Without the microenvironment and its supply of chemokines, cytokines, and growth factors, the MCL cells cannot proliferate, and they either die within a few days and/or eventually are cleared from the circulation (Fig. 7C).[53]

Approximately 70% of MCL patients in phase 1/2 and phase 2 clinical trials of ibrutinib have had objective clinical responses.[26;27] Our results provide important insight into the mechanism of this anti-tumor effect; inhibition of adhesion and chemokine and BCR signaling downstream of BTK; resulting in egress of malignant cells from tissues and lymphoid organs. Such effects may be inferred from the clinical observation of transiently increasing MCL cells in the PB, with concurrently

decreasing lymphatic masses; here, these effects are clearly substantiated by *in vitro* inhibition of chemotaxis, adhesion, and migration of cells beneath stromal cells (pseudo-emperipoiesis). Moreover, we demonstrated that ibrutinib directly inhibits downstream signaling from receptors for stromal adhesion (CXCL12 and CXCL13) as well as through BCR ligation. Once MCL cells are displaced from their microenvironment, local contact and soluble factor exposure are likely to be suboptimal for supporting cellular proliferation and survival. The cells with decreased CXCR4 expression are impaired in chemotaxis and thus fail to reenter and home into tissues. In addition, production of crucial chemokines important for homing to tissues was also apparently inhibited *in vitro* and *in vivo*. To the best of our knowledge, this is the first report that shows BTK activity to be essential for the homing of MCL cells into secondary lymphoid organs, and its inhibition results in an egress of malignant cells into PB.

Acknowledgment
The authors thank medical illustrator Jacqueline Schaffer, M.A.M.S., for editing the figures and creating Fig. 7C. This work was supported by the intramural research program of the National Heart, Lung, and Blood Institute (A.W.) and the National Cancer Institute (W.W.).

Authorship contributions
Contribution: B.Y.C. conceived, directed, and supervised the studies, analyzed the data, and wrote the manuscript; M.F., M.F.M.D.R., P.M., S.M.S., M.M.H., A.K., S.E.M.H., and S.C. performed the experiments, analyzed the data, and contributed to the manuscript; M.S. and S.T.P. analyzed the data and contributed to the manuscript; W.W. collected samples from patients; L.E. and J.J.B. conceived the study and coordinated the collaborations between institutes; M.S., A.W., W.W., S.E.M.H., S.M.S., M.F., J.J.B., and L.E. reviewed and revised the manuscript; and all authors read and approved the final manuscript.

Disclosure of conflicts of interest
B.Y.C., M.F., P.M., M.M.H., S.C., J.J.B., and L.E. hold stock and/or stock options at Pharmacyclics, Inc. Pharmacyclics, Inc. owns patents and patent applications covering various aspects of and relating to ibrutinib. S.M.S. consults for Pharmacyclics. The remaining authors declare no competing financial interests.

References

1. Argatoff LH, Connors JM, Klasa RJ, Horsman DE, Gascoyne RD. Mantle cell lymphoma: a clinicopathologic study of 80 cases. *Blood* 1997;89(6):2067-2078.
2. Hoster E, Dreyling M, Klapper W et al. A new prognostic index (MIPI) for patients with advanced-stage mantle cell lymphoma. *Blood* 2008;111(2):558-565.
3. Perez-Galan P, Dreyling M, Wiestner A. Mantle cell lymphoma: biology, pathogenesis, and the molecular basis of treatment in the genomic era. *Blood* 2011;117(1):26-38.
4. Burger JA, Kipps TJ. Chemokine receptors and stromal cells in the homing and homeostasis of chronic lymphocytic leukemia B cells. *Leuk.Lymphoma* 2002;43(3):461-466.
5. De Rooij MF, Kuil A, Geest CR et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* 2012;119(11):2590-2594.
6. Kurtova AV, Tamayo AT, Ford RJ, Burger JA. Mantle cell lymphoma cells express high levels of CXCR4, CXCR5, and VLA-4 (CD49d): importance for interactions with the stromal microenvironment and specific targeting. *Blood* 2009;113(19):4604-4613.
7. Lenz G, Wright G, Dave SS et al. Stromal gene signatures in large-B-cell lymphomas. *N.Engl.J.Med.* 2008;359(22):2313-2323.
8. Burger JA, Ford RJ. The microenvironment in mantle cell lymphoma: cellular and molecular pathways and emerging targeted therapies. *Semin.Cancer Biol.* 2011;21(5):308-312.
9. Medina DJ, Goodell L, Glod J et al. Mesenchymal stromal cells protect mantle cell lymphoma cells from spontaneous and drug-induced apoptosis through secretion of B-cell activating factor and activation of the canonical and non-canonical nuclear factor kappaB pathways. *Haematologica* 2012;97(8):1255-1263.
10. Ansel KM, Ngo VN, Hyman PL et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000;406(6793):309-314.
11. Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood* 1999;94(11):3658-3667.
12. de Gorter DJ, Beuling EA, Kersseboom R et al. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity.* 2007;26(1):93-104.
13. Okada T, Ngo VN, Ekland EH et al. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *J.Exp.Med.* 2002;196(1):65-75.
14. Pals ST, de Gorter DJ, Spaargaren M. Lymphoma dissemination: the other face of lymphocyte homing. *Blood* 2007;110(9):3102-3111.
15. von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. *Nat.Rev.Immunol.* 2003;3(11):867-878.

16. Burger JA. Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. *Hematology.Am.Soc.Hematol.Educ.Program.* 2011;2011:96-103.
17. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J.Exp.Med.* 2003;198(10):1539-1550.
18. Herishanu Y, Perez-Galan P, Liu D et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* 2011;117(2):563-574.
19. Buggy JJ, Elias L. Bruton tyrosine kinase (BTK) and its role in B-cell malignancy. *Int.Rev.Immunol.* 2012;31(2):119-132.
20. Parekh S, Weniger MA, Wiestner A. New molecular targets in mantle cell lymphoma. *Semin.Cancer Biol.* 2011;21(5):335-346.
21. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood* 2011;118(16):4313-4320.
22. Pighi C, Gu TL, Dalai I et al. Phospho-proteomic analysis of mantle cell lymphoma cells suggests a pro-survival role of B-cell receptor signaling. *Cell Oncol.(Dordr.)* 2011;34(2):141-153.
23. Psyrri A, Papageorgiou S, Liakata E et al. Phosphatidylinositol 3'-kinase catalytic subunit alpha gene amplification contributes to the pathogenesis of mantle cell lymphoma. *Clin.Cancer Res.* 2009;15(18):5724-5732.

24. Boyd RS, Jukes-Jones R, Walewska R et al. Protein profiling of plasma membranes defines aberrant signaling pathways in mantle cell lymphoma. *Mol.Cell Proteomics.* 2009;8(7):1501-1515.
25. Rinaldi A, Kwee I, Taborelli M et al. Genomic and expression profiling identifies the B-cell associated tyrosine kinase Syk as a possible therapeutic target in mantle cell lymphoma. *Br.J.Haematol.* 2006;132(3):303-316.
26. Advani RH, Buggy JJ, Sharman JP et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J.Clin.Oncol.* 2013;31(1):88-94.
27. Wang LH, Martin P, Blum KA et al. The Bruton's tyrosine kinase inhibitor PCI-32765 is highly active as single-agent therapy in previously-treated mantle cell lymphoma (MCL): preliminary results of a phase II trial. *Blood* 2011;118(21):203-204.
28. Chang BY, Huang MM, Francesco M et al. The Bruton tyrosine kinase inhibitor PCI-32765 ameliorates autoimmune arthritis by inhibition of multiple effector cells. *Arthritis Res.Ther.* 2011;13(4):R115.
29. de Gorter DJ, Reijmers RM, Beuling EA et al. The small GTPase Ral mediates SDF-1-induced migration of B cells and multiple myeloma cells. *Blood* 2008;111(7):3364-3372.
30. Honigberg LA, Smith AM, Sirisawad M et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc. Natl.Acad.Sci.U.S.A.* 2010;107(29):13075-13080.
31. Chiorazzi N. Implications of new prognostic markers in chronic lymphocytic leukemia. *Hematology. Am.Soc.Hematol.Educ.Program.* 2012;2012:76-87.

32. Malavasi F, Deaglio S, Damle R et al. CD38 and chronic lymphocytic leukemia: a decade later. *Blood* 2011;118(13):3470-3478.
33. Determann O, Hoster E, Ott G et al. Ki-67 predicts outcome in advanced-stage mantle cell lymphoma patients treated with anti-CD20 immunochemotherapy: results from randomized trials of the European MCL Network and the German Low Grade Lymphoma Study Group. *Blood* 2008;111(4):2385-2387.
34. Allen CD, Ansel KM, Low C et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat.Immunol.* 2004;5(9):943-952.
35. Reif K, Ekland EH, Ohl L et al. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* 2002;416(6876):94-99.
36. Calissano C, Damle RN, Marsilio S et al. Intraclonal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Mol.Med.* 2011;17(11-12):1374-1382.
37. Ghobrial IM, Bone ND, Stenson MJ et al. Expression of the chemokine receptors CXCR4 and CCR7 and disease progression in B-cell chronic lymphocytic leukemia/ small lymphocytic lymphoma. *Mayo Clin.Proc.* 2004;79(3):318-325.
38. Vlad A, Deglesne PA, Letestu R et al. Down-regulation of CXCR4 and CD62L in chronic lymphocytic leukemia cells is triggered by B-cell receptor ligation and associated with progressive disease. *Cancer Res.* 2009;69(16):6387-6395.
39. Burger JA, Montserrat E. Coming full circle: 70 years of chronic lymphocytic leukemia cell redistribution, from glucocorticoids to inhibitors of B-cell receptor signaling. *Blood* 2013;121(9):1501-1509.
40. Ek S, Bjorck E, Hogerkorp CM et al. Mantle cell lymphomas acquire increased expression of CCL4, CCL5 and 4-1BB-L implicated in cell survival. *Int.J.Cancer* 2006;118(8):2092-2097.
41. Hattori N, Nakamaki T, Ariizumi H et al. Over-expression of CCL3 MIP-1alpha in a blastoid mantle cell lymphoma with hypercalcemia. *Eur.J.Haematol.* 2010;84(5):448-452.
42. Visser HP, Tewis M, Willemze R, Kluin-Nelemans JC. Mantle cell lymphoma proliferates upon IL-10 in the CD40 system. *Leukemia* 2000;14(8):1483-1489.
43. Friedberg JW, Sharman J, Sweetenham J et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* 2010;115(13):2578-2585.
44. Furman RR, Byrd JC, Brown JR et al. CAL-101, an isoform-selective inhibitor of phosphatidylinositol 3-kinase p110 delta, demonstrates clinical activity and pharmacodynamic effects in patients with relapsed or refractory chronic lymphocytic leukemia. *Blood* 2010;116(21):31.
45. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood* 2009;114(16):3367-3375.
46. Stein H, Bonk A, Tolksdorf G et al. Immunohistologic analysis of the organization of normal lymphoid tissue and non-Hodgkin’s lymphomas. *J.Histochem.Cytochem.* 1980;28(8):746-760.

47. Tsukada N, Burger JA, Zvaifler NJ, Kipps TJ. Distinctive features of “nurselike” cells that differentiate in the context of chronic lymphocytic leukemia. *Blood* 2002;99(3):1030-1037.
48. Deaglio S, Mallone R, Baj G et al. CD38/CD31, a receptor/ligand system ruling adhesion and signaling in human leukocytes. *Chem.Immunol.* 2000;75:99-120.
49. Deaglio S, Vaisitti T, Zucchetto A, Gattei V, Malavasi F. CD38 as a molecular compass guiding topographical decisions of chronic lymphocytic leukemia cells. *Semin.Cancer Biol.* 2010;20(6):416-423.
50. Deaglio S, Vaisitti T, Serra S et al. CD38 in chronic lymphocytic leukemia: from bench to bedside? *Mini.Rev.Med.Chem.* 2011;11(6):503-507.
51. Lund FE, Solvason NW, Cooke MP et al. Signaling through murine CD38 is impaired in antigen receptor-unresponsive B cells. *Eur.J.Immunol.* 1995;25(5):1338-1345.
52. Medd PG, Clark N, Leyden K et al. A novel scoring system combining expression of CD23, CD20, and CD38 with platelet count predicts for the presence of the t(11;14) translocation of mantle cell lymphoma. *Cytometry B Clin.Cytom.* 2011;80(4):230-237.
53. Herman SE, Gordon AL, Hertlein E et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* 2011;117(23):6287-6296.

Supplemental appendix

PTID	Age	Sex	CHMOLEC	# Prior Trt	Baseline ALC	Baseline LDH	Baseline ECOG	BTK Dose
PCYC-2-00F-001	63	Male	t (11;14) by cytogenetics/FISH	1	1.508	145	0	560mg
PCYC-2-00F-004	76	Male	t (11;14) by cytogenetics/FISH	4	4.88	268	2	560mg
PCYC-2-00F-005	65	Male	Cyclin D1 expression by IHC	1	7.245	326	0	560mg
PCYC-2-0CH-001	82	Male	Both t (11;14) by cytogenetics/FISH and Cyclin D1 expression by IHC	2	1.338	238	0	560mg
PCYC-2-0CH-003	57	Male	Flow cytometry	2	1.55	194	0	560mg
PCYC-2-B00-004	50	Male	Both t (11;14) by cytogenetics/FISH and Cyclin D1 expression by IHC	1	0.876	176	0	560mg
PCYC-2-B00-005	78	Female	Both t (11;14) by cytogenetics/FISH and Cyclin D1 expression by IHC	4	3.27	333	1	560mg
PCYC-2-B00-007	81	Female	Cyclin D1 expression by IHC	2	1.606	174	2	560mg
PCYC-2-B00-008	54	Male	Cyclin D1 expression by IHC	3	1.96	160	1	560mg
PCYC-2-BA0-001	67	Male	Cyclin D1 expression by IHC	5	2.69	373	1	560mg
PCYC-2-BA0-003	84	Male	Cyclin D1 expression by IHC		0.095	217	1	560mg
PCYC-2-BAG-004	56	Male	Cyclin D1 expression by IHC	3	0.534	67	0	560mg
PCYC-2-BAG-005	61	Male	t (11;14) by cytogenetics/FISH	2	3.619	163	0	560mg
PCYC-2-BAG-006	79	Male	Both t (11;14) by cytogenetics/FISH and Cyclin D1 expression by IHC	1	0.595	232	2	560mg
PCYC-2-BAG-007	65	Male	Cyclin D1 expression by IHC	3	1.498	163	2	560mg
PCYC-2-BAG-008	70	Male	Both t (11;14) by cytogenetics/FISH and Cyclin D1 expression by IHC		1.126	244	0	560mg
PCYC-2-BAG-009	80	Female	t (11;14) by cytogenetics/FISH		4	598	2	560mg
PCYC-2-CDC-003	61	Male	Cyclin D1 expression by IHC	2	0.897	161	0	560mg
PCYC-2-CDC-004	59	Male	Cyclin D1 expression by IHC	1	1.114	517	0	560mg
PCYC-2-CDC-005	72	Male	Cyclin D1 expression by IHC	5	0.254	731	0	560mg
PCYC-2-CDC-007	74	Male	Cyclin D1 expression by IHC		1.327	541	0	560mg
PCYC-2-CE0-001	60	Male	Cyclin D1 expression by IHC		0.234	393	2	560mg

PTID: Patient identity; CHMOLEC: Molecular characteristics; Trt: Treatment; ALC: Absolute lymphocyte count; LDH: Lactate dehydrogenase; ECOG: Eastern cooperative oncology group status

Table S1.

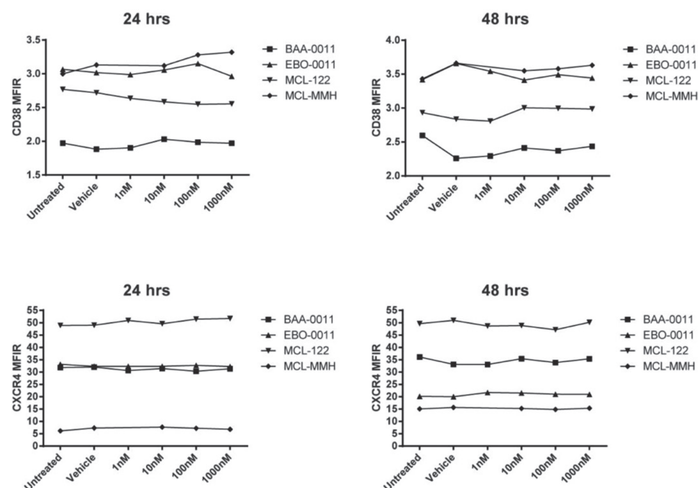
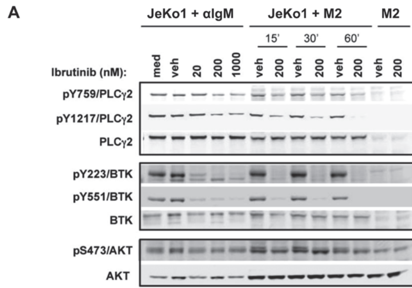


Figure S1.



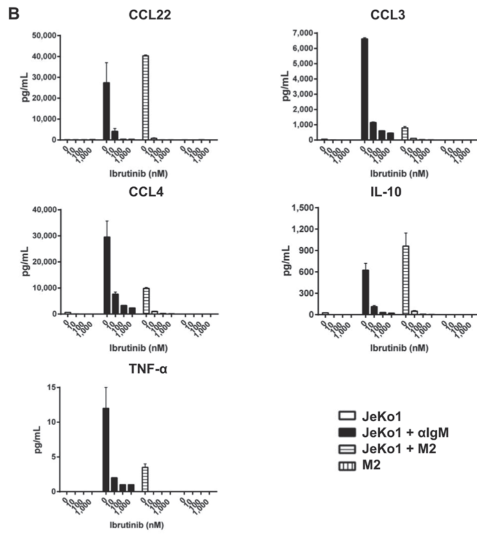


Figure S2.

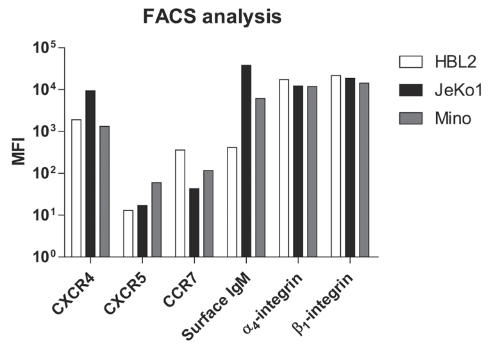


Figure S3.

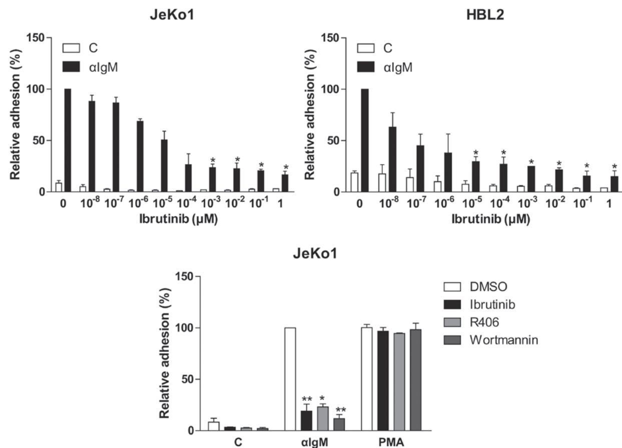
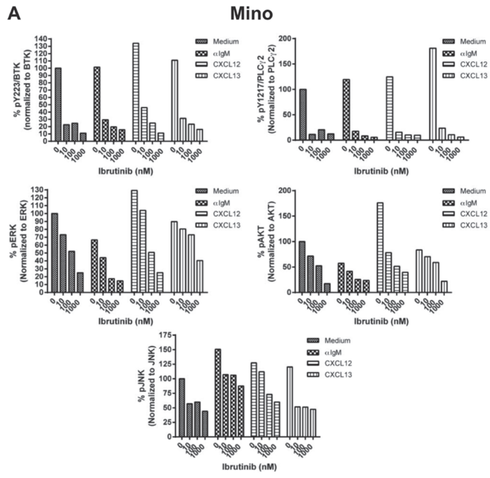


Figure S4.

A

Mino



JeKo1

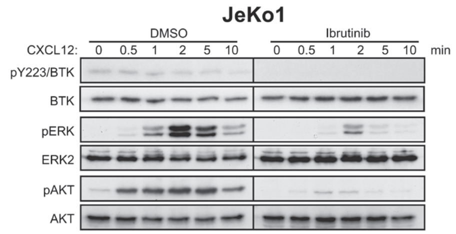


Figure S6.

B

JeKo1

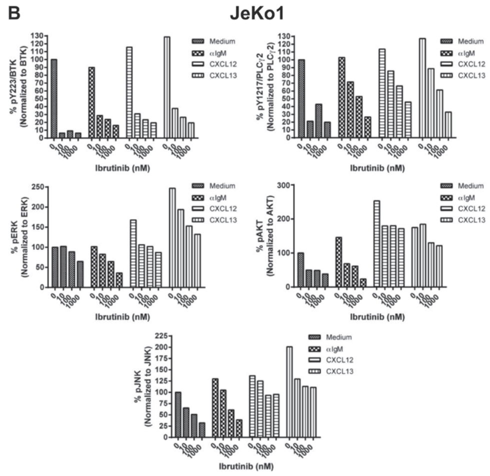


Figure S5.

Ki-67 (Proliferation marker) in CD19+ CD5+ cells

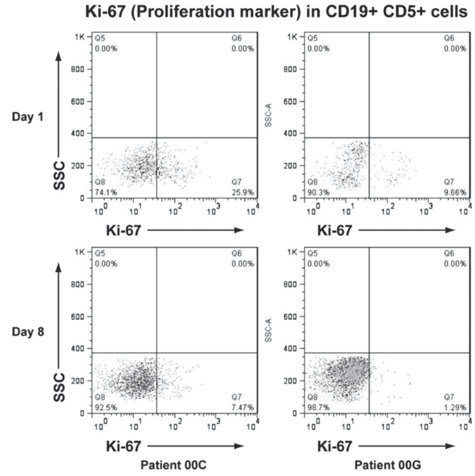


Figure S7.

Chapter 4

BTK inhibitors in chronic lymphocytic leukemia: a glimpse to the future

Marcel Spaargaren[1,4], Martin F.M. de Rooij[1], Arnon P. Kater[2,4], and Eric Eldering[3,4*]

Departments of [1]Pathololology, [2]Hematology and [3]Experimental Immunology, Academic Medical Center, Amsterdam, the Netherlands. [4]Lymphoma and Myeloma Center Amsterdam – LYMMCARE.

Oncogene (2014) 34:2426-36

ONCOGENE (2014) 34:2426-36

Abstract

The treatment of chronic lymphocytic leukemia (CLL) with inhibitors targeting B cell receptor signaling and other survival mechanisms holds great promise. Especially the early clinical success of ibrutinib, an irreversible inhibitor of Bruton's tyrosine kinase (BTK), has received widespread attention. In this review we will focus on the fundamental and clinical aspects of BTK inhibitors in CLL, with emphasis on ibrutinib as the best studied of this class of drugs. Furthermore, we summarize recent laboratory as well as clinical findings relating to the first cases of ibrutinib resistance. Finally, we address combination strategies with ibrutinib, and attempt to extrapolate its current status to the near future in the clinic.

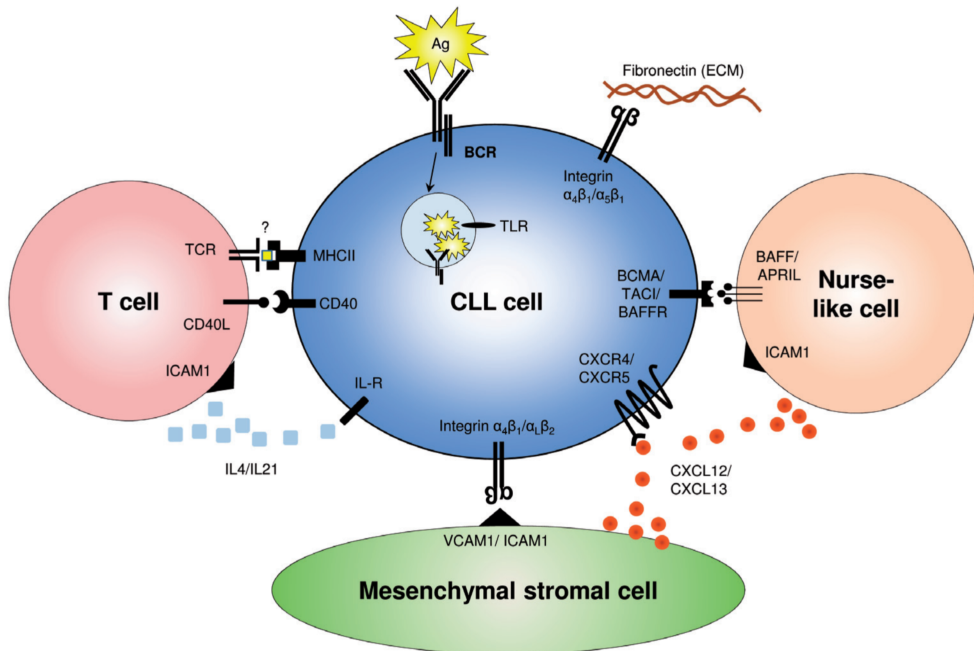
Introduction to chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL), the most common adult leukemia in western countries, is a neoplastic disease of monoclonal $CD5^+$ B cells, which accumulate in blood, marrow, and secondary lymphoid tissues. The median age at diagnosis lies between 65 and 70 years. More male than female patients are affected by this disease.[1] The disease is very heterogeneous; in less than 30% of all patients the disease has a very indolent course, with patients eventually dying from causes unrelated to CLL. About 15% of patients die rapidly -within 2-3 years from diagnosis- from CLL and/or treatment related causes. In the remaining proportion of patients the disease has a relative indolent course during the first 5 to 10 years, followed by a terminal phase of active disease and relapses following therapy, resulting in morbidity, both from the disease itself and from complications of therapy.[2]

Specific genomic alterations, especially deletions on the long arm of chromosome 13 (del(13q14)) are thought to be on the basis of leukemic transformation. Later during disease course, additional aberrations occur, most frequently deletion of the long arm of chromosome 11 (del(11q)) and deletion of the short arm of chromosome 17 (del(17p)).[3] Especially del11q and del17p, which are strongly associated with p53 dysfunction, predict for poor outcome.[4] More recently, next-generation sequencing studies have uncovered novel recurrent somatic mutations in CLL. Of these, mutations affecting the genes encoding *NOTCH1, BIRC3* and *SF3B1* seem to be the most common and to have prognostic impact.[5;6]

CLL cells are highly dependent on external stimuli for survival and proliferation. Despite their malignant nature CLL cells retain their susceptibility to external signals in lymph nodes and bone marrow, largely resembling mature healthy B cells. These interactions are collectively referred to as the microenvironment. Healthy B cells become activated upon antigen ligation to the B cell receptor (BCR), resulting in proliferation and differentiation. This can be further enhanced by cytokine stimulation and co-stimulation. The various signals from the microenvironment together orchestrate the activation of B cells and likewise of CLL cells. The lymph nodes and bone marrow thus provide a protective niche for CLL cells, enabling progression of the disease.[7]

A large number of factors have been studied that may contribute to the activation of CLL cells. Factors that potentially contribute include cell-cell contact, chemokines, cytokines and activation of the BCR (Fig. 1, for an overview, adapted from the study by de Weerdt *et al.*[8]). While many of these factors appear to contribute *in vitro*, it is yet to be defined which factors are relevant for CLL cell activation *in vivo*.



4

Figure 1: The microenvironment of CLL cells in lymphoid tissues.
CLL cells interact via integrins $\alpha_4\beta_1$ and $\alpha_L\beta_2$ to VCAM1 and ICAM1, respectively, which are expressed on mesenchymal stromal cells, nurse-like cells (tumor-associated macrophages) and T cells, and via integrin $\alpha_4\beta_1/\alpha_5\beta_1$ to extracellular matrix (ECM) components, for example, fibronectin. The activation of these integrins is controlled by chemokines (CXCL12/13 and CCL19/21 (not shown)) and B-cell antigen receptor (BCR) signaling. BCR signaling, either antigen (Ag)-stimulated or selfactivated, has an important role in CLL pathogenesis. Internalized antigens can activate intracellular Toll-like receptors (TLR), and can be presented to the T cells via MHC class II molecules. Furthermore, the proliferation and survival of CLL cells can be stimulated by CD40L and IL4/21 provided by the T cells, and by BAFF and APRIL derived from the nurse-like cells. Combined, these microenvironmental interactions have critical roles in the regulation of proliferation and survival of CLL cells in specialized niches within the lymphoid tissues, the proliferation centers.

Brief overview of current treatment options and patient outcomes

Standard therapy for fit patients is currently a combination of chemotherapeutics with monoclonal antibodies. Especially purine analogies with alkylating agents together with monoclonal antibodies directed against CD20 have prolonged progression free survival and improved overall survival.[9] Very recently it was shown that addition of anti-CD20 antibodies to chlorambucil is also highly beneficial for the treatment of unfit patients with CLL.[10]

With such regimens, most patients will have an initial complete or partial response resulting in a median progression free survival of up to 5 years for fit patients and up to 2.2 years for unfit patients. However, disease relapse invariably occurs after treatment has been discontinued. At relapse, mutations that affect p53 function occur at increased frequencies, resulting in chemoresistant disease.[11] Two monoclonal antibodies have shown activity in patients with chemoresistant disease. Treatment of relapsed and refractory patients with alemtuzumab, a recombinant, fully humanized monoclonal antibody against CD52 resulted in response rates of 30% to 50%, with a median duration of response ranging from 8.7 to 15.4 months. Due to its T-cell depleting features, (serious) infectious complications are common.[1] Ofatumumab, a fully humanized antibody targeting CD20, is active in very high-risk patients that are fludarabine and alemtuzumab refractory or have bulky disease. Response rates (all partial) are around 50% with a median duration of response of around half a year.[12]

These clinical data indicate an unanswered need specifically in refractory patients for effective treatment regimens, with a toxicity profile that is acceptable for the majority of elderly and less fit patients. It was envisioned that small molecule drugs targeting the BCR signalosome, in particular the cytoplasmic tyrosine kinase BTK, might fulfill these demands. Recently, as outlined below, the BTK inhibitor ibrutinib indeed seems to live up to these expectations, as excellent results were obtained in clinical trials with CLL patients. To provide insight into why BTK was anticipated to be such a successful target for CLL therapy, we will first review the physiological role of BTK, followed by the role of BCR-signaling and BTK in CLL.

The physiological role of BTK

BTK in B cell development, differentiation, and function

In 1952, the pediatrician Ogdon Bruton described X-linked agammaglobulinemia (XLA), an inherited disease characterized by the absence of antibodies, resulting in recurrent bacterial infections and sepsis early in childhood. In a milder form, a similar disease was also observed in mice (X-linked immunodeficiency, Xid). Forty-one years later, the gene underlying XLA was identified as agammaglobulinemia tyrosine kinase (ATK)[13] and B-cell progenitor kinase (BPK).[14] Xid mice were found to carry a mutation in the same gene,[15;16] and this gene was renamed Bruton's tyrosine kinase (BTK).

BTK is a non-receptor tyrosine kinase of the TEC-family. The difference in severity between XLA and Xid can (partially) be explained by Btk-redundancy with

the related kinase Tec in mice.[17] As can be concluded from XLA, loss-of-function mutations in *BTK* are detrimental for human B cell development. BTK expression is induced early in B cell development,[18] even before the pro-B cell stage. In the pro-B cell stage the immunoglobulin heavy chain locus will rearrange, after which it will be expressed together with a surrogate light chain. Expression of this pre-BCR induces allelic exclusion, proliferation (large pre-B cell), and subsequently light chain rearrangements (small pre-B cell).[19] In XLA-B cells, the differentiation halts at the pro-to-pre B cell transition.[20] Actually, the B cells do not recognize that heavy chain rearrangement is successful: XLA-B cells have rearrangements at both immunoglobulin alleles and do not proliferate at the pre-B cell stage, whereas proliferation in early pro-B cell stage is not affected.[21;22] Like in normal pro-B cells that do not succeed in heavy chain rearrangements, XLA-B cells will die by apoptosis. As a result, all mature B cells from female carriers have the same X-chromosome inactivation pattern.[23]

Apart from early B cell development, BTK also has critical roles in mature B cells. Mature B cells recirculate between secondary lymphoid organs and blood. The influx of B cells into B cell zones of secondary lymphoid organs is controlled by the chemokines CXCL12/13 and CCL19/21 and is integrin-dependent, whereas the egress is controlled by S1P and is integrin-independent.[24] Upon chemokine binding, the chemokine receptor will be desensitized and internalized, and due to this mechanism, influx and egress signaling are not tangled.[25] In the lymphoid tissues, when a cognate antigen is recognized by the BCR, the recirculation is arrested: CD69 is quickly upregulated, which acts as an inhibitor for the S1P-receptor and prevents egress,[26] and integrins are activated.[27] Upon inhibition of BTK, both processes do not take place,[28;29] so recirculation cannot be arrested. Furthermore, BTK expression is upregulated upon BCR engagement, CD40-, and TLR-stimulation,[30] most likely through NFκB activation, as BTK is a direct NFκB-target.[31] In Xid mice, somatic hypermutation and selection in the germinal center are unaffected, however memory B cell and plasma cell formation in the primary response are diminished.[32] In mice, ectopic overexpression of BTK in B cells promotes germinal center formation, plasma cell production, and autoimmunity,[30] suggesting that BTK has an instructive role in activated B cells. In terminally differentiated B cells, that is, plasma cells, BTK-expression is down regulated.[18;33] So, BTK expression closely resembles (pre) BCR-expression, and is a prerequisite for its signaling.

BTK in BCR-signaling

For BTK activation, two signals are required: a recruitment signal by PI3K, and an activation signal by LYN/SYK. Upon BCR engagement, the tyrosine kinase LYN

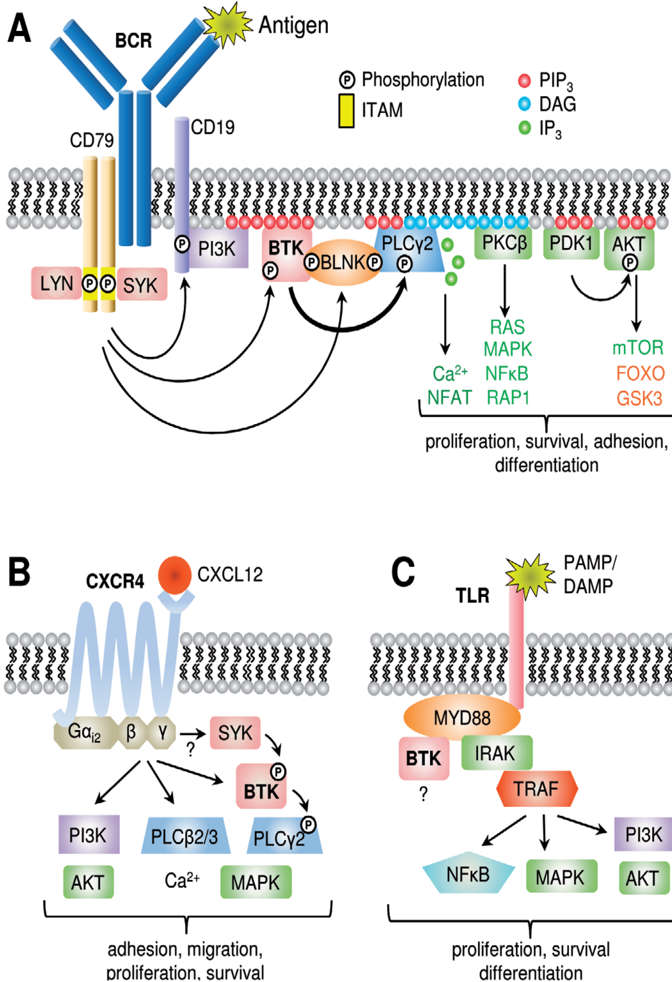


Figure 2: BTK-dependent signaling pathways.
Involvement of BTK in B-cell antigen receptor (BCR) signaling (A), chemokine (CXCR4) signaling (B) and Toll-like receptor (TLR) signaling (C). See text for details. ITAM, immunoreceptor tyrosine-based activation motif; PIP_3, phosphatidylinositol-3,4,5-triphosphate; DAG, diacylglycerol; IP_3, inositol-1,4,5-triphosphate; Ca^{2+}, calcium ions; PAMP, pathogen-associated molecular pattern; DAMP, danger-associated molecular pattern; IRAK, IL1 receptor-associated kinase; TRAF, TNF receptor-associated factor. Tyrosine kinases are in pink, serine/threonine kinases in green.

phosphorylates the ITAM (immunoreceptor tyrosine-based activation motif-containing BCR-subunit CD79, which then recruits the tyrosine kinase SYK. LYN (and/or SYK) phosphorylates CD19, resulting in the binding and activation of PI3Kδ, the major class I PI3K isoform expressed in B cells,[34] which in turn generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 production results in the recruitment of PH-domain containing proteins, including BTK and PLCγ2, to the membrane.[35] In parallel,

SYK phosphorylates BLNK, a scaffold protein which upon phosphorylation binds both BTK and PLCγ2. Subsequently, LYN or SYK phosphorylates BTK on Y551, an activating phosphorylation (Fig. 2A).[36;37] BTK also autophosphorylates on Y223, however, the function of this autophosphorylation is not exactly known: a Y223F mutation which cannot be phosphorylated, does not prevent (pre)BCR-controlled BTK-activity,[38] but rather enhances BTK-activity,[39] suggesting phosphorylation of Y223 is an autoinhibitory signal. Another important regulatory phosphorylation site on BTK is S180, which is phosphorylated by PKC, and acts as a negative feedback loop.[40]

Direct substrates of BTK include PLCγ2 and TFII-I (BAP-135). Upon SYK-mediated phosphorylation of the docking protein BLNK (SLP-65), both BTK and PLCγ2 are recruited to BLNK, and BTK activates PLCγ2 by phosphorylation on Y753/Y759.[41;42] Activated PLCγ2 produces diacylglycerol and inositol-1,4,5-trisphosphate (IP_3) in which diacylglycerol activates RAS/MAPK-signaling, PKC/NFκB-signaling, and RAP1-signaling, a hallmark for integrin activation, whereas IP_3-mediated calcium release from the endoplasmatic reticulum induces NFAT-signaling and cytoskeleton rearrangements (Fig. 2A).[43-49] Furthermore, upon BCR-engagement BTK recruits PIP5K, which is involved in production of PIP_2, the substrate for BTK's upstream regulator PI3K as well as its downstream effector PLCγ2.[50] Another important BCR-controlled pathway is the PI3K/PKB-pathway, which controls mTOR- and FOXO signaling, however, this pathway is collateral and independent of BTK.[27] A second pathway induced by BTK is the TFII-I pathway.[51] Upon BCR engagement, TFII-I is phosphorylated by BTK and subsequently translocate to the nucleus and acts as a transcription factor.[52] In B cells, TFII-I together with Bright (ARID3A) is involved in regulation of immunoglobulin heavy chain expression,[53-55] and in BCR-controlled cell cycle arrest.[56] At the molecular level, the BCR-controlled pathways are responsible for B cell activation, proliferation, survival, and differentiation, and BTK is a critical hub controlling these pathways.

The role of BTK in myeloid cells

Apart from B cells, the BTK-PLCγ2-axis is present in all myeloid lineages. In these cells, BTK and PLCγ2 act downstream of ITAM-containing receptors, for example, in Fcε-receptor signaling in mast cells,[57;58] Fcγ-receptor signaling in macrophages, (plasmacytoid) dendritic cells and natural killer cells,[59-61] ITAM-containing adaptors mediated signaling in osteoclasts,[62] and in collagen-, non-integrin receptors- and CD32-signaling in platelets.[63] Because XLA patients do not have defects in the myeloid system,[64-66] and myeloid cells of XLA-carriers are not selected against

the unaffected X-chromosome,[23;66] BTK does not have a critical role in myeloid development and function.

BTK in chemokine- and TLR-signaling

In B cells and myeloid cells, apart from BCR- and other ITAM-involved pathways, BTK is associated with chemokine-,[67] and Toll like receptor (TLR) signaling.[68-71] BTK can interact with $G\alpha_{12}$- and Gβγ-subunits of the G protein-coupled receptors-pathway,[72-74] and with TLRs, MYD88, IRAK1, and MAL of the TLR-pathway.[68;75] In chemokine signaling, BTK Y551-phosphorylation and activation is also PI3K- and SYK-dependent, and BTK phosphorylates and activates PLCγ2,[28;67] suggesting possible cross-talk between chemokine- and BCR signaling in the activation of BTK (Figs. 2B-C). Regarding TLR signaling, it remains elusive how BTK is activated, what it substrates are, and what the functional role of BTK may be.[64;76] In this respect, it is perhaps noteworthy that in CLL also mutations in MYD88 have been found.[77]

The role of BTK in CLL

BCR signaling in CLL

In CLL cells, BCR-signaling has the same role as in activated B cells and there is strong evidence that (antigen-stimulated) BCR-signaling has a critical role in CLL pathogenesis. Many CLL-BCRs are auto/polyreactive,[78] and half of CLL patients express a stereotypic BCR-repertoire, indicating that the BCRs of different CLL patients recognize the same antigen.[79] Indeed, for a stereotypic subgroup the cognate antigen was recently discovered, namely a yeast cell wall component.[80] However, evidence has also been provided for cell-autonomous antigen-independent BCR signaling in CLL, being driven by recognition of an epitope in the BCR itself.[81] Several studies provide indirect evidence that antigens have a critical role in CLL pathogenesis. In CLL, there are two compartments: cells that reside in lymphoid tissues, and cells that are circulating in blood. CLL cells that just entered the circulation have low surface IgM expression, whereas cells that are in the circulation for a longer time have high surface IgM expression,[82] suggesting that CLL cells experience active (antigen-driven) BCR-signaling in the lymphoid tissues. Also in gene expression profiling studies it was found that tissue-derived CLL cells have stronger BCR- and NFκB signaling than blood-derived CLL cells.[83] In the lymphoid microenvironment, however, NFκB-signaling is not only driven by BCR-, but also by CD40L-, TLR-, and BAFF/APRIL signaling.[84]

Apart from direct activation of NFκB, BCR signaling is also involved in

the TLR- and CD40-mediated pathways leading to NFκB. In CLL cells, the most abundantly expressed TLRs are TLR-7 and -9,[85-87] which are localized in lysosomes. Upon BCR-mediated uptake and processing of antigens, these TLRs are activated.[88;89] CD40L-signals are provided by activated cognate T cells, which interact with the B cells upon TCR-MHCII-engagement. This also requires BCR-mediated uptake of antigen. The proper uptake and processing of antigens requires actin reorganization, which is BTK-dependent.[90] CLL cells still have the machinery to load MHCII with antigen fragments,[91;92] and in a xenograft mouse model CLL cells only grow in the presence of autologous T cells.[93] This suggests that cognate T cells have a role in CLL-pathogenesis, which may specifically involve tissue-derived CLL cells as it has been reported that blood-derived CLL cells are poor antigen-presenting cells.[94] This notion is reinforced by our recent studies that suggest a role for IL21 derived from follicular T helper cells in the CLL lymph node environment.[95]

BTK in CLL pathogenesis

Because either tonic, chronic, or antigen-driven BCR signaling is involved in the pathogenesis of most types of B-cell malignancies, the BCR signalosome is a rational therapeutic target.[96] Regarding selectivity and clinical safety, BTK was considered a particularly promising target: it is a key component of the BCR signaling pathway, only critical for B cells, and loss of BTK function is not lethal (for example, XLA patients).[97] In line with the important role of either antigen-stimulated and/or chronic BCR signaling in CLL cells, BTK has a critical role in CLL development and maintenance. As outlined below, this can be concluded from various recent studies, employing translational mouse models for CLL, *in vitro* studies on primary human CLL cells, and clinical studies with CLL patients.

Several mouse models display strong CLL-like features and have been used also to study the role of BTK. Kill *et al*. used the IgH.ETμ CLL mouse model, which is based on sporadic expression of the simian oncovirus SV40 T-antigen in mature B cells. Crossing IgH.ETμ mice either with *Btk*-deficient mice or with human *BTK* transgenic mice, has demonstrated that *Btk* deficiency fully abrogates CLL formation in the IgH.ETμ mice, that in *Btk* haplo-insufficient female mice leukemias only formed if their active X-chromosome carried the wild-type *Btk* allele, and that *BTK* overexpression accelerated CLL onset.[98] Likewise, Woyach *et al*. used the Eμ-TCL1 (TCL1) transgenic mouse model of CLL, which results in spontaneous leukemia development. Crossing the TCL1 mice with Xid mice significantly delays the development of CLL.[99] Thus, Btk expression is critical for development (and expansion) of CLL-like disease in these mouse models. Similar results were obtained upon treatment of TCL1-CLL mice with the BTK inhibitor ibrutinib.[99;100] These results

also support the conclusion that BCR-signaling is essential for CLL development and maintenance.

In CLL cells, BTK is a crucial regulator of BCR- and NFκB signaling. Upon treatment of CLL patients with the BTK-inhibitor ibrutinib the lymph node- and blood-derived CLL cells display strongly decreased expression of BCR- and NFκB-target genes and of activation markers, for example, CD69 and CD86.[101] In addition, plasma levels of the BCR-targets CCL3 and CLL4 are decreased.[100] Thus, like in normal B cells, BTK is also a critical hub in BCR-signaling in CLL cells.

Following treatment of CLL patients with ibrutinib, a rapid and sustained decrease in lymphadenopathy is observed, accompanied by transient lymphocytosis.[102] This lymphocytosis can persist for more than 12 months but is not linked with a poorer response.[103] In fact, a recent study argued that in patients under treatment with BCR inhibitors, the peripheral lymphocyte count is not a valid surrogate for disease burden.[104] Lymphocytosis also occurs in TCL1-CLL mice when ibrutinib was applied after tumor development, albeit not as strong as in humans,[100] and in a xenograft mice model.[105] The observed lymphocytosis upon BTK inhibition suggests that the CLL cells do not die directly upon BTK inhibition, but are rather mobilized from lymphoid tissues to blood. Indeed, we and others demonstrate that inhibition of BTK, at concentrations of ibrutinib that can also be accomplished *in vivo*, is not cytotoxic for unstimulated blood-derived CLL cells.[28;100;106;107]

In CLL cells, BTK regulates BCR-controlled integrin-mediated interactions with the microenvironment. We have recently demonstrated that BCR-engagement of CLL cells induces strong integrin-mediated adhesion to fibronectin (an extracellular matrix ligand) and VCAM1 (a cellular ligand) in a BTK-dependent manner. The cells that arise in the peripheral blood of CLL patients upon ibrutinib treatment display phenotypic characteristics of lymphoid organ-derived CLL cells.[28] As naive B cells, which do not experience stimulated BCR-signaling, are not mobilized from lymphoid tissues upon ibrutinib treatment,[108;109] this indicates that BTK inhibition can specifically disrupt adhesion of CLL cells because this is actively controlled by stimulated BCR-signaling. Interestingly, also chemokine-controlled signaling, adhesion and migration is (partially) reduced upon *in vitro* inhibition of BTK in CLL cells.[28;100]

In heavy water studies it was found that CLL has a high turnover rate:[110] CLL cells proliferate in the lymphoid tissues,[111;112] translocate to blood and eventually die. Indeed, CLL cells of patients treated with a BTK-inhibitor show reduced expression of proliferation markers.[101;107] Furthermore, in a coculture with stromal cells,[107] and in a xenograft mouse model,[105] proliferation of CLL cells was reduced upon inhibition of BTK. This may be the consequence of compromised integrin-mediated interactions of the CLL cells with the microenvironment (*in vivo*) or stromal cells (*in vitro*), resulting

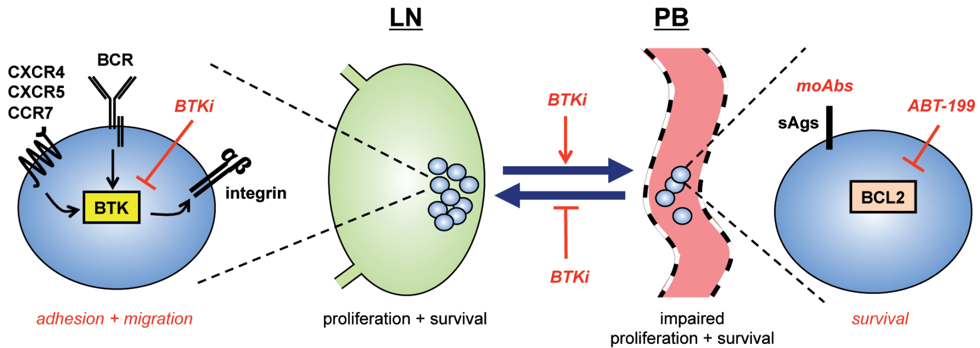


Figure 3: Proposed model for future combination therapies.
Inhibition of BTK impairs BCR-controlled integrin-mediated adhesion and chemokine (CXCL12, CXCL13, and CCL19)-induced adhesion and migration of CLL cells. Consequently, BTK inhibitors (BTKi) overcome BCR and chemokine-controlled integrin-mediated retention of the CLL cells in their growth- and survival-supporting lymph node (LN) and bone marrow microenvironment, resulting in their egress from these protective niches into the circulation (PB), resulting in CLL regression. As the CLL cells do not die directly, combination therapies with cytotoxic drugs, for example, monoclonal antibody therapies (moAbs) against specific surface antigens (sAgs) or BH3-only mimetics (ABT-199), will most likely be highly efficacious (adapted from de Rooij et al.[28]).

in mitigated acquisition of proliferation signals.

Taken together, BTK has an essential role in the regulation of BCR-controlled integrin-mediated adhesion of CLL cells and their retention within the growth and survival promoting lymphoid microenvironment. Inhibition of BTK appears to induce the premature egress of CLL cells from the lymphoid tissue into the circulation, thereby depriving the CLL cells from growth and survival signals provided by the lymphoid microenvironment, resulting in impaired proliferation and survival, and disease regression (Fig. 3).

Historic highlights on the clinical development of the BTK inhibitor ibrutinib

In 2007, scientists from Celera Genomics reported the development of small molecules that could inactivate BTK by binding irreversibly to the Cys-481 residue in its ATP-binding domain.[113] As Pharmacyclics acquired Celera's BTK inhibitor program, initially mainly aiming at targeted therapy for rheumatoid arthritis, ibrutinib was selected for follow-up studies because of its high potency (IC_{50} = 0.5nM) and selectivity for BTK in a screen with a large panel of kinases. In July 2010, Honigberg *et al.* [29] reported the first *in vivo* results on ibrutinib, showing that it is well tolerated and efficacious upon oral administration in murine autoimmune models for rheumatoid

arthritis and SLE and in a canine B-cell lymphoma model.

At the ASCO meeting in June 2010,[114] updated at the ASH meeting in December 2010,[115] the first early results of a phase 1 study with ibrutinib in patients with various relapsed/refractory B-cell malignancies were reported, showing clinical safety and promising durable objective responses particularly in CLL and mantle cell lymphoma (MCL) patients. In most CLL and MCL patients enrolled in this phase 1 study, typically a rapid and sustained reduction in lymphadenopathy was observed, which was, however, frequently accompanied by lymphocytosis. Despite this sign of disease progression, being counterbalanced by the frequently observed impressive reduction in lymphadenopathy and rapid improvement of the physical condition of several patients, the trial was not aborted. During this first phase 1 trial, with a treatment regimen of a daily oral capsule for 4 weeks followed by 1 week off-drug (drug holiday), the absolute lymphocyte count displayed a sawtooth-pattern that coincides with each treatment cycle, that is, the initial rise in absolute lymphocyte count was followed by a drop during the drug holiday (reversible) and by another rise as treatment was re-started.[102] During a phase 1b trial for CLL patients, with continuous daily dosing of ibrutinib, typically the absolute lymphocyte count was enhanced during the first weeks of treatment but gradually declined during continuation of the treatment.[116] In the end, in most CLL patients, both with intermittent or continuous ibrutinib treatment regimen, the absolute lymphocyte count normalized and dropped below baseline levels after approximately 8 months of treatment.[102;116] Thus, the observed lymphocytosis turned out to be reversible and transient. On the basis of these early clinical observations it was suggested that the observed lymphocytosis could be the consequence of a redistribution of the CLL cells from the lymphoid organs into the peripheral blood, rather than of disease progression.

In 2011, Herman *et al*.[106] reported that *in vitro* treatment of CLL cells with ibrutinib induced only modest cytotoxicity/apoptosis (at relatively high concentrations, that is, μM range) but abolished CpG-induced TLR-mediated proliferation (at 100nM). In early 2012, Ponader *et al*.[100] reported that ibrutinib inhibits CLL cell chemotaxis *in vitro* and delayed disease progression (accompanied by lymphocytosis) in a TCL1 adoptive transfer SCID mouse model of CLL. We demonstrated that ibrutinib treatment impairs BCR- and chemokine-controlled integrin-mediated adhesion and chemokine-controlled migration of CLL cells, and that the cells in the peripheral blood of CLL patients upon ibrutinib treatment display phenotypic characteristics of lymphoid organ-derived CLL cells.[28] Later we presented similar results for MCL.[108] These findings provided a molecular and mechanistic explanation for the observed clinical responses and the clinical efficacy of ibrutinib. Inhibition of BTK by

ibrutinib overcomes BCR- and chemokine-controlled retention (and homing) of the malignant B cells in the lymphoid organs, resulting in reduced lymphadenopathy and (transient) lymphocytosis; upon egress from the lymphoid organs into the circulation the malignant cells are deprived of critical growth- and survival-signals provided by the lymphoid microenvironment, resulting in lymphoma regression (Fig. 3).[28] These novel insights into the action of ibrutinib, in particular regarding the observed lymphocytosis response, instigated revision of the clinical response criteria for CLL, that is, on the use of blood lymphocyte counts as a surrogate marker for tumor burden.[117] It was concluded that in trials with ibrutinib and similar agents targeting the BCR signalosome, such as fostamatinib and idelalisib, lymphocytosis is not a sign of progressive disease but rather reflects an on-target effect.

In January 2013, the final results of the phase 1 trial were published. This report on 56 patients with various relapsed/refractory B cell malignancies demonstrated the highest response rates in 11 out of 16 CLL (69%) and 7 of 9 MCL patients (78%), and high clinical safety.[102] Furthermore, the daily oral administration of this irreversible covalent-binding kinase inhibitor resulted in rather unique pharmacokinetics and pharmacodynamics: ibrutinib is rapidly adsorbed (the mean peak plasma concentration of 300nM is reached after 1-2h), cleared from the plasma (upon reaching peak-value the initial mean half-life is 2-3h) and eliminated (the mean terminal half-life is 4-8h), but due to its irreversible covalent binding BTK remained fully occupied for at least 24h. Thus, without compromising effective targeting of BTK, the restricted exposure will limiting off-target effects of ibrutinib, which, combined with the restricted expression pattern of BTK, contributes to the excellent safety profile of this compound.

Because of the outstanding trial results in MCL, CLL and WM, unprecedented for any monotherapy in these malignancies, in February 2013 the FDA granted a “Breakthrough Therapy” designation to expedite the development and review of ibrutinib as a monotherapy for patients with relapsed/refractory MCL and for patients with WM, and in April 2013 also for the treatment of patients with CLL or SLL with deletion of the short arm of chromosome 17 (del17p). In June 2013, this was followed by the results of a phase 1b and 2 study specifically in 85 relapsed or refractory CLL patients, showing an overall response rate of 71%,[116] and the results of a phase 2 study in 111 patients with relapsed/refractory MCL with a response rate of 68%.[118]

In November 2013, less than 3.5 years (!) after the first publication of its effect on lymphoma in an animal model, the FDA granted approval to ibrutinib (Imbruvica) under the agency’s “Accelerated Approval” program for the treatment of patients with MCL who have received at least one prior therapy, and in February

Agent	ONO-4059	Dasatinib	CC-292	Ibrutinib
Developer	ONO	Bristol-Myers Squibb	Celgene	Pharmacyclics and Janssen
Mechanism, target	Reversible, Tyr223	Reversible, multiple targets including LYN and BTK	Irreversible, Cys481	Irreversible, Cys481
Potency	IC_{50} = 2nM	IC_{50} = 5nM	IC_{50} <0.5nM	IC_{50} = 0.5nM
Response data from monotherapy trials in CLL	*R/R n=16 (ref. 143)* PR: 20% nPR: 70% CR: 0%	*R/R n=15 (ref. 144)* PR: 20% nPR: unknown CR: 0%	*R/R n=83 (ref. 145)* PR: 42% nPR: 62-67% CR: 0%	*R/R n=85 (ref. 116)* PR: 71% nPR: 89% CR: 0% *Naive n=31 (ref. 146)* PR: 55% nPR: 13% CR: 13%

Table 1: Overview of BTK inhibitors in clinical trials.

2014 the FDA expanded approval to ibrutinib to include the treatment of patients with CLL who have received at least one previous therapy. See table 1 for a summary of clinical trials with ibrutinib and other BTK inhibitors.

Potential drawbacks of long-term BTK inhibition

Despite considerable clinical success leading to assignment of breakthrough status and the recent FDA approval of ibrutinib, there are some emerging disadvantages associated with its long-term clinical application.

Side effects

Reported toxicity resulting from ibrutinib treatment is modest,[116] with grade 1-2 diarrhea as the most frequent event (47%) and grade 3-4 in 2% of patients. Cases of chronic diarrhea after prolonged, continuous treatment (>6 months), are rare with ibrutinib but have been reporter for idelalisib, the PI3Kδ inhibitor which also targets BCR signaling pathway and has likewise shown positive clinical responses in CLL.[119;120] Diarrhea and nausea appear quite bearable as low-grade side-effects in the context of conventional chemotherapy, which is meant to be temporary. When experienced on a permanent basis as a result of daily pills, such toxicities will however become a burden of increasing severity and eventually affect quality of life quite strongly. This is also the case for the reported fatigue which occurred in approximately 30% of patients, the large majority as grade 1-2. Ibrutinib is quite a selective agent, but does target other kinases with affinities within physiological range,[29] so potential side effects may be related to off-target properties. One possibility might be its proven ability to also target ITK, the kinase involved in especially T helper 2 (Th2) signaling. This property has been reported to contribute to its clinical efficacy in a positive sense, by shifting the Th2 skewed immune system in CLL back towards a balanced Th1/Th2 status, thereby restoring a healthy immune system.[121] Hypothetically after

long term application of ibrutinib, this induced Th1 shift in the intestinal tissues might lead to inflammatory disturbances. Although colitis has not been reported in association with ibrutinib, a contribution of skewed Th1 responses is also seen in Crohn's disease and inflammatory bowel disease.[122] Another speculative cause for side-effects might be that ibrutinib inhibits BTK also in myeloid cells such as tissue macrophages and dendritic cells[59-61] and that this may, over time, cause problems by disturbing homeostasis.

Resistance

Selection or outgrowth of resistant clones is a major problem in cancer treatment. Both for conventional chemotherapeutics and novel targeted drugs such as ibrutinib, there is considerable selection pressure for the cancer cells to escape elimination. Ibrutinib has been continuously applied in clinical trial setting for more than two years in significant numbers of CLL patients. In a minority of patients progressive disease occurred after initial responses. Three recent meeting reports detailed the emergence and molecular characteristics of such resistant clones. Chang *et al.*[123] examined 20 progressive patients from a total of 246 (8%) in various clinical trials. Of the three patients that did not undergo Richter's transformation, RNA sequencing and whole-exome sequencing was performed. The results were striking and showed in two patients a mutation at cysteine-481 in BTK, the site of attack of ibrutinib. The remaining patient displayed an activating mutation in PLCγ2, an important downstream substrate of BTK (see also figure 2). Furman *et al.*[124] reported that recombinant expression of the mutant BTK protein rendered the cells resistant to ibrutinib, but not to dasatinib -which can also inhibit BTK[125]- or SYK inhibitors. These data emphasize the importance of BTK signaling in CLL, as well as the considerable selective pressure exerted by targeting it. Similar analyses reported at ASH 2013 on 3 out of 11 relapsed patients (13%) emphasized that other mechanisms of escape also have a role.[77] In these patients no mutations in BTK or its downstream targets were apparent, although in subclonal fractions (<15%) PLCγ2 mutations were detected. Such small fractions seemed unable to explain the clinical resistance. Remarkably, other driver mutations associated with poor prognosis were detected, notably in the splicing factor SF3B1, that apparently contributed to the acquired resistance by nonspecific adverse clonal evolution.

An important question regarding resistance to ibrutinib, or any drug, is whether the mutations involved are induced *de novo* under selective pressure, or are selected from (very minor) subfractions of the malignant clone. In the latter case, the prospect of successful long-term treatment or even cure with monotherapy becomes

less likely. With the growing application of next-generation sequencing techniques, capable of very deep probing of the cancer genome, the answer to this question is within reach. A first glimpse afforded by this type of analysis in fludarabine-refractory CLL, at a maximal resolution of three mutated cells per 1000, demonstrated that minor subclonal *TP53* mutations are already present at diagnosis and relatively rapidly selected under treatment.[126] An earlier investigation using Sanger sequencing reached similar conclusions.[127]

Resistance to a particular drug, be it fludarabine or ibrutinib, can be mediated by mutations in distinct genes, as illustrated by *TP53* and *ATM* which together account for only approximately 40% of fludarabine resistant cases.[128] The next-generation sequencing efforts of recent years have underlined that also mutations in *SF3B1*, *NOTCH1*, and *BIRC3* are associated with poor response to therapy,[5] through as yet unknown mechanism(s). As illustrated above, in the case of ibrutinib-resistance, the situation appears similar, in the sense that mutations in BTK and direct downstream effectors can lead to resistance. This notion, combined with the emerging insight that subclonal mutations that may afford resistance are predominantly pre-existing, does not bode very well for permanent or long-term application of monotherapy with ibrutinib or similar targeted drugs. Thus, in addition to the looming financial burden of protracted treatment with targeted drugs, combination therapy seems a sensible route to prevent emergence of resistance.

Ibrutinib combination therapies -ongoing and hypothetical

The novel insights regarding drugs targeting BTK, for example, ibrutinib, may not only provide support for their further use as monotherapy, exploiting the microenvironment-dependence as the Achilles' heel of CLL, but also for their exploration as rational combination therapy. Once the malignant B cells egress from their protective niches into circulation they may become better accessible and more vulnerable for chemo- and antibody-therapy (for example, rituximab). This would make a promising and highly efficacious combination therapy, possibly resulting in even greater benefit for the CLL patients or even complete eradication of their malignancy (Fig. 3).

Combinations with rituximab and bendamustin in clinical trials

To date, two clinical studies have described combination therapies with ibrutinib in CLL. Data on a phase II trial of ibrutinib plus rituximab[129] and a Phase I trial of the combination ibrutinib, rituximab and bendamustin[130] were presented at ASH2013.

Both studies treated relapsed/refractory patients and reported favorable toxicity profiles. In the presence of rituximab (plus bendamustin), the lymphocytosis normally observed after ibrutinib administration was less pronounced and resolved faster. As a result, overall response rates (still defined as a 50% reduction in lymphocyte count) of the combinations were higher than with ibrutinib monotherapy. Whether this translates into longer progression-free and overall survival will be addressed in larger, randomized trials (NCT01611090 and NCT01886872). It is worth mentioning that recent *in vitro* studies indicated that ibrutinib unexpectedly antagonized rituximab-mediated natural killer cell cytotoxicity.[131] This was tentatively ascribed to inhibition of ITK in natural killer cells. Although a potential antagonism would clearly be unfavorable, the first clinical data do not seem to support it. At present, no data on other combination treatments with Ibrutinib are available.

Combinations with other BCR-targeting drugs, proteasome inhibitors or BH3-mimetics

Laboratory tests for various combinations of ibrutinib with other drugs, either targeting within or outside the BCR signaling pathway, have been reported for MCL cell lines and patient samples.[132] The results demonstrated synergy with several drugs that target survival pathways other than BCR signaling. The read-out in this system was cytotoxicity and especially for proteasome inhibitors (carfilzomib and to a lesser extent bortezomib) and the BH3 mimetic ABT-199 a significant synergy was noted. This has relevance for CLL as the direct cytotoxic activity of ibrutinib in CLL is modest at best[100;106] and was reported otherwise only in the context of BCR signaling,[100] or under serum-free conditions.[133] Proteasome inhibitors are presumed to inhibit pro-survival NFκB signaling, which has an important part in the microenvironmental stimulation that CLL cells receive within the lymph nodes of patients.[83] Furthermore, recently it has been reported that indeed ibrutinib prevents the NFκB gene signature *in vivo,*[101] and conversely, that mutations in CLL that affect NFκB signaling confer resistance to ibrutinib.[133] Thus, it might be expected that in CLL, similar to MCL, simultaneous application of ibrutinib to inhibit BCR-mediated NFκB signals, with proteasome inhibitors to block additional co-stimulatory signals, could be beneficial. It should be noted that proteasome inhibitors are very cytotoxic to CLL cells *in vitro,*[134-136] yet bortezomib displayed hardly any clinical benefit when administered as single agent in patients.[137;138] This discrepancy remains unexplained thus far, but may be linked with another proposed mechanism of action of bortezomib in multiple myeloma cells, namely the induction of a lethal unfolded protein response,[139] which would not be expected to occur in CLL cells as they do not secrete immunoglobulins.

Ibrutinib inhibits various signaling pathways emanating from, or linked with, the BCR-signalosome, including a very effective inhibition of BCR-controlled integrin-mediated adhesion. However, chemokine-controlled adhesion/migration is only partially impaired.[28] Given the importance of overcoming lymphoid organ localization in the clinical activity of ibrutinib, the treatment efficacy could be further improved by combinations with agents that block this aspect of lymph node retention and homing more efficiently. From this perspective, the PI3Kδ inhibitor idelalisib could be a promising candidate.

Another possibility is to combine ibrutinib with ABT-199, the BCL2-specific BH3 mimetic[140] which as single agent shows already very promising activity in CLL.[141] On the basis of available knowledge, ibrutinib (or other BCR-signaling inhibitors) plus ABT-199 might prove even more efficient than the separate agents, especially in view of long-term application. In this scenario, ibrutinib's proven ability to force the CLL cells out of the protective lymph node environment would be complemented by the cytotoxicity of ABT-199 against CLL cells in the circulation that rely on high BCL2 expression for survival (Fig. 3). The available clinical data with ABT-199 show significant efficacy also with respect to nodal responses in CLL.[141;142] Yet, it might be argued that as the lymph node environment induces expression of MCL1, BCL-XL and BFL1,[134;136] which are not targeted by ABT-199, its long-term application might select for resistant niches. Thus, the combination with ABT-199 would not only limit the lymphocytosis resulting from ibrutinib treatment, but may also be expected to reduce the probability that resistance against either agent occurs. The proposed synergy between agents targeting BCR- and/or adhesion signaling in the lymph node with ABT-199 to kill the cells that have moved to the blood is illustrated in figure 3.

Concluding remarks

It seems clear that with the arrival of ibrutinib and other kinase inhibitors, as well as BH3 mimetics such as ABT-199, a new treatment era for CLL is about to begin.[1] Despite the obvious clinical success and general optimism, we have also sketched the contours of the first warning signs that even these highly efficient therapeutics may present problems of side effects and development of resistance. These may be linked with the requirement of permanent medication of patients. Even if toxicity or resistance occurs in only a minority of patients, further research into these aspects is warranted. As is evident from ongoing clinical trials, the expectation in the field appears to be that a possible solution to such problems is the development of

combination therapies with improved efficacy. Surely, within the next few years we will obtain the answers to these emerging questions.

Authorship contributions

M.S. wrote “Historic highlights on the clinical development of the BTK inhibitor ibrutinib”, and designed the figures. M.F.M.d.R. wrote “The physiological role of BTK” and “The role of BTK in CLL”, and designed the figures. A.P.K. wrote “Introduction to chronic lymphocytic leukemia” and “Brief overview of current treatment options and patient outcomes”, and designed the table. E.E. wrote “Potential drawbacks of long-term BTK inhibition” and “Ibrutinib combination therapies -ongoing and hypothetical”.

Disclosure of conflicts of interest

MS has received research support from Pharmacyclics. The other authors declare no conflict of interest.

References

1. Hallek M. Signaling the end of chronic lymphocytic leukemia: new frontline treatment strategies. *Blood* 2013;122(23):3723-3734.
2. Cramer P, Hallek M. Prognostic factors in chronic lymphocytic leukemia-what do we need to know? *Nat.Rev.Clin.Oncol.* 2011;8(1):38-47.
3. Dohner H, Stilgenbauer S, Benner A et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N.Engl.J.Med.* 2000;343(26):1910-1916.
4. Pospisilova S, Gonzalez D, Malcikova J et al. ERIC recommendations on TP53 mutation analysis in chronic lymphocytic leukemia. *Leukemia* 2012;26(7):1458-1461.
5. Messina M, Del Giudice I, Khiabanian H et al. Genetic lesions associated with chronic lymphocytic leukemia chemo-refractoriness. *Blood* 2014;123(15):2378-88.
6. Landau DA, Carter SL, Stojanov P et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* 2013;152(4):714-726.
7. Burger JA, Gribben JG. The microenvironment in chronic lymphocytic leukemia (CLL) and other B cell malignancies: insight into disease biology and new targeted therapies. *Semin.Cancer Biol.* 2014;24:71-81.
8. de Weerdt I, Eldering E, van Oers MH, Kater AP. The biological rationale and clinical efficacy of inhibition of signaling kinases in chronic lymphocytic leukemia. *Leuk.Res.* 2013;37(7):838-847.
9. Hallek M, Fischer K, Fingerle-Rowson G et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet* 2010;376(9747):1164-1174.
10. Goede V, Fischer K, Busch R et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. *N.Engl.J.Med* 2014;370(12):1101-1110.
11. Zenz T, Krober A, Scherer K et al. Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. *Blood* 2008;112(8):3322-3329.
12. Wierda WG, Kipps TJ, Mayer J et al. Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. *Journal of Clinical Oncology* 2010;28(10):1749-1755.
13. Vetrie D, Vorechovsky I, Sideras P et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature* 1993;361(6409):226-233.
14. Tsukada S, Saffran DC, Rawlings DJ et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 1993;72(2):279-290.
15. Thomas JD, Sideras P, Smith CI et al. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science* 1993;261(5119):355-358.
16. Rawlings DJ, Saffran DC, Tsukada S et al. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science* 1993;261(5119):358-361.

17. Ellmeier W, Jung S, Sunshine MJ et al. Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. *J.Exp.Med.* 2000;192(11):1611-1624.
18. de Weers M., Verschuren MC, Kraakman ME et al. The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur.J.Immunol.* 1993;23(12):3109-3114.
19. Martensson IL, Rolink A, Melchers F et al. The pre-B cell receptor and its role in proliferation and Ig heavy chain allelic exclusion. *Semin.Immunol.* 2002;14(5):335-342.
20. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. *Blood* 2008;112(5):1570-1580.
21. Mensink EJ, Schuurman RK, Schot JD, Thompson A, Alt FW. Immunoglobulin heavy chain gene rearrangements in X-linked agammaglobulinemia. *Eur.J.Immunol.* 1986;16(8):963-967.
22. Campana D, Farrant J, Inamdar N, Webster AD, Janossy G. Phenotypic features and proliferative activity of B cell progenitors in X-linked agammaglobulinemia. *J.Immunol.* 1990;145(6):1675-1680.
23. Fearon ER, Winkelstein JA, Civin CI, Pardoll DM, Vogelstein B. Carrier detection in X-linked agammaglobulinemia by analysis of X-chromosome inactivation. *N.Engl.J.Med.* 1987;316(8):427-431.
24. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu.Rev.Immunol.* 2005;23:127-159.

25. Lo CG, Xu Y, Proia RL, Cyster JG. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. *J.Exp.Med.* 2005;201(2):291-301.
26. Shiow LR, Rosen DB, Brdickova N et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 2006;440(7083):540-544.
27. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J.Exp.Med.* 2003;198(10):1539-1550.
28. de Rooij MF, Kuil A, Geest CR et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* 2012;119(11):2590-2594.
29. Honigberg LA, Smith AM, Sirisawad M et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc.Natl.Acad.Sci.U.S.A.* 2010;107(29):13075-13080.
30. Kil LP, de Bruijn MJ, van NM et al. Btk levels set the threshold for B-cell activation and negative selection of autoreactive B cells in mice. *Blood* 2012;119(16):3744-3756.
31. Yu L, Mohamed AJ, Simonson OE et al. Proteasome-dependent autoregulation of Bruton tyrosine kinase (Btk) promoter via NF-kappaB. *Blood* 2008;111(9):4617-4626.
32. Ridderstad A, Nossal GJ, Tarlinton DM. The xid mutation diminishes memory B cell generation but does not affect somatic hypermutation and selection. *J.Immunol.* 1996;157(8):3357-3365.

33. Genevier HC, Hinshelwood S, Gaspar HB et al. Expression of Bruton's tyrosine kinase protein within the B cell lineage. *Eur.J.Immunol.* 1994;24(12):3100-3105.
34. Ramadani F, Bolland DJ, Garcon F et al. The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development. *Sci.Signal.* 2010;3(134):ra60.
35. Saito K, Scharenberg AM, Kinet JP. Interaction between the Btk PH domain and phosphatidylinositol-3,4,5-trisphosphate directly regulates Btk. *J.Biol.Chem.* 2001;276(19):16201-16206.
36. Rawlings DJ, Scharenberg AM, Park H et al. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* 1996;271(5250):822-825.
37. Kurosaki T, Kurosaki M. Transphosphorylation of Bruton's tyrosine kinase on tyrosine 551 is critical for B cell antigen receptor function. *J.Biol.Chem.* 1997;272(25):15595-15598.
38. Middendorp S, Dingjan GM, Maas A, Dahlenborg K, Hendriks RW. Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity. *J.Immunol.* 2003;171(11):5988-5996.
39. Park H, Wahl MI, Afar DE et al. Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity.* 1996;4(5):515-525.
40. Kang SW, Wahl MI, Chu J et al. PKCbeta modulates antigen receptor signaling via regulation of Btk membrane localization. *EMBO J.* 2001;20(20):5692-5702.
41. Kim YJ, Sekiya F, Poulin B, Bae YS, Rhee SG. Mechanism of B-cell receptor-induced phosphorylation and activation of phospholipase C-gamma2. *Mol.Cell Biol.* 2004;24(22):9986-9999.
42. Takata M, Kurosaki T. A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-gamma 2. *J.Exp.Med.* 1996;184(1):31-40.
43. de Gorter DJ, Vos JC, Pals ST, Spaargaren M. The B cell antigen receptor controls AP-1 and NFAT activity through Ras-mediated activation of Ral. *J.Immunol.* 2007;178(3):1405-1414.
44. Rawlings DJ. Bruton's tyrosine kinase controls a sustained calcium signal essential for B lineage development and function. *Clin.Immunol.* 1999;91(3):243-253.
45. Genevier HC, Callard RE. Impaired Ca2+ mobilization by X-linked agammaglobulinaemia (XLA) B cells in response to ligation of the B cell receptor (BCR). *Clin.Exp.Immunol.* 1997;110(3):386-391.
46. Petro JB, Rahman SM, Ballard DW, Khan WN. Bruton's tyrosine kinase is required for activation of IkappaB kinase and nuclear factor kappaB in response to B cell receptor engagement. *J.Exp.Med.* 2000;191(10):1745-1754.
47. Antony P, Petro JB, Carlesso G et al. B-cell antigen receptor activates transcription factors NFAT (nuclear factor of activated T-cells) and NF-kappaB (nuclear factor kappaB) via a mechanism that involves diacylglycerol. *Biochem.Soc.Trans.* 2004;32(Pt 1):113-115.
48. McLeod SJ, Ingham RJ, Bos JL, Kurosaki T, Gold MR. Activation of the Rap1 GTPase by the B cell antigen receptor. *J.Biol.Chem.* 1998;273(44):29218-29223.
49. Petro JB, Khan WN. Phospholipase C-gamma 2 couples Bruton's tyrosine kinase to the NF-kappaB signaling pathway in B lymphocytes. *J.Biol.Chem.* 2001;276(3):1715-1719.

50. Saito K, Tolias KF, Saci A et al. BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity.* 2003;19(5):669-678.
51. Yang W, Desiderio S. BAP-135, a target for Bruton's tyrosine kinase in response to B cell receptor engagement. *Proc.Natl.Acad.Sci.U.S.A.* 1997;94(2):604-609.
52. Novina CD, Kumar S, Bajpai U et al. Regulation of nuclear localization and transcriptional activity of TFII-I by Bruton's tyrosine kinase. *Mol.Cell Biol.* 1999;19(7):5014-5024.
53. Webb CF, Yamashita Y, Ayers N et al. The transcription factor Bright associates with Bruton's tyrosine kinase, the defective protein in immunodeficiency disease. *J.Immunol.* 2000;165(12):6956-6965.
54. Rajaiya J, Hatfield M, Nixon JC, Rawlings DJ, Webb CF. Bruton's tyrosine kinase regulates immunoglobulin promoter activation in association with the transcription factor Bright. *Mol.Cell Biol.* 2005;25(6):2073-2084.
55. Rajaiya J, Nixon JC, Ayers N et al. Induction of immunoglobulin heavy-chain transcription through the transcription factor Bright requires TFII-I. *Mol.Cell Biol.* 2006;26(12):4758-4768.
56. Ashworth T, Roy AL. Cutting Edge: TFII-I controls B cell proliferation via regulating NF-kappaB. *J.Immunol.* 2007;178(5):2631-2635.
57. Kawakami Y, Yao L, Miura T et al. Tyrosine phosphorylation and activation of Bruton tyrosine kinase upon Fc epsilon RI cross-linking. *Mol.Cell Biol.* 1994;14(8):5108-5113.
58. Hata D, Kawakami Y, Inagaki N et al. Involvement of Bruton's tyrosine kinase in FcepsilonRI-dependent mast cell degranulation and cytokine production. *J.Exp.Med.* 1998;187(8):1235-1247.
59. Rock J, Schneider E, Grun JR et al. CD303 (BDCA-2) signals in plasmacytoid dendritic cells via a BCR-like signalosome involving Syk, Slp65 and PLCgamma2. *Eur.J.Immunol.* 2007;37(12):3564-3575.
60. Jongstra-Bilen J, Puig CA, Hasija M et al. Dual functions of Bruton's tyrosine kinase and Tec kinase during Fcgamma receptor-induced signaling and phagocytosis. *J.Immunol.* 2008;181(1):288-298.
61. Wang D, Feng J, Wen R et al. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity.* 2000;13(1):25-35.
62. Shinohara M, Koga T, Okamoto K et al. Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals. *Cell* 2008;132(5):794-806.
63. Oda A, Ikeda Y, Ochs HD et al. Rapid tyrosine phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking. *Blood* 2000;95(5):1663-1670.
64. Zorn CN, Keck S, Hendriks RW et al. Bruton's tyrosine kinase is dispensable for the Toll-like receptor-mediated activation of mast cells. *Cell Signal.* 2009;21(1):79-86.
65. Gagliardi MC, Finocchi A, Orlandi P et al. Bruton's tyrosine kinase defect in dendritic cells from X-linked agammaglobulinaemia patients does not influence their differentiation, maturation and antigen-presenting cell function. *Clin.Exp.Immunol.* 2003;133(1):115-122.
66. Futatani T, Watanabe C, Baba Y, Tsukada S, Ochs HD. Bruton's tyrosine kinase is present in normal platelets and its absence identifies patients with X-linked agammaglobulinaemia and carrier females. *Br.J.Haematol.* 2001;114(1):141-149.

67. de Gorter DJ, Beuling EA, Kersseboom R et al. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity.* 2007;26(1):93-104.
68. Jefferies CA, Doyle S, Brunner C et al. Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor kappaB activation by Toll-like receptor 4. *J.Biol.Chem.* 2003;278(28):26258-26264.
69. Jefferies CA, O'Neill LA. Bruton's tyrosine kinase (Btk)-the critical tyrosine kinase in LPS signalling? *Immunol.Lett.* 2004;92(1-2):15-22.
70. Horwood NJ, Page TH, McDaid JP et al. Bruton's tyrosine kinase is required for TLR2 and TLR4-induced TNF, but not IL-6, production. *J.Immunol.* 2006;176(6):3635-3641.
71. Horwood NJ, Mahon T, McDaid JP et al. Bruton's tyrosine kinase is required for lipopolysaccharide-induced tumor necrosis factor alpha production. *J.Exp.Med.* 2003;197(12):1603-1611.
72. Jiang Y, Ma W, Wan Y et al. The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. *Nature* 1998;395(6704):808-813.
73. Tsukada S, Simon MI, Witte ON, Katz A. Binding of beta gamma subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase. *Proc.Natl.Acad.Sci.U.S.A*. 1994;91(23):11256-11260.
74. Lowry WE, Huang XY. G Protein beta gamma subunits act on the catalytic domain to stimulate Bruton's agammaglobulinemia tyrosine kinase. *J.Biol.Chem.* 2002;277(2):1488-1492.
75. Yang G, Zhou Y, Liu X et al. A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenstrom macroglobulinemia. *Blood* 2013;122(7):1222-1232.
76. Perez de DR, Lopez-Granados E, Pozo M et al. Bruton's tyrosine kinase is not essential for LPS-induced activation of human monocytes. *J.Allergy Clin.Immunol.* 2006;117(6):1462-1469.
77. Burger JA, Landau D, Hoellenriegel J et al. Clonal evolution in patients with chronic lymphocytic leukemia (CLL) developing resistance to BTK inhibition. *Blood* 2013;122(21):866.
78. Ghia P, Scielzo C, Frenquelli M, Muzio M, Caligaris-Cappio F. From normal to clonal B cells: Chronic lymphocytic leukemia (CLL) at the crossroad between neoplasia and autoimmunity. *Autoimmun.Rev.* 2007;7(2):127-131.
79. Messmer BT, Albesiano E, Efremov DG et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. *J.Exp.Med.* 2004;200(4):519-525.
80. Hoogeboom R, van Kessel KP, Hochstenbach F et al. A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *J Exp.Med* 2013;210(1):59-70.
81. Duhren-von MM, Ubelhart R, Schneider D et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* 2012;489(7415):309-312.
82. Coelho V, Krysov S, Steele A et al. Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function. *Blood* 2013;122(15):2664-2672.

83. Herishanu Y, Perez-Galan P, Liu D et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-{kappa}B activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* 2011;117(2):563-574.
84. Burger JA. Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. *Hematology.Am.Soc.Hematol.Educ.Program.* 2011;2011:96-103.
85. Spaner DE, Masellis A. Toll-like receptor agonists in the treatment of chronic lymphocytic leukemia. *Leukemia* 2007;21(1):53-60.
86. Rozkova D, Novotna L, Pytlik R et al. Toll-like receptors on B-CLL cells: expression and functional consequences of their stimulation. *Int.J.Cancer* 2010;126(5):1132-1143.
87. Wolska A, Cebula-Obrzut B, Smolewski P, Robak T. Effects of Toll-like receptor 7 and Toll-like receptor 9 signaling stimulators and inhibitors on chronic lymphocytic leukemia cells ex vivo and their interactions with cladribine. *Leuk.Lymphoma* 2013;54(6):1268-1278.
88. Peng SL. Signaling in B cells via Toll-like receptors. *Curr.Opin.Immunol.* 2005;17(3):230-236.
89. Monroe JG, Keir ME. Bridging Toll-like- and B cell-receptor signaling: meet me at the autophagosome. *Immunity.* 2008;28(6):729-731.
90. Sharma S, Orlowski G, Song W. Btk regulates B cell receptor-mediated antigen processing and presentation by controlling actin cytoskeleton dynamics in B cells. *J.Immunol.* 2009;182(1):329-339.
91. Souwer Y, Chamuleau ME, van de Loosdrecht AA et al. Detection of aberrant transcription of major histocompatibility complex class II antigen presentation genes in chronic lymphocytic leukaemia identifies HLA-DOA mRNA as a prognostic factor for survival. *Br.J.Haematol.* 2009;145(3):334-343.
92. Kremer AN, van der Meijden ED, Honders MW et al. Human leukocyte antigen-DO regulates surface presentation of human leukocyte antigen class II-restricted antigens on B cell malignancies. *Biol. Blood Marrow Transplant.* 2014; 20(5):742-7.
93. Bagnara D, Kaufman MS, Calissano C et al. A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease. *Blood* 2011;117(20):5463-5472.
94. Dazzi F, D'Andrea E, Biasi G et al. Failure of B cells of chronic lymphocytic leukemia in presenting soluble and alloantigens. *Clin.Immunol.Immunopathol.* 1995;75(1):26-32.
95. Pascutti MF, Jak M, Tromp JM et al. IL-21 and CD40L signals from autologous T cells can induce antigen-independent proliferation of CLL cells. *Blood* 2013;122(17):3010-3019.
96. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood* 2011;118(16):4313-4320.
97. Conley ME, Dobbs AK, Farmer DM et al. Primary B cell immunodeficiencies: comparisons and contrasts. *Annu.Rev.Immunol.* 2009;27:199-227.
98. Kil LP, de Bruijn MJ, van Hulst JA et al. Bruton's tyrosine kinase mediated signaling enhances leukemogenesis in a mouse model for chronic lymphocytic leukemia. *Am.J.Blood Res.* 2013;3(1):71-83.
99. Woyach JA, Bojnik E, Ruppert AS et al. Bruton's tyrosine kinase (BTK) function is important to the development and expansion of chronic lymphocytic leukemia (CLL). *Blood* 2014;123(8):1207-1213.

100. Ponader S, Chen SS, Buggy JJ et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood* 2012;119(5):1182-1189.

101. Herman SE, Mustafa RZ, Gyamfi JA et al. Ibrutinib inhibits B-cell receptor and NF-kappaB signaling and reduces tumor proliferation in tissue-resident cells of patients with chronic lymphocytic leukemia. *Blood* 2014;22;123(21):3286-95.

102. Advani RH, Buggy JJ, Sharman JP et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J.Clin.Oncol.* 2013;31(1):88-94.

103. Woyach JA, Smucker K, Smith LL et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood* 2014;123(12):1810-1817.

104. Herman SEM, Niemann CU, Farooqui M et al. Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukemia: correlative analyses from a phase II study. *Leukemia* 2014;28(11):2188-96.

105. Herman SE, Sun X, McAuley EM et al. Modeling tumor-host interactions of chronic lymphocytic leukemia in xenografted mice to study tumor biology and evaluate targeted therapy. *Leukemia* 2013;27(12):2311-2321.

106. Herman SE, Gordon AL, Hertlein E et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* 2011;117(23):6287-6296.

107. Cheng S, Ma J, Guo A et al. BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. *Leukemia* 2014;28(3):649-657.

108. Chang BY, Francesco M, de Rooij MF et al. Egress of $CD19^{+}CD5^{+}$ cells into peripheral blood following treatment with the Bruton tyrosine kinase inhibitor ibrutinib in mantle cell lymphoma patients. *Blood* 2013;122(14):2412-2424.

109. Wiestner A. Emerging role of kinase-targeted strategies in chronic lymphocytic leukemia. *Blood* 2012;120(24):4684-4691.

110. Messmer BT, Messmer D, Allen SL et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J.Clin.Invest.* 2005;115(3):755-764.

111. Schmid C, Isaacson PG. Proliferation centres in B-cell malignant lymphoma, lymphocytic (B-CLL): an immunophenotypic study. *Histopathology* 1994;24(5):445-451.

112. van Gent R, Kater AP, Otto SA et al. In vivo dynamics of stable chronic lymphocytic leukemia inversely correlate with somatic hypermutation levels and suggest no major leukemic turnover in bone marrow. *Cancer Res.* 2008;68(24):10137-10144.

113. Pan Z, Scheerens H, Li SJ et al. Discovery of selective irreversible inhibitors for Bruton's tyrosine kinase. *Chem.Med.Chem.* 2007;2(1):58-61.

114. Advani R, Sharman JP, Smith SM et al. Effect of Btk inhibitor PCI-32765 monotherapy on responses in patients with relapsed aggressive NHL: Evidence of antitumor activity from a phase I study. *Journal of Clinical Oncology* 2010;28(15):8012.

115. Fowler N, Sharman JP, Smith SM et al. The Btk inhibitor, PCI-32765, induces durable responses with minimal toxicity in patients with relapsed/refractory B-cell malignancies: results from a phase I study. *Blood* 2010;116(21):425.
116. Byrd JC, Furman RR, Coutre SE et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N.Engl.J.Med.* 2013;369(1):32-42.
117. Cheson BD, Byrd JC, Rai KR et al. Novel targeted agents and the need to refine clinical end points in chronic lymphocytic leukemia. *J.Clin Oncol.* 2012;30(23):2820-2822.
118. Wang ML, Rule S, Martin P et al. Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. *N.Engl.J.Med.* 2013;369(6):507-516.
119. Brown JR, Byrd JC, Coutre SE et al. Idelalisib, an inhibitor of phosphatidylinositol 3 kinase p110delta, for relapsed/refractory chronic lymphocytic leukemia. *Blood* 2014;123(22):3390-7.
120. Furman RR, Sharman JP, Coutre SE et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *N.Engl.J Med.* 2014;370(11):997-1007.
121. Dubovsky JA, Beckwith KA, Natarajan G et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. *Blood* 2013;122(15):2539-2549.
122. Schulzke JD, Ploeger S, Amasheh M et al. Epithelial tight junctions in intestinal inflammation. *Ann.N.Y.Acad.Sci.* 2009;1165:294-300.

123. Chang BY, Furman RR, Zapatka M et al. Use of tumor genomic profiling to reveal mechanisms of resistance to the BTK inhibitor ibrutinib in chronic lymphocytic leukemia (CLL) . *J.Clin.Oncol.* 2013;31:7014.
124. Furman RR, Cheng S, Lu P et al. A novel mutation in Bruton tyrosine kinase confers acquired resistance to ibrutinib (PCI-32765) in CLL. *Blood* 2013;122(21):4914.
125. Hantschel O, Rix U, Schmidt U et al. The Btk tyrosine kinase is a major target of the Bcr-Abl inhibitor dasatinib. *Proc.Natl.Acad.Sci.U.S.A.* 2007;104(33):13283-13288.
126. Rossi D, Khiabanian H, Spina V et al. Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia. *Blood* 2014;123(14):2139-2147.
127. Ouillette P, Saiya-Cork K, Seymour E et al. Clonal evolution, genomic drivers, and effects of therapy in chronic lymphocytic leukemia. *Clin Cancer Res* 2013;19(11):2893-2904.
128. Zenz T, Habe S, Denzel T et al. Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, TP53 mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. *Blood* 2009;114(13):2589-2597.
129. Keating MJ, Wierda WG, Hoellenriegel J et al. Ibrutinib in combination with rituximab (iR) is well tolerated and induces a high rate of durable remissions in patients with high-risk chronic lymphocytic leukemia (CLL): new, updated results of a phase II trial in 40 patients. *Blood* 2013;122(21):675.
130. Barrientos JC, Barr PM, Flinn I et al. Ibrutinib in combination with bendamustine and rituximab is active and tolerable in patients with relapsed/refractory CLL/SLL: final results of a phase 1b study. *Blood* 2013;122(21):525.

131. Kohrt HE, Sagiv-Barfi I, Rafiq S et al. Ibrutinib antagonizes rituximab-dependent NK cell-mediated cytotoxicity. *Blood* 2014;123(12):1957–1960.
132. Axelrod M, Ou Z, Brett LK et al. Combinatorial drug screening identifies synergistic co-targeting of Bruton/'s tyrosine kinase and the proteasome in mantle cell lymphoma. *Leukemia* 2014;28(2):407-410.
133. Tiao G, Kiezun A, Wang Y et al. NF-κB pathway mutations modulate cell survival and ibrutinib response in chronic lymphocytic leukemia. *Blood* 2013;122(21):670.
134. Hallaert DY, Jaspers A, van Noesel CJ et al. c-Abl kinase inhibitors overcome CD40-mediated drug resistance in CLL: implications for therapeutic targeting of chemoresistant niches. *Blood* 2008;112(13):5141-5149.
135. Kelley TW, Alkan S, Srkalovic G, Hsi ED. Treatment of human chronic lymphocytic leukemia cells with the proteasome inhibitor bortezomib promotes apoptosis. *Leuk.Res.* 2004;28(8):845-850.
136. Smit LA, Hallaert DY, Spijker R et al. Differential Noxa/Mcl-1 balance in peripheral versus lymph node chronic lymphocytic leukemia cells correlates with survival capacity. *Blood* 2007;109(4):1660-1668.
137. O'Connor OA, Wright J, Moskowitz C et al. Phase II clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non-Hodgkin's lymphoma and mantle cell lymphoma. *J.Clin.Oncol.* 2005;23(4):676-684.
138. Faderl S, Rai K, Gribben J et al. Phase II study of single-agent bortezomib for the treatment of patients with fludarabine-refractory B-cell chronic lymphocytic leukemia. *Cancer* 2006;107(5):916-924.
139. Obeng EA, Carlson LM, Gutman DM et al. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. *Blood* 2006;107(12):4907-4916.
140. Souers AJ, Leverson JD, Boghaert ER et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat.Med.* 2013;19(2):202-208.
141. Davids MS, Pagel JM, Kahl BS et al. Bcl-2 inhibitor ABT-199 (GDC-0199) monotherapy shows anti-tumor activity including complete remissions in high-risk relapsed/refractory (R/R) chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL). *Blood* 2013;122(21):872.
142. Tam CCS, Seymour JF, Bell A et al. Selective Bcl-2 inhibition with ABT-199 is highly active against chronic lymphocytic leukemia (CLL) irrespective of TP53 mutation or dysfunction. *Blood* 2013;122(21):1304.
143. Karlin L, Rule S, Shah N et al. A phase I study of the oral BTK inhibitor ONO-4059 in patients with relapsed/refractory and high risk chronic lymphocytic leukaemia (CLL). *Blood* 2013;122(21):676.
144. Amrein PC, Attar EC, Takvorian T et al. Phase II study of dasatinib in relapsed or refractory chronic lymphocytic leukemia. *Clin Cancer Res* 2011;17(9):2977-2986.
145. Brown JR, Hill BT, Gabrilove J et al. Phase 1 study of single agent CC-292, a highly selective Bruton's tyrosine kinase (BTK) Inhibitor, in relapsed/refractory chronic lymphocytic leukemia (CLL). *Blood* 2013;122(21):1630.

146. O'Brien S, Furman RR, Fowler N et al. The Bruton tyrosine kinase (BTK) inhibitor ibrutinib (PCI-32765) monotherapy demonstrates long-term safety and durability of response in chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) patients in an open-label extension study. *Blood* 2013;122(21):4163.

Chapter 5

Ibrutinib and idelalisib synergistically target BCR-controlled adhesion in MCL and CLL: a rationale for combination therapy

Martin F.M. de Rooij[1], Annemieke Kuil[1], Arnon P. Kater[2,3], Marie José Kersten[2,3], Steven T. Pals[1,3]*, and Marcel Spaargaren[1,3]*

Departments of [1]Pathology and [2]Hematology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. [3]Lymphoma and Myeloma Center Amsterdam – LYMMCARE. *S.T.P. and M.S. contributed equally to this study.

Blood (2015) 125:2306-9

BLOOD (2015) 125:2306-9

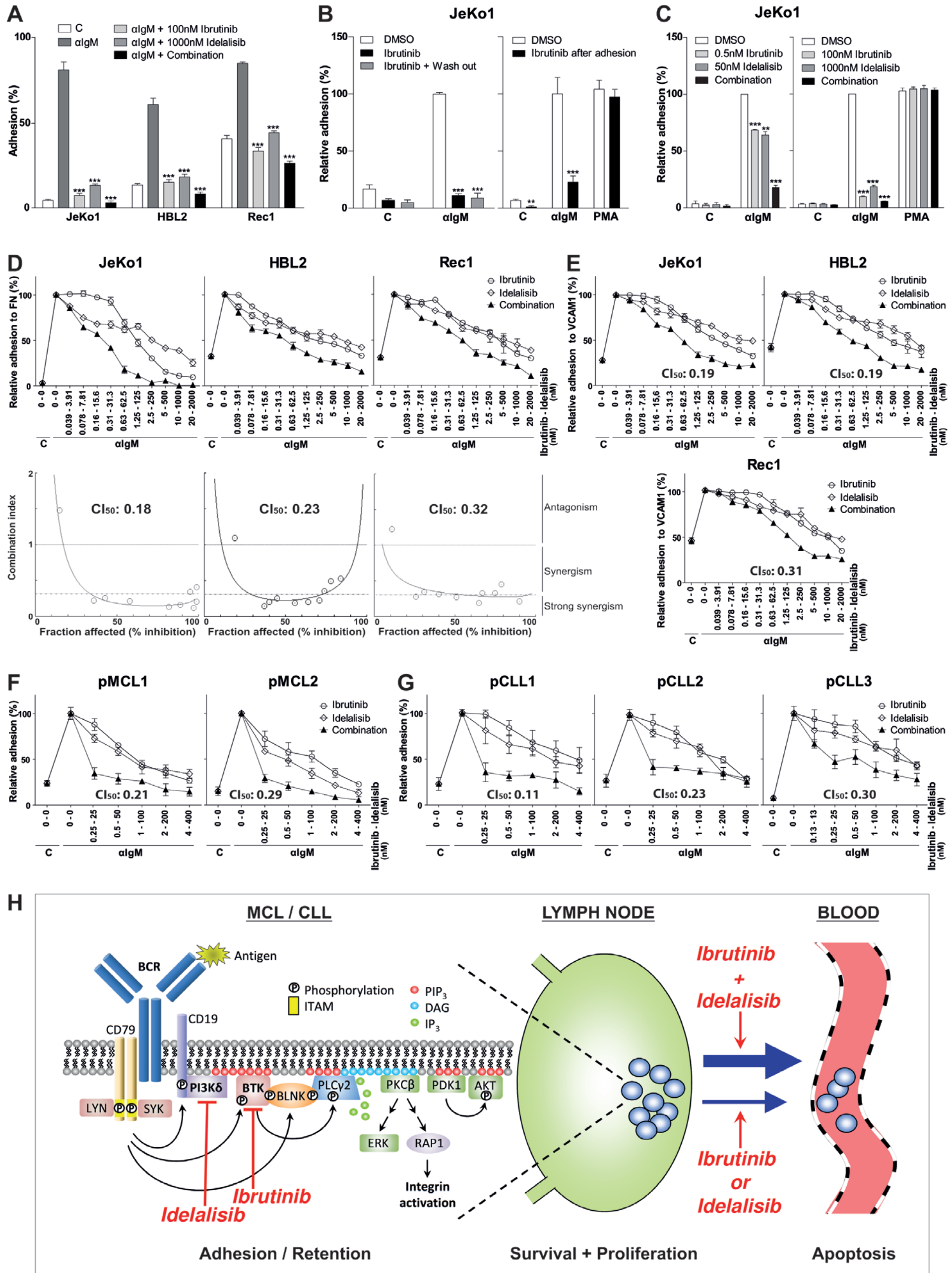
Letter to the editor

Most B-cell malignancies are dependent upon signaling by the B-cell receptor (BCR) and other growth and survival signals provided by the tumor microenvironment. Two recently US Food and Drug Administration approved drugs that target the BCR signalosome, the Bruton tyrosine kinase (BTK) inhibitor ibrutinib, and the phosphatidylinositol-3-kinase δ (PI3Kδ) inhibitor idelalisib, show unprecedented clinical activity, in particular in mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL).[1-5] In these malignancies, both drugs result in a rapid and sustained reduction of lymphadenopathy, which is unexpectedly accompanied by transient lymphocytosis.[1;4;6-8] We have previously demonstrated that ibrutinib targets BCR- and chemokine-controlled integrin-mediated adhesion (retention) and migration (homing) of the malignant cells in their growth- and survival-supporting lymphoid organ microenvironment, resulting in their mobilization from these protective niches into the circulation; this deprives the malignant cells of critical growth and survival factors, resulting in CLL and MCL regression.[6-8]

Unfortunately, approximately 30% of MCL and CLL patients show primary resistance against ibrutinib.[1;2] Furthermore, a subset of patients that do respond and receive prolonged treatment with ibrutinib relapse[1;2] because of recurrent mutations of BTK and its substrate phospholipase C-γ2 (PLCγ2).[9] Combination therapy may be more effective and prevent or overcome this therapy resistance. Because targeting BCR-controlled integrin-mediated adhesion is of major relevance for the clinical efficacy of ibrutinib,[6-8] and because BTK and PI3K differentially regulate BCR signaling,[8] we investigated the effect of idelalisib, either alone or in combination with ibrutinib, on this pathway.

In the MCL cell lines JeKo1, HBL2, and Rec1, BCR stimulation strongly induced integrin-mediated adhesion to fibronectin, which was severely impaired upon treatment with selective and clinically relevant concentrations of ibrutinib (100nM) and idelalisib (1μM) (Fig. 1A). The BTK dependence and specificity of the ibrutinib effect is strongly supported by wash-out experiments that exploit the irreversible interaction between ibrutinib and BTK (Fig. 1B). Furthermore, BCR-controlled adhesion was not only prevented, but also reversed by ibrutinib (Fig. 1B); this is of major importance because cell detachment underlies/precedes the clinically observed egress of the malignant cells from lymphoid organs (lymphocytosis). Interestingly, the combination of ibrutinib and idelalisib displayed stronger inhibition of adhesion compared with either drug alone (Fig. 1A), which became more evident at suboptimal concentrations of ibrutinib (0.5nM) and idelalisib (50nM) (Fig. 1C).

To establish if the combination of ibrutinib and idelalisib is synergistic, we



5

Figure 1: Ibrutinib and idelalisib inhibit BCR-controlled integrin-mediated adhesion of MCL and CLL cells in a strongly synergistic manner.
(A) JeKo1, HBL2, and Rec1 cells, pretreated with 100nM ibrutinib and/or 1µM idelalisib for 1 hour, were stimulated with anti-immunoglobulin M (αIgM) and allowed to adhere to fibronectin-coated surfaces for 30 minutes. Nonadherent cells were removed by extensive washing, and adherent cells were quantified. (B) Left panel: JeKo1 cells, pretreated for 1 hour with 100nM ibrutinib, were washed to remove unbound ibrutinib, stimulated with αIgM, and allowed to adhere to fibronectin-coated surfaces. Right panel: JeKo1 cells were stimulated with αIgM and allowed to adhere to fibronectin-coated surfaces. After 30 minutes, cells were treated with 100nM ibrutinib for 2 hours. DMSO, dimethylsulfoxide. (C) Left panel: JeKo1 cells, pretreated with suboptimal concentrations of ibrutinib (0.5nM) and/or idelalisib (50nM), were stimulated with αIgM and allowed to adhere to fibronectin-coated surfaces (n = 3 independent experiments). Right panel: JeKo1 cells, pretreated with 100nM ibrutinib and/or 1µM idelalisib, were stimulated with αIgM or PMA and allowed to adhere to fibronectin-coated surfaces (n = 3 independent experiments). (D,E) JeKo1, HBL2, and Rec1 cells, pretreated with different concentrations of ibrutinib and/or idelalisib were stimulated with αIgM and allowed to adhere to fibronectin (FN)-coated (D) or VCAM-1–coated (E) surfaces. Upper panel: adhesion plots. Lower panel: FaCI (Chou-Talalay) plots. (F,G) Primary MCL cells (F) and CLL cells (G), pretreated with different concentrations of ibrutinib and/or idelalisib, were stimulated with αIgM and allowed to adhere to fibronectin-coated surfaces. All graphs are presented as normalized means ± standard error of the mean (100% = stimulated cells without inhibitors). C, control (absence of stimulus); CI_{50}, combination index at 50% inhibition/inhibitory concentration. *$P < .05$; **$P < .01$; ***$P < .001$, significantly different from DMSO controls (one-way analysis of variance followed by Dunnett t-test). Details regarding materials, MCL cell lines, primary MCL and CLL cell isolation, adhesion assays, and synergy calculations are described in the supplemental methods section. Approval was obtained from the Academic Medical Center Institutional Review Board for these studies, and informed consent was provided according to the Declaration of Helsinki. (H) Ibrutinib and idelalisib synergistically target BCR-controlled integrin-mediated retention of MCL and CLL cells in lymphoid organs. (Left panel) Antigen-stimulated BCR signaling activates PI3Kδ, which produces PIP_3, resulting in the membrane recruitment of BTK, PLCγ2, and PDK1/AKT through their PH domains. LYN/SYK-mediated phosphorylation of the adaptor protein BLNK brings BTK and PLCγ2 in close proximity of each other. BTK is activated by LYN/SYK-mediated tyrosine phosphorylation, and subsequently BTK phosphorylates and activates PLCγ2. PLCγ2 produces DAG, a recruitment signal for PKC, and inositol-1,4,5-triphosphate (IP_3), which causes the release of calcium from intracellular stores, resulting in PKC activation. PKC activates the RAS/extracellular signal-regulated kinase (ERK) pathway and RAP1, a switch for integrin activation. Targeting of PI3Kδ by idelalisib inhibits membrane recruitment of BTK, PLCγ2, and PDK1/AKT, and targeting of BTK by ibrutinib inhibits activation of PLCγ2. Furthermore, ibrutinib and idelalisib both inhibit BCR-controlled integrin-mediated adhesion. Moreover, the combination of ibrutinib and idelalisib results in strongly synergistic inhibition of integrin-mediated adhesion. The point of synergy between ibrutinib and idelalisib appears to lie upstream of PKC, because neither ibrutinib nor idelalisib alone nor their combination affects PMA-controlled adhesion (see panel C). Indeed, ibrutinib inhibits BTK activity, but ibrutinib-bound BTK can still bind (and block) PIP_3, the product of PI3K, and idelalisib inhibits PIP_3 formation, which is required for translocation and activation of BTK and its substrate PLCγ2. (Right panel) Ibrutinib and idelalisib overcome retention of MCL and CLL cells in their growth- and survival-supporting lymph node and bone marrow microenvironment, resulting in their egress from these protective niches into the circulation (lymphocytosis) and in lymphoma regression. The strongly synergistic targeting of BCR-controlled adhesion, which will result in more efficient mobilization of MCL and CLL cells, provides a strong rationale for clinical studies on combination therapy with ibrutinib and idelalisib, both from the perspective of therapy efficacy as well as drug resistance (see text for further details). DAG, diacylglycerol; ITAM, immunoreceptor tyrosine-based activation motif. Adapted with permission from de Rooij *et al*.[6] and Spaargaren *et al*.[8].

employed the quantitative combination index (CI) theorem of Chou-Talalay (CI <1.0 = synergy; <0.3 strong synergy). The concentration range was centered around the determined 50% inhibition/inhibitory concentration of ibrutinib (~1nM) and idelalisib (~100nM), using the corresponding constant molar ratio (1:100). Ibrutinib and idelalisib inhibited BCR-controlled adhesion of all 3 MCL cell lines to fibronectin and vascular cell adhesion molecule-1 (VCAM-1) in a strongly synergistic manner, with CI_{50} values typically below 0.3 (Figs. 1D-E). This effect is specific for proximal BCR signaling because stimulation of integrin-mediated adhesion with the protein kinase C (PKC) activator phorbol-12-myristate-13-acetate (PMA), which circumvents BTK and PI3Kδ activation, was not affected (Fig. 1C). Moreover, also in primary MCL and CLL, ibrutinib and idelalisib inhibit BCR-stimulated integrin-mediated adhesion in a strongly synergistic manner (Figs. 1F-G).

Apparently, because ibrutinib and idelalisib target BCR signaling by different means, they enforce each other's effect. Consequently, combination therapy is expected to result in a more prominent mobilization of malignant MCL and CLL cells from their growth and survival promoting niches (Fig. 1H). From the perspective of drug clearance and protein turnover, the effect may also be stronger and prolonged because BTK and PI3Kδ do not have to be fully occupied when the combination is used. Furthermore, lower doses can be given, which might be beneficial for the efficacy/toxicity ratio. Of major importance, however, targeting more than one key component of a pathway may overcome innate and overcome or prevent acquired (mono)therapy resistance. For example, ibrutinib may still be beneficial in (relapsed) MCL patients that aberrantly express the PI3Kδ-redundant PI3Kα,[10] and idelalisib may still be beneficial in the ibrutinib-treated patients that relapse from mutations in BTK and/or PLCγ2,[9] because phosphatidylinositol-3,4,5-triphosphate (PIP_3) association is required for both BTK and PLCγ2 activity. Moreover, if applied simultaneously, to develop therapy resistance by the previously mentioned mechanisms, a cell would have to contain or concurrently acquire two independent mutations: a highly unlikely event.

Taken together, from the perspective of therapeutic efficacy and drug resistance, our preclinical observations provide a rationale for (sequential or) combination therapy with ibrutinib and idelalisib in the treatment of MCL and CLL, but also of other B-cell malignancies that depend upon active BCR signaling and/or the tumor microenvironment.

Acknowledgements

The authors thank Eric Eldering and Rachel Thijssen for providing CLL patient samples. This study was supported by a grant from the Dutch Cancer Society (KWF).

Authorship contributions

M.F.M.d.R. designed the research, performed experiments, analyzed the data, designed the figure, and wrote the manuscript; A.K. performed experiments; A.P.K. and M.J.K. provided patient material and reviewed the manuscript; S.T.P. cosupervised the study and reviewed the manuscript; and M.S. designed the research, supervised the study, and wrote the manuscript.

Disclosure of conflicts of interest

M.S. has previously received research support from Pharmacyclics Inc. and Johnson & Johnson. M.J.K. has previously received honoraria from Janssen Pharmaceuticals. The remaining authors declare no competing financial interest.

References

1. Byrd JC, Furman RR, Coutre SE et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N.Engl.J.Med.* 2013;369(1):32-42.
2. Wang ML, Rule S, Martin P et al. Targeting BTK with ibrutinib in relapsed or refractory mantle cell lymphoma. *N.Engl.J.Med.* 2013;369(6):507-516.
3. Byrd JC, Brown JR, O'Brien S et al. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *N.Engl.J.Med.* 2014;371(3):213-223.
4. Brown JR, Byrd JC, Coutre SE et al. Idelalisib, an inhibitor of phosphatidylinositol-3-kinase p110δ, for relapsed/refractory chronic lymphocytic leukemia. *Blood* 2014;123(22):3390-3397.
5. Kahl BS, Spurgeon SE, Furman RR et al. A phase 1 study of the PI3Kδ inhibitor idelalisib in patients with relapsed/refractory mantle cell lymphoma. *Blood* 2014;123(22):3398-3405.
6. de Rooij MF, Kuil A, Geest CR et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* 2012;119(11):2590-2594.
7. Chang BY, Francesco M, de Rooij MF et al. Egress of $CD19^{+}CD5^{+}$ cells into peripheral blood following treatment with the Bruton's tyrosine kinase inhibitor ibrutinib in mantle cell lymphoma patients. *Blood* 2013;122(14):2412-2424.
8. Spaargaren M, de Rooij MF, Kater AP, Eldering E. BTK inhibitors in chronic lymphocytic leukemia: a glimpse to the future. *Oncogene* 2015;34(19):2426-36.
9. Woyach JA, Furman RR, Liu TM et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N.Engl.J.Med.* 2014;370(24):2286-2294.
10. Iyengar S, Clear A, Bodor C et al. p110α-mediated constitutive PI3K signaling limits the efficacy of p110δ-selective inhibition in mantle cell lymphoma, particularly with multiple relapse. *Blood* 2013;121(12):2274-2284.

Supplementary appendix

Supplementary materials and methods

Cell lines and primary cells

The mantle cell lymphoma lymphoma (MCL) cell lines: JeKo1 and HBL2 (kindly provided by Dr. Lydia Visser, Department of Pathology, University Medical Center Groningen, the Netherlands with permission of Dr. Wolfram Klapper, Department of Pathology, University of Kiel, Germany), and Rec1 (DSMZ) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine and pen/strep. Peripheral blood-derived MCL and CLL cells were obtained after routine diagnostic or follow-up procedures at the departments of Hematology or Pathology of the Academic Medical Center (AMC) Amsterdam, and were purified using Ficoll and eventually by B cell isolation kit (negative selection) (Miltenyi Biotec). Purified MCL and CLL samples contained 85-99% $CD5^+$/$CD19^+$ cells. This study was conducted and approved by the AMC Medical Committee on Human Experimentation. Informed consent was obtained in accordance with the Declaration of Helsinki.

Adhesion assays

The cell adhesion assays were performed essentially as described.[1;2] In detail, adhesion assays were done in triplicate on EIA/RIA 96-well plates (Costar) coated with PBS containing 10µg/ml fibronectin (Sigma-Aldrich) or 500ng/ml VCAM1 (R&D Systems) at 4°C overnight, or with 1mg/ml poly-L-lysine (PLL; Sigma-Aldrich) at 37°C for 15 minutes, and blocked with 4% BSA/RPMI at 37°C for 1h. Cells were pretreated with DMSO, ibrutinib (Selleck Chemicals), and/or idelalisib (Selleck Chemicals) in 1% BSA/RPMI at 37°C for 1h. If required (wash-out experiment), ibrutinib was washed out for 3 times 5 min with 1% BSA/RPMI. Subsequently, cells were stimulated with either 200ng/ml goat F(ab')2 anti-human IgM (LE/AF; Southern Biotech), or 50ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich), and $1.5x10^5$ cells in 100µl were immediately plated and incubated at 37°C for 30 minutes. If required (detachment experiment), only now the cells were treated with ibrutinib, for 2h. After extensive washing of the plate with 1% BSA/RPMI to remove non-adhered cells, the adherent cells were fixed for 10 minutes with 10% glutaraldehyde/PBS and subsequently stained for 45 minutes with 0.5% crystal violet/20% methanol/water. After extensive washing with water, the dye was eluted in methanol and absorbance was measured after 40 minutes at 570nm on a spectrophotometer (Multiskan RC spectrophotometer, Thermo labsystems). Background absorbance (no cells added) was subtracted. Absorbance due to nonspecific adhesion, as determined in wells

coated with 4% BSA, was always less than 10% of the absorbance of anti-IgM-stimulated cells. Maximal adhesion (100%) was determined by applying the cells to wells coated with PLL, without washing the wells before fixation. Adhesion of the non-pretreated anti-IgM-stimulated cells was normalized to 100% and the bars represent the means + SEM of at least three independent experiments, each assayed in triplicate, or means + SEM of a representative experiment of at least two independent experiments, assayed in triplicate.

Synergistic calculations

CompuSyn (ComboSyn, Inc.), was used for calculating combination indices and creating FaCI-plots, and were based on the Chou-Talalay method.[3]

Statistics

Graphpad Prism (GraphPad Software Inc.) was used for all graphs and statistics. All multicomparisons were analyzed by a one-way analysis of variance (ANOVA). A post hoc Dunnett's t-test was carried out following a significant ANOVA, comparing the drugs treatments to the DMSO-controls. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

References

1. De Rooij MF, Kuil A, Geest CR et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* 2012;119(11):2590-2594.
2. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J.Exp.Med.* 2003;198(10):1539-1550.
3. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* 2010;70(2):440-446.

Chapter 6

Ibrutinib and idelalisib target B cell receptor- but not CXCL12/CXCR4-controlled integrin-mediated adhesion in Waldenström macroglobulinemia

Martin F.M. de Rooij[1], Annemieke Kuil[1], Willem Kraan[1], Marie José Kersten[2,3], Steven P. Treon[4], Steven T. Pals[1,3]*, and Marcel Spaargaren[1,3]*

Department of [1]Pathology, Academic and [2]Hematology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; [3]Lymphoma and Myeloma Center Amsterdam – LYMMCARE; [4]Bing Center for Waldenström's Macroglobulinemia, Dana-Farber Cancer Institute, Boston, MA. *S.T.P. and M.S. contributed equally to this study.

Heamatologica (2016) 101:e111-5

HEAMATOLOGICA (2016) 101:E111-5

Letter to the editor

The Bruton tyrosine kinase (BTK) inhibitor ibrutinib and the phosphatidylinositol-3-kinase δ (PI3Kδ) inhibitor idelalisib show promising clinical efficacy in the treatment of Waldenström macroglobulinemia (WM), a lymphoplasmacytic lymphoma.[1-3] Very recently, ibrutinib became the first FDA- and EMA-approved treatment for WM patients. Herein, we investigated the molecular and cellular mechanisms underlying the clinical efficacy of ibrutinib and idelalisib in WM patients. We show that, at clinically relevant concentrations, idelalisib, but not ibrutinib, reduced proliferation of WM cells, whereas cytotoxicity was not observed. Furthermore, we demonstrate that WM cells express a signaling-competent B-cell antigen receptor (BCR) which controls integrin-mediated adhesion, and that ibrutinib and idelalisib both inhibit BCR-controlled signaling and integrin-mediated adhesion, whereas chemokine (CXCL12/CXCR4)-controlled signaling, adhesion and migration are not affected. Our data indicate that ibrutinib and idelalisib target BCR-controlled retention of WM cells in the lymphoid organs, resulting in the clinically observed mobilization of malignant cells from these protective niches into the circulation; this may deprive the WM cells from essential microenvironmental growth and survival factors, resulting in WM regression. In addition, our results provide a molecular explanation for the relative ibrutinib resistance of WM patients with gain-of-function CXCR4 mutations.

WM, a lymphoplasmacytic lymphoma, is characterized by the accumulation of post-germinal center B cells in bone marrow, spleen, liver and lymph nodes, which produce a monoclonal IgM M-protein. Apart from genetic lesions in the malignant cells, such as MYD88 and CXCR4 mutations, the bone marrow and lymphoid microenvironments play a critical role in the survival and proliferation of these cells. The CXCL12/CXCR4-axis plays a major role in the homing of WM cells to these protective niches. Furthermore, WM cells express a biased IgH_V repertoire,[4] suggesting that (antigen-dependent) BCR signaling plays a role in the pathogenesis of WM.

In CLL and MCL, BCR signaling plays a prominent role in the regulation of integrin-mediated retention of malignant cells in lymphoid organs.[5-8] In these patients, the BCR signalosome inhibitors ibrutinib and idelalisib induce a rapid decrease in lymphadenopathy, accompanied by transient lymphocytosis.[6;9-11] In CLL and MCL, we have previously demonstrated that ibrutinib and idelalisib target BCR-controlled -and ibrutinib also chemokine-controlled- integrin-mediated adhesion, resulting in mobilization of the malignant cells from their protective niches in the lymphoid organs into the circulation, followed by lymphoma regression.[5;6;8] Recently, ibrutinib received FDA and EMA approval for the treatment of CLL and MCL, and idelalisib for small lymphocytic lymphoma and follicular lymphoma. Clinical trials for WM were

also very promising, with an overall response rate of 90.5% (n = 63) for ibrutinib,[3] and 55–80% (n = 9 and n = 10) for idelalisib,[1;2] and recently, ibrutinib became the first ever FDA-approved treatment for WM patients. Interestingly, in WM patients ibrutinib also induces lymphocytosis, but patients with CXCR4 mutations are relatively resistant against ibrutinib.[3] Herein, we investigated the molecular and cellular mechanisms underlying the clinical efficacy of ibrutinib and idelalisib in WM patients.

First we assessed the possible effect of ibrutinib and idelalisib on cell growth in the WM cell lines MWCL-1 and BCWM.1, which both carry the WM characteristic MYD88^{L265P} mutation (Fig. S1A).[12] Cell growth was already reduced at 10-100nM idelalisib, but only at 1μM ibrutinib (Figs. 1A and S2A-B). The observed dose-dependency of ibrutinib was in agreement with Yang et al.[13] Distinguishing between proliferation and viability revealed that at clinically relevant/achievable concentrations (i.e., c_{max} ibrutinib 170nM (dose 420 mg/day)[14] and idelalisib 6μM (dose 350mg/day)[10]) only idelalisib inhibited proliferation, whereas neither drug affected cell viability (Figs. 1B-C and S2A-B). The differential effect of idelalisib and ibrutinib may reflect the capacity of PI3K to regulate not only BTK- but also AKT-mediated signaling (Fig. 2A), including mTOR, GSK3 and FOXO

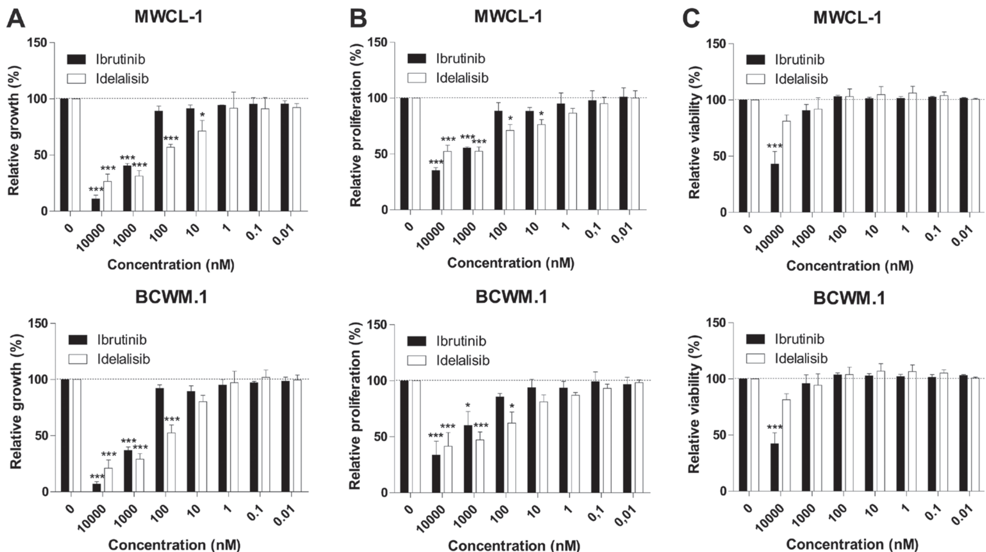


Figure 1. Idelalisib, but not ibrutinib, strongly inhibits WM proliferation.
MWCL-1 and BCWM.1 cells were labelled with CFSE and cultured in the presence of different concentrations of ibrutinib or idelalisib. After 5 days, the numbers of viable cells were counted (A), proliferation was measured by analyzing the CFSE-dilution (B), and the viability was determined (C), (n = 3 independent experiments). Graphs are presented as normalized means + SEM (100% = cells treated with only DMSO). *P < .05; **P < .01; ***P < .001, significantly different from DMSO controls (one-way ANOVA followed by Dunnett's t-test).

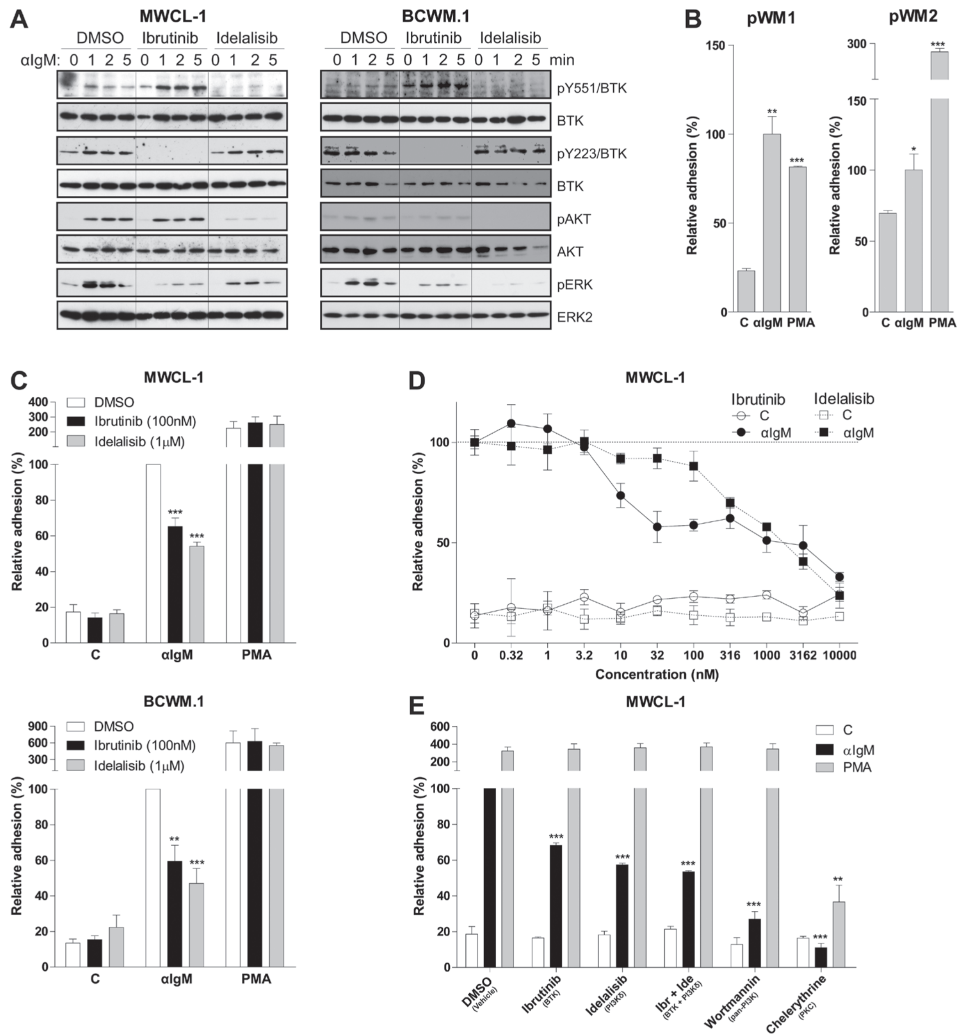


Figure 2. Ibrutinib and idelalisib target BCR-controlled signaling and integrin-mediated adhesion of WM cells. (A) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib or 1μM idelalisib were stimulated with 500ng/ml αIgM, and immunoblotted for p-BTK (pY551 and pY223), p-AKT, and p-ERK. Total BTK, AKT, and ERK2 were used as loading controls. (B) Bone marrow mononuclear cells from 2 WM patients were stimulated with αIgM or PMA, and allowed to adhere to fibronectin-coated surfaces for 30 minutes. Adherence of CD19+ WM cells was quantified by flow cytometry. (C) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib or 1μM idelalisib were stimulated with αIgM or PMA, and allowed to adhere to fibronectin-coated surfaces for 30 minutes. The means are from 6 (BCWM.1) or 10 (MWCL-1) independent experiments. (D) MWCL-1 cells pretreated with different concentrations of ibrutinib or idelalisib were stimulated with αIgM and allowed to adhere to fibronectin-coated surfaces for 30 minutes. (E) MWCL-1 cells pretreated with different BCR-signalosome inhibitors were stimulated with αIgM or PMA, and allowed to adhere to fibronectin-coated surfaces for 30 minutes (n = 3 independent experiments). Graphs are presented as normalized means + SEM (100% = stimulated cells without inhibitors). *$P < .05$; **$P < .01$; ***$P < .001$, significantly different from DMSO controls (one-way ANOVA followed by Dunnett's *t*-test).

pathways. Furthermore, it is tempting to suggest that aberrant NFκB activation by mutant MYD88 may compensate for ibrutinib treatment, since combining IRAK inhibitors with ibrutinib enhances NFκB inhibition and WM cytotoxicity.[13]

BCR signaling controls survival, proliferation, and adhesion of B cells. After having established that the BCR is expressed (Figs. S1B-C) and functional in MWCL-1 and BCWM.1 cells (Figs. 2A and C) and in primary WM cells (Fig. 2B), we investigated how ibrutinib and idelalisib affect BCR-controlled signaling. Ibrutinib completely abrogated BTK autophosphorylation (Y223), whereas phosphorylation of the activating LYN/SYK substrate site Y551 of BTK was actually augmented (Fig. 2A). A similar potentiation of BTK-Y551 phosphorylation upon ibrutinib treatment, indicative for the inhibition of BTK-mediated negative feedback of proximal BCR signaling, has been observed in other B cell lines [(*e.g.* Namalwa Burkitt's cells,[5] and MCL cell lines (de Rooij, MFM *et al*., manuscript in preparation)]. Interestingly, this occurred in the absence of additional BCR cross-linking of the WM cells (Fig. 2A), suggesting substantial basal BCR signaling (tonic/chronic BCR signaling). In addition, ERK activation was inhibited by ibrutinib, but AKT activation was not affected. Previously, we and others have reported that ibrutinib abrogated AKT signaling in CLL and MCL cells,[5;6] but we have recently demonstrated that this was not related to specific BTK inhibition (de Rooij, MFM *et al*., manuscript in preparation). Idelalisib completely abrogated AKT activation. The activation of BTK by LYN/SYK, which requires PIP_3-mediated membrane association, and the activation of ERK were also inhibited by ibrutinib, but BTK autophosphorylation was not affected (Fig. 2A).

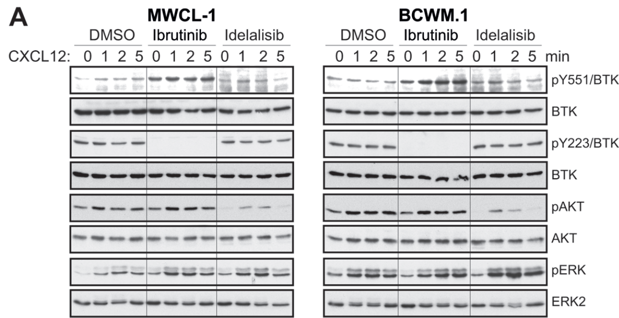
An important function of the BCR, which is of major relevance for the clinical efficacy of ibrutinib and idelalisib in CLL and MCL, is the control of integrin-mediated adhesion/retention. Indeed, BCR stimulation induced adhesion of the WM cell lines as well as primary WM cells to the extracellular matrix component fibronectin and the cell adhesion molecule VCAM-1 (Figs. 2B-C and S3A), which is mediated by integrin $\alpha_4\beta_1$ (being expressed on MWCL-1 and BCWM.1 cells (Fig. S1C)). Moreover, BCR-controlled adhesion was inhibited by 40-50% upon ibrutinib and idelalisib treatment (Figs. 2C and S3A). Adhesion in response to the PKC activator phorbol-12-myristate-13-acetate (PMA) was not attenuated (Figs. 2C and S3A), demonstrating that the observed effects of ibrutinib and idelalisib on BCR-controlled integrin activation were selective, and not caused by cytotoxicity. The inhibition of adhesion by ibrutinib and idelalisib was already observed at 3.2nM and 100nM, respectively (Fig. 2D), well within their clinically achievable ranges. Furthermore, the ibrutinib effect persisted upon wash out (Fig. S3B), indicating it is BTK-specific, involving covalent irreversible binding of ibrutinib to BTK. Unlike in CLL and MCL,[8] combining the drugs did not

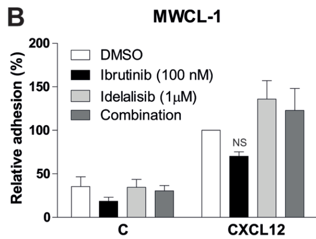
enhance the inhibitory effect (Figs. 2E and S4A-B). Nevertheless, combination (or sequential) therapy could still be beneficial in WM as it may possibly prevent or overcome single drug resistance, *e.g.* due to mutations in BTK or PLCγ2. The partial effects of ibrutinib and idelalisib on adhesion can be explained by the involvement of parallel pathways, implicating other kinases. In support of this, the pan-PI3K inhibitor wortmannin and the more distally acting PKC inhibitor chelerythrine completely abolished BCR-controlled adhesion (Fig. 2E). Given the previously reported interaction of BTK with MYD88^{L265P},[13] it is tempting to speculate that BTK engaged in either the TLR or BCR signalosome may control different cellular functions.

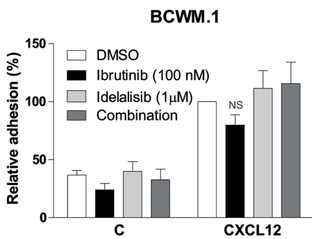
Many components of the BCR signalosome are also involved in CXCL12/CXCR4-signaling. CXCR4 is expressed on MWCL-1 and BCWM.1 cells, although at low levels (Figs. S1C-E). Membrane expression and CXCL12-induced internalization of CXCR4 were not affected by ibrutinib and idelalisib (Fig. S5). As mentioned, treatment with ibrutinib completely abrogated BTK autophosphorylation (Y223), whereas phosphorylation of Y551 was augmented; however, both occurred already in the absence of CXCL12, indicating that it may rather reflect the inhibition of (tonic) BCR signaling (Fig. 3A). Furthermore, ibrutinib did not affect CXCL12-induced activation of ERK and AKT. Idelalisib reduced AKT phosphorylation, but again already in the absence of CXCL12, most likely reflecting inhibition of (tonic) BCR signaling (Fig. 3A). The activation of BTK and ERK were not affected. Moreover, CXCL12-induced adhesion and migration were not (specifically) inhibited by ibrutinib and idelalisib (Figs. 3B–D); the effect of ibrutinib on CXCL12-induced adhesion was reversible upon wash out, which demonstrates it does not reflect a BTK-specific action of ibrutinib (Fig. 3C). Together, these data indicate that the BCR signalosome components are not critical for CXCL12-induced responses in WM.

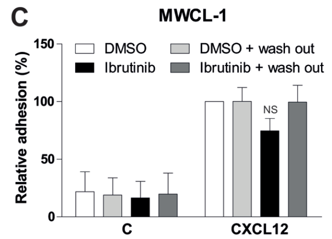
Although CXCL12 is important for homing, most likely it is not involved in retention: in lymphoid tissues CXCL12 is abundantly expressed, causing CXCR4 desensitization and internalization. Interestingly however, approximately 30% of WM patients carry WHIM-like mutations in CXCR4 (*e.g.* S338X); this mutation prevents CXCR4 desensitization,[15] which may result in aberrant CXCL12-controlled adhesion and sustained retention of WM cells in lymphoid organs (Fig. 3E). Combined with our observation that ibrutinib (or idelalisib) does not target CXCL12-controlled adhesion of WM cells, this may explain why WM patients with these gain-of-function CXCR4 mutations show a strongly reduced lymphocytosis upon, and are less responsive to, ibrutinib treatment as compared to patients with wild-type CXCR4.[3]

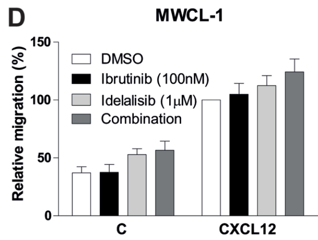
Taken together, our data show that ibrutinib and idelalisib target tonic and antigen-controlled BCR signaling in WM cells, thereby inhibiting BCR-controlled integrin-mediated adhesion (Fig. 3E). In vivo this would result in impaired retention of

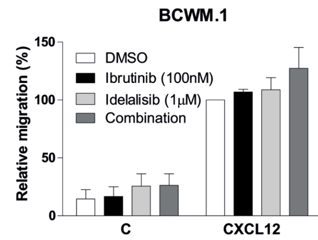

A
MWCL-1
BCWM.1
DMSO
Ibrutinib
Idelalisib
CXCL12:
0 1 2 5 0 1 2 5 0 1 2 5
min
pY551/BTK
BTK
pY223/BTK
BTK
pAKT
AKT
pERK
ERK2


B
MWCL-1
Relative adhesion (%)
DMSO
Ibrutinib (100 nM)
Idelalisib (1μM)
Combination
NS
C
CXCL12


BCWM.1
Relative adhesion (%)
DMSO
Ibrutinib (100 nM)
Idelalisib (1μM)
Combination
NS
C
CXCL12


C
MWCL-1
Relative adhesion (%)
DMSO
Ibrutinib
DMSO + wash out
Ibrutinib + wash out
NS
C
CXCL12


D
MWCL-1
Relative migration (%)
DMSO
Ibrutinib (100nM)
Idelalisib (1μM)
Combination
C
CXCL12


BCWM.1
Relative migration (%)
DMSO
Ibrutinib (100nM)
Idelalisib (1μM)
Combination
C
CXCL12

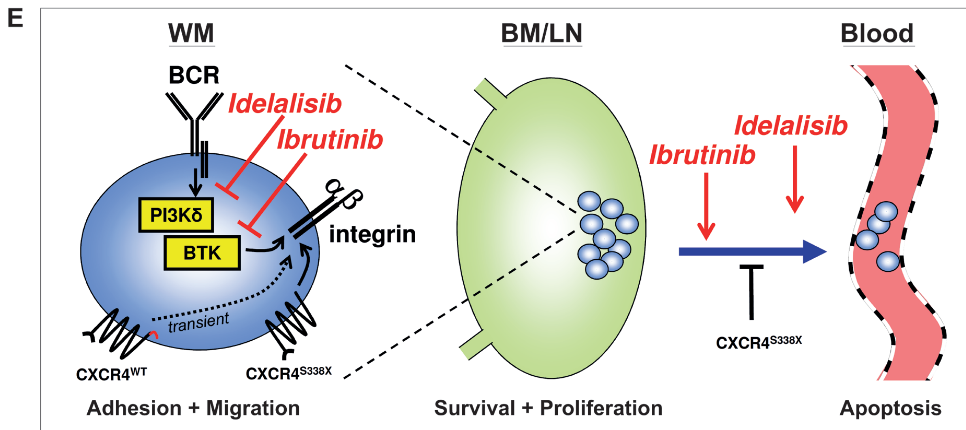

E
WM
BM/LN
Blood
BCR
Idelalisib
Ibrutinib
PI3Kδ
BTK
integrin
transient
CXCR4WT
CXCR4S338X
Idelalisib
Ibrutinib
CXCR4S338X
Adhesion + Migration
Survival + Proliferation
Apoptosis

Figure 3. Ibrutinib and idelalisib do not target CXCL12-controlled integrin-mediated adhesion and migration of WM cells.
(A) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib or 1μM idelalisib were stimulated with 100ng/ml CXCL12, and immunoblotted for p-BTK (pY551 and pY223), p-AKT, and p-ERK. Total BTK, AKT, and ERK2 were used as loading controls. (B) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib and/or 1μM idelalisib were allowed to adhere to VCAM-1– and CXCL12-cocoated surfaces for 5 minutes (n = 3 independent experiments). (C) MWCL-1 cells pretreated with 100nM ibrutinib were subsequently washed (to remove any non-covalently bound ibrutinib) and allowed to adhere to VCAM-1– and CXCL12-cocoated surfaces for 5 minutes (n = 3 independent experiments). (D) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib and/or 1μM idelalisib were allowed to migrate towards CXCL12 in VCAM-1–coated transwells for 5 hours (n = 3 independent experiments). Graphs are presented as normalized means + SEM (100% = stimulated cells without inhibitors). NS: not significantly different from DMSO controls (one-way ANOVA). (E) Model of the mechanism of action of ibrutinib and idelalisib in WM. Inhibition of BTK by ibrutinib or PI3Kδ by idelalisib impairs BCR-controlled integrin-mediated adhesion of WM cells in bone marrow (BM) and lymph nodes (LN), which results in their egress from these protective niches into the circulation, resulting in WM regression. The homing receptor CXCR4 is normally desensitized upon binding of CXCL12, which is highly expressed within the lymphoid organ microenvironment; however, this is prevented by the WHIM-like mutation (S338X), lacking the regulatory domain. Consequently, CXCR4S338X might aberrantly support retention of WM cells in the lymphoid organs. Since CXCR4-controlled integrin-mediated adhesion is insensitive to ibrutinib and idelalisib, this would counteract their ability to inhibit BCR-controlled integrin activation, thus explaining the clinically observed ibrutinib-resistance of WM patients with the CXCR4S338X mutation.

WM cells in lymphoid tissues, explaining the lymphocytosis observed upon ibrutinib treatment.[3] Thus, our data indicate that ibrutinib and idelalisib do not directly kill WM cells but rather target their BCR-controlled adhesion, thereby causing mobilization of the malignant cells from the protective niches in the lymphoid organs into the circulation, resulting in the deprivation of microenvironmental growth and survival factors, and clinically evident WM regression.

Acknowledgement
The authors would like to thank Yang Cao for providing the WM cell lines, Leonie Grüneberg, Monique Oud and Esther Schilder-Tol for characterization of the WM cell lines, and the WM patients for donating primary WM cells. This work was supported by grants from the Dutch Cancer Society, Lymph&Co, and Fonds Stimulans.

Authorship contributions
M.F.M.d.R. designed the research, performed experiments, analyzed the data, designed the figures and wrote the manuscript; A.K. and W.K. performed experiments; M.J.K. and S.P.T. provided material and reviewed the manuscript; S.T.P. co-

supervised the study and reviewed the manuscript and M.S. designed the research, supervised the study, analyzed the data, and wrote and revised the manuscript.

Disclosure of conflicts of interest

M.S. has previously received research support from Pharmacyclics Inc. and Johnson & Johnson. M.J.K. has previously received honoraria from Janssen Pharmaceuticals. S.P.T. has previously received active research funding, consulting fees, and/or speaking honoraria from Janssen Pharmaceuticals Inc., Pharmacyclics Inc., and Gilead Pharmaceuticals. The remaining authors declare no competing financial interest.

References

1. Flinn IW, Kahl BS, Leonard JP, et al. Idelalisib, a selective inhibitor of phosphatidylinositol 3-kinase-delta, as therapy for previously treated indolent non-Hodgkin lymphoma. *Blood* 2014;123(22):3406-3413.
2. Gopal AK, Kahl BS, de Vos S, et al. PI3Kdelta inhibition by idelalisib in patients with relapsed indolent lymphoma. *N.Engl.J.Med.* 2014;370(11):1008-1018.
3. Treon SP, Tripsas CK, Meid K, et al. Ibrutinib in previously treated Waldenstrom's macroglobulinemia. *N.Engl.J.Med.* 2015;372(15):1430-1440.
4. Martin-Jimenez P, Garcia-Sanz R, Balanzategui A, et al. Molecular characterization of heavy chain immunoglobulin gene rearrangements in Waldenstrom's macroglobulinemia and IgM monoclonal gammopathy of undetermined significance. *Haematologica* 2007;92(5):635-642.
5. de Rooij MF, Kuil A, Geest CR, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* 2012;119(11):2590-2594.
6. Chang BY, Francesco M, De Rooij MF, et al. Egress of CD19^{+}CD5^{+} cells into peripheral blood following treatment with the Bruton tyrosine kinase inhibitor ibrutinib in mantle cell lymphoma patients. *Blood* 2013;122(14):2412-2424.
7. Spaargaren M, De Rooij MF, Kater AP, Eldering E. BTK inhibitors in chronic lymphocytic leukemia: a glimpse to the future. *Oncogene* 2015;34(19):2426-2436.
8. de Rooij MF, Kuil A, Kater AP, Kersten MJ, Pals ST, Spaargaren M. Ibrutinib and idelalisib synergistically target BCR-controlled adhesion in MCL and CLL: a rationale for combination therapy. *Blood* 2015;125(14):2306-2309.
9. Advani RH, Buggy JJ, Sharman JP, et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J.Clin.Oncol.* 2013;31(1):88-94.
10. Brown JR, Byrd JC, Coutre SE, et al. Idelalisib, an inhibitor of phosphatidylinositol 3 kinase p110delta, for relapsed/refractory chronic lymphocytic leukemia. *Blood* 2014;123(22):3390-3397.
11. Kahl BS, Spurgeon SE, Furman RR, et al. A phase 1 study of the PI3Kd inhibitor idelalisib in patients with relapsed/refractory mantle cell lymphoma (MCL). *Blood* 2014;123(22):3398-3405.
12. Treon SP, Xu L, Yang G, et al. MYD88 L265P somatic mutation in Waldenstrom's macroglobulinemia. *N.Engl.J.Med.* 2012;367(9):826-833.
13. Yang G, Zhou Y, Liu X, et al. A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenstrom macroglobulinemia. *Blood* 2013;122(7):1222-1232.
14. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N.Engl.J.Med.* 2013;369(1):32-42.
15. Balabanian K, Lagane B, Pablos JL, et al. WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood* 2005;105(6):2449-2457.

Supplemental appendix

Supplementary materials and methods

Materials

The following reagents were used in this study: the phosphorylation state-specific antibodies phospho-p44/42-MAPK [T202/Y204] against p-ERK1 and -2, phospho-AKT [Ser473] against p-AKT (Cell Signaling Technology), phospho-BTK [Y551] against p-BTK (BD Biosciences), phospho-BTK [Y223] against p-BTK (Epitomics); anti-ERK2 (Santa Cruz Biotechnology), anti-AKT (Santa Cruz Biotechnology), anti-BTK (BD Bioscience), goat F(ab')$_2$ anti-human IgM (LE/AF; Southern Biotech), horseradish peroxidase (HRP)-conjungated rabbit anti-mouse and HRP-conjungated goat anti-rabbit (Dako); phycoerythrin-conjungated rabbit F(ab')$_2$ anti-IgM (Dako), mouse anti-CD79β (SN8; Dako), mouse anti-integrin α_4 (HP2/1; Immunotech), mouse anti-integrin β_1 (4B4; Coulter), phycoerythrin-conjungated goat F(ab')$_2$ anti-kappa (Cytognos), and rabbit F(ab')$_2$ anti-lambda (Dako). Control mouse IgG$_1$ or IgG$_{2a}$ (Dako),phycoerythrin-conjungated goat anti-mouse IgG$_1$ or IgG$_{2a}$ (Southern Biotech), mouse anti-CXCR4 (BD Biosciences), allophycocyanin-conjungated mouse anti-CD19 (HD37; Dako); the pharmacological inhibitors ibrutinib and idelalisib (Selleck Chemicals), wortmannin and chelerythrine (Sigma-Aldrich); human plasma fibronectin (Sigma-Aldrich, BSA (fraction V; Roche), and recombinant human sVCAM-1 (R&D Systems), carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen), poly-L-lysine (PLL; Sigma-Aldrich).

Cell lines and primary material

The Waldenström macroglobulinemia (WM) cell lines MWCL-1[1] and BCWM.1[2], which both carry the WM-characteristic MYD88^{L265P} mutation (Fig. S1A), are IgL-κ$^+$ and IgL-λ$^+$, respectively (Fig. S1C), and are both monoclonal, as determined by *IGH* GeneScan analysis (Fig. S1B), were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine and pen/strep. Bone marrow aspirations from 2 WM patients were obtained after routine diagnostic or follow-up procedures at the departments of Hematology of the Academic Medical Center (AMC) Amsterdam, and mononuclear cells were purified using Ficoll. This study was conducted and approved by the AMC Medical Committee on Human Experimentation. Informed consent was obtained in accordance with the Declaration of Helsinki.

Signal transduction assays

Immunoblotting was performed essentially as described.[3,4] In detail, 10^7 cells/ml serum free RPMI were pretreated with DMSO, ibrutinib or idelalisib at 37°C for 1h. After stimulation with 500ng/ml goat $F(ab')_2$ anti-human IgM or 100ng/ml CXCL12, after 1, 2 and 5 minutes the cells were directly lysed in SDS-PAGE sample buffer. Lysates (2.10^5 cells/lane) were applied on a 10% SDS-PAGE gel, blotted and incubated with rabbit anti-phospho-ERK1/2, rabbit anti-phospho-AKT, mouse anti-phospho-Y551-BTK, and rabbit anti-phospho-Y223-BTK in 5% milk/TBST followed by HRP-conjugated goat anti-rabbit or rabbit anti-mouse and developed by enhanced chemoluminescence (Amersham Pharmacia). To confirm equal expression and loading, the blots were stripped, and incubated with the antibodies rabbit anti-ERK2 rabbit anti-AKT, and mouse anti-BTK. Blots are representative of at least three independent experiments.

Growth, proliferation, and viability assays

Cell number, CFSE, and viability were measured in individual wells. Cells were washed with PBS, and labeled with 1μM CFSE for 15 minutes at 37°C. CFSE was quenched with FCS, and cells were plated (10^4) in a 96-well plate with 10%FCS/RPMI and DMSO, ibrutinib, and/or idelalisib. At day 0 and 5, numbers of viable cells were counted using a FACS Canto II flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v 6.0), and analyzed with Flow Jo (v7.2.1), the percentage of growth of untreated cells at day 5 was normalized to 100%; CFSE was measured in FITC channel, the geometric mean of the untreated cells at day 5 was normalized to 1.0; and viability was determined using FSC-SSC gating (which correlated completely with Annexin V- or $DiOC_6$-stainings in all cell lines), the percentage viable cells of untreated cells at day 5 was normalized to 100%. The bars represent the means + SEM of at least three independent experiments, each assayed in triplicate.

Adhesion assays

The cell adhesion assays were performed essentially as described.[3,4] In detail, adhesion assays were performed in triplicate on EIA/RIA 96-well plates (Costar) coated with PBS containing 10μg/ml fibronectin or 500ng/ml VCAM-1 at 4°C overnight, or with 1mg/ml poly-L-lysine (PLL) at 37°C for 15 minutes, and blocked with 4% BSA/RPMI at 37°C for 1h. Cells were pretreated with DMSO, 100nM ibrutinib, 1μM idelalisib, 100nM wortmannin, or 1μM chelerythrine in 1% BSA/RPMI at 37°C for 1h. If indicated, unbound ibrutinib was washed out 3 times with 1% BSA/RPMI. Subsequently, cells were stimulated with either 500ng/ml goat F(ab')2 anti-

human IgM, or 50ng/ml PMA, and $1.5.10^5$ BCWM.1 or MWCL.1 cells, or 4.10^5 bone marrow mononuclear cells from WM patients in 100µl were immediately plated and incubated at 37°C for 30 minutes. After extensive washing of the plate with 1% BSA/RPMI to remove non-adhered cells, the adherent cells were fixed for 10 minutes with 10% glutaraldehyde in PBS and subsequently stained for 45 minutes with 0.4% crystal violet/20% methanol/water. After extensive washing with water, the dye was eluted in methanol and absorbance was measured after 40 minutes at 570nm on a spectrophotometer (Multiskan RC spectrophotometer, Thermo labsystems). Background absorbance (no cells added) was subtracted. Absorbance due to nonspecific adhesion, as determined in wells coated with 4% BSA, was always less than 10% of the absorbance of anti-IgM-stimulated cells. Maximal adhesion (100%) was determined by applying the cells to wells coated with PLL, without washing the wells before fixation. For analysis of primary WM cells, cells were detached with 2mM EDTA/0.5% BSA/0.02% NaN_3/PBS, followed by an allophycocyanin-conjungated anti-CD19 staining. The numbers of adhered $CD19^+$ WM cells were analyzed on a FACScanto II flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v6.0), and analyzed with Flow Jo (v7.2.1). Adhesion of the nonpretreated anti-IgM-stimulated cells was normalized to 100% and the bars represent the means + SEM of at least three independent experiments, each assayed in triplicate.

Chemokine-mediated adhesion was assayed as described above, except that the chemokine CXCL12 (50ng/ml) was co-immobilized with 500ng/ml VCAM-1. The plates were spun directly after applying the cells, and the cells were allowed to adhere for 5 minutes.

Migration assays

The cell migration assays were performed essentially as described.[5] In detail, migration assays were performed in triplicate with transwells (pore size 8µm) coated with 1µg/ml VCAM-1 or uncoated. The lower compartment contained 100ng/ml CXCL12 in 0.5% BSA/RPMI. 5.10^5 cells/100ul 0.5% BSA/RPMI, pretreated with DMSO, ibrutinib and/or idelalisib at 37°C for 1h, were applied to the upper compartment and allowed to migrate for 5h at 37°C. The amount of viable migrated cells was determined by FACS and expressed as a percentage of the input. The migration of nonpretreated cells on VCAM-1–coated transwells in the presence of CXCL12 was normalized to 100%, and the bars represent the means + SEM of at least three independent experiments, each assayed in triplicate.

PCR

The MYD88 mutation was determined as described.[6] DNA was isolated with the QIAamp DNA Micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Screening DNA for the *MYD88* L265P mutations was performed with allele-specific PCR assays, employing primers that were designed to specifically anneal with their 3'-terminal nucleotide to either the mutated or wild-type base. The primers were: *MYD88* (L265P;*T794C*) Fw: TGCCAGGGGTACTTAGATGG + Rv: CCTTGTACTTGATGGGGATCG and *MYD88* (WT): Fw: GTGCCCATCAGAA GCGACT + Rv: GGGCCTCAGAACAGTCTTCA. The *IGH* GeneScan analysis was performed as described.[7]

Flow cytometry

10^5 cells were stained with phycoerythrin-conjungated anti-IgM, anti-kappa, anti-lambda, or with mouse anti-CXCR4, anti-CD79β, anti-α_4-integrin, anti-β_1-integrin, or control mouse IgG_1 or IgG_{2a} (isotype control), and secondary stained with phycoerythrin-conjungated goat anti-mouse IgG_1 or IgG_{2a} for 30 min on ice and washed. Cell staining was measured on a FACScanto II flow cytometer system (BD Biosciences, San Jose, CA, USA) interfaced to FACS Diva software (v6.0), and analyzed with Flow Jo (v7.2.1). For CXCR4 internalization, the cells were pretreated with DMSO, 100nM ibrutinib, and/or 1μM idelalisib for 1h, and subsequently with 100nM CXCL12 for 5 minutes, and immediately kept on ice.

Synergy calculations

CompuSyn (ComboSyn, Inc.) was used for calculating combination indices, based on the Chou-Talalay method.[8]

Statistical analysis

Graphpad Prism (GraphPad Software Inc.) was used for all graphs and statistics. The one sample *t*-test was used to determine the significance of differences between means and normalized values. All multicomparisons were analyzed by a one-way analysis of variance (ANOVA). A post hoc Dunnett's t-test was carried out following a significant ANOVA, comparing the drugs treatments to the DMSO-controls. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

References

1. Hodge LS, Novak AJ, Grote DM, et al. Establishment and characterization of a novel Waldenstrom macroglobulinemia cell line, MWCL-1. Blood 2011;117 (19):e190-7
2. Ditzel Santos D, Ho AW, Tournilhac O, et al. Establishment of BCWM.1 cell line for Waldenström's macroglobulinemia with productive in vivo engraftment in SCID-hu mice. Exp. Hematol. 2007;35(9):1366-75.
3. de Rooij MF, Kuil A, Geest CR, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. Blood 2012;119(11):2590-2594.
4. Spaargaren M, Beuling EA, Rurup ML, et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. J.Exp.Med. 2003;198(10):1539-1550.
5. de Gorter DJ, Beuling EA, Kersseboom R, et al. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. Immunity. 2007;26(1):93-104.
6. Kraan W, Horlings HM, van KM, et al. High prevalence of oncogenic MYD88 and CD79B mutations in diffuse large B-cell lymphomas presenting at immune-privileged sites. Blood Cancer J. 2013;3:e139.
7. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17(12):2257-317.
8. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010;70(2):440-446.

Supplementary figures

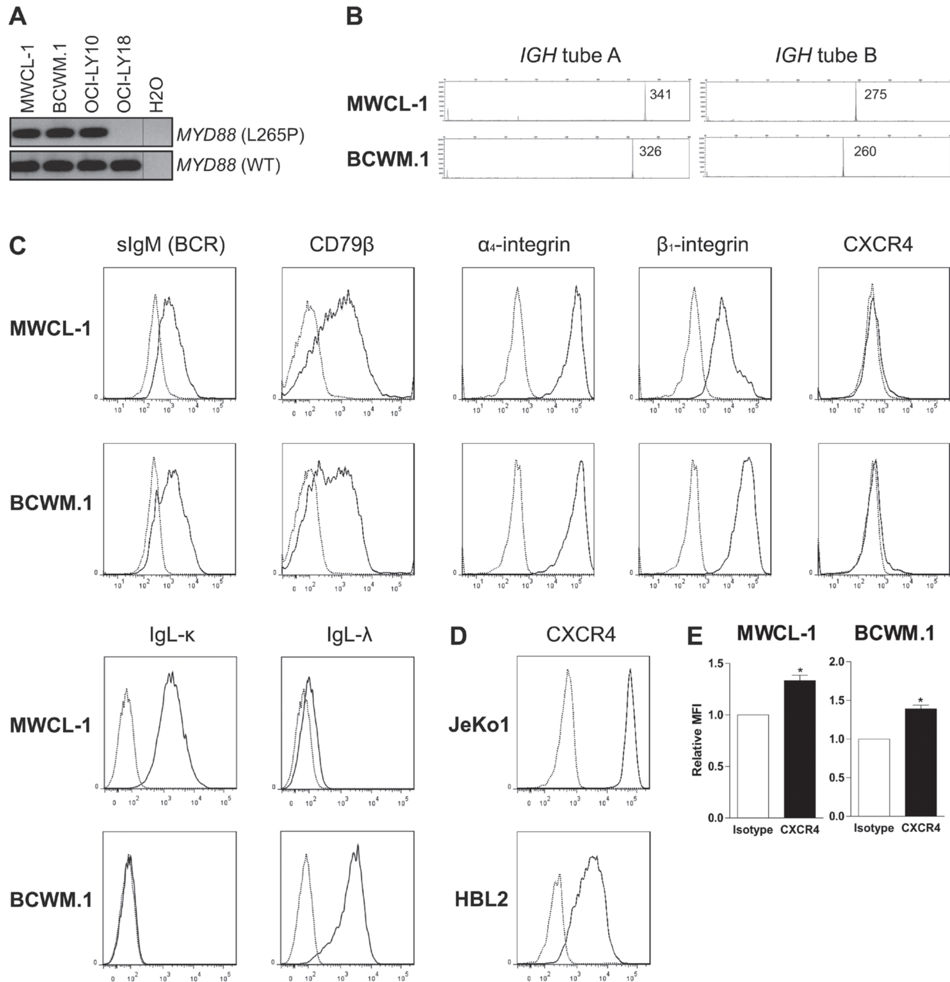


Figure S1. BCR, integrin, and CXCR4 expression in WM.
(A) MWCL-1 and BCWM.1 were heterozygous for MYD88 (L265P; *T794C*), a characteristic of WM. The DLBCL samples OCI-LY10 and OCI-LY18 are positive and negative controls for MYD88 (L265P), respectively. (B) GeneScan analysis of Ig heavy chain (IGH) rearrangements from MWCL-1 and BCWM.1.(C) MWCL-1 and BCWM.1 were stained for surface IgM (BCR), the BCR-coreceptor CD79β, α4-integrin, β1-integrin, the chemokine receptor CXCR4, and the kappa and lambda immunoglobulin light chains (solid lines), or isotype controls (dashed lines) and analyzed by flow cytometry. (D) The MCL cell lines JeKo1 and HBL2 (used as positive control) were stained for CXCR4 (solid lines), or isotype control (dashed lines) and analyzed by flow cytometry. (E) MWCL-1 (n = 3) and BCWM.1 (n = 4) were stained for CXCR4 or isotype control and analyzed by flow cytometry. Graphs are presented as normalized means + SEM (1.0 = MFI from isotype staining). *P < .05; **P < .01; ***P < .001 significantly different (one sample *t*-test).

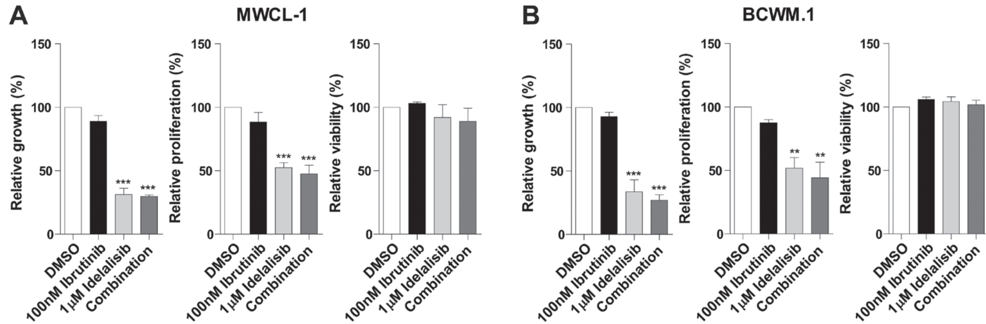


Figure S2. Idelalisib, but not Ibrutinib, strongly inhibits WM proliferation.
MWCL-1 (A) and BCWM.1 (B) cells were labelled with CFSE and cultured in the presence of ibrutinib and/or idelalisib in combination. After 5 days, the numbers of viable cells were counted (left), proliferation was measured by analyzing the CFSE-dilution (middle) and the viability was determined (right) (n = 3 independent experiments). Graphs are presented as normalized means + SEM (100% = cells treated with only DMSO). *P < .05; **P < .01; ***P < .001, significantly different from DMSO controls (one-way ANOVA followed by Dunnett's t-test).

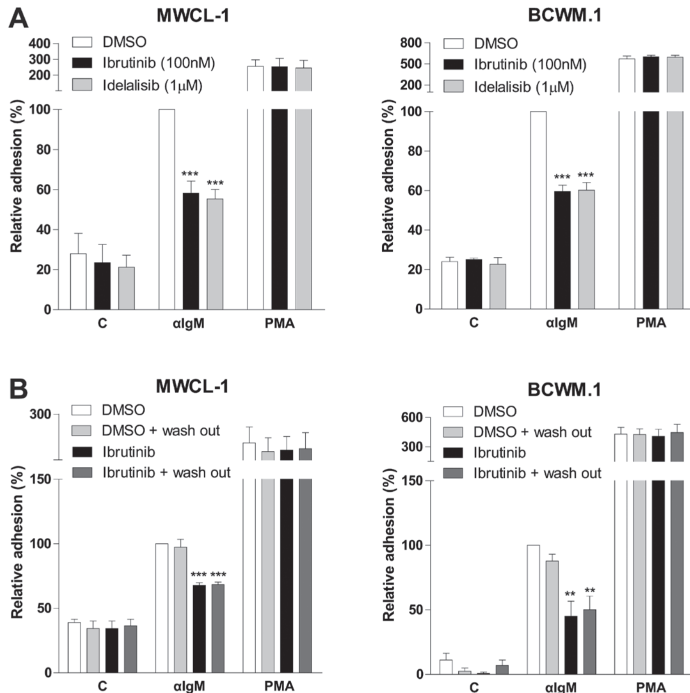


Figure S3. Ibrutinib and idelalisib target BCR-controlled integrin-mediated adhesion of WM cells.
(A) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib or 1µM idelalisib were stimulated with αIgM or PMA, and allowed to adhere to VCAM-1–coated surfaces for 30 minutes (n = 3 independent experiments). (B) MWCL-1 and BCWM.1 cells pretreated with 100nM ibrutinib were subsequently washed (to remove any non-covalently bound ibrutinib) and stimulated with αIgM, and allowed to adhere to fibronectin-coated surfaces for 30 minutes (n = 3 independent experiments). Graphs are presented as normalized means + SEM (100% = stimulated cells without inhibitors). *P < .05; **P < .01; ***P < .001, significantly different from DMSO controls (one-way ANOVA followed by Dunnett's t-test).

Figure S4. Ibrutinib and idelalisib do not act in a synergistic manner in WM.
(A) MWCL-1 and BCWM.1 cells pretreated with different concentrations of ibrutinib, and/or idelalisib were stimulated with αIgM, and allowed to adhere to fibronectin-coated surfaces for 30 minutes. Combination indices (CI) were determined by the Chou-Talalay theorem of synergy calculations. (B) MWCL-1 and BCWM.1 cells pretreated with different concentrations of ibrutinib, and/or idelalisib were stimulated with αIgM, and allowed to adhere to VCAM-1–coated surfaces for 30 minutes. Combination indices (CI) were determined by the Chou-Talalay method of synergy calculations. CI_{25}: CI at IC_{25} (→ ~50% of maximal inhibition).

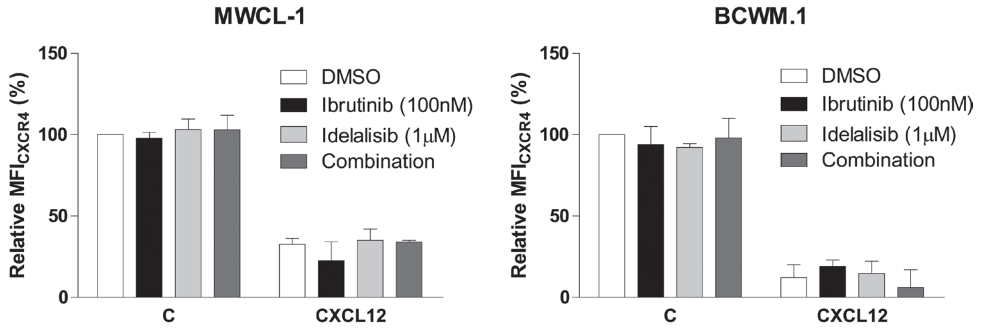


Figure S5. Ibrutinib and idelalisib do not affect CXCR4 internalization in WM.
MWCL-1 (n = 3) and BCWM.1 (n = 3) cells pretreated with 100nM ibrutinib and/or 1μM idelalisib were stimulated with CXCL12 for 5 minutes, and were stained for surface CXCR4 and analyzed by flow cytometry. Graphs are presented as normalized means + SEM (100% = MFI from DMSO-treated cells). No significant differences were observed due to ibrutinib and/or idelalisib treatments (one-way ANOVA).

Chapter 7

General discussion

7

General discussion

Chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and Waldenström macroglobulinemia (WM) are B cell malignancies which are still incurable. Therefore, novel treatment strategies are needed to cure these patients. CLL, MCL, and WM originate from different B cell differentional stages, but a common feature of these three malignancies is that they respond in a remarkable way to the novel B cell antigen receptor (BCR) signalosome inhibitors fostamatinib (spleen tyrosine kinase (SYK) inhibitor), ibrutinib (Bruton tyrosine kinase (BTK) inhibitor), and idelalisib (phosphoinositide-3-kinase δ-isoform (PI3Kδ) inhibitor). Treatment with these BCR signalosome inhibitors, result in a rapid and sustained reduction of lymphadenopathy, which unexpectedly is accompanied by transient lymphocytosis.[1-6] This lymphocytosis can persist for more than 12 months during continuous treatment.[7] In the circulation, the cells lack proliferation and survival signals, and will be cleared in time (Fig. 1). Because tumor lysis syndrome is rare,[8;9] it is likely that the tumor cells disappear by natural clearance, rather than massive cell death.

In this thesis, we focused on the molecular and cellular mechanisms underlying the action of these drugs. We did not detect strong cytotoxic effects. We found very strong inhibition of BCR-controlled integrin-mediated adhesion, which was consistent between the lymphoma types. In addition, we found partial inhibition of chemokine-controlled adhesion and migration in CLL and MCL, but not in WM. Furthermore, we found strong synergism between ibrutinib and idelalisib in inhibiting BCR-controlled integrin-mediated adhesion in CLL and MCL. CLL and MCL have strongly active BCR signaling in lymphoid tissues compared to circulating cells,[10;11] and BCR signaling induces strong integrin-mediated adhesion.[12] Consequently, active BCR signaling is an important retention factor, which is abrogated by these BCR signalosome inhibitors. By analyzing blood-derived cells before and after ibrutinib treatment of CLL and MCL patients, we found that the malignant cells which accumulate in the circulation upon treatment have a 'tissue-localized phenotype', suggesting that they are mobilized from the lymphoid tissues. Next, we discuss the effect of BCR-signalosome inhibitors in cell signaling, growth, adhesion and migration in more detail, and examine future therapies to improve treatment and overcome resistance.

BCR signalosome inhibitors in BCR signaling

The BCR signals either in a tonic, chronic, or antigen-dependent manner.[13] Our data suggest that Namalwa (Burkitt lymphoma cell line), and the MCL and WM cell lines

have autonomous (tonic or chronic) BCR signaling, because we always detected a basal level of phosphorylation of the proximal downstream kinases BTK and PLCγ2 (Chapters 2, 3, 6, and data not shown). The basal phosphorylation of PLCγ2 was completely inhibited by fostamatinib (data not shown), suggesting that it is activated by the BCR pathway. In addition, treatment of MCL cells with pervanadate, a general phosphatase inhibitor, induced very strong phosphorylation of BTK and PLCγ2 within minutes, which was also abolished by fostamatinib (data not shown). So, these cells have strong autonomous BCR signaling, which is partially repressed by negative feedback pathways.

Chronic BCR signaling can be caused by activating mutations in the BCR pathway. In some cases of WM, gain-of-function mutations in CD79,[14;15] and loss-of-function mutations in LYN have been detected.[15] Both mutations can enhance BCR signaling.[16] In MCL, SYK and PI3Kα are frequently amplified.[17;18] In CLL, no activating mutations in the BCR pathway have been described, but CLL BCRs can signal autonomously by recognizing an intrinsic epitope of the BCR itself.[19]

There is strong evidence that antigen-induced BCR signaling plays a role in the lymphoid tissues in CLL and MCL.[10;11;20;21] For WM it is currently unknown whether antigen-dependent BCR signaling plays a role in tumorigenesis. Nevertheless, WM cells also show a biased IgV_H usage, although true stereotypy was not found.[22] Upon BCR cross linking by anti-IgM, which mimics antigen-dependent BCR signaling, the activation of downstream kinases (BTK, PLCγ2, ERK and AKT) was strongly enhanced in CLL, MCL, and WM cell lines and primary cells (Chapters 2, 3 and 6), indicating that tonic/chronic BCR signaling is far from the maximal.

In the BCR pathway, the proximal kinases LYN, SYK, PI3Kδ, and BTK, are almost exclusively expressed in hematopoietic cells, while the more downstream kinases are more ubiquitously expressed. As a consequence, the proximal kinases are interesting candidates for novel targeted therapies, with the exception of LYN, because it also induces important inhibitory signals.[23] Note that targeting LYN could still be interesting, because LYN has a crucial role in nurse-like cells in a CLL mouse model.[24]

In MCL and WM cell lines, inhibition of SYK by fostamatinib or inhibition of PI3Kδ by idelalisib completely abrogated BCR downstream signaling (Chapter 6 and data not shown). Both inhibitors strongly inhibited activation of BTK, ERK, and AKT, which was as expected, and in line with other studies.[25-29]

Inhibition of BTK by ibrutinib abrogated parts of the BCR pathway. In Namalwa and MCL and WM cell lines, activation of ERK was completely inhibited, whereas at specific concentrations of ibrutinib, AKT activation was unaffected, and BTK activation (phosphorylation of Y551) was even hyperactivated (Chapters 2

and 6, and data not shown). The latter most likely reflects abrogation of negative feedback. We and others[30-32] found that, at high concentrations of ibrutinib, activation of AKT also was inhibited (Chapters 2 and 3). However, this inhibition was abolished upon wash-out of ibrutinib, during which BTK remains inhibited due to its irreversible covalent interaction (data not shown). This could be explained by inhibition of off targets, as at least 18 other kinases have an IC_{50} below 100nM for ibrutinib, and most of these kinases cannot form an irreversible interaction.[33] In our MCL study, we found that ibrutinib inhibits the LYN/SYK phosphorylation site on BTK (Y551) (Chapter 3). These experiments were performed in serum-free conditions. In the absence of the plasma proteins albumin and α_1-acid glycoprotein, the active (unbound) concentration of ibrutinib is much higher, since 97.3% of ibrutinib binds to these plasma proteins,[34] and therefore in the absence of serum (or bovine serum albumin) more off targets are inhibited. Ibrutinib-mediated inhibition of AKT and Y551-BTK phosphorylation could reflect inhibition of LYN, because LYN has also some affinity for ibrutinib (IC_{50} of 200nM).[33] It is not likely that this happens *in vivo,* because the maximal plasma concentration is 170nM upon intake (dose 420mg/ day), and is quickly cleared.[3] Due to the covalent binding, ibrutinib remains bound to BTK during clearance, and after 24 hours still more than 90% of BTK is bound by ibrutinib (Chapter 3), while plasma levels are almost null.[1;3] This was also seen in CLL patients.[1] So, only at very high (and clinically irrelevant) concentrations, ibrutinib reversibly targets kinases upstream of BTK. This conclusion is also supported by previous work from our group, in which the BTK knock-out DT40 chicken Burkitt cells showed normal AKT activation upon BCR ligation.[12] In addition, we found that stimulation with PMA activates ERK, but not AKT in MCL cell lines (data not shown). Therefore, the AKT pathway is not downstream of BTK and PKC in B cells.

Another interesting signaling event is the autophosphorylation of BTK (Y223). Y223 is supposed to be phosphorylated upon BTK activation.[35] However, we always detect strong phosphorylation of this site in unstimulated cells, and no (or only a small) increase upon BCR ligation (Chapters 3, 6, and data not shown). In addition, upon SYK or PI3Kδ inhibition, which inhibits Y551-BTK phosphorylation, the autophosphorylation site Y223-BTK still remained phosphorylated. Phosphorylation of Y223 was only inhibited upon BTK inhibition (*e.g.* by ibrutinib (Chapters 3, 6, and data not shown) or dasatinib (data not shown)). Thus, phosphorylation of BTK at Y223 does not appear to require recruitment to the membrane and activation of BTK via Y551 phosphorylation. Possibly, this site is typically in a phosphorylated state, and is transiently dephosphorylated upon BTK activation. This fits a model proposing that phosphorylation of this site represents an inhibitory signal.[36]

Several studies including from our own lab, indicate that BTK is involved in

multiple other pathways besides BCR signaling, *e.g.* chemokine (Chapters 2 and 3), CD40, BAFF and APRIL, TLR, HGF, integrin outside-in and stromal cell-induced signaling.[30;32;37-40] Because these factors activate the same pathways as the BCR does (*e.g.* MAPK, PI3K, and NFκB), and no study was performed in the (complete) absence of tonic/chronic BCR signaling, it could be that tonic/chronic BCR signaling collaborates with the other signaling pathways, rather than that other pathways induce BCR signaling. Interestingly, BTK is an NFκB target,[41] and its expression is upregulated upon BCR and CD40 signaling.[42] So tonic/chronic BCR signaling might be enhanced by NFκB activation. In support, most lymphomas do not loose BCR expression,[43] stressing the importance of BCR signaling itself. Another explanation could be that CLL and MCL BCRs are polyreactive, and that they are cross linked by multiple (auto-)antigens.

BCR signalosome inhibitors in cell growth

Since the MCL and WM cell lines display strong tonic/chronic BCR signaling, we studied the effect of the BCR signalosome inhibitors on cell growth. We used 6 MCL cell lines (HBL2, JeKo1, Mino, Rec1, Granta-519, and UPN1), and 2 WM cell lines (BCWM.1 and MWCL-1). Not all cell line data are shown in this thesis. Ibrutinib (100nM), idelalisib (1μM), and fostamatinib (1μM) did not affect cell viability in 5 out of 6 MCL cell lines (with the exception of Rec1), nor in both WM cell lines (Chapter 6 and data not shown). Ibrutinib and idelalisib also do not affect cell viability in primary CLL cells.[40] These findings are in line with the data showing that tonic BCR-mediated cell survival is mainly mediated via the PI3K(α/δ)-AKT pathway, and not via BTK.[44-46] Regarding SYK which is also involved in tonic BCR signaling, fostamatinib affected the viability of all MCL cell lines at 10μM (data not shown). Interestingly, Wodarz *et al.* calculated that most CLL cells in ibrutinib-treated patients already die by apoptosis before being mobilized to the circulation.[47] In light of our own data, the tissue-resident CLL cells could either die directly due to loss of active BCR-signaling (which is relatively low in blood-resident cells),[10;11] or loss of integrin-mediated outside-in signaling (“anoikis”),[48] and/or indirectly as a consequence of being deprived of tissue-derived survival factors upon mobilization (“death-by-neglect”); in all scenarios the majority of cells may already die within the lymph node or lymphatic vasculature before reaching the blood circulation. Indeed, ibrutinib causes more cell death in lymph nodes than in the circulation.[49]

Ibrutinib and idelalisib cause a differential effect on proliferation in MCL and WM cells. Ibrutinib (100nM) moderately inhibits proliferation of 5 out of 6 MCL cell lines (with the exception of HBL2), but not of the WM cell lines (Chapter 6 and data

not shown). On the other hand, the WM cells were more sensitive to idelalisib (1μM) than the MCL cells. The observed ibrutinib sensitivity of JeKo1, Mino, and Rec1 is in agreement with Rahal *et al.*[50] and Cinar *et al.*[51], and the effect on BCWM.1 and MWCL-1 with Yang *et al.*[38] Furthermore, fostamatinib (1μM) inhibited proliferation of 5 out of 6 MCL cell lines (with the exception of HBL2), and of both WM cell lines (data not shown).

In MCL, *PIK3CA* is frequently amplified,[18] and PTEN is frequently lost.[52] Both mutations result in hyperactivation of the PI3K pathway. PI3K activity is not only important for BTK activation, but also for AKT activation. This pathway has strong survival and mitogenic activities. Due to high activity of PI3Kα, MCL and diffuse large B-cell lymphoma (DLBCL) cells are insensitive to PI3Kδ inhibition by idelalisib, regarding cell growth.[53;54]

In WM, more than 95% of WM patients have gain-of-function mutations in MYD88,[55] which facilitates TLR and IL1 signaling, in which NFκB activation is the major downstream pathway. Both WM cell lines contain the L265P MYD88 mutation, driving strong NFκB signaling, which may enable the WM cells can resist BTK inhibition by ibrutinib. Indeed, the WM cells are more sensitive to MYD88 knock-down, than BTK inhibition.[38]

The effect of ibrutinib on MCL proliferation is in line with recent reports which demonstrate that CLL and MCL cells are dependent on the BTK/PKC/NFκB-axis for their growth.[50;56] Interestingly, this pathway is even more important in a subgroup of activated B-cell type of DLBCL (ABC-DLBCL) patients, which rely on chronic active BCR signaling. The growth and survival of these cells is strongly driven by this pathway. Many of them are extremely sensitive to ibrutinib treatment *in vitro* as well as in clinical trials.[16;57]

The reduced proliferation in our *in vitro* MCL study corroborates the observations of reduced expression of proliferation markers (Ki67, CD38) by $CD19^{+}CD5^{+}$ cells from ibrutinib-treated MCL patients (Chapter 3). This is also seen in a xenograft mouse model of CLL,[58] and in CLL patients.[8;30;31;56] It should be noted that it is hard to conclude whether the effect on proliferation *in vivo* is due to a direct effect on the tumor cells or to indirect inhibition due to loss of tumor-stroma interactions.

In a WM patient study, the presence of gain-of-function MYD88 mutations highly correlated with ibrutinib sensitivity.[5] It was concluded that BTK activity is directly regulated by MYD88. We observed that WM cell growth *in vitro* is not sensitive to ibrutinib. Both cell lines had an IC_{50} of 1μM (Chapter 6), which is far above clinically observed blood concentrations and BTK-selective concentrations. Possibly, enhanced TLR/MYD88 signaling induces high BTK expression via NFκB

signaling *in vivo*, and that therefore MYD88-mutated tumors could be more sensitive to BTK inhibition than MYD88 wild type tumors.

Because the *in vitro* effects of ibrutinib and idelalisib on proliferation and survival in MCL, CLL, and WM cells does not correlate with the impressive clinical responses, direct cytotoxicity is not the main effect in MCL, CLL, and WM.

BCR signalosome inhibitors in BCR-controlled adhesion

The most interesting effect of BCR signalosome inhibitors was the rapid induced lymphocytosis in CLL, MCL, and WM.[1-6] This suggests that these drugs cause inhibition of homing and/or retention signals in lymphoid tissues. *In vitro*, ibrutinib, idelalisib, and fostamatinib caused strong inhibition of BCR-controlled integrin-mediated adhesion. These data together with the gene expression profiling of CLL and MCL cells,[10;11] which demonstrated that there is active BCR signaling in lymph node resident CLL and MCL cells, revealed that the BCR induces important retention signals in CLL and MCL cells. For WM, these gene expression profiling data are lacking, but the observed lymphocytosis upon ibrutinib treatment suggests that a similar mechanism is active.

Of great interest, we detected strongly synergistic inhibition of BCR-controlled integrin-mediated adhesion by the combination of ibrutinib and idelalisib in MCL cell lines as well as in primary CLL and MCL cells (Chapter 5). This strong synergism was observed for integrin-mediated adhesion to the extracellular matrix component fibronectin as well as to the cellular ligand VCAM-1. The combination effect was synergistic by the Chou-Talalay model,[59] but not by the Bliss independence model.[60] This can be explained by the fact that these drugs target the same pathway, and alone can inhibit the pathway completely at high doses. In line with our observation, synergistic interactions were reported between ibrutinib and several PI3K inhibitors in killing of ABC-DLBCL cell lines, *e.g.* with AZD-8835 (PI3Kα/δ), GDC-094 (PI3Kα/δ), and duvelisib (PI3Kγ/δ).[61;62]

The point of synergy between ibrutinib and idelalisib appears to lie upstream of PKC, since neither ibrutinib or idelalisib alone nor their combination affects PMA-controlled adhesion (Chapter 5). Indeed, ibrutinib inhibits BTK-activity, but ibrutinib-bound BTK can still bind (and block) PIP_3, the product of PI3K, and idelalisib inhibits PIP_3 formation, which is required for translocation and activation of BTK, but also of its substrate PLCγ2. Many enzymes act as di- or multimers, which causes allostery.[63] When one partner is inhibited, the other molecule is also affected. When two consecutive kinases in the same pathway are partially inhibited, this might have tremendous effect on the pathway output, resulting in synergistic inhibition.

In WM, we did not find this synergistic effect (Chapter 6). It could be that this is a technical issue, since the WM cell lines have lower adhesion rates than the MCL cells upon BCR ligation (data not shown), and complete inhibition of adhesion was not obtained, so the window to detect synergism was limited. These results also suggest that there are redundant pathways active in addition to the PI3Kδ/BTK/integrin axis. It would be of great interest to identify these pathways.

Due to the strongly synergistic inhibition of BCR-controlled integrin-mediated adhesion by ibrutinib and idelalisib, we expect that in patients this combination therapy will result in a more prominent egress of malignant CLL and MCL cells from the growth and survival promoting niches. From the perspective of drug-clearance and turnover of the targeted proteins (BTK and PI3Kδ), the effect of ibrutinib and idelalisib combined can be stronger and more prolonged since BTK and PI3Kδ do not have to be fully occupied when the combination is used. In addition, lower drug doses can be given, which might be beneficial for the efficacy/toxicity ratio, and could prevent resistance. Interestingly, there are now dual inhibitors under development which targets both BTK and PI3Kδ.[64;65]

BCR signalosome inhibitors in chemokine-controlled adhesion and migration

An alternative explanation for the observed ibrutinib-induced lymphocytosis is aberrant homing. Normally, homing of B cells to lymphoid tissues is mediated by chemokine-controlled adhesion and migration, in which BTK is involved.[37] We demonstrated that ibrutinib targets chemokine-controlled adhesion in CLL (Chapter 2), MCL (Chapter 3), and normal B cells (data not shown), but not in WM (Chapter 6). While BCR-controlled adhesion was almost always completely inhibited by ibrutinib, chemokine-controlled adhesion was only partially inhibited, and the amount differs per cell line -from more than half (*e.g.* Mino and Daudi) to nothing (*e.g.* WM cell lines)-. Also the SYK inhibitor fostamatinib and the pan-PI3K inhibitor wortmannin were able to partially inhibit chemokine-controlled adhesion. In contrast, idelalisib does not inhibit CXCL12-controlled adhesion of MCL cells at all (data not shown). The partial effect of ibrutinib and lack of effect of idelalisib on chemokine-controlled adhesion could be a consequence of collaboration between tonic/chronic BCR-signaling and chemokine signaling. Upon stimulation with CXCL12, we found some upregulation of phosphorylated Y551-BTK, (Chapters 2 and 3) while upregulation of the BTK substrate pY759-PLCγ2 was not detectable (data not shown). Hence, chemokine signaling enhance BCR signaling, but not as strong as the BCR itself. In antigen-independent (tonic/chronic) BCR signaling, PI3Kα and -δ play redundant roles, while in antigen-dependent BCR-signaling the δ-isoform is crucial.[46;66] In this

scenario, idelalisib cannot inhibit chemokine-controlled adhesion, but can inhibit BCR-controlled adhesion. It is tempting to speculate that the phosphatase CD45 represents the link between chemokine and BCR signaling, as it is involved in BCR and chemokine signaling.[67;68]

We and others[31] showed that ibrutinib targets chemokine-controlled migration in CLL and MCL (Chapters 2 and 3), but not in WM (Chapter 6). The amount of inhibition was also dependent on cell line, -from almost 50% inhibition (CLL and Daudi), to nothing (*e.g.* Namalwa and WM cell lines)-. At the time of our CLL and MCL studies (Chapters 2 and 3), the clinical relevant concentrations of ibrutinib were not yet known, and ibrutinib was supposed to be specific for BTK up to 1μM, because at lower concentrations T cell activation was not affected.[33] However, regarding the specificity of ibrutinib (*e.g*. inhibition of the BTK-independent phosphorylation sites pY551-BTK, pY1217-PLCy2, and pAKT), the complete inhibition of migration in JeKo1, Mino, and JVM1 was probably due to off targets (Chapter 3). In contrast to Hoellenriegel *et al.*[26], we did not find inhibition of migration by idelalisib in CLL,[69] MCL (data not shown), and WM cell lines (Chapter 6). However, our data are in line with a mouse study in which CXCL12-controlled migration was not affected in PI3Kδ-deficient murine B cells,[70] or by other PI3Kδ-inhibitors.[71] Together, the data suggest that chemokine-controlled adhesion and migration are not as consistently inhibited as BCR-controlled adhesion by ibrutinib and idelalisib.

BCR signalosome inhibitors selectively mobilize malignant over normal B cells in CLL and MCL, a key role for BCR-controlled adhesion and retention

Another interesting observation was that only malignant MCL cells were mobilized by ibrutinib, but not normal B cells (Chapter 3). This was also reported for CLL.[72] Normal and malignant B cells recirculate between lymphoid tissues and blood. B cells home to lymphoid tissues via the homeostatic chemokines CCL21, CLL19, CXCL13 and CXCL12.[73] These chemokines induce integrin-dependent cell adhesion and migration. Via sphingosine-1-phosphate (S1P) gradients they move back to the lymph and blood circulation, which is integrin independent.[74] The corresponding receptors of these chemokines are members of the G protein-coupled receptors (GPCR) family. An important property of GPCRs is desensitization upon activation, which is the driving force of lymphocyte recirculation.[73-75] Abrogation of this system is involved in lymphomagenesis, *e.g.* mutations in CXCR4 that inhibit desensitization are found in WM,[15] and a similar mutation is found in CCR4 in T cell lymphoma.[76] These recirculation events are completely independent of the BCR.

Upon contact with cognate antigens, the BCR induces integrin activation, which retain the B cell in the lymph node. The integrins are continuously kept in an activated state, as ibrutinib did also detach previously adhered MCL cells (Chapter 5). *In vitro*, ibrutinib and idelalisib inhibited BCR-controlled integrin-mediated adhesion almost completely in CLL and MCL (Chapter 5), and also in normal B cells (data not shown). In contrast to the clonal malignant CLL and MCL cells, normal B cells rarely meet a cognate antigen. So the BCR signalosome inhibitors selectively affect B cells which have BCR-controlled retention, which include the malignant CLL/MCL/WM cells, but not the bulk of normal recirculating B cells (Fig. 1).

Apart from BCR-controlled integrin-mediated adhesion, the BCR controls retention via another mechanism. Upon BCR-ligation the early activation marker CD69 is quickly upregulated. CD69 in an inhibitor of the S1P receptor 1 (S1P1), the receptor which is involved lymph node egress.[77] Honigberg *et al.* showed that ibrutinib prevents antigen-induced BCR-controlled upregulation of CD69 in normal B cells.[33] Interestingly, in CLL both ibrutinib and idelalisib decrease CD69 expression,[8;78] and

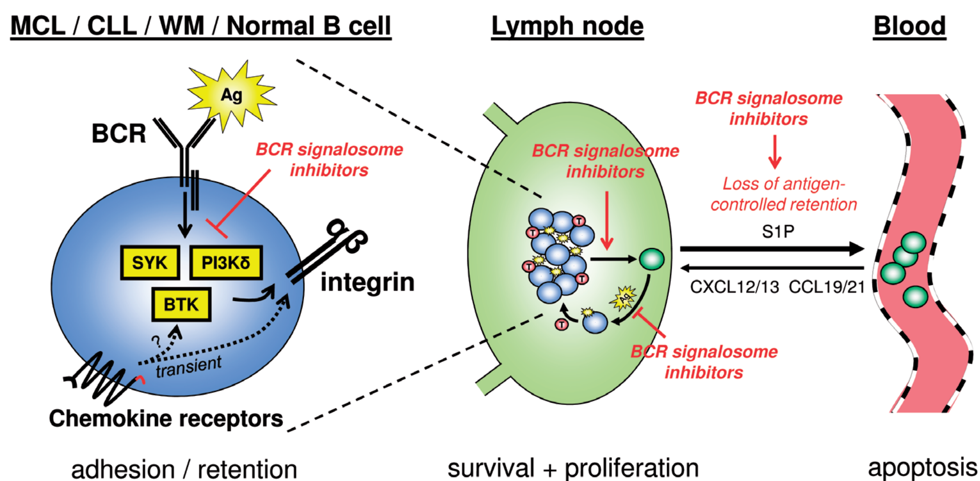


Figure 1: BCR signalosome inhibitors inhibit BCR-controlled retention of malignant B cells
Controlled by chemokines, normal B cells and malignant CLL, MCL, and WM cells home to lymphoid tissues, and due to desensitization of the chemokine receptors, they will recirculate between lymphoid tissues and blood (green cells). In the presence of a cognate antigen (blue cells), the B cells are retained in the lymph nodes, and proliferation will be stimulated by the microenvironment (*e.g.* cognate follicular T cells). BCR signalosome inhibitors prevent the retention of the (malignant) B cells in this niche, resulting in mobilization to the blood, which indirectly prevents proliferation of the B cells. Because CLL, MCL, and WM cells obtain antigens in the lymphoid microenvironment, and normal B cells only sporadically during infection, only the malignant B cells in the lymphoid tumors are mobilized (only the blue cells are affected). This mobilization results in a decrease in lymphadenopathy accompanied by a lymphocytosis from the malignant B cells. The mobilized cells are cleared by normal turn over, and after a while the lymphocytosis shrinks.

thereby could prevent lymph node retention.

Apart from BCR-controlled adhesion and retention, ibrutinib causes a partial inhibition of chemokine-controlled adhesion and migration in CLL and MCL (Chapters 2 and 3), as well as in normal B cells (data not shown). So this does not correlate with the observation that only malignant cells are mobilized. In addition, idelalisib did not affect chemokine-controlled adhesion and migration in CLL[69] and MCL (data not shown), and ibrutinib and idelalisib did not target chemokine-controlled adhesion and migration in WM (Chapter 6) at all. Since both ibrutinib and idelalisib causes a mobilization of CLL, MCL, and WM cells,[1-3;5;6] this suggests strongly that the mobilization is predominantly mediated by BCR-controlled retention rather than by chemokine-controlled homing.

In CLL and MCL patients treated with ibrutinib, we observed decreased levels of CXCR4 on the mobilized cells (Chapters 2 and 3). This suggests that these cells just left lymphoid tissues,[10] since the chemokine receptors are quickly desensitized and internalized due to high chemokine levels.[73-75] In the CLL study (Chapter 2), samples were collected 4 hours after treatment, while in the MCL study (Chapter 3) 7 days after treatment. At both time points a decrease of CXCR4 expression was observed. This suggests that the egress from tissues is a rapid and long term ongoing effect. We do not have data which actually show whether or not cells can home back after mobilization. However, the fact that CXCR4 expression is still lower after a week of treatment, suggests that the cells may not stay for long times in the circulation, because in that scenario a CXCR4 re-expression would be expected. In contrast to a TCL1-CLL mouse model,[79] ibrutinib does not directly decrease CXCR4 membrane expression in human CLL and MCL cells (Chapters 2 and 3). Hence, there is no evidence that *in vivo* the chemokine-pathways are strongly affected by ibrutinib.

The mobilization of malignant cells by BCR signalosome inhibitors strongly correlates with BCR usage by the lymphoma cells. The ibrutinib and idelalisib-induced lymphocytosis is the strongest in CLL compared to other studied B cell malignancies, and is also frequently seen in MCL and WM.[1-3;5;6] This effect probably correlates with the BCR signaling strength, since CLL and MCL both have antigen-induced BCR signaling[10;11;13;20;21] (for WM this is yet unknown). Recently, an ibrutinib-induced lymphocytosis was also observed in marginal zone lymphoma.[80] This type of lymphoma has stereotypic BCRs like in CLL and MCL, suggesting that (auto) antigens, and thus BCR signaling, play a role.[81] Another BTK inhibitor (ONO-4059) induced also a lymphocytosis in a FL patient.[82] The variable region of BCRs of FL patients is frequently glycosylated and can be cross linked by lectins.[83] This reflects

(super)antigen-induced BCR signaling.

The response to ibrutinib treatment within a given lymphoma type also correlates with BCR usage. Mutated IgH_V CLL have less active BCR signaling than unmutated IgH_V CLL, and ibrutinib treatment is more effective in the unmutated subtype (objective response rate (ORR) of 33% vs 68% respectively).[3] Furthermore, in DLBCL the ABC-subtype which rely on chronic active BCR signaling is more sensitive to ibrutinib than the germinal center subtype which just has tonic BCR signaling (ORR 37% vs 5% respectively).[45;57]

We expect that BCR signalosome inhibitors are also promising drugs in other B cell malignancies in which BCR signaling plays a crucial role. This can be predicted by the presence of stereotypic BCR subgroups within the lymphoma type, or by mutations in the BCR pathway.

Resistance and side effects of BCR signalosome inhibitors

Many CLL, MCL, and WM patients become resistant against ibrutinib and idelalisib. In CLL, disease progression or Richter's transformation frequently occurs.[84] Compagno *et al.* have suggested that idelalisib and to a lesser extent ibrutinib enhance genomic instability via upregulation of AID.[85] PI3Kδ knock-out mice do not have activated B and T cells, and lack germinal centers.[66] However, when BCR signaling is blocked at germinal center stage, somatic hypermutation may not be terminated, as the germinal center cell cannot detect expressible (right open reading frame and signaling competent) BCRs.

Progression of CLL is frequently caused by mutations of the binding site of ibrutinib (most frequently C481S), or in the autoregulatory domain of PLCγ2 (most frequently R665W).[84] These mutations are also found in ibrutinib resistant MCL patients.[86] Interestingly, these mutations are only found *in vivo*, but not *in vitro* in ibrutinib resistant MCL and WM cell lines.[32;87] This is in line with our model, that inhibition of BCR-controlled retention is more important than direct cytotoxicity *in vivo*.

Woyach *et al.* showed that BTK with the C481S mutation is still inhibited by ibrutinib *in vitro*, but not upon a washout.[88] Thus this mutation only prevents covalent binding of ibrutinib. Since cysteine-481 mutations are the most common acquired ibrutinib-resistance mutation, a different BTK inhibitor which does not target cysteine-481, can be used. For patients with PLCγ2 mutations, a specific PLCγ2 inhibitor could be developed. PLCγ2 knock-out mice are viable, but are immunodeficient, and have problems with bone (osteclastogenesis) and blood/lymph vessel development.[89-91] These problems are probably negligible in adults. Because

PLCγ2 with these mutations is still responsible to BCR-ligation,[88] it is likely that this is regulated by PI3K-mediated (PIP_3) recruitment of PLCγ2 to the membrane. This can be targeted by PI3K inhibitors, such as idelalisib. Interestingly, recent studies showed that idelalisib and the PI3Kγ/δ-inhibitor duvelisib can inhibit proliferation and signaling in cells carrying the BTK mutation (C481S).[92;93] To prevent acquiring of these resistance mutations, a dual treatment of ibrutinib and idelalisib might be ideal, because than the malignant cell should acquire two mutations at the same moment. Interestingly, apart from BTK and PLCγ2 mutations, an ibrutinib resistant WM patient is found with a CARD11 mutation.[94] This mutation is downstream of BTK, and signals to NFκB, rather than integrins. This patient also has the CXCR4 mutation, so the BCR-integrin axis is not so important for retention in this case. Furthermore, some ibrutinib resistant MCL patients obtained loss of function mutations in S1P1, the receptor for S1P.[95] This means that the ibrutinib-induced mobilization is facilitated by S1P-mediated egress, like in normal B cell egress, and suggests again that BCR signals are responsible for retention signals.

There are also patients which acquire mutations not involving the BCR signaling pathway. Some CLL patient transform into DLBCL (Richter's transformation),[84;96] and other patients develop progressive CLL due to other driver mutations, like EP300, MLL2 and EIF2A mutations.[97] Probably these lymphoma cells become independent of BCR signaling, and other treatment strategies have to be developed.

Resistance mechanisms for idelalisib are not published yet. There were no recurrent mutations detected,[98] but *PIK3CA* amplifications (PI3Kα) are expected.[99] Fostamatinib is not further studied in lymphomas, because the company Rigel Therapeutics focused on autoimmune diseases.

A strategy to improve the mobilization of the malignant cells could be to combine BCR signalosome inhibitors with specific $G\alpha_{i2}$ inhibitors, or with blocking antibodies against integrins, because chemokine-mediated homing is only partially or not inhibited. Another strategy could be to kill all the cells which are mobilized by BCR signalosome inhibitors. Many cytotoxic drugs do not kill all tumor cells. It is believed that some cells in specialized niches are hard to kill, because of cell adhesion mediated drug resistance, in which the microenvironment provides strong survival signals.[100;101] Therefore, drugs which mobilize cells from these microenvironments together with cytotoxic drug would be interesting. This can be done by a combination of BCR signalosome inhibitors in combination with anti-CD20 antibodies, like rituximab or ofatumumab, or with pro-apoptotic drugs, like the BCL2 inhibitor venetoclax (also discussed in chapter 4). Furthermore, *in vitro* ibrutinib has synergy with among others venetoclax,[62;102] several PI3K inhibitors as previously

described, and with epigenetic drugs such as BET and HDAC inhibitors.[62;103;104] Clinical trials in the near future will tell which combinations are also effective in clinic. Finally, ibrutinib improves T cell numbers and function in CLL patients, which makes it very interesting to combine ibrutinib with immunotherapy.[105]

A problem of BCR signalosome inhibitors are the side effects. Both ibrutinib and idelalisib causes pancytopenia, resulting in anemia, bleedings and infections, however idelalisib stronger than ibrutinib.[106] Since PI3Kδ is also important in other immune cells (*e.g.* T cells, neutrophils, macrophages), and BTK plays a less important role in macrophages and platelets, the combination of a lower dose of ibrutinib and idelalisib could make the effect more B cell specific than idelalisib alone. Another common side effect of ibrutinib and idelalisib is diarrhea. This is probably an on target effect, because PI3Kδ inactivated mice also develop diarrhea and inflammatory bowel disease. So this should be treated by symptom repression.

Conclusion

BCR signalosome inhibitors are very interesting new tools in the treatment of several types of B cell malignancies. Unfortunately, complete remissions are not frequently observed. The clinical responses to these inhibitors are diverse. In some B cell lymphoma types which rely on chronic BCR-NFκB signaling (*e.g.* ABC-DLBCL), these drugs are cytotoxic in a direct manner. On the other hand, in B cell lymphomas which have antigen-mediated BCR-controlled retention in lymphoid tissues (*e.g.* CLL, MCL, and WM) the malignant cells are mobilized into the circulation, where they are deprived of proliferation and survival signals, and eventually die in an indirect manner. With this knowledge, novel combination therapies can be developed with these drugs to improve clinical responses.

References

1. Advani RH, Buggy JJ, Sharman JP et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J.Clin.Oncol.* 2013;31(1):88-94.
2. Brown JR, Byrd JC, Coutre SE et al. Idelalisib, an inhibitor of phosphatidylinositol 3-kinase p110delta, for relapsed/refractory chronic lymphocytic leukemia. *Blood* 2014;123(22):3390-3397.
3. Byrd JC, Furman RR, Coutre SE et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N.Engl.J.Med.* 2013;369(1):32-42.
4. Friedberg JW, Sharman J, Sweetenham J et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* 2010;115(13):2578-2585.
5. Treon SP, Tripsas CK, Meid K et al. Ibrutinib in previously treated Waldenstrom's macroglobulinemia. *N.Engl.J.Med.* 2015;372(15):1430-1440.
6. Wang ML, Rule S, Martin P et al. Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. *N.Engl.J.Med.* 2013;369(6):507-516.
7. Woyach JA, Smucker K, Smith LL et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood* 2014;123(12):1810-1817.
8. Herman SE, Niemann CU, Farooqui M et al. Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukemia: correlative analyses from a phase II study. *Leukemia* 2014;28(11):2188-2196.
9. Titus-Rains KS, Brown JN, Hammond JM. Ibrutinib-associated tumor lysis syndrome in chronic lymphocytic leukemia/small lymphocytic lymphoma and mantle cell lymphoma: A case series and review of the literature. *J.Oncol.Pharm.Pract.* 2017; doi: 10.1177/1078155217718617.
10. Herishanu Y, Perez-Galan P, Liu D et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* 2011;117(2):563-574.
11. Saba NS, Liu D, Herman SE et al. Pathogenic role of B-cell receptor signaling and canonical NF-kappaB activation in mantle cell lymphoma. *Blood* 2016;128(1):82-92.
12. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J.Exp.Med.* 2003;198(10):1539-1550.
13. Niemann CU, Wiestner A. B-cell receptor signaling as a driver of lymphoma development and evolution. *Semin.Cancer Biol.* 2013;23(6):410-421.
14. Poulain S, Roumier C, Galiegue-Zouitina S et al. Genome wide SNP array identified multiple mechanisms of genetic changes in Waldenstrom macroglobulinemia. *Am.J.Hematol.* 2013;88(11):948-954.

15. Hunter ZR, Xu L, Yang G et al. The genomic landscape of Waldenstrom macroglobulinemia is characterized by highly recurring MYD88 and WHIM-like CXCR4 mutations, and small somatic deletions associated with B-cell lymphomagenesis. *Blood* 2014;123(11):1637-1646.
16. Davis RE, Ngo VN, Lenz G et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. *Nature* 2010;463(7277):88-92.
17. Rinaldi A, Kwee I, Taborelli M et al. Genomic and expression profiling identifies the B-cell associated tyrosine kinase Syk as a possible therapeutic target in mantle cell lymphoma. *Br.J.Haematol.* 2006;132(3):303-316.
18. Psyrri A, Papageorgiou S, Liakata E et al. Phosphatidylinositol 3'-kinase catalytic subunit alpha gene amplification contributes to the pathogenesis of mantle cell lymphoma. *Clin.Cancer Res.* 2009;15(18):5724-5732.
19. Duhren-von MM, Ubelhart R, Schneider D et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* 2012;489(7415):309-312.
20. Fais F, Ghiotto F, Hashimoto S et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J.Clin.Invest.* 1998;102(8):1515-1525.
21. Hadzidimitriou A, Agathangelidis A, Darzentas N et al. Is there a role for antigen selection in mantle cell lymphoma? Immunogenetic support from a series of 807 cases. *Blood* 2011;118(11):3088-3095.
22. Martin-Jimenez P, Garcia-Sanz R, Balanzategui A et al. Molecular characterization of heavy chain immunoglobulin gene rearrangements in Waldenstrom's macroglobulinemia and IgM monoclonal gammopathy of undetermined significance. *Haematologica* 2007;92(5):635-642.
23. Nishizumi H, Horikawa K, Mlinaric-Rascan I, Yamamoto T. A double-edged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. *J.Exp.Med.* 1998;187(8):1343-1348.
24. Nguyen PH, Fedorchenko O, Rosen N et al. LYN kinase in the tumor microenvironment is essential for the progression of chronic lymphocytic leukemia. *Cancer Cell* 2016;30(4):610-622.
25. Herman SE, Gordon AL, Wagner AJ et al. Phosphatidylinositol 3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood* 2010;116(12):2078-2088.
26. Hoellenriegel J, Meadows SA, Sivina M et al. The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood* 2011;118(13):3603-3612.
27. Hoellenriegel J, Coffey GP, Sinha U et al. Selective, novel spleen tyrosine kinase (Syk) inhibitors suppress chronic lymphocytic leukemia B-cell activation and migration. *Leukemia* 2012;26(7):1576-1583.
28. Lannutti BJ, Meadows SA, Herman SE et al. CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. *Blood* 2011;117(2):591-594.
29. Suljagic M, Longo PG, Bennardo S et al. The Syk inhibitor fostamatinib disodium (R788) inhibits tumor growth in the Emu- TCL1 transgenic mouse model of CLL by blocking antigen-dependent B-cell receptor signaling. *Blood* 2010;116(23):4894-4905.

30. Herman SE, Gordon AL, Hertlein E et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* 2011;117(23):6287-6296.
31. Ponader S, Chen SS, Buggy JJ et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood* 2012;119(5):1182-1189.
32. Zhao X, Lwin T, Silva A et al. Unification of de novo and acquired ibrutinib resistance in mantle cell lymphoma. *Nat.Commun.* 2017;8:14920.
33. Honigberg LA, Smith AM, Sirisawad M et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc. Natl.Acad.Sci.U.S.A.* 2010;107(29):13075-13080.
34. de JJ, Skee D, Hellemans P et al. Single-dose pharmacokinetics of ibrutinib in subjects with varying degrees of hepatic impairment. *Leuk.Lymphoma* 2017;58(1):185-194.
35. Mohamed AJ, Yu L, Backesjo CM et al. Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. *Immunol.Rev.* 2009;228(1):58-73.
36. Park H, Wahl MI, Afar DE et al. Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity.* 1996;4(5):515-525.
37. de Gorter DJ, Beuling EA, Kersseboom R et al. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity.* 2007;26(1):93-104.
38. Yang G, Zhou Y, Liu X et al. A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenstrom macroglobulinemia. *Blood* 2013;122(7):1222-1232.
39. Singhal E, Kumar P, Sen P. A novel role for Bruton's tyrosine kinase in hepatocyte growth factor-mediated immunoregulation of dendritic cells. *J.Biol.Chem.* 2011;286(37):32054-32063.
40. Slinger E, Thijssen R, Kater AP, Eldering E. Targeting antigen-independent proliferation in chronic lymphocytic leukemia through differential kinase inhibition. *Leukemia* 2017; doi: 10.1038/leu.2017.129.
41. Yu L, Mohamed AJ, Simonson OE et al. Proteasome dependent auto-regulation of Bruton's tyrosine kinase (Btk) promoter via NF-{kappa}B. *Blood* 2008;111(9):4617-4626.
42. Kil LP, de Bruijn MJ, van Nimwegen M et al. Btk levels set the threshold for B-cell activation and negative selection of autoreactive B cells in mice. *Blood* 2012;119(16):3744-3756.
43. Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat.Rev.Cancer* 2005;5(4):251-262.
44. Srinivasan L, Sasaki Y, Calado DP et al. PI3 kinase signals BCR-dependent mature B cell survival. *Cell* 2009;139(3):573-586.
45. Havranek O, Xu J, Kohrer S et al. Tonic B-cell receptor signaling in diffuse large B-cell lymphoma. *Blood* 2017; doi: 10.1182/blood-2016-10-747303.

46. Ramadani F, Bolland DJ, Garcon F et al. The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development. *Sci.Signal.* 2010;3(134):ra60.
47. Wodarz D, Garg N, Komarova NL et al. Kinetics of CLL cells in tissues and blood during therapy with the BTK inhibitor ibrutinib. *Blood* 2014;123(26):4132-4135.
48. Koopman G, Keehnen RM, Lindhout E et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. *J.Immunol.* 1994;152(8):3760-3767.
49. Burger JA, Li KW, Keating MJ et al. Leukemia cell proliferation and death in chronic lymphocytic leukemia patients on therapy with the BTK inhibitor ibrutinib. *JCI.Insight.* 2017;2(2):e89904.
50. Rahal R, Frick M, Romero R et al. Pharmacological and genomic profiling identifies NF-kappaB-targeted treatment strategies for mantle cell lymphoma. *Nat.Med.* 2014;20(1):87-92.
51. Cinar M, Hamedani F, Mo Z et al. Bruton tyrosine kinase is commonly overexpressed in mantle cell lymphoma and its attenuation by Ibrutinib induces apoptosis. *Leuk.Res.* 2013;37(10):1271-1277.
52. Rudelius M, Pittaluga S, Nishizuka S et al. Constitutive activation of Akt contributes to the pathogenesis and survival of mantle cell lymphoma. *Blood* 2006;108(5):1668-1676.
53. Iyengar S, Clear A, Bodor C et al. P110alpha-mediated constitutive PI3K signaling limits the efficacy of p110delta-selective inhibition in mantle cell lymphoma, particularly with multiple relapse. *Blood* 2013;121(12):2274-2284.
54. Paul J, Soujon M, Wengner AM et al. Simultaneous Inhibition of PI3Kdelta and PI3Kalpha Induces ABC-DLBCL Regression by Blocking BCR-Dependent and -Independent Activation of NF-kappaB and AKT. *Cancer Cell* 2017;31(1):64-78.
55. Treon SP, Xu L, Yang G et al. MYD88 L265P somatic mutation in Waldenstrom’s macroglobulinemia. *N.Engl.J.Med.* 2012;367(9):826-833.
56. Cheng S, Ma J, Guo A et al. BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. *Leukemia* 2014;28(3):649-657.
57. Wilson WH, Young RM, Schmitz R et al. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. *Nat.Med.* 2015;21(8):922-926.
58. Herman SE, Sun X, McAuley EM et al. Modeling tumor-host interactions of chronic lymphocytic leukemia in xenografted mice to study tumor biology and evaluate targeted therapy. *Leukemia* 2013;27(12):2311-2321.
59. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* 2010;70(2):440-446.
60. Bliss CI. The toxicicity of poisons applied jointly. *Ann.Appl.Biol.* 1939;26(3):585-615.
61. Erdmann T, Klener P, Lynch JT et al. Sensitivity to PI3K and AKT inhibitors is mediated by divergent molecular mechanisms in subtypes of DLBCL. *Blood* 2017;130(3):310-322.
62. Schaffer M, Chaturvedi S, Davis C et al. Identification of potential ibrutinib combinations in hematological malignancies using a combination high-throughput screen. *Leuk.Lymphoma* 2017; doi: 10.1080/10428194.2017.1349899.

63. Perutz MF. Stereochemistry of cooperative effects in haemoglobin. *Nature* 1970;228(5273):726-739.
64. Alfaro J, Perez de AF, Belmar S et al. Dual inhibition of Bruton's tyrosine kinase and phosphoinositide-3-kinase p110delta as a therapeutic approach to treat non-Hodgkin's B cell malignancies. *J.Pharmacol. Exp.Ther.* 2017;361(2):312-321.
65. Pujala B, Agarwal AK, Middya S et al. Discovery of pyrazolopyrimidine derivatives as novel dual inhibitors of BTK and PI3Kdelta. *ACS Med.Chem.Lett.* 2016;7(12):1161-1166.
66. Okkenhaug K, Bilancio A, Farjot G et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* 2002;297(5583):1031-1034.
67. Fernandis AZ, Cherla RP, Ganju RK. Differential regulation of CXCR4-mediated T-cell chemotaxis and mitogen-activated protein kinase activation by the membrane tyrosine phosphatase, CD45. *J.Biol.Chem.* 2003;278(11):9536-9543.
68. Yanagi S, Sugawara H, Kurosaki M et al. CD45 modulates phosphorylation of both autophosphorylation and negative regulatory tyrosines of Lyn in B cells. *J.Biol.Chem.* 1996;271(48):30487-30492.
69. Thijssen R, Ter BJ, van Bochove GG et al. The pan phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor SAR245409 (voxtalisib/XL765) blocks survival, adhesion and proliferation of primary chronic lymphocytic leukemia cells. *Leukemia* 2016;30(2):337-345.
70. Reif K, Okkenhaug K, Sasaki T et al. Cutting edge: differential roles for phosphoinositide 3-kinases, p110gamma and p110delta, in lymphocyte chemotaxis and homing. *J.Immunol.* 2004;173(4):2236-2240.
71. Niedermeier M, Hennessy BT, Knight ZA et al. Isoform-selective phosphoinositide 3'-kinase inhibitors inhibit CXCR4 signaling and overcome stromal cell-mediated drug resistance in chronic lymphocytic leukemia: a novel therapeutic approach. *Blood* 2009;113(22):5549-5557.
72. Wiestner A. Emerging role of kinase-targeted strategies in chronic lymphocytic leukemia. *Blood* 2012;120(24):4684-4691.
73. Stein JV, Nombela-Arrieta C. Chemokine control of lymphocyte trafficking: a general overview. *Immunology* 2005;116(1):1-12.
74. Lo CG, Xu Y, Proia RL, Cyster JG. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. *J.Exp. Med.* 2005;201(2):291-301.
75. Arnon TI, Xu Y, Lo C et al. GRK2-dependent S1PR1 desensitization is required for lymphocytes to overcome their attraction to blood. *Science* 2011;333(6051):1898-1903.
76. Nakagawa M, Schmitz R, Xiao W et al. Gain-of-function CCR4 mutations in adult T cell leukemia/lymphoma. *J.Exp.Med.* 2014;211(13):2497-2505.
77. Shiow LR, Rosen DB, Brdickova N et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 2006;440(7083):540-544.
78. Montraveta A, Lee-Verges E, Roldan J et al. CD69 expression potentially predicts response to bendamustine and its modulation by ibrutinib or idelalisib enhances cytotoxic effect in chronic lymphocytic leukemia. *Oncotarget.* 2016;7(5):5507-5520.

79. Chen SS, Chang BY, Chang S et al. BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia. *Leukemia* 2016;30(4):833-843.
80. Noy A, De VS, Thieblemont C et al. Targeting Bruton tyrosine kinase with ibrutinib in relapsed/refractory marginal zone lymphoma. *Blood* 2017;129(16):2224-2232.
81. Bende RJ, Janssen J, Wormhoudt TA et al. Identification of a novel stereotypic IGHV4-59/IGHJ5-encoded B-cell receptor subset expressed by various B-cell lymphomas with high affinity rheumatoid factor activity. *Haematologica* 2016;101(5):e200-e203.
82. Walter HS, Rule SA, Dyer MJ et al. A phase 1 clinical trial of the selective BTK inhibitor ONO/GS-4059 in relapsed and refractory mature B-cell malignancies. *Blood* 2016;127(4):411-419.
83. Coelho V, Krysov S, Ghaemmaghami AM et al. Glycosylation of surface Ig creates a functional bridge between human follicular lymphoma and microenvironmental lectins. *Proc.Natl.Acad.Sci.U.S.A.* 2010;107(43):18587-18592.
84. Ahn IE, Underbayev C, Albitar A et al. Clonal evolution leading to ibrutinib resistance in chronic lymphocytic leukemia. *Blood* 2017;129(11):1469-1479.
85. Compagno M, Wang Q, Pighi C et al. Phosphatidylinositol 3-kinase delta blockade increases genomic instability in B cells. *Nature* 2017;542(7642):489-493.
86. Balasubramanian S, Schaffer M, Deraedt W et al. Mutational analysis of patients with primary resistance to single-agent ibrutinib in relapsed or refractory mantle cell lymphoma (MCL). *Blood* 2014;124(21):78.
87. Paulus A, Akhtar S, Yousaf H et al. Waldenstrom macroglobulinemia cells devoid of BTKC481S or CXCR4WHIM-like mutations acquire resistance to ibrutinib through upregulation of Bcl-2 and AKT resulting in vulnerability towards venetoclax or MK2206 treatment. *Blood Cancer J.* 2017;7(5):e565.
88. Woyach JA, Furman RR, Liu TM et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N.Engl.J.Med.* 2014;370(24):2286-2294.
89. Wang D, Feng J, Wen R et al. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity.* 2000;13(1):25-35.
90. Ichise H, Ichise T, Ohtani O, Yoshida N. Phospholipase Cgamma2 is necessary for separation of blood and lymphatic vasculature in mice. *Development* 2009;136(2):191-195.
91. Mao D, Epple H, Uthgenannt B, Novack DV, Faccio R. PLCgamma2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2. *J.Clin.Invest.* 2006;116(11):2869-2879.
92. Cheng S, Guo A, Lu P et al. Functional characterization of BTK(C481S) mutation that confers ibrutinib resistance: exploration of alternative kinase inhibitors. *Leukemia* 2015;29(4):895-900.
93. Dong S, Guinn D, Dubovsky JA et al. IPI-145 antagonizes intrinsic and extrinsic survival signals in chronic lymphocytic leukemia cells. *Blood* 2014;124(24):3583-3586.
94. Xu L, Tsakmaklis N, Yang G et al. Acquired mutations associated with ibrutinib resistance in Waldenstrom macroglobulinemia. *Blood* 2017;129(18):2519-2525.

95. Wu C, de Miranda NF, Chen L et al. Genetic heterogeneity in primary and relapsed mantle cell lymphomas: Impact of recurrent CARD11 mutations. *Oncotarget.* 2016;7(25):38180-38190.
96. Chan KL, Blombery P, Jones K et al. Plasmablastic Richter transformation as a resistance mechanism for chronic lymphocytic leukaemia treated with BCR signalling inhibitors. *Br.J.Haematol.* 2017;177(2):324-328.
97. Burger JA, Landau DA, Taylor-Weiner A et al. Clonal evolution in patients with chronic lymphocytic leukaemia developing resistance to BTK inhibition. *Nat.Commun.* 2016;7:11589.
98. Ghia P, Ljungstrom V, Tausch E et al. Whole-exome sequencing revealed no recurrent mutations within the PI3K pathway in relapsed chronic lymphocytic leukemia patients progressing under idelalisib treatment. *Blood* 2016;128(22):2770.
99. Woyach JA. Patterns of resistance to B cell-receptor pathway antagonists in chronic lymphocytic leukemia and strategies for management. *Hematology Am.Soc.Hematol.Educ.Program.* 2015;2015:355-360.
100. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* 1999;93(5):1658-1667.
101. de la Fuente MT, Casanova B, Moyano JV et al. Engagement of alpha4beta1 integrin by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. *J.Leukoc.Biol.* 2002;71(3):495-502.
102. Kuo HP, Ezell SA, Schweighofer KJ et al. Combination of Ibrutinib and ABT-199 in Diffuse Large B-Cell Lymphoma and Follicular Lymphoma. *Mol.Cancer Ther.* 2017;16(7):1246-1256.
103. Bernasconi E, Gaudio E, Lejeune P et al. Preclinical evaluation of the BET bromodomain inhibitor BAY 1238097 for the treatment of lymphoma. *Br.J.Haematol.* 2017;doi: 10.1111/bjh.14803.
104. Amengual JE, Prabhu SA, Lombardo M et al. Mechanisms of acquired drug resistance to the HDAC6 selective inhibitor ricolinostat reveals rational drug-drug combination with ibrutinib. *Clin.Cancer Res.* 2017;23(12):3084-3096.
105. Long M, Beckwith K, Do P et al. Ibrutinib treatment improves T cell number and function in CLL patients. *J.Clin.Invest.* 2017;127(8):3052-3064.
106. Falchi L, Baron JM, Orlikowski CA, Ferrajoli A. BCR signaling inhibitors: an overview of toxicities associated with ibrutinib and idelalisib in patients with chronic lymphocytic leukemia. *Mediterr.J.Hematol.Infect.Dis.* 2016;8(1):e2016011.

Chapter 8

Summary

PhD portfolio

Nederlandse samenvatting

Curriculum vitae

Dankwoord

Summary

Upon infection, lymph nodes can swell considerably. In the lymph nodes, B cells traffic in and out, to find their cognate antigen. When there is a match, the B cells will proliferate and produce antibodies to attack the pathogen. A subset of these B cells will optimize the affinity of its B cell antigen receptor (BCR) in a germinal center reaction. Here, the B cells proliferate and mutate their BCR loci. Subsequently they are selected for the best antigen presentation to T cells, and thus, indirectly, by the best affinity for the antigen. At the end, the B cells differentiate into plasma cells which produce antibodies (secreted version of the BCR) to attack the pathogens, and into memory cells which can quickly respond upon a second infection with the same antigen. Proliferation, mutation and differentiation are highly controlled events, and mistakes will promote lymphomagenesis.

Chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and Waldenström macroglobulinemia (WM) are B cell malignancies which are still incurable. Therefore, novel treatment strategies are needed to cure these patients. In these lymphomas, the cells proliferate in specialized niches in lymphoid tissues, in which they are provided by stromal derived growth and survival factors. At least for CLL and MCL, several studies suggest that BCR signaling is active in these niches. The first clinical trials with the novel BCR signalosome inhibitors fostamatinib (SYK inhibitor), ibrutinib (BTK inhibitor), or idelalisib (PI3Kδ inhibitor) were very promising. Unexpectedly, these inhibitors act in a remarkable way. In CLL patients a rapid and sustained reduction of lymphadenopathy accompanied by transient lymphocytosis was observed, which was reversible upon temporary drug deprivation.

In **chapter 2** we studied the mechanism of action of the BTK inhibitor ibrutinib (f.k.a. PCI-32765) at a molecular and cellular level. We established that ibrutinib did not target the viability of CLL cells in a direct manner. Ibrutinib strongly inhibited BCR-controlled signaling and integrin-mediated adhesion of CLL cells. Furthermore, ibrutinib partially inhibited chemokine-induced signaling, adhesion, and migration of CLL cells. Our data suggest that inhibition of BTK by ibrutinib overcomes BCR- and chemokine-controlled integrin-mediated retention and homing of malignant B cells in their growth- and survival-supporting lymph node and bone marrow microenvironment, which results in clinically evident CLL regression.

In **chapter 3** we demonstrated that this mechanism was not specific for CLL, but applies to MCL. We also observed an ibrutinib-induced lymphocytosis in MCL patients. We confirmed the hypothesis that the accumulation of blood-resident cells originates from lymphoid tissue-derived cells. Furthermore, we established that only the malignant MCL cells were mobilized, whereas normal B cells were not. *In*

8

vitro, we performed the same signaling, adhesion, and migration experiments as in **chapter 2**, and found the same molecular and cellular mechanisms for MCL as in CLL.

In **chapter 4**, we reviewed the role of BTK in BCR signaling and CLL pathogenesis, discuss the clinical application and drawbacks of BTK inhibitors in CLL, and discuss about ongoing and future combination therapies with BTK inhibitors.

Apart from BTK inhibitors, the PI3Kδ inhibitor idelalisib is also very promising in CLL and MCL patients. Idelalisib gave similar clinical responses as ibrutinib, which is, a decrease in lymphadenopathy which is accompanied by a transient lymphocytosis. This suggests that idelalisib also affects integrin-mediated retention. In **chapter 5**, we showed that idelalisib is able to inhibit BCR-controlled integrin-mediated adhesion, and that the combination ibrutinib and idelalisib inhibit BCR-controlled adhesion in a strongly synergistic manner in CLL and MCL. We speculate that the combination would not only give better clinical responses, but is also beneficial to prevent resistance.

In **chapter 6**, we studied ibrutinib and idelalisib in WM. In these patients, a lymphocytosis was also observed upon treatment with ibrutinib. In this type of lymphoma, we demonstrated that BCR-controlled signaling and integrin-mediated adhesion was targeted by ibrutinib and idelalisib, but not chemokine-controlled signaling, adhesion and migration.

Taken together, our data suggest that inhibition of BCR-controlled integrin-mediated adhesion and retention is the major pathway which is targeted by BCR signalosome inhibitors in CLL, MCL and WM, because this was consistently inhibited by ibrutinib in these lymphomas. This assumption is supported by our unpublished data that idelalisib did not affect chemokine-controlled adhesion and migration, and that ibrutinib also affected chemokine-controlled adhesion of normal B cells, which are not mobilized in patients.

The novel BCR signalosome inhibitors are very interesting new tools in the treatment of several types of B cell malignancies. The clinical responses of these inhibitors are divergently. In some B cell lymphoma types which rely on chronic BCR-NFκB signaling (*e.g.* ABC-DLBCL), these drugs are cytotoxic in a direct manner. On the other hand, in B cell lymphomas which have antigen-mediated BCR-controlled retention in lymphoid tissues (*e.g.* the studied B cell malignancies in this thesis: CLL, MCL, and WM) the malignant cells are mobilized into the circulation, in which there is a lack of proliferation and survival signals, and eventually die in an indirect manner. With this knowledge, novel combination therapies can developed with these drugs to improve clinical responses.

PhD portfolio

Name PhD student: Martin F.M. de Rooij
PhD period: March 2009 - May 2014
Name PhD supervisor: Prof. dr. Steven T. Pals

	Year	Workload (ECTS)
Courses		
Intensive English (AmbassyCES), Cambridge, UK	2009	3
Advanced immunology (UvA/VU/Sanquin), Amsterdam, NL	2011	3
Conferences		
PhD retreat immunology (3I), Kameryck, NL	2009,2013	2
PhD retreat oncology (OOA), Texel, NL	2009	1
Tumor cell biology conference (KWF), Lunteren, NL	2009-2013	5
Dutch hematology conference (NVvH), Papendal, NL	2010-2014	5
Cell adhesion and migration in inflammation and cancer (NCMLS), Amsterdam, NL	2011	1
European heamatology congress (EHA), Amsterdam, NL	2012	1
Fibronectin, integrins, and related molecules (GRC) - Ventura Beach CA, USA	2013	2
Seminars		
Oncology seminar series (AMC)	2009-2014	4
Immunology seminar series (AMC)	2009-2014	7.5
Pathology seminar series (AMC)	2009-2014	4
Ruysch lectures (AMC)	2009-2014	0.5
Work discussions		
Weekly work discussion / journal club (LYMMCARE)	2009-2014	7.5
Weekly work discussion (Spaargaren/Pals/Guikema/Noesel)	2009-2014	7.5
Weekly work discussion (Spaargaren/Pals)	2009-2014	7.5
Weekly work discussion (Spaargaren)	2009-2014	7.5
Poster presentations		
PhD retreat immunology (3I), Kameryck, NL	2009	0.5
PhD retreat oncology (OOA), Texel, NL	2009	0.5
Cell adhesion and migration in inflammation and cancer (NCMLS), Amsterdam, NL	2011	0.5
European heamatology congress (EHA), Amsterdam, NL	2012	0.5
Fibronectin, integrins, and related molecules (GRC) - Ventura Beach CA, USA	2013	0.5
Oral presentations		
Hematology seminar (AMC)	2010-2013	2
Immunology seminar (AMC)	2011,2012	1
Tumor cell biology conference (KWF), Lunteren, NL	2011-2013	1.5
Oncology seminar (AMC)	2012	0.5
Pathology seminar (AMC)	2012,2012	1
Dutch hematology conference (NVvH), Papendal, NL	2012,2014	1
PhD retreat immunology (3I), Kameryck, NL	2013	0.5
Teaching		
Supervision of 3 master students	2011-2013	12
Practicum assistant, The Amsterdam International Medical Summer School (AMC)	2011	2

Publications by the author

Groen RW, **de Rooij MF**, Kocemba KA, Reijmers RM, de Haan-Kramer A, Overdijk MB, Aalders L, Rozemuller H, Martens AC, Bergsagel PL, Kersten MJ, Pals ST, Spaargaren M (2011). N-cadherin-mediated interaction with multiple myeloma cells inhibits osteoblast differentiation. *Haematologica* 96(11):1653-61

de Rooij MF, Kuil A, Geest CR, Eldering E, Chang BY, Buggy JJ, Pals ST, Spaargaren M (2012). The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* 119(11):2590-4

Chang BY, Francesco M, **de Rooij MF**, Magadala P, Steggerda SM, Huang MM, Kuil A, Herman SE, Chang S, Pals ST, Wilson W, Wiestner A, Spaargaren M, Buggy JJ, Elias L (2013). Egress of $CD19^{+}CD5^{+}$ cells into peripheral blood following treatment with the BTK inhibitor ibrutinib in mantle cell lymphoma patients. *Blood* 122(14):2412-24

Spaargaren M, **de Rooij MF**, Kater AP, Eldering E (2014). BTK inhibitors in chronic lymphocytic leukemia: a glimpse to the future. *Oncogene* 34(19):2426-36

de Rooij MF, Kuil A, Kater AP, Kersten MJ, Pals ST, Spaargaren M (2015). Ibrutinib and idelalisib synergistically target BCR-controlled adhesion in MCL and CLL: a rationale for combination therapy. *Blood* 125(14):2306-9

Thijssen R, Ter Burg J, van Bochove GG, **de Rooij MF**, Kuil A, Jansen MH, Kuijpers TW, Baars JW, Virone-Oddos A, Spaargaren M, Egile C, van Oers MH, Eldering E, Kersten MJ, Kater AP (2016). The pan phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor SAR245409 (voxtalisib/XL765) blocks survival, adhesion and proliferation of primary chronic lymphocytic leukemia cells. *Leukemia* 30(2):337-45

de Rooij MF, Kuil A, Kraan W, Kersten MJ, Treon SP, Pals ST, Spaargaren M (2016). Ibrutinib and idelalisib target B cell receptor- but not CXCL12/CXCR4-controlled integrin-mediated adhesion in Waldenström macroglobulinemia. *Haematologica* 101(3):e111-5

Nederlandse samenvatting

Bij een infectie kunnen de lymfeklieren flink opzwellen. In deze lymfeklieren worden de antigenen van het pathogeen (bacterie, virus, toxine, etc.) gepresenteerd aan B cellen die af en aan reizen van lymfeklier naar lymfeklier om deze antigenen te zoeken. Wanneer de B cellen met hun B cel receptor (BCR) een antigeen herkennen, krijgen ze een signaal om in deze lymfeklier te blijven en te gaan vermenigvuldigen. Vervolgens gaan ze hun BCR verder muteren en worden de B cellen geselecteerd die de BCR met de hoogste affiniteit voor het antigeen hebben verkregen. Uiteindelijk differentiëren ze in antilichaam producerende plasmacellen en in geheugencellen. De antilichamen binden aan het pathogeen, dat het onschadelijk maakt door middel van neutralisatie, complement activatie en/of activatie van fagocytose. Als het antigeen verdwenen is uit het lichaam worden de B cellen niet meer getriggerd en gaan de meeste B cellen dood, waardoor de lymfeklier weer hersteld. Een aantal geheugen- en plasmacellen blijven leven, zodat een tweede infectie met hetzelfde antigen gemakkelijk te bestrijden is.

In chronische lymfatische leukemie (CLL), mantelcel lymfoom (MCL), en Waldenström macroglobulinemia (WM) delen kwaadaardige B cellen ongecontroleerd in de lymfeklieren en beenmerg. Deze celgroei is niet helemaal autonoom, want als ze uit deze micro-omgeving gehaald worden (bv in een kweekflesje) delen ze totaal niet. In de lymfeklieren worden ze namelijk gestimuleerd door verschillende cellen; zoals T cellen, macrofagen en stromale cellen. Deze cellen produceren belangrijke groei- en overlevingsfactoren voor de B cellen. Daarnaast is de BCR ook van groot belang voor de overleving en het delen van de B cellen. Recente studies laten zien dat CLL en MCL cellen specifiek in de lymfeklieren actieve BCR signalering hebben. Voor sommige CLL subgroepen is zelfs opgehelderd welke antigenen de BCR herkennen.

De Amerikaanse farmaceutische bedrijven Pharmacyclics Inc. en Gilead Science hebben medicijnen ontwikkeld om BCR signalering te remmen, namelijk respectievelijk ibrutinib en idelalisib. Ibrutinib remt het enzym Bruton's tyrosine kinase (BTK), en idelalisib de delta isovorm van phosphoinositide-3-kinase (PI3Kδ). BTK en PI3Kδ zijn een essentiële schakels om het BCR signaal in de cel door te geven. Bij afwezigheid van PI3Kδ kunnen de cellen niet geactiveerd raken, en bij afwezigheid van BTK worden er zelfs helemaal geen rijpe B cellen gevormd. Het idee was dat door remming van BTK en PI3Kδ en dus van BCR-signalering de kwaadaardige B cellen zouden stoppen met delen, of zelfs dood zouden gaan. In diffuus grootcellig B cel lymfoom patiënten was dit daadwerkelijk het geval. In dit type lymfoom gaan de cellen direct dood door ibrutinib, doordat ze verslaafd zijn aan BCR signalering.

Echter, bij het testen van dit medicijn in CLL, MCL en WM patiënten deden ze een opmerkelijke bevinding: de lymfeklierzwelling werd weliswaar sterk verminderd maar de maligne cellen accumuleerde in het bloed. De cellen gingen dus niet direct dood, maar worden uit hun proliferatieve omgeving gemobiliseerd. In dit proefschrift hebben we dit mechanisme op moleculair en cellulair niveau onderzocht.

Wij hebben gevonden dat ibrutinib de BCR-geïnduceerde adhesie (hechting) van CLL, MCL en WM cellen remt. De retentie van CLL en MCL cellen in de lymfeklieren wordt dus net als bij normale geactiveerde B cellen gereguleerd door de BCR. Door dit te remmen verliezen de maligne B cellen hun houvast in de lymfeklieren en stromen ze met het lymfevocht mee naar de bloedbaan. Wij vonden geen directe cytotoxiciteit van ibrutinib en idelalisib op de CLL, MCL en WM cellen. Doordat deze cellen in de bloedbaan echter niet langer hun groei- en overlevingssignalen krijgen, zoals in de lymfeklieren, gaan ze op den duur dood. Door ibrutinib te combineren met idelalisib zien we zelfs een sterk synergistisch effect op remming van de adhesie. Dit betekent dat als patiënten behandeld worden met een combinatie van ibrutinib met idelalisib, de kwaadaardige cellen mogelijk beter uit de lymfeklieren gejaagd worden.

Of we op korte termijn CLL, MCL en WM kunnen genezen is nog niet te voorspellen. Ibrutinib als monotherapie kan in ruim 70% van de CLL, MCL en WM patiënten de ziekte sterk onderdrukken, maar complete genezing blijft meestal uit. Recent is ook gebleken dat veel lymfoompatiënten resistent worden tegen ibrutinib en idelalisib. We zijn er dus nog niet. Als beide drugs in combinatie gebruikt worden, wordt de kans op resistentie mogelijk kleiner. Doordat door dit onderzoek het werkingsmechanismen van ibrutinib en idelalisib is opgehelderd, kunnen in de toekomst slimme combinaties met andere medicijnen bedacht en toegepast worden om CLL, MCL en WM volledig te bestrijden.

Curriculum vitae

Martin de Rooij begon zijn laboratorium carrière in 1998 met de opleiding tot klinisch chemisch analist aan de ROC van Eindhoven. Om de opleiding af te ronden heeft hij een ministage gelopen in het klinisch chemisch en hematologisch laboratorium van longcentrum Dekkerswald te Groesbeek onder leiding van Hettie Sjabbens, en een lange stage bij de afdeling chemische endocrinologie van UMC St. Radboud te Nijmegen onder leiding van Henk J.W. Ariaens. Hierna heeft hij zich verder verdiept in de biochemie aan de Hogeschool van Arnhem en Nijmegen. Hiervoor heeft hij een onderzoeksstage verricht bij de afdeling moleculaire dierfysiologie van de Raboud Universiteit te Nijmegen onder leiding van drs. Dorien M. de Groot en Prof. dr. Gerard J.M. Martens. Hij ging hier op zoek naar het substraat van het eiwit neuroserpine. Vervolgens ging hij medische biologie studeren aan de Radboud Universiteit te Nijmegen. In zijn master heeft hij 2 onderzoeksstages verricht, de eerste bij de afdeling pathologie van UMC St. Radboud te Nijmegen onder leiding van dr. Léon C.L. van Kempen, waar hij de interactie van melanoom cellen met fibroblasten bestudeerde, en de tweede bij klinische chemie en hematologie van UMC Utrecht, onder leiding van dr. Martine J. Hollestelle en Prof. dr. Phillip G. de Groot, waarbij hij de rol van de von-Willebrand factor in acuut myocard infarct bestudeerde. In 2009 behaalde hij zijn doctoraal. Vervolgens heeft hij het zuiden des lands moeten verlaten om in de Randstad zijn promotietraject af te leggen aan de Universiteit van Amsterdam. Tot 2014 verrichtte hij hiervoor wetenschappelijk onderzoek in het laboratorium van dr. Marcel Spaargaren en Prof. dr. Steven T. Pals op de afdeling Pathologie van het Academisch Medisch Centrum te Amsterdam. Hij onderzocht daar het werkingsmechanisme van de nieuwe veelbelovende medicijnen ibrutinib en idelalisib tegen chronische lymfatisch leukemie, mantelcel lymfoom en Waldenström macroglubulinemia, dat in dit proefschrift beschreven is. Vervolgens heeft hij 2 jaar gewerkt in het lab van dr. Roderick L. Beijersbergen en Prof. Dr. René Bernards op het Nederlands Kanker Insitituut te Amsterdam. Hier heeft hij deels wetenschappelijk onderzoek verricht naar epigenetica in borstkanker, en deels geassisteerd in de robot- en screeningsfaciliteit. Begin 2017 is hij weer teruggekeerd naar zijn vorige werkgevers in het Academisch Medisch Centrum te Amsterdam, waar hij de kennis van beide onderzoeken combineert, en op zoek is gegaan naar nieuwe targets die betrokken zijn bij groei, overleving, adhesie en migratie van B lymfoom cellen door middel van genetische screens.

Dankwoord

Omdat mijn PhD-traject (en zelfs mijn voornaam) grotendeels met **Martine** overlapte, kan ik natuurlijk gewoon naar haar dankwoord refereren, dus zie: van Keimpema M. The FOX and the mutants 1:203-205.

Haha, dan wordt er helemaal geen hoofdstuk gelezen van dit boekje. Laat ik als eerste mijn copromotor **Marcel Spaargaren** en promotor **Steven Pals** bedanken voor het aannemen van mij als PhD-student, en de verdere begeleiding wat heeft geleid tot dit boekje. Van beide kanten lijkt dit goed bevallen, aangezien ik weer terug gekomen ben. Vervolgens wil ik **Annemieke Kuil** en **Anneke Kramer** bedanken, waarvan ik de meeste technieken geleerd heb, **Annemieke Kuil** nogmaals, en **Willem Kraan** voor de experimentele bijdrage aan dit boekje, en **Avital Amir** voor het reviewen van de introductie. Verder nog **Eric Eldering, Arnon Kater, Marie José Kersten, Christian Geest, Rachel Thijssen en Robbert Hoogeboom** die het mogelijk gemaakt hebben om patiënten materiaal te verkrijgen, wat heel belangrijk is om je bevindingen in goede tijdschriften te publiceren, en uiteraard **Joseph Buggy** en **Betty Chang** van Pharmacyclics Inc. die ons hebben gemotiveerd om weer aan BTK te gaan werken: het project dat compleet beschreven is in dit boekje. Als laatste wil ik nog de overige co-auteurs bedanken voor hun bijdrages, en **Nike Claessen** voor alle bestellingen en lab-management. En niet te vergeten **Eelco Roos** en **Wim van Est** voor de layout en kaft van dit boekje.

Hiernaast heb ik ook nog hulp gekregen van 3 master studenten, namelijk van **Marc Burgers, Guus de Wilde** en **Koen van de Ven**. **Marc**: we wachten nog steeds op je verslag!, veel succes met je geneeskunde studie. **Guus**: ben je een emotionele drinker als je van koud bier houd?, trouwens leuk dat je mijn paranimf wil zijn. **Koen**: jouw stagecijfer verraad natuurlijk al dat je uit de beste regio van Nederland komt! Daarnaast krijg ik hulp tijdens mijn verdediging van mijn paranimfen **Guus de Wilde** en **Linda Slot**. **Guus**: ook leuk dat je mijn student Wilde zijn. **Linda**: hoewel je helemaal aan de andere kant van Nederland vandaan komt als ik, hadden we toch een gezamenlijk Hyves vriend!, daarnaast hebben we nog veel meer gemeen, o.a. hebben we allebei in het publiek bij Ik hou van Holland gezeten (daarentegen ging jij dan weer wel naar huis met fantastische prijzen), de ideale paranimf dus.

Naast serieus onderzoek verrichten, zit er, zoals de functieomschrijving PhD-student al suggereert, een studenten kant aan. Daar had ik natuurlijk op alle niveau's al ervaring mee, van MLO, HLO, tot universitair, en nu dus ook op PhD niveau. Eigenlijk is het, hoe hoger het niveau, hoe meer er gedronken wordt. Op de MLO stages ging je gewoon altijd stipt om 5 uur naar huis, op de HLO en master

stages had je soms een borrel na je werk, en als PhD-student kan je er zelf altijd één organiseren als er niet genoeg borrels zijn. Soms met een reden, zoals je verjaardag (met dank aan **Leonie, Patrick, Miriam** en **Zemin** om in dezelfde week jarig te zijn als ik), kerstborrel (met dank aan **Per**; ik denk niet dat wij nog een kerstdiner mogen organiseren, omdat we laatst dubbel zoveel drank hadden besteld dan er op ging, ach het kwam dan wel weer ten goede aan de nieuwjaarsborrel), of bijvoorbeeld als er een goede literaire film in de bioscoop draait, als New Kids Nitro of Bro's Before Ho's, -deze films waren trouwens goed te combineren het nuttigen van de 010-se delicatesse: "De Kapsalon"-. Ook de afscheidsborrel van **Kinga** in het Oude Gasthuis is nog in mijn geheugen blijven hangen, vooral vanwege de schitterende foto's die ik het liefste hier in het dankwoord had willen plakken. Soms ook zonder goede reden, als bijvoorbeeld lekker BBQen op het Hoge Dijkse strand als het zonnetje schijnt, of meters bier halen in de legendarische Epstein-Bar. De mooiste herinneringen van de Epstein-Bar waren altijd de dag erna, als iemand (eigenlijk iedereen wel, behalve ik :p) niet meer zo goed konden fietsen of in de bus of trein in slaap waren gevallen, en natuurlijk **Richard**, die met **Leonie's** jas aan naar huis was gegaan. Een ander slecht idee was om op stap te gaan in Groningen. Met weinig respect naar Brabanders toe was dit gepland op de dag na carnaval. Ach een dagje meer of minder feesten, geen probleem. En dat was het ook niet. Alleen de dag erna werken zat er voor mij niet meer in, 24 uur slapen wel. Dat borrelen houdt je trouwens wel lekker jong. Gelukkig heb ik me nooit zo verlaagd tot het infantiele gedrag als naambordjes verwisselen, computer beeldschermen omwisselen, toetsenbord knopjes verwisselen, etc :p.

Omdat ik ergens diep in de provincie ben gaan wonen, had ik na half 12 geen vervoer meer naar huis (ja 's nachts dan slapen de paarden en ezels natuurlijk!). Omdat de kroegen in Amsterdam tot vroeg in de ochtend open zijn, wil ik **Martine, Linda, Harmen, Marcel J, Anand, Elena, Jesper, Martin, Iris** en **Erik** heel erg bedanken voor de gastvrijheid om van jullie logeerbed gebruik te hebben mogen maken. **Linda** ook nog bedankt voor de heerlijke versgebakken broodjes, en **Martine** voor de heerlijke pompoenensoep en de kakkerlakkensalade!

Van al dat borrelen krijg je natuurlijk een hartstikke dikke buik, dus als oplossing daarvoor moest er wel wat gesport worden op de UvA-sportscholen. Echter, als we naar de sportschool in het Science Park gingen, schoten we soms het doel een beetje mis, en gingen we hooguit een kwartiertje sporten, en daarna lekker chillen in de sauna, en vervolgens een goeie burger eten bij de Polder, met een lekker triple'tje erbij. Voor deze gezelligheid wil ik **Martine, Katarina, Lotte, Linda** bedanken, en vooruit, ook de heren: **Wilco, Hans** en **Marcel J**. Daarnaast hebben ik en **Wilco**, en ik snap nog steeds niet waarom, met **Martine, Leonie** en **Anneke** ons Zumba talent geshowed, man man, man, wat duurde dat uurtje lang!, ik hoop

dat mijn verdediging beter zal gaan. Qua andere sportiviteiten was ik een belangrijke speler tijdens de jaarlijkse AMC hockeytoernooien, niet zozeer vanwege mijn fraaie flatsen (meestal deed ik niets eens mee), maar wel om op tijd de bierkannen te vullen, zodat niemand uitgedroogd raakt. Volgens mij zijn we daardoor ook wel eens een keertje niet laatste geworden. Ook heb ik ontzettend veel verstand van voetbal. Dit resulteerde zelfs een keer in een prijs tijdens de EK/WK pools (met dank aan **Sander, Harmen** en **Robbert** voor de organisatie). Ik had zo weinig punten, dat ik wel een kenner moest zijn. Als ik alles omgekeerd had ingevuld was ik zeker eerste geworden. Nu we het toch over voetbal hebben, we zijn ook twee keer naar de Amsterdam Arena geweest. Een keer naar jong AJAX – jong PSV, en een keer naar de Johan Cruijff Schaal AJAX – PSV. In beide gevallen had natuurlijk de beste club gewonnen!, dat is ook logisch, want de winnaar heeft altijd als beste gespeeld!

Een ander uitje om onverwachte resultaten even te laten bezinken was een weekendje Pinkpop. Eigenlijk houd ik helemaal niet zo van die 3FM muziek. Al vraag ik me wel heel erg af of dat geruis nou wel muziek is, of dat die zender niet zo'n goeie ontvangst heeft. Op Pinkpop kwam ik erachter dat die herrie wordt veroorzaak door heel hard op een gitaren te raggen, en snoeihard op trommels te rammen. Om dit wetenschappelijk te bevestigen zijn we 3 keer (n = 3) naar Pinkpop geweest. Ik moet zeggen, sommige optredens zal ik nooit meer vergeten, als The Prodigy, Rammstein en Linkin Park! En ook niet te vergeten P!nk, die in een string stond op te treden met: "What do you want from me?". De camping was ook kei gezellig, met stevig knakworst met bier ontbijt, en lekker niet douchen. Voor deze gezelligheden wil ik **Tessa** en **Wilco**, **Martine** en **Maarten, Katarina** en **Peter, Stijntje** en **Marcel J**, **Lorenza** en **Hans**, **Jeroen (B&G)** en **Jesper** bedanken. Uiteindelijk ben ik 3FM toch niet gaan waarderen, en lukte me het soms om de radiozender in de celkweek te veranderen naar Slam FM of Q-music.

Daarnaast hadden we ook officiële jaarlijkse dagjes uit, wat belangrijk is voor team building. Soms met de hele pathologie groep, en soms met alleen met de B cellen. Hoogtepunten waren het langlaufen in de Ardennen, als ruim 50 Hell's Angels op de Solex door de duistere havens van Rotterdam rijden, en de kanotocht naar Oost-Knollendam. Bij dat laatste kan ik me nog herinneren dat ik hier met **Sander** een wedstrijdje had, niet wie het eerste aan de overkant is, maar om elkaars kano te laten zinken! Daarnaast moet ik ook nog de hond van **Annemieke** bedanken om mijn hand er niet af te bijten, anders had ik dit proefschrift nooit bij elkaar kunnen pipetteren. Ook hadden we elk jaar een fotowedstrijd, met lekkere gebakjes erbij :). Nadat ik de fotografen **Wim** en **Eelco** door had (je moet juist een foto met zo min mogelijk kleur maken), is het mij met **Linda** ook een keer gelukt om die prijs te winnen (door AMC met bacteriën in een agarplaat te schrijven).

Uitjes met een wetenschappelijk karakter waren er ook, namelijk congressen en PhD-retraites. Een van mijn eerste was de oncologie retraite op het exotische Texel. Daarvoor had ik een heel bijpassende poster gemaakt, met een zonnetje en palmbomen. Helaas werd hij niet door iedereen gewaardeerd, anders had ik hem zo als kaft voor dit proefschrift kunnen gebruiken. Een ander hoogtepunt was mijn TV debuut op oncologie TV, waarvoor ik gevraagd was bij het hematologie congres in de RAI. Andere hoogtepunten zijn natuurlijk de foute Bioké disco's bij Tumor Cell Biology (c.k.a. Cancer Biology) in Lunteren, biertjes drinken bij het kampvuur bij de immunologie retraite in Kamerik, en hoe slecht dan ook, partycrashen bij de disco van het NVVI-congres in Noordwijkerhout, want daar woonde ik toch om de hoek :p. Het toppunt was natuurlijk het integrine congres in Ventura Beach in de buurt van Los Angeles. Zeeleeuwen, dolfijnen en walvissen gespot, de getto's (ik had het me heel anders voorgesteld) van Hollywood bezocht, en een stukje van de beroemde pacific coast highway gereden. Hiervoor wil ik mijn baas **Marcel S** nog heel erg bedanken. Daarnaast wil ik **Martine**, **Jeroen G** en **Carel** bedanken, die toevallig op hetzelfde tijdstip naar Keystone waren, om de Amerika reis samen af te sluiten met een gezellig weekendje New York, New York!

Mensen die ik niet wil bedanken zijn **Yvonne, Marthe, Hildo, Naomi** en **Jerry** :p. Dit natuurlijk omdat ze er nog niet waren tijdens mijn PhD tijd. Ik wens jullie heel veel succes met jullie PhD. Volgens mij heb ik van de B cellen **Chiel, Alex, Anna** en **Thera** nog niet specifiek genoemd, nou bij deze, en **Timon** en **Mahnoush** ook nog niet, van horen zeggen waren jullie een heel leuk 'koppel', veel succes met jullie PhD's, en **Harmen, Zemin, Katarina** en **Linda** natuurlijk ook.

Ten slotte wil ik naast mijn hele lieve vriendin **Anne Hafkemeijer**, nog ________* in het speciaal bedanken voor de prettige werksfeer, interessante wetenschappelijke discussies, gezellige koffiepauzes, legendarische borrels, omdat je meer PSV spelers kent dan ik, of gewoon omdat het kan.

*Vul hier je naam in.