INAUGURAL DISSERTATION

for

obtaining the doctoral degree

of the

Combined Faculty of Mathematics, Engineering and Natural Sciences

of the

Ruprecht – Karls – University

Heidelberg

Presented by

Michael Rudi Otto Persicke, M.Med.Sc.

born in Eberswalde-Finow, Germany, 23rd August 1989

Oral examination: 8th May 2024

THE ROLE OF THE INOSITOL PHOSPHATASE SHIP1 IN B-CELL RECEPTOR SIGNALLING AND CLL CELL ADHESION

Referees:

Prof Dr Stefan Wiemann

Prof Dr Peter Lichter

‚When he [the scientist] designs his experiments or executes them with devoted attention to the details he may say to himself. "This is my composition; the pipette is my clarinet". And the orchestra may include instruments of the most subtle design. To others, however, his music is as silent as the music of the spheres. (…) The scientist has in common with the artist only this: that he can find no better retreat from the world than his work and also no stronger link with the world than his work.' Max Delbrück

Declaration

The work and results of this dissertation were performed and obtained from June 2017 to September 2022 under the supervision of PD Dr Daniel Mertens at the Division of Molecular Genetics and the junior research group Mechanisms of Leukemogenesis at the German Cancer Research Center (DKFZ), Heidelberg, Germany. Parts of the methods, results and discussion of this study have been published in a joint manuscript Wolf, Maus, Persicke et al. Contributions of authors other than myself are indicated within the figure legends. I hereby declare that I have written and submitted this dissertation myself, and in the process have not used any other sources or materials other than those indicated. I declare that I have not applied to be examined at any other institution, nor used the dissertation in this or any other form at any other institution as an examination paper or submitted it to any other faculty as a dissertation.

Michael Persicke

Abstract

Chronic lymphocytic leukaemia (CLL) is a mature B-cell malignancy with accumulating B-cells in the peripheral blood and secondary lymphoid tissue. CLL cell proliferation is highly dependent on B-cell receptor (BCR) signalling and microenvironmental support. BCR pathway-activated genes are most prominently expressed in CLL cells in lymphatic tissue. Lymphatic tissue is at the same time the major site of CLL cell proliferation. The crucial signalling node of the BCR pathway is PI3K. PI3K activity is countered by the inositol phosphatases Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN (PTEN) and Src homology domain 2-containing inositol-5'-phosphatase 1 (SHIP1). PTEN reverses PI3K activity by enzymatically converting PIP_3 to $PI(4,5)P_2$. In contrast, SHIP1 catalyses the removal of another phosphate residue, thereby degrading PIP_3 to phosphatidylinositol-3,4-bisphosphate.

Based on the analysis of a developed model to depict interconnections between signalling factors and to predict beneficial combination treatments, further regulating signalling factors were included in a panel of BCR stimulation dynamics. Through the present work, the impact of PTEN and SHIP1 on stimulation-dependent BCR signalling in CLL and on the adhesion capacity of CLL cells to cells of the microenvironment were investigated. As CLL cells accumulate in two distinct niches in the patients, the fractions of distinct adhesion capacity were separated in flow cytometry measurements and microscopically imaged in a modified and CLL-optimised adhesion under flow assay.

The results of this work indicate that PTEN and SHIP1 control BCR signalling in distinct stimulation conditions. SHIP1 could be shown to be highly relevant in determining the (re-)adhesion capacity of peripheral CLL cells. Furthermore, the von-Hippel Lindau factor was observed to regulate the adhesion of CLL cells and directed future work towards integrins on the CLL cell surface.

Taken together, the present work sheds light on the potential of SHIP1 and related factors as targets for future combination treatment approaches.

Zusammenfassung

Chronisch lymphatische Leukämie (CLL) ist ein B-Zell-Malignom differenzierter B-Zellen, bei dem B-Zellen im peripheren Blut und im sekundären lymphatischen Gewebe akkumulieren. Die Proliferation der CLL-Zellen hängt in hohem Maße von der Signalübertragung durch den B-Zell-Rezeptor (BCR) und der Mikroumgebung ab. Genexpressionsdaten haben gezeigt, dass der BCR-Signalweg bei CLL-Zellen im lymphatischen Gewebe, dem Ort der CLL-Zellvermehrung, am stärksten aktiviert ist. Der entscheidende Signalknoten des BCR-Signalwegs ist PI3K. Die PI3K-Aktivität wird durch die Inositol-Phosphatasen Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN (PTEN) sowie die Src homology domain 2-containing inositol-5'-phosphatase 1 (SHIP1) gehemmt. Während PTEN die PI3K-Aktivität direkt umkehrt, um $PI(4,5)P_2$ zu erzeugen, katalysiert SHIP1 die Umwandlung von $PI(3,4,5)P_3$ in Phosphatidylinositol-3,4-bisphosphat.

Auf der Grundlage der Analyse eines entwickelten Modells zur Darstellung der Interkonnektivität zwischen BCR-Signalfaktoren und zur Vorhersage vorteilhafter Kombinationsbehandlungen wurden weitere regulierende Signalfaktoren in ein Panel der durchflusszytometrischen Analyse der BCR-Stimulationsdynamik aufgenommen. In der vorliegenden Arbeit wurden die Auswirkungen von PTEN und SHIP1 auf die stimulationsabhängige BCR-Signalaktivität bei CLL und auf die Adhäsionsfähigkeit von CLL-Zellen an Zellen der Mikroumgebung untersucht. Da CLL-Zellen bei den Patienten in zwei verschiedenen Nischen akkumulieren, wurden die Fraktionen mit unterschiedlicher Adhäsionskapazität in durchflusszytometrischen Messungen getrennt und in einem modifizierten und für CLL optimierten Adhäsionsassay mikroskopisch abgebildet.

Die Ergebnisse dieser Arbeit deuten darauf hin, dass PTEN und SHIP1 die BCR-Signalaktivität unter verschiedenen Stimulationsbedingungen kontrollieren. Es konnte gezeigt werden, dass SHIP1 bei der Bestimmung der (Re-)Adhäsionsfähigkeit von peripheren CLL-Zellen von großer Bedeutung ist. Darüber hinaus wurde festgestellt, dass der von-Hippel-Lindau-Faktor die Adhäsion von CLL-Zellen reguliert, was künftige Arbeiten auf Integrine auf der Oberfläche von CLL-Zellen lenkt.

Insgesamt wirft die vorliegende Arbeit ein Licht auf das Potenzial von SHIP1 und verwandten Faktoren als Ziele für künftige Kombinationstherapieansätze.

1 Table of contents

List of Figures

List of tables

List of abbreviations (A-Z)

AKT	Protein kinase B
BCR	B-cell receptor
BTK	Bruton's tyrosine kinase
CLL	Chronic lymphocytic leukaemia
CR	Complete response
ERK1/2	Extracellular signal-related kinases 1 and 2
HSC	Hematopoietic stem cell
IGHV	Immunoglobulin heavy chain variable region
M-CLL	Mutated IGHV CLL
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PFS	Progression-free survival
Phospho-flow	Phospho-specific flow cytometry
PI3K	Phosphoinositide 3-kinase
PLCy2	Phospholipase C, gamma 2
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN (Phosphatase and tensin homologue)
PTPs	Protein tyrosine phosphatases
SHIP1	SH2 domain-containing inositol 5'-phosphatase 1 (Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1)
SYK	Spleen tyrosine kinase
U-CLL	Unmutated IGHV CLL
VHL	von Hippel-Lindau disease tumour suppressor

1 Introduction

1.1 Aberrant signalling in cancer

Cancer is a disease of uncontrolled survival and proliferation of the body's cells. Cancer is defined by several characteristics that include overproliferation, escaping apoptosis and migration outside of the respective cellular niche[1,2]. In cells outside of their niches, apoptosis is induced through homeostatic controls. These homeostatic controls can only be escaped through a malignant transformation with genetic and epigenetic alterations[3]. Genetic and epigenetic alterations often affect signalling pathways that control cellular growth, division, apoptosis, differentiation, and motility. Altered signalling pathways may also affect non-malignant cells of the tumour microenvironment (TME), for example pathways that regulate angiogenesis and inflammation[3]. The recruitment of inflammatory factors and angiogenesis is controlled by the PI3K/AKT signalling pathway[4,5] among others. The PI3K/AKT signalling pathway is one of the pathways most frequently affected by mutations driving cancer progression[6]. Mutations aberrantly activating the PI3K/AKT pathway may be present in several genes that include PI3KCA, phosphatases Phosphatase and tensin homolog deleted on chromosome ten (PTEN), AKT, TSC1, and mTOR[7]. Several strategies to inhibit the tumour promoting nodes of PI3K/AKT signalling networks were developed. The PI3K inhibitor idelalisib and the BTK inhibitor ibrutinib are the most prominent examples of clinically approved inhibitors while *e.g.* for BTK two more have been approved by the FDA[8,9]. The recent advancements in the clinics by applying PI3K/AKT pathway inhibitors have improved clinical parameters such as overall survival (OS). The current challenges consist of a better understanding of cancer type specificity, appropriate combination treatments and beneficial patient stratification. Patient stratification as well as in-depth analysis of the signalling network interconnections have therefore come into focus of current research[3,8,10].

1.2 The tumour microenvironment is a hallmark of cancer

In the next generation of *the hallmarks of cancer* the TME was considered crucial for tumour formation[2]. As the tumour develops in a complex surrounding tissue, three types of cells have been associated with cancer progression: infiltrating immune cells, angiogenic vascular cells, and cancer-associated fibroblasts (CAFs)[11]. CAFs contribute to the establishment of a tumour-

promoting environment, while normal fibroblasts contribute to tumour suppression[12]. The tumour-promoting environment also consists of a milieu of chronic inflammation. Chronic inflammation drives mesenchymal stem cell transition towards CAF formation[13]. Stromal fibroblasts and other tumour-promoting cells of the TME deliver proliferative signals, induce angiogenesis[14] and metabolic reprogramming, activate or facilitate migration, and assist in evading growth suppressors and immune destruction[11]. While the immune control of cancer cells is evaded, CAFs further support the tumour-promoting niche of chronic inflammation through NF-kB signalling[15]. The signalling pathways involved in TME interaction have moved into research focus to find new therapeutic options.

1.3 Chronic Lymphocytic Leukaemia, an exceptional malignancy

CLL is in many respects an exceptional malignancy. CLL cells remain in cell cycle arrest while circulating in the peripheral blood. The clinical course is highly variable including patients subgroups that do not require therapy and remain without further symptoms for decades while other subgroups suffer from rapid progression and high risk[16].

1.3.1 Epidemiology, diagnosis and clinical characteristics

In the Western world, CLL has the highest diagnosis rate among adult leukaemia with an incidence of 4.1 per 100 000 inhabitants[17]. This leukaemia usually affects the elderly, the median age at diagnosis being 72 years[18], and the initial disease development may occur over a long period of time[19,20]. During the disease development, blood count of CD5+ CD19+ B-cells increases as well as the presence of these cells in secondary lymphoid tissue[9]. At diagnosis, blood count surpasses 5×10^9/L CLL cells[21] with characteristically small cells in the blood smear. These CLL cells are CD19, CD20, CD23, and CD5 positive[22–24]. The disease staging follows the Rai (0, I/II, and III/IV) or Binet (A, B and C) systems. Both systems group the patients into low, intermediate, and high risk according to the levels of lymphocytosis, lymphadenopathy, anaemia, and thrombocytopenia[25,26]. Additional biomarkers of prognostic value include ZAP70, CD38, CD49d which are associated to poorer outcome[27–30]. The most prominent patient stratification is based on the degree of somatic mutation in the immunoglobulin (Ig) heavy chain gene (IGHV). IGHV unmutated CLL (U-CLL) is associated with more aggressive CLL and lower overall median survival[27,31,32] than CLL with >2%

germline difference IGHV (mutated IGHV, M-CLL). M-CLL also differs from U-CLL in the underlying genetic lesions, clonal evolution, epigenetic dysregulation and hyperactive signalling pathways[33–35]. These differences have led to the assumption that U-CLL and M-CLL cells relate to a different cell type as cell of origin[36]. The different characteristics of U-CLL and M-CLL cells also span to the kind of interactions established with the cells of the TME[33,37].

1.3.2 The development and function of normal B-cells

The B-cell formation is initiated in the bone marrow (BM). In the BM, hematopoietic stem cells (HSCs) differentiate into common lymphoid progenitor cells[38]. The lymphoid progenitor cells undergo epigenetical changes that lead to differentiation into the B or T cell lineage[39,40]. In the B-cell lineage, a chronologically tightly controlled process of Ig H and L chain loci rearrangements further transition the pro-B-cells to pre-B-cells[41]. In pre-B-cells, a complete and unique B-cell receptor (BCR) locus is assembled[42,43]. Of the resulting expressed BCRs, 50%-75% are specific to self-antigens[44,45]. A self-reactive BCR leads to further receptor editing[46,47], anergy[48], induction of apoptosis[49,50] or exclusion from follicular niches[51]. Immature B-cells migrate from BM to B-cell follicles in secondary lymphoid tissue, i.e. lymph nodes (LNs) and spleen. In the lymphoid tissue, antigen binding leads to B-cell activation. Activated B-cells engage into a coordinated crosstalk with different cell types to form the germinal center (GC)[52,53]. Within these GCs, stromal cells produce gradients of CXCR4 and CXCR5 that establish the GC light and dark zones[54]. In the GC dark zone, B-cells undergo proliferation at a rate unparalleled in human tissue and diversify the immunoglobulin variable region (IgV) genes by somatic hypermutation. Together, hypermutation and proliferation lead to an extreme differentiation of the BCR, thereby generating mutant clones increasing the antigen recognition repertoire by several magnitudes covering very variable affinities for the immunizing antigen. The affinity of GC-derived B-cells increases, a phenomenon called affinity maturation. Affinity maturation is possible through an effective selection process that excludes inferior or self-reactive IgV mutants in the GC light zone through positive selection of and thereby proliferative outcompetition by high-affinity BCRs. High-affinity BCR-expressing B-cells are fast and effectively produced in the specialized GC microenvironment where rapid movement and close interaction of different cell types is facilitated[52,53]. The activated B-cells undergo several iterative rounds of further mutation and affinity selection and differentiate into memory B-cells[52] or antibody-secreting plasma cells[55,56]. These memory B-cells and plasma cells may also have switched Ig classes, *i.e.* replacing IgM and IgD with IgA, IgG or IgE[57]. Further BCR

recruitment is not necessary to maintain memory B-cells. Mature B-cells including follicular (B-2) and marginal zone B-cells in the secondary lymphoid tissue almost exclusively originate from the GC reaction and belong to the adaptive immune system[58]. In the peritoneal and pleural cavities, described as part of the innate immune system, and independent from T cell interaction, another type of mature B-cells was found. These so-called B-1 cells are CD5+. The CD5+ non-malignant B-cells detected in human PB are still poorly understood in terms of their origin and function[59].

1.3.3 CLL origin and development

As mentioned in the section 1.3.1, U-CLL and M-CLL appear to develop from different cells of origin[36] which has not been finally resolved as the exact normal B-cell counterpart remains under controversial debate[60–62]. Initial genetic lesions that result in CLL formation were observed in HSCs[63] and in multipotent progenitor cells of CLL patients[64]. As CLL cells express CD5 on the cell surface, the hypothesis arose that the B-1 cell lineage harbours the cell of origin[65]. But the cell of origin, according to recent findings based on transcriptomics and BCR signalling analysis, may rather be derived from a marginal zone B-cell[66,67]. Marginal zone B-cells can express either mutated or unmutated IGHV. The proportion of IGHV-mutated and –unmutated marginal zone B-cells differs between immunisation status and different anatomic sites[68] reaching 70%-80% mutated IGHV in the spleen[69]. In the spleen, the marginal zone is located at the outermost layer of the white pulp. Outside the white pulp, marginal zone-like tissues exist in tonsil subepithelial regions, dome regions of Peyer patches, and in the subcapsular part of LNs[70]. Marginal zone B-cells are characterised by IgM^{high} IgD^{low} expression and respond to bacterial polysaccharides independently from T cells[71,72]. Independent from antigen encounter are also occasional IGHV mutations and class switch recombinations occurring in marginal zone B-cells[73,74]. These CD27+ marginal zone B-cells have also been observed to share a similar gene expression profile with CLL cells, irrespective of the IGHV mutation status[36,67]. The IGHV mutation status was associated to different DNA methylation patterns. Analysing the DNA methylation patterns, U-CLL cells were observed to cluster with CD5+ naïve B-cells and M-CLL cells with memory B-cells[35]. CLL cells may also derive from a continuum of B-cell developmental stages, another DNA methylation analysis indicated[75]. The development of CLL from a non-malignant B-cell occurs through a multistep process of genetic and epigenetic alterations, clonal selection and expansion, escape from homeostatic controls, and microenvironmental survival and proliferation signals. A precursor of CLL is the monoclonal B-cell lymphocytosis that may remain for years before CLL symptoms develop[20]. The malignant transformation of CLL appears to be supported by antigen-driven

selective pressure as both U-CLL and M-CLL express highly restricted and biased BCR repertoires[76–78]. Stereotyped BCRs can be found in approximately one third of CLL patients, reflecting similar genetic, epigenetic and clinical characteristics[76,77,79]. Characteristically, U-CLL BCRs show low affinity and poly-reactivity or self-reactivity while M-CLL BCRs are usually oligo- or mono-reactive, likely high affine to exogenous antigens[37,80].

1.3.4 Genetic alterations

CLL is a malignancy that sporadically develops in elderly patients. The few patients with familial predisposition usually bear one or several mutations in over 40 CLL-associated genetic loci. While these loci are located in open chromatin, their role in CLL pathogenesis is poorly understood[81–83]. Using whole-exome and whole-genome sequencing, the genetic landscape of CLL has been studied extensively. The main genetic alterations, unlike in other cancer entities, do not appear to be restricted to a small set of driver mutations but span a broad range of genes[19,84,85]. In M-CLL there are on average slightly more somatic mutations detected than in U-CLL but CLL in general has a lower mutational burden when compared to solid tumours or other haematological malignancies[6,19,84]. The combination of genetic and epigenetic alterations with chromosomal aberrations, tumour suppressor inactivation, oncogene hyperactivation, and dysregulated miRNA expression allows further predictive and prognostic patient stratification[9,16]. Patient stratification has been proposed to follow a hierarchical model based on chromosomal abnormalities. Cytogenetic alterations are detectable in approximately 80% of CLL patients[86]. With >50% of CLL cases, the deletion 13q14.3 displays the most frequent genetic lesion and at the same time predicts a favourable course of the disease[86,87]. The 13q14.3 deletion affects the loci of the DLEU2-mir-15a/16-1 cluster which control apoptosis and genes of the cell cycle control[88,89]. The second-most frequent lesion is the deletion 11q. Deletion 11q is present in <20% of CLL patients and associated to resistance to chemotherapy and overall poor prognosis. The poor prognosis derives from frequently associated alterations of ataxia telangiectasia mutated (ATM), a crucial factor in the DNA repair machinery. Loss of a functional DNA damage response through genetic loss or dysfunction of ATM or TP53 leads to genomic instability[86,90]. Another lesion affecting approximately 15% of CLL patients is trisomy 12. Trisomy 12 is associated with a higher risk of developing Richter transformation. Richter transformation is the pathogenesis of a high-grade lymphoma in CLL or small lymphocytic lymphoma[91,92]. CLL with trisomy 12 is also more likely to develop secondary tumours but the full mechanism on how trisomy 12 drives CLL pathogenesis is still to be resolved[93,94]. Another prominent lesion is the deletion of 17p, found in <10% of CLL

patients. Deletion 17p involves TP53 inactivation. TP53 inactivation as mentioned before causes genomic instability[19,86].

Genomic instability can also be caused by somatic mutations, *e.g.* again involving TP53 and ATM pathways. Other pathways commonly affected by somatic mutations in CLL are RNA splicing, chromatin modification, Notch signalling, inflammatory response, and B-cell activity pathways[19,84,85,95]. Some somatic mutations are of prognostic value, e.g. mutations affecting NOTCH1 or SF3B1[96]. SF3B1 but also TP53 mutations also serve as predictive markers for disease progression. During disease progression and after initial treatment success, subclones bearing such driver mutations expand and require treatment strategy changes[9,19].

1.3.5 Treatment

Treatment of CLL is only initiated when an active, symptomatic disease is diagnosed. An early stage disease without symptoms is monitored without treatment as treatment does not provide any benefit except for (rapidly) progressing disease[9,24]. When disease progression or symptoms are diagnosed, treatment is initiated. Treatment options depend on the age, patient fitness and a set of prognostic factors[9,97]. Younger patients may benefit from allogeneic stem cell transplantation, the only to-date therapy considered curative but challenged by limited donor availability, graft-versus-host disease and immunosuppression[98,99]. For decades, standard treatment for CLL was the application of alkylating agents with chlorambucil being the gold standard[9,100]. Compared to chlorambucil, treatment with the purine analogue fludarabine resulted in more remissions and more complete responses (CRs) without improving overall survival (OS)[101]. The treatment of several B-cell malignancies including CLL was significantly improved when, in 1998, CD20-targeting antibodies became available[102,103]. CD20 is a membrane protein expressed in mature B-cells where its function is yet to be investigated though suspected to act as a calcium channel[104]. The combination of anti-CD20 therapy with chemotherapeutics has led to the introduction of chemoimmunotherapy to CLL treatment improving both OS and progression-free survival (PFS) and thereby the combination of fludarabine, cyclophosphamide, and rituximab became the new first-line therapy standard in a large subset of patients[9,105]. Another subset of patients, including active disease U-CLL as well as Binet C or Rai III-IV, benefit from more recently developed BCR inhibitors as front-line therapy and relapsed or refractory patients in second line[9,24]. The importance of the BCR in CLL pathogenesis is explained in section 1.3.6. In short, the treatment in CLL was revolutionised by small molecule inhibitors of BCR signalling pathway kinases[16,106]. The most prominent kinases are Bruton's tyrosine kinase (BTK) and Phosphatidylinositol-3 kinase delta

(PI3Kd), being inhibited *e.g.* by the compounds ibrutinib and idelalisib, respectively[107,108]. Another small molecule inhibitor, venetoclax, targets B-cell lymphoma 2 (BCL-2)[109] and has been approved for second-line treatment of relapsed and refractory CLL, showing improved response rates especially in del(17) patients[110,111]. During the last years, also immunotherapies and immune checkpoint inhibitors were tested in clinical trials[112–114]. While the clinical trials using CAR T cells against CD19 led to durable remissions[114], immune checkpoints as single agents failed to improve therapy outcome[112]. The therapy outcome of combination treatments of immune checkpoint blockade with kinase inhibitors is investigated currently or has been recently (NCT03331198, NCT02329847, NCT02332980). In general, the largest efforts in current clinical trials are made to determine the benefit of fixed duration combination therapies compared to monotherapies especially in the light of BCR signalling inhibitors, such as BTK inhibitors. BTK and BCL2 inhibitor double-refractory disease is still in urgent need of alternative therapies.

1.3.6 BCR signalling

In B-cells, proliferation, selection and differentiation are promoted upon BCR antigen engagement[115]. The antigen is engaged through specific Ig and Ig-a/Ig-b heterodimer that compose the BCR. BCR binding to the antigen leads to the cytoplasmic phosphorylation of immunoreceptor tyrosine-based activation motifs of the Ig-a/Ig-b tails[116]. This phosphorylation of the Ig-a/Ig-b tails, BCR oligomerisation and microcluster growth initiate the signalling cascade via docking and protein phosphorylation of the Spleen tyrosine kinase (SYK)[117,118], Phospholipase C, gamma 2 (PLCγ2) and Phosphoinositide 3-kinase (PI3K)[119]. Phosphorylated SYK, PLCγ2, and PI3K signal to calcium mobilization[120] and membrane localization of Protein kinase B (AKT) and Bruton's tyrosine kinase (BTK)[121–123]. AKT and BTK amplify the signal towards the transcription factors NF-ΚB and NFAT[124,125] and activate the extracellular signal-related kinases 1 and 2 (ERK1/2) pathway[126]. In CLL cells, the BCR pathway was the most prominently activated in samples from lymphatic tissue, the site of CLL cell proliferation[127,128], and was associated with poor prognosis[129]. Poor prognosis subgroups are also associated with unmutated IGHV status, thus classified based on BCR characteristics. As mentioned in section 1.3.3, U-CLL and M-CLL show different levels of affinity and specificity[37,80,130]. The specificity of BCRs is in many cases identical among unrelated CLL patients[131,132], indicating that these stereotype BCRs recognise common antigen(s). It is a common trait in many malignant cell types that excessive proliferation is linked to hyperactive PI3K/AKT signalling[3]. In CLL, the BCR signalling pathway is a central factor in the

pathomechanism[133,134] and has thus become one of the main targets in CLL treatment[106,108,135,136]. CLL treatment has benefited from this approach through the development of new therapeutics inhibiting BCR-related kinases such as BTK[107,136], PI3K[108], LYN[137], or SYK[138,139]. Successful inhibition of the BCR leads to LN and spleen shrinkage as well as initial lymphocytosis in the PB. This lymphocytosis is most likely a result of the mobilisation of CLL cells from the secondary lymphoid organs to the periphery[140–142]. Despite the great benefit of these kinase inhibitors, resistances and other counter indications still produce challenges for the treatment of CLL[143–146]. These challenges can be tackled by different approaches. One approach is the further characterisation of available inhibitors in terms of optimal sequencing and combination strategies[9]. Combination strategies promise great potential as the inhibition of several signalling nodes synergistically silences BCR signalling and cannot be circumvented in healthy B-cells or CLL cells regardless of IGHV subgroup (Figure 1)[10]. Resistance to treatment may occur through clonal evolution[145], microenvironmental protection[143] or mutations in the targeted kinase or downstream effector genes[144,146]. Thus, alternative strategies are required for refractory CLL cases. Advances in targeting the opponents of kinases, phosphatases[147,148], have raised the interest in these effectors with mainly inhibitory function.

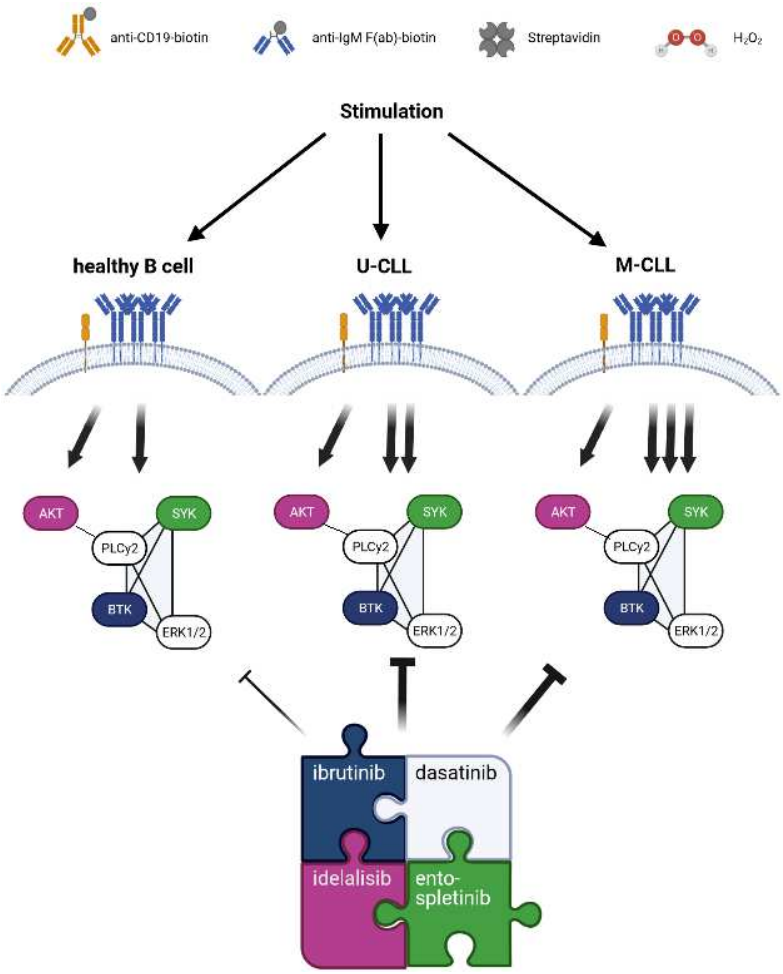


Figure 1 The BCR signaling pathway in healthy B-cells as well as U-CLL and M-CLL cells remains stable with or without BCR kinase inhibitor treatment. *The stimulation with crosslinking of a-CD19 and a-IgM antibodies through biotin-streptavidin interaction resulted in different degrees of BCR pathway activation between healthy B-cells, U-CLL and M-CLL cells. Different kinase inhibitor combinations reduced the phosphorylation levels of BCR pathway components beyond additive effects. The graph was taken and modified from Wolf, Maus, Persicke et al. 2022 originally created by myself*[10].

1.3.7 Inositol phosphatases

The central intracellular second messenger molecule in the BCR signalling pathway is phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 is released from PI3K by phosphorylating phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$). $PI(4,5)P_2$ is an abundant molecule at the plasma membrane and locally enriched at the Ig tails in quiescent B-cells[149,150]. In quiescent B-cells there is no PIP_3 detectable. PIP_3 recruits AKT and BTK to the plasma membrane where phosphorylation occurs to initiate downstream signalling cascades. This phosphorylation is controlled by the

degradation of PIP_3 through inositol phosphatases. The inositol phosphatases PTEN and Src homology domain 2-containing inositol-5'-phosphatase 1 (SHIP1) are activated by membrane localisation[151,152]. While PTEN and SHIP1 share the common substrate, PIP3, there is no sequential or structural homology. PTEN catalyses the dephosphorylation of protein tyrosine residues and additionally functions as a phosphatidylinositol 3,4,5-triphosphate 3-phosphatase[153]. SHIP1 is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase[154,155]. Thus, PTEN directly reverses PI3K activity to generate $PI(4,5)P_2$, while SHIP1 catalyses the conversion of PIP_3 to phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$)[156]. $PI(3,4)P_2$ in contrast to $PI(4,5)P_2$ is a dynamic second messenger binding a different set of proteins, thereby recruiting further downstream effectors[157].

1.3.7.1 PTEN

PTEN is a *bona fide* tumour suppressor being deleted or mutated in a manifold of malignant entities[158–160]. While in many cancer entities, *PTEN* is one of the most frequently mutated genes, the second most after *TP53* considering all cancer types[161], some entities are not found to be associated to a loss of *PTEN*[160,162]. A loss of PTEN generally leads to hyperactivated, constitutive AKT signalling[158]. While hyperactivated AKT signalling is crucial in the development of CLL, mutations in the *PTEN* locus are observed rarely if at all[162,163]. About 20% of CLL cases though do show a loss of heterozygosity in the genetic region of the PTEN locus at 10q23.3, while the gene itself is not affected by these mutations[162]. Without inactivating mutations, the loss of control of AKT phosphorylation in CLL cells by PTEN was found to be caused by transcriptional, translational and spatial repression[162–164]. Transcriptional repression has already been linked to poor prognosis[165]. Independent from PTEN expression levels, its subcellular localisation to the CLL cell nucleus may induce apoptosis. The induction of apoptosis was also reported to be independent from the TP53 mutation status[164]. While PTEN also functions as a protein phosphatase, its tumour suppressor function is associated to the inositol phosphatase activity, removing the 3' phosphate of phosphoinositides[166], thereby antagonising PI3K activity. The phosphatase activity of PTEN is reversibly inactivated by H_2O_2[167].

1.3.7.2 SHIP1

Another antagonist of PI3K is a 5'-inositol phosphatase: SHIP1. Similar to PTEN, SHIP1 cleaves PIP_3, a messenger molecule in the cell membrane. The cleavage produces phosphatidylinositol $PI(3,4)P_2$. SHIP1 does not only function through its phosphatase activity. It was shown to function as scaffold protein interfering with signalling cascades of the MAPK and other pathways[168,169]. SHIP1 is crucial in B-cell development[170] and together with PTEN, these phosphatases are thought to cooperatively suppress the malignant transformation of B-cells[171]. The suppression of excessive BCR-mediated activation is achieved through the conversion of PIP_3 to $PI(3,4)P_2$, thereby antagonising the membrane localisation of BCR signalling components, *e.g.* BTK[172,173] or AKT[174]. But the role of SHIP1 in the formation and maintenance of malignant B-cells is ambiguous. Both tumour suppressor[175,176] and pro-proliferative[177,178] characteristics have been reported. Counterintuitively, SHIP1 inhibition has also been reported to trigger apoptosis in hematopoietic cancer cells[179]. The ambiguous role of SHIP appears to be due to the 5' phosphatase activity as $PI(3,4)P_2$ is capable if not required to enhance AKT phosphorylation and hence activation of the PI3K/AKT pathway[177,178]. In another report, SHIP1 was shown to inhibit AKT activation in B-cells[175] and SHIP1 mutations found in leukaemia associated to impaired enzymatic activity led to increased activation of the PI3K/AKT pathway[176]. SHIP1 has served as target in the pioneering development of phosphatase activators in therapies of multiple myeloma[148,180] *inter alia*[181,182]. Interestingly, in myeloma cells PTEN but not SHIP1/2 suppresses the PI3K/AKT pathway[183]. The SHIP1 activators are pelorol derivates[184] and the most recent generation has entered preclinical evaluation in several B-cell malignancies[185]. With this recent preclinical advances, the role of SHIP1 and its targetability in CLL became one focus of this thesis project.

1.3.8 The TME in CLL

In response to infection, normal B-cells interact with the microenvironment. The microenvironment facilitates antigen presentation, differentiation and maturation, and antibody secretion[127,186]. Different cell types interact with B-cells, including mesenchymal stromal cells, endothelial cells, fibroblasts, neutrophils, monocytes, macrophages, dendritic cells, T cells and NK cells. These different cell types enter a crosstalk with B-cells through adhesion molecules, endocrine processes, and ligand-receptor interactions. These interactions are not yet fully elucidated[187,188]. In CLL, genetic lesions and epigenetic reprogramming predispose malignant B-

cells to increased life spans and proliferation advantage *in vivo*. *In vitro*, CLL cells undergo apoptosis within hours to days. The apoptosis is most likely initiated as pro-survival factors from the microenvironment are no longer present[187]. Factors contributing to CLL survival, proliferation and migration are cytokines, chemokines, proangiogenic factors, and components of the extracellular matrix. The survival and proliferation signals provided by the microenvironment can be mimicked *in vitro* by co-culture with BM stromal cells[189,190]. These BM stromal cells interact with CLL cells through adhesion molecules. Adhesion molecule types include integrins, vascular cell adhesion molecule 1 (VCAM1), VEGF and others[191–194]. Stromal cells also release growth factors signalling on the CXCR4-CXCL12 axis. This interaction protects CLL cells from spontaneous as well as drug-induced apoptosis[189–191,195,196]. Though, apoptosis measurements in co-culture studies have to be considered with care as confounding phagocytosis has recently been reported[197]. The interaction between CLL cells and stromal cells leads to a change in the expression pattern of both cell types. One such change in expression affects the C-C motif chemokine ligand 3 (CCL3) and 4 (CCL4) that recruit other types of lymphocytes, *e.g.* T cells[198,199]. The region of close interaction with the microenvironment, the pseudofollicles of secondary lymphoid tissue, were identified as the site of CLL cell proliferation *in vivo*[127,200]. CLL cells in lymphoid tissue have an upregulated expression of BCR-related and NF-kB and NFAT target genes compared to CLL cells in other tissue, *i.e.* PB and BM[127]. In PB, nurse-like cells could be extracted that protected CLL cells from spontaneous apoptosis in a similar manner as stromal cells[201]. The interaction of CLL cells to cells of the microenvironment reshapes the tissue organisation as well as cellular function. T cell function is affected by IL-10 and TGF-b release as well as PD-L1 surface expression, rendering T cells incapable of controlling excessive CLL cell proliferation[202–204].

1.4 The impact of shear stress on lymphocytes

Throughout the entire human body, cell surfaces interact with watery extracellular milieu. The extracellular fluid impacts on the cell surface. The most relevant mechanical impact on the cell surface is known as shear stress. Shear stress is a mechanical force applied in parallel to the respective surface. The SI unit for shear stress is Pascal (Pa) while in biological conditions, dynes/cm^2 is used with 1 Pa = 10 dyn/cm^2. Shear stress on the cell surface induces mechanotransduction processes. These mechanotransduction processes impact on cellular function and activation by cell morphology modulation, induction of proliferation or differentiation, altering metabolic pathways and extracellular matrix formation[205,206]. Especially in

the development of endothelial cells in the blood, shear stress plays an important role and is the highest in the arteries reaching 15 dyn/cm^2 in physiological conditions or >80 dyn/cm^2 in hypertension patients[207–209]. While the effects of shear stress on endothelial cells are well studied in the vascular system[207,210], the effects on circulating cells in blood, lymph and intercellular fluid are not completely elucidated, yet. Between these body fluids, lymphocytes are able to migrate from one to another[211–213]. In these different physiological environments, very different levels of shear stress apply to the lymphocytes. Different levels of shear stress are also found within the respective body fluid, *e.g.* areas of lower or higher shear stress within the LNs[214–216]. Responsible for changing levels of shear stress is also the lymph nodal vasomotion. Lymph nodal vasomotion is regulated through prostanoids[217]. The changing shear stress regulates calcium signalling, ATP metabolism, and subsequent pathways also affecting the permeability of lymphoid stromal cells[215,218,219]. Leukocytes leaving the lymphoid microenvironment, re-circulating to the blood stream with high shear stress, in general show a lower level of cellular activation. The lower activation levels are indicated by the loss of pseudopods and the reduction in integrin surface expression[220,221]. An example of shear stress-dependent integrin surface expression in B-cells is CD49d. CD49d is an interactor of the vascular cell adhesion molecule 1 (VCAM-1) which is expressed by endothelial cells[222,223]. The upregulation of CD49d is associated to increased transendothelial migration capacity[224] and to the escape from apoptosis of CLL cells[191,225]. CLL cells are primed for the transendothelial migration under shear stress by increased expression of CD62L, CXCR4, CD5, and CD69[224]. CD62L is a selectin that tethers the initial adhesion to endothelial cell walls through its ligand PNAd[226]. The receptor of stromal cell-derived factor 1, CXCR4, is responsible for the chemotaxis towards the protective microenvironment[227]. In the case of CD69, upregulation was also observed when cells were brought into close contact with the protective microenvironment and was found to predict CLL prognosis[127,228]. Thus, CLL cells exhibit phenotypical differences under shear stress conditions[224,229].

1.4.1 CLL and cellular adhesion

Survival and proliferation of B-cells and CLL cells are dependent on the interaction with a supportive microenvironment. In the microenvironment of the LNs, CLL as the highest rate of proliferation[128]. The microenvironment can *in vitro* be mimicked by co-culture with marrow stromal cells[189–191,230], follicular dendritic cells[231] and nurse-like cells[201]. Without supportive co-culture, CLL cells undergo spontaneous apoptosis *in vitro*. *In vivo*, the development of resistance

to treatment can in part be explained by microenvironmental protection of CLL cells[143,232–234], in addition to clonal evolution[145,146]. The activity of the BCR antagonists PTEN and SHIP1 impact on B-cell adhesion, motility and migration[235–239]. CLL cell adhesion in lymphoid tissue decreases when BCR signalling inhibitors are applied. In fact, the treatment success of these inhibitors of BCR signalling factors is associated with lymphocytosis during the first weeks after initial drug administration[240,241]. It is thus the BCR signalling that is crucial in the retention of CLL cells in the supportive microenvironment through the adhesion onto stromal cells[242].

2 Aims of thesis

My PhD project aimed to shed light onto the mechanisms and dynamics involved in phosphatase activity in the BCR signalling. In particular, I measured the quantity and quality of the inhibitory or modulating effect of PTEN and SHIP1 on key components of the BCR signalling pathway including the messenger lipid PIP_3. Therefore, I performed phospho-flow cytometry analysis of cell lines with modified phosphatase expression and primary CLL cells. Furthermore, I investigated methods to measure quantity and quality of CLL cell to stromal cell interactions. This work might result in a significant impact on *ibrutinib* or other kinase inhibitor treatment strategies that might include phosphatase agonists or antagonists.

2.1 Involvement of PTEN and SHIP1 in BCR signalling

The biochemical function of both PTEN and SHIP1 differs in catalysing the removal of phosphate residues at the positions 3 or 5, respectively. The removal of either phosphate residue renders the phosphatidyl-inositol molecule to interact with other signalling molecules than uncatalysed PIP_3. While research had focused on the function of the resulting signalling molecules $PI(3,4)P_2$ and $PI(4,5)P_2$, the dynamics of PTEN and SHIP1-catalysed inhibition of stimulated BCR signalling has not been exhaustively addressed. Here, I shed light on the control of BCR dynamics by these two phosphatases PTEN and SHIP1.

2.2 BCR signalling and adhesion of CLL cells to cells of the protective microenvironment

The adhesion of CLL cells to cells of secondary lymphoid tissue is believed to be crucial for CLL cell survival and proliferation. The mechanisms of CLL cell retention and re-adherence is not yet understood. As the recent advancements in targeting CLL cells in their protective niche produced promising but ultimately short-falling clinical results, the further understanding of molecular mechanisms causing CLL cells to remain or re-enter the protective lymphoid tissue is necessary in the search for additional targets in improved treatments. With this project, the hypothesis is tested that the BCR is tightly interlinked to the adhesive capacity of CLL cells. Furthermore, the

role of SHIP1 was closely investigated to evaluate its potential as one of the future targets in CLL therapy. To make future evaluation of molecular mechanisms and targets adequately measurable, an adhesion under flow assay was optimised and established mimicking the protective lymphoid tissue niche.

3 Materials and Methods

3.1 Materials

3.1.1 Human Samples

Peripheral blood samples from CLL patients were obtained after informed consent and according to the guidelines of the University Hospital of Ulm Ethics Committee, the Ethics Committee of the University of Heidelberg, and the Declaration of Helsinki. Patients with CLL were diagnosed following the World Health Organization (WHO) classification criteria[21]. Clinical information of the patients whose cells were analysed in this work are summarised in Table 1. Healthy donor PBMCs were thankfully received from the Blutbank (bloodbank) of the University Hospital of Heidelberg. B-cells of healthy control PBMCs were extracted for further experiments.

Table 1 Clinical data of CLL patients and healthy controls.

Patient ID	VH status	Gender	FISH karyotype
B448	mutated (V4-34, productive; 93,59%)	male	normal
B510	mutated (V3-23 productive: 88,89%)	female	13q deletion
B342	mutated (V1-3 productive;93.6%)	female	13q deletion
B457	mutated (V4-30.4 productive: 81,48%)	female	normal
B505	mutated (V3-7, productive; 97,22%)	female	13q deletion
B480	mutated V3-23; productive, 92,16%)	male	13q deletion
B501	NA	male	normal

B454	mutated (V4-34: 91,78%)	male	13q deletion
B444	unmutated (V3-9, productive, 100%)	male	normal
B343	unmutated (V3-21, productive;100%)	female	13q deletion
B464	unmutated (V1-69, productive; 100%)	male	13q deletion
Healthy controls	not tested	unknown	not tested

3.1.2 Cell lines

Table 2 Cell lines

Name	Experiment/ Purpose	Supplier	Reference
HG3	Flow cytometry, Flow assay, Western Blot	DSMZ	Lanemo Myhrinder et al.[243]
HS-5	Flow assay	ATCC	Roecklein, Torok-Storb[244]
LCL-WEI	Flow cytometry, Western Blot	DSMZ	NA
JEKO-1	Flow cytometry, Western Blot	DSMZ	Drexler, Macleod[245]
PGA-1	Flow cytometry, Western Blot	DSMZ	Lewin et al[246]
CII	Flow cytometry	Amsterdam University Medical Center	Fialkow et al.[247]

3.1.3 Antibodies

Table 3 Flow cytometry antibodies

Name	Experiment/ Purpose	Supplier	Reference
Akt (pS473)	Flow cytometry	Becton Dickinson	560343
Biotin F(ab´) Goat anti-human IgM	Stimulation	Jackson Immuno Research	109-066-006
BTK	Flow cytometry	Becton Dickinson	558528
BTK (pY551)/ITK (pY511)	Flow cytometry	Becton Dickinson	558129
CD19	Flow cytometry	Becton Dickinson	560728
CD20	Flow cytometry	Becton Dickinson	561171
CD5	Flow cytometry	Becton Dickinson	341109
ERK1/2 (pT202/pY204)	Flow cytometry	Becton Dickinson	562644
F(ab´) Goat anti-human IgG	Stimulation	Jackson Immuno Research	109-006-006
F(ab´) Goat anti-human IgM	Stimulation	Jackson Immuno Research	109-006-129
IgG2a, k Isotype Control	Flow cytometry	Becton Dickinson	555576
IgG2a, k Isotype Control	Flow cytometry	Becton Dickinson	555748
IgG1, k Isotype Control	Flow cytometry	Becton Dickinson	555574
IgG2a, k Isotype Control	Flow cytometry	Becton Dickinson	560167
IgG1, k Isotype Control	Flow cytometry	Becton Dickinson	560373
IgG1, k Isotype Control	Flow cytometry	Becton Dickinson	556652
IgG2a, k Isotype Control	Flow cytometry	Becton Dickinson	557872
IgG1, k Isotype Control	Flow cytometry	Becton Dickinson	558020

IgG2a, k Isotype Control	Flow cytometry	Becton Dickinson	580894
IgM	Flow cytometry	Becton Dickinson	555782
PLCy2(pY759)	Flow cytometry	Becton Dickinson	558507
PTEN Monoclonal Antibody (1B8)	Flow cytometry	Life Technologies	MA515560
SHIP1	Flow cytometry	Biolegends	656604
Zap70 (pY319)/Syk (pY352)	Flow cytometry	Becton Dickinson	561458

Table 4 Western Blot antibodies

Name	Experiment/ Purpose	Supplier	Reference
GAPDH	Western Blot	ThermoFisher Scientific	PA1-987
PTEN	Western Blot	Cell Signaling Technology	9552
SHIP1	Western Blot	ThermoFisher Scientific	MA1-10450

3.1.4 Chemicals and other reagents

Table 5 Chemicals and reagents

Name	Experiment/ Purpose	Supplier	Reference
Dimethylsulfoxid (DMSO)	Cryopreservation	Sigma Aldrich, Steinheim	41639
Dulbecco's Phosphate Buffered Saline (PBS)	Miscellanous	Sigma Aldrich, Steinheim	D8537
Ethanol	Miscellanous	Fisher Scientific, Schwerte	10342652
Fetal Bovine Serum (FBS)	Miscellanous	Sigma Aldrich, Steinheim	F7524

GeneRuler 1 kb Plus DNA Ladder	PCR	Fisher Scientific Oy, Vantaa	11581625
Gibco L-Glutamine (200 mM)	Cell culture	Thermo Fisher Scientific, Waltham	25030024
Gibco Penicillin/Streptomycin (10,000 U/mL)	Cell culture	Thermo Fisher Scientific, Waltham	15140122
PKH26 Green Fluorescent Cell Linker	Flow assay	VWR International	SAFSMIDI26
PKH26 Red Fluorescent Cell Linker	Flow assay	VWR International	SAFSMIDI26
Plerixafor	Flow assay	Biozol Diagnostica, Eching	APE-A23025-5.1
RPMI 1640	Cell culture	Sigma Aldrich, Steinheim	R8758

3.1.5 Buffers and solutions

Table 6 Buffers and solutions

Name	Experiment/ Purpose	Composition/Supplier	Reference
HEPES	Flow assay	Life Technologies	15630056
Biocoll	PBMC isolation	Biochrom	BS.L6115
FACS buffer	Flow cytometry	PBS + 1%BSA + 0.1% NaN_3	NA
Freezing media	Cryopreservation	90% FBS + 10% DMSO	NA
RIPA	Protein extraction	10 mM TRIS-HCl (pH 7.4), 1 mM EDTA, 1% NP-40, 0.1% Sodium Deoxycholate, 0.1% SDS, 140 mM NaCl, 10 mM NaF, 1 mM PSMF,	NA

Running buffer	Western Blot	25 mM TRIS-HCl, 192 mM glycine, 1% SDS; pH 8.3	NA
Transfer buffer	Western Blot	25 mM TRIS-HCl, 192 mM glycine, 20% MeOH, 0.1% SDS	NA

3.1.6 Kits

Table 7 Kits

Name	Experiment/ Purpose	Supplier	Reference
Lightning Link APC/Cy7 Labeling Kit	Flow cytometry	Biozol Diagnostica, Eching	INB-765-0005
PIP3 Mass ELISA Kit	Inositol phosphate assay	Echelon Biosciences	K-2500S
PI(3,4)P2 Mass ELISA Kit	Inositol phosphate assay	Echelon Biosciences	K-3800
PI(4,5)P2 Mass ELISA Kit	Inositol phosphate assay	Echelon Biosciences	K-4500

3.1.7 Consumables

Table 8 Consumables

Name	Experiment/ Purpose	Supplier	Reference
96-Well U Bottom Plate	BCR stimulation / Miscellaneous	Techno Plastic Products (TPP), Trasadingen	TPP92197
Hose clamp	Flow assay	neoLab Migge GmbH, Heidelberg	KL-1423

Injection port	Flow assay	IMPROMEDIFORM, Lüdenscheid	MF1542
T connector	Flow assay	IMPROMEDIFORM, Lüdenscheid	MF4701
µ-Slide 0.8 ibiTreat	Flow assay	ibidi GmbH, Gräfelfing	80196
X100 Spritze 5ML Luer Zentriert	Flow assay	Fisher Scientific/Terumo, Illkirch (France)	11760465
Perfusion Set White	Flow assay	ibidi GmbH, Gräfelfing	10963
Elbow Luer Connector Male	Flow assay	ibidi GmbH, Gräfelfing	10802
Cell scraper 237 mm	Flow assay, miscellaneous	Kisker Biotech	330097
Einmalspritzen 2 ml Luer	Flow assay	Fisher Scientific/Terumo	12798
Spritzenvorsatzfilter	Flow assay	Neolab Migge	101263153
Cell culture flask TC T75 Suspen.	Cell culture	Sarstedt AG	83.3911.502
Electroporation cuvette	siRNA knock-down	Sigma-Aldrich Chemie, Taufkirchen	Z706086-50EA
LLG-Trockenperlen	Flow assay	Faust Lab Science	NA

3.1.8 Equipment and devices

Table 9 Equipment and devices

Name	Experiment/ Purpose	Supplier
Zeiss		
ibidi pump		
BD FACSCanto II	Flow Cytometry Staining	BD (Becton, Dickinson & Company), Franklin Lakes
BD LSRFortessaTM	Flow Cytometry Staining	BD (Becton, Dickinson & Company), Franklin Lakes

Cell Culture Safety Cabinet, Herasafe KS	Cell culture	Thermo Fisher Scientific, Waltham
Centrifuge 5810 R	Miscellaneous	Eppendorf, Hamburg
Centrifuge Heraeus Fresco 17	Miscellaneous	Thermo Fisher Scientific, Waltham
Heracell 150i Incubator	Cell culture	Thermo Fisher Scientific, Waltham
Heracell 240i Incubator	Cell culture	Thermo Fisher Scientific, Waltham
Mithras plate reader	ELISA	MITRAS
PIPETMAN L Multichannel P8x200L, 20-200 µL	Miscellaneous	Gilson, Middleton
Pipettes (2 µL, 20 µL, 100 µL, 200 µL, 1000 µL)	Miscellaneous	Gilson, Middleton
Vi-CELL XR 2.03	Cell counting	Beckman Coulter Inc.,Brea
Vortex Mixer Neolab 7-2020	Miscellaneous	neoLab Migge, Heidelberg
Water Bath Julabo SW-20C	Miscellaneous	Julabo, Seelbach

3.1.9 Software

Table 10 Software

Name	Experiment/ Purpose	Supplier	Version/reference
BD FACSDivaTM	Flow cytometry data acquisition	BD (Becton, Dickinson & Company), Franklin Lakec	3149011B
FlowJo	Flow cytometry analysis	BD (Becton, Dickinson & Company), Franklin Lakes	X 10.0.7
ImageJ	Flow Assay/ picture processing	NIH	1.52 and previous versions
Microsoft Excel	Miscellaneous	Microsoft, Redmond	2016
Microsoft PowerPoint	Miscellaneous	Microsoft, Redmond	2016
Microsoft Word	Miscellaneous	Microsoft, Redmond	2016
SigmaPlot			14.0
ibidi flow			NA
Zotero	Bibliography management		6
Zeiss ZEN	Microscopy	Zeiss Group	NA

3.2 Methods

3.2.1 Human tissue processing and conservation

Peripheral blood samples of CLL patients were separated by Ficoll-Paque sedimentation (GE-Healthcare, GE17-1440-02). The PBMCs were resuspended in heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, F7524) containing 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, D4540) and cryopreserved. Buffy coats from healthy donors were obtained from the German Red Cross in accordance with the Declaration of Helsinki. These buffy coats from healthy donors were enriched for CD19-positive cells via negative magnetic enrichment using EasySepTM Human B-cell Enrichment Kit (Stemcell Technologies, Vancouver, Canada). Samples of isolated PBMCs and CD19-sorted cells were analysed by flow cytometry using anti-CD5-FITC and anti-CD19-APC antibodies (BD Biosciences). If not otherwise indicated, all samples were cryopreserved in liquid nitrogen until further handling.

3.2.2 Cell culture, treatment and siRNA transfection

After thawing, cells were cultured in Roswell Park Memorial Institute (RPMI 1640, Sigma-Aldrich, R8758) medium with 10% FBS and 1% Penicillin/Streptomycin (1000 U/ml, Life Technologies, Darmstadt, Germany).

PBMCs were isolated from blood samples by density centrifugation in Biocoll. Primary cells from PBMCs were either used freshly or after thawing in case stored after isolation from the donors. After thawing, cells were rolled in RPMI 1640 medium for 2 h for recovery. After recovery or when used freshly, cells were cultured in RPMI 1640 medium supplemented with anti-IgM and anti-CD19 30 min prior to fixation. When H_2O_2 (3.3 mM) was added, the stimulation was performed at 28 min, 21 min, 15 min, 10 min, 6 min, and 3 min before fixation. After fixation in 2% PFA, cells were permeabilised and prepared for flow cytometry as published[10] (**Figure 2**).

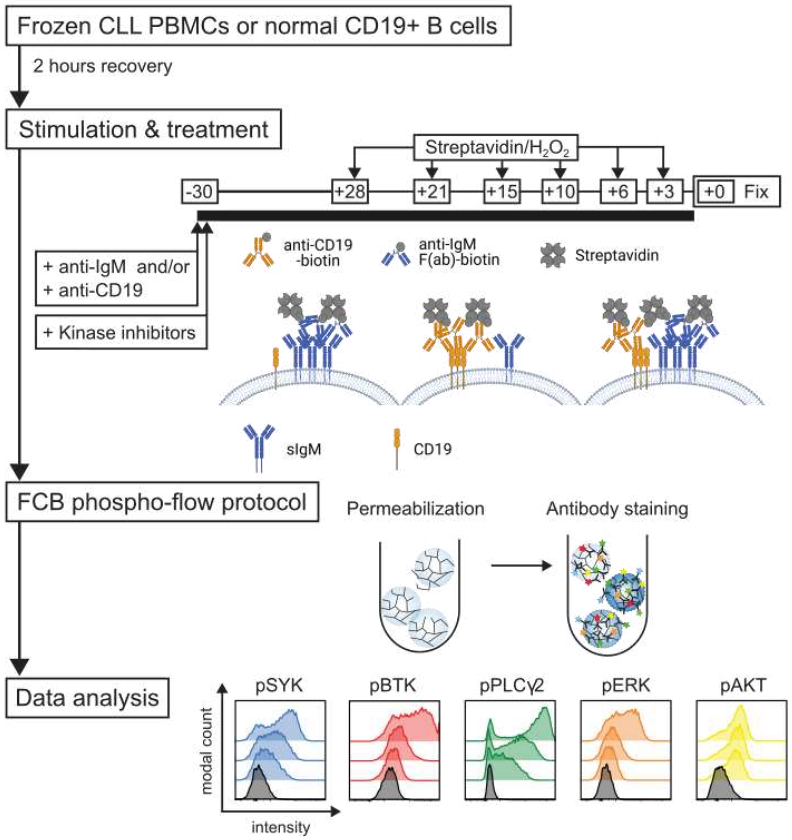


***Figure 2 Preparation, measurement and analysis of CLL and B-cells**, modified after Wolf, Maus, Persicke et al. 2022[10].*

3.2.3 Inositol phosphate assay

Cells were harvested and lysed according to the manufacturer's instructions. The incubation and reading plates were prepared accordingly. In short, cells were harvested by centrifugation and mixed and incubated with cold 0.5 M TCA. The collected pellet was further processed at room temperature and washed with 5% TCA, 1 mM EDTA buffer. The lipids in the lysate were separated with methanol (MeOH): chloroform (CHCl3) [2:1] (neutral lipids), and with MeOH:CHCl3: 12 N HCl (80:40:1; acidic lipids). The acidic lipids were phase separated with CHCl3 and 0.1 N HCl, and dried in a vacuum dryer. For detection, the extracted phosphatidyl inositol (PI)-derivates were brought into aqueous solution and incubated with the detector protein. Once incubated with the detector protein, the samples were transferred to the respectively PIP_3, $PI(3,4)P_2$, or $PI(4,5)P_2$ coated plate. The signal was measured using a plate reader at 450 nm.

3.2.4 Flow Cytometry analysis

Single-cell suspensions were treated with equal amounts of pre-warmed 4% paraformaldehyde solution (PFA) to reach a final concentration of 2% PFA. The PFA was pre-warmed to 37 °C just before application. After centrifugation at 300 g, cells were resuspended in PBS and washed twice. The cells were the then permeabilised with -20 °C-cold methanol (MeOH) for 30 min in the dark. The MeOH was removed by centrifugation and cells washed in FACS buffer. The immunostaining with antibodies against cytosolic and cell surface proteins (Table 3) was applied in FACS buffer containing 0.1% sodium azide (NaN_3).

3.2.4.1 Data acquisition and analysis

The cells' fluorescence levels were assessed using the BD LSRFortessa™ (BD Biosciences) flow cytometer with the running and pre-analysis software FACSDiva™. The full data analysis was done using the FlowJo X 10.0.7 software (FlowJo). Single staining samples were used to compensate fluorophore spectral overlaps. The spectral overlap compensation and sample analysis was controlled with fluorescence minus one (FMO) controls.

3.2.5 Flow assay

To elucidate the mechanisms involved in CLL and B-cell adhesion, I optimised an adhesion and shear stress assay to mimic the physiological conditions of the lymphoid tissue. In this assay, a layer of stromal cells was seeded into a microscopy slide chamber (µ slide) and left to adhere for 24 h. The inside of the chamber was coated with collagen or cell culture-optimised agents (ibiTreat, ibidi GmbH, Martinsried) to facilitate adherence. This slide was connected to tubing of defined length and diameter. The tubing was itself connected to two reservoirs filled with cell culture medium (RPMI with 10% FCS and 10 mM HEPES). By applying defined pressure, alternating to one or the other reservoir, and alternating opening and closing of valves, steady and controlled shear stress was applied to the layer of stromal cells. Lymphocytes were injected into the system through an injection port leading onto the layer of stromal cells in the channel slide. After an initial adhesion phase of 30 min, increasing shear stress was applied over time and during constant microscopy imaging. Cell adhesion was measured as a function of image area occupied by

lymphocytes and normalised to the initial area occupied before either drug administration or start of shear stress. Two of these systems could run in parallel imaging. Images were exported to subsequent data analysis.

3.2.5.1 Staining and cell preparation

Primary CLL cells, healthy B-cells or immortal cells were stained with PKH26 red or PKH26 green according to manufacturer's instructions. In short, thawed or fresh cells were washed in PBS, centrifuged at 400 x g and washed in diluent (Diluent C, Sigma Aldrich) before resuspension in diluent. The resuspended cells were quickly mixed with equal amounts of 2x staining solution ($4x10^{-6}$ M). The staining procedure was stopped with equal amounts of FCS after 2 min. Before insertion into the flow chamber, cells were centrifuged and resuspended in RPMI with 10% FCS media. The cell suspension was injected into the system with syringes through injection ports directly upstream of the flow chamber.

3.2.5.2 Microscopy and data acquisition

Microscopy images were taken using the Cell Observer® microscope (Zeiss). The microscope was built into a heating chamber with CO_2 supply. Two hours prior to image acquisition, the heating chamber was warmed up to 37 °C. CO_2 supply was activated to 5% at the inlet right before image acquisition. For image acquisition, two chamber slides were mounted onto a plate-holder and 10 parallel positions were selected for imaging in each slide. Images were taken every 5 min.

3.2.6 Statistical analysis

Statistical tests were calculated using the SigmaPlot software (Systat Software). The Mann-Whitney U test was applied to determine the significance of the differences between two groups, and one-way analysis of variance (ANOVA) was used for more than two groups. In the case of flow assay microscopy image quantifications, a linear function was calculated for each shear stress level and only corresponding levels compared as two groups. For all statistical analyses, the confidence interval was set to 95% (p-value <0.05) below which the null hypothesis could be rejected.

4 Results

4.1 BCR stimulation causes long-term changes in downstream factor phosphorylation

Both CLL cells and PB B-cells respond to BCR stimulation. The BCR stimulation dynamics vary largely between B-cells and the IGHV-classified subgroups of CLL cells[10,248,249]. To understand the mechanisms leading to the phenotypical changes between anergic and activated CLL cells, healthy B-cells, M-CLL and U-CLL cells were subjected to anti-IgM, anti-CD19 and/or H_2O_2 treatment. The level of activation was measured by phospho-flow[250] of BCR downstream effectors SYK, AKT, BTK, PLCy2, and ERK1/2. Additionally, SHIP1 and PTEN expression levels were measured, thereby complementing the measurements of BCR signalling upon stimulation[248,249].

Stimulation effects were tested in B-cell-derived cell lines including malignant origins JEKO-1, PGA-1, MEC1, and LCL-WEI. The phosphorylation levels of BTK and AKT represented BCR downstream pathway activation[10,121–123]. Phosphorylation levels were assessed by phospho-flow cytometry (Figure 3A). Considering cell lines as representatives of single biological samples, the statistical power of cell line-derived signalling measurements was limited but subpopulations of stimulated cells showed a visible shift in phospho-BTK and phospho-AKT levels (Figure 3A-C). Throughout all tested cell lines, AKT phosphorylation after 10 min of H_2O_2-stimulation compared to unstimulated control was not significant ($p=0.1951$), while BTK phosphorylation was significantly different ($p=0.0387$).

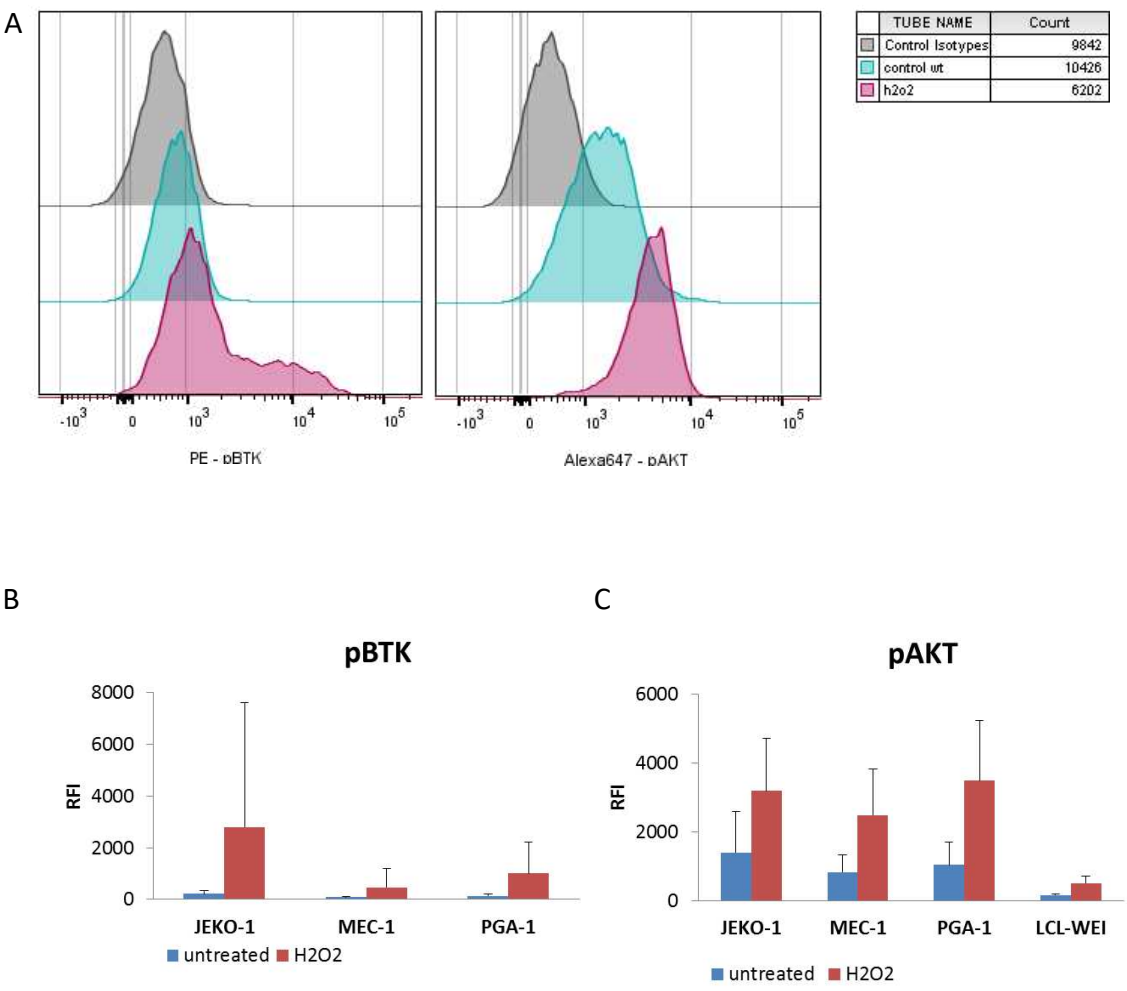


Figure 3 Intracellular levels of phospho-BTK and phospho-AKT. *(A) Representative flow cytometry analysis of pBTK and pAKT in JEKO-1 cells; (B) pBTK and (C) pAKT levels in malignant or lymphoid B-cell-derived cell lines.*

When mapping the levels of SHIP1, PTEN, phospho-BTK, phospho-S6, phospho-ERK1/2, and phospho-PLCγ2 across several time points after stimulation (0 min, 3 min, 6 min, 15 min, 28 min, 120 min, 360 min) in a t-distributed stochastic neighbor embedding (tSNE), thus reducing these factors to two dimensions, the cell populations clustered into subgroups with similar phosphorylation and expressions patterns, respectively. Cluster analysis based on single contributor comparisons revealed that low SHIP1 expression was found in these cell subpopulations that showed the highest increase in BTK and PLCγ2 phosphorylation. PTEN levels were less variable between subgroups and did not show correlations to stimulation-dependent phosphorylation levels (Figure 4).

As phosphorylation levels upon stimulation had previous been assessed only in time points up to 28 min[10], the question arose whether stimulation has a longer effect on the BCR signalling

landscape. Thus, I determined BCR signalling pathway activation levels in primary CLL cells up to 360 min after H_2O_2 treatment. The phosphorylation levels of AKT, BTK, ERK1/2, PLCy2, and SYK were determined at time points of 3 min, 6 min, 10 min, 15 min, 21 min, 28 min, 120 min, and 360 min in M-CLL patient samples. Four distinct stimulation patterns were applied and normalised to unstimulated control. AKT, after an initial peak in phosphorylation levels, showed decreased phosphorylation levels six hours after stimulation. While, as reported[10], initial phosphorylation patterns of BTK, ERK1/2, PLCy2, and SYK were similar though timely distinct, long-term phosphorylation levels differed whether increased or decreased compared to the pre-stimulation levels. BTK showed low but increased, ERK1/2 continuously higher phosphorylation levels six hours after stimulation, whereas PLCy2 phosphorylation levels returned to the pre-stimulation levels. SYK phosphorylation levels were seen to depend on stimulation condition, with H_2O_2 only leading to a decrease in long-term phosphorylation in contrast to anti-IgM and anti-CD19-assisted stimulation with H_2O_2 that maintained the phosphorylation levels on higher levels (

Figure *5*).

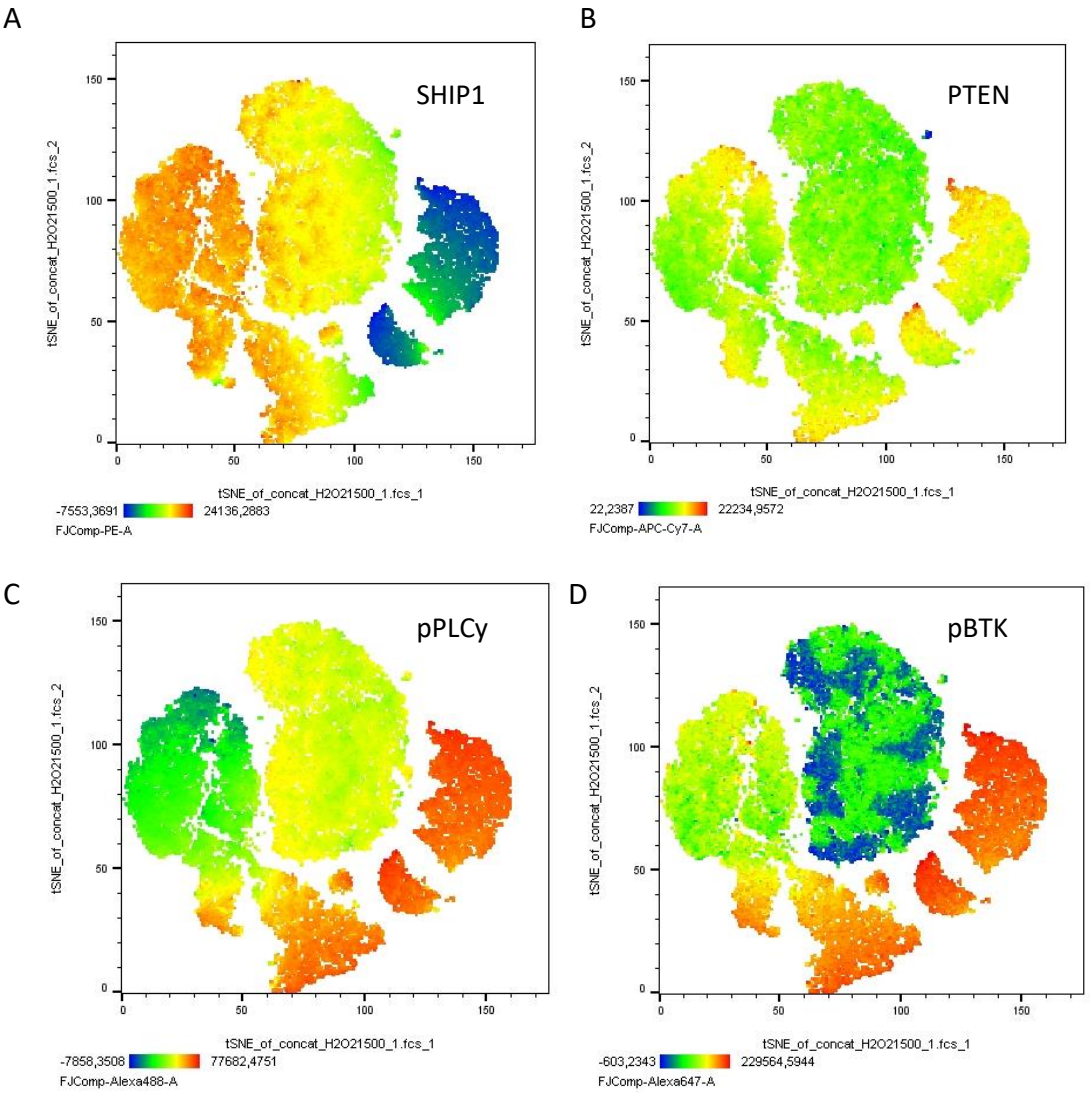


Figure 4 Expression and phosphorylation levels of BCR signalling pathway factors. *(A) SHIP1; (B) PTEN; (C) phospho-PLCy; and (D) phospho-BTK levels indicated in tSNE of cell populations after H_2O_2 stimulation.*

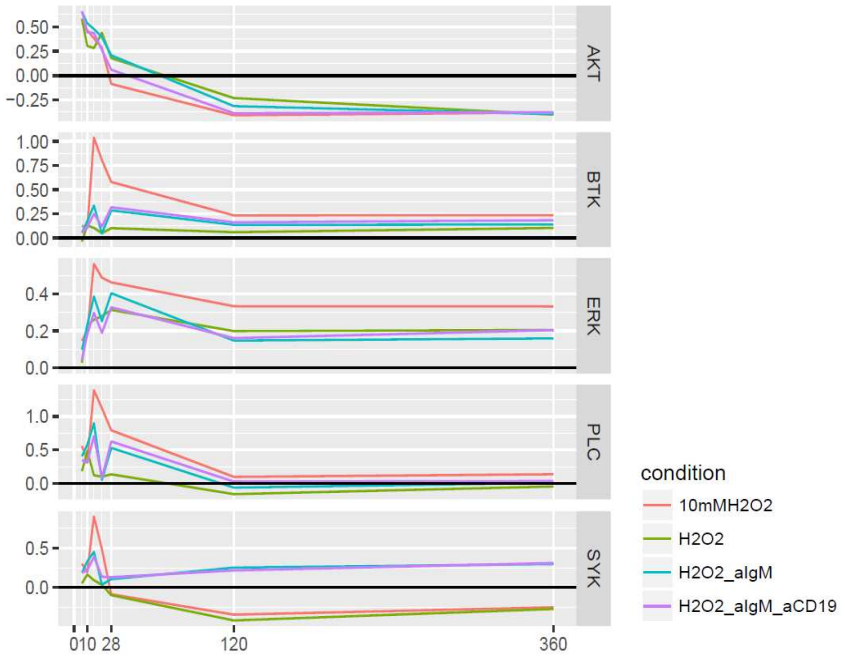


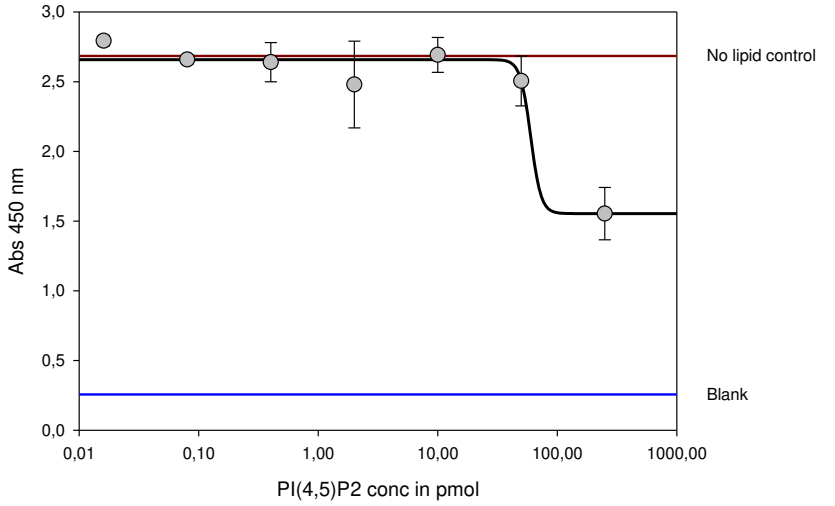
Figure 5 Average phosphorylation levels of BCR signaling pathway components AKT, BTK, ERK1/2, PLCγ2, and SYK under different stimulation conditions, normalised to the unstimulated control, joint data analysis with Carsten Maus.

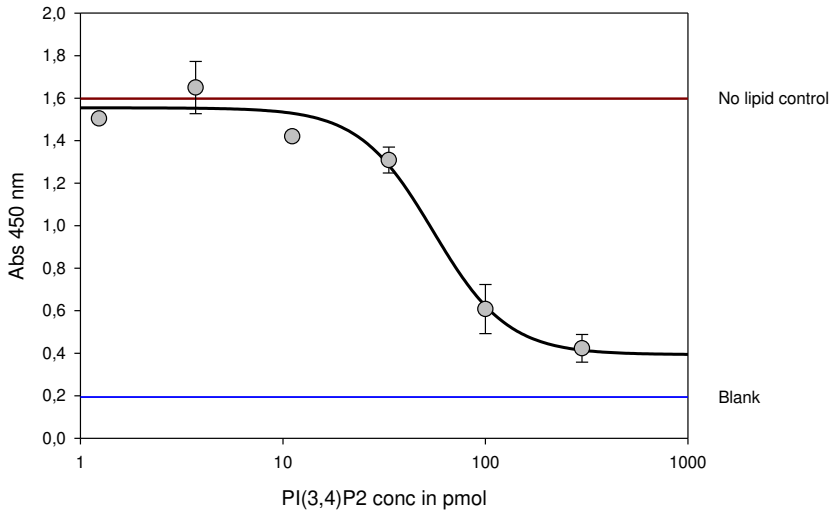
4.2 BCR signalling is controlled by phosphatases

After observing that signaling intensities correlated with H_2O_2 levels (

Figure *5*), the activity of the BCR pathway was sought to be validated. As one of the upstream events in BCR signaling, PI3K is activated upon BCR and CD19 clustering[251]. PI3K phosphorylates $PI(4,5)P_2$ into PIP_3, the key messenger molecule of the BCR pathway. PTEN is the natural antagonist of PI3K signaling by reversing the phosphorylation from PIP_3 to $PI(4,5)P_2$, while SHIP1 dephosphorylates PIP_3 at position 5, producing $PI(3,4)P_2$. $PI(3,4)P_2$ was reported to have crucial messenger functions in the AKT signaling pathway[177]. To quantitatively measure cellular levels of these messenger molecules, ELISAs were performed. $PI(4,5)P_2$ is the most abundant PI in cells[252,253] despite subcellular, local membrane cluster concentration differences[150]. $PI(4,5)P_2$ was thus aimed to be measured as a normalisation control. As $PI(4,5)P_2$ is an abundant, highly conserved metabolite within the animal kingdom, the specificity of the currently available antibodies is insufficient and tests on defined $PI(4,5)P_2$ concentrations (standard curve) failed to deliver signals over the required detection range (Figure 6A). $PI(3,4)P_2$ and PIP_3 could be measured between 1 pmol and 1000 pmol and between 0.01 pmol to 1000 pmol, respectively (Figure 6B,C). As normalization to $PI(4,5)P_2$ was impossible, samples were normalized to the initial cell number. Interestingly, H_2O_2 stimulation alone did not result in the detection of PIP_3 in HG3 cells at any

measured time point and did not significantly elevate PI(3,4)P_2 levels. Anti-IgM/anti-CD19-assisted stimulation did result in elevated PI(3,4)P2 (Figure 7A) and PIP3 (Figure 7B) levels as soon as five minutes after stimulation initiation. Thus, the combined stimulation of H_2O_2 and anti-IgM/anti-CD19 was able to activate BCR signaling the CLL-derived cell lines.





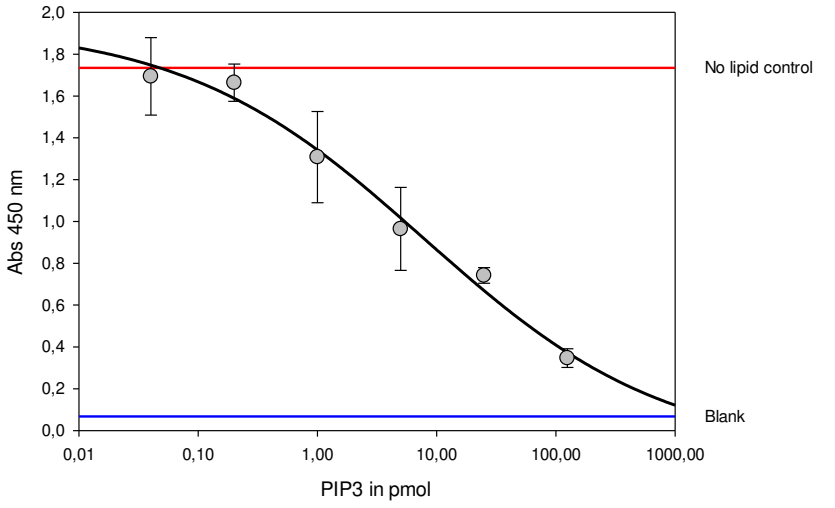


Figure 6 Assay validation for the detection *of $PI(3,4)P_2$ (top), $PI(4,5)P_2$ (centre), and PIP_3 (bottom).*

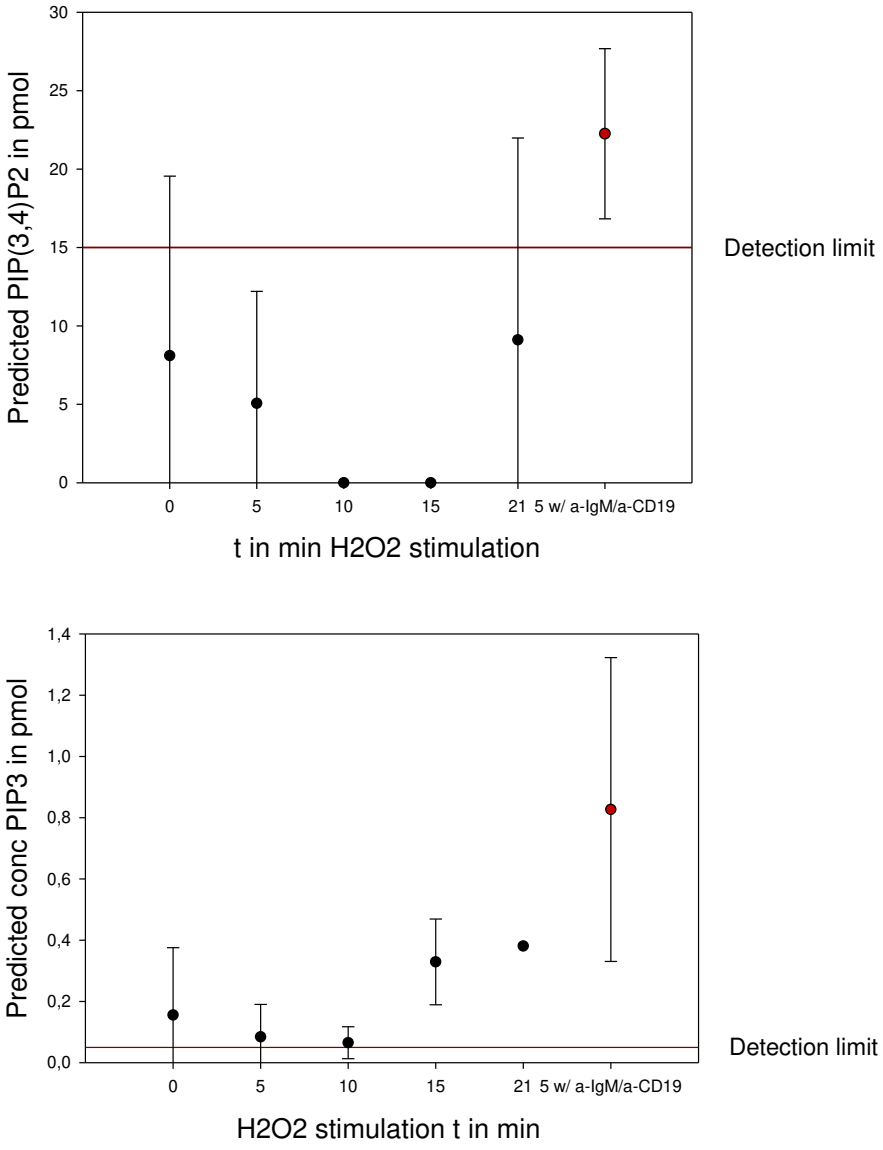


Figure 7 *PI(3,4)P_2 (top) and PIP_3 (bottom) levels in HG3 cells after H_2O_2 stimulation alone or in combination with anti-IgM and anti-CD19 at indicated time points.*

4.2.1 Minor correlation found between phosphatase expression and regulation of targeting miRNAs in CLL

First, transcriptional and translational dysregulation of phosphatases were investigated. MiRNA target site prediction[254] revealed 16 miRNAs with conserved target motifs inside the PTEN transcript while only one was detected for the major INPP5D (SHIP1) transcript isoform. Based on previous publications and RNAseq data availability[163,255,256], a selection of miRNAs for expression analysis in CLL was made. The correlation between the expression of these miRNAs with the expression of phosphatases INPP5D (SHIP1), PTEN, PTPN6 (SHP1) or PTPN11 (SHP2) were determined (Figure 8A-D). Only one passenger miRNA showed significant negative correlation to the target phosphatase guide strand, miR-186* to PTPN11 (SHP2). Despite miR-155 being frequently reported to drive cancer and found to be responsible for SHIP1 downregulation[257–259], the RNAseq data of 56 CLL patients did not reveal a negative correlation. MiR-155 expression in this patient cohort was highly homogeneous (Figure 8E) while SHIP1 expression followed normal distribution (Figure 8F).

Secondly, the correlation between SHIP1 and PTEN expression was analysed. No significant co-expression patterns were found using microarray data (data provided by Johannes Bloehdorn, University Hospital Ulm). The microarray data indicated that PTEN and SHIP1 are not co-controlled on transcriptional level.

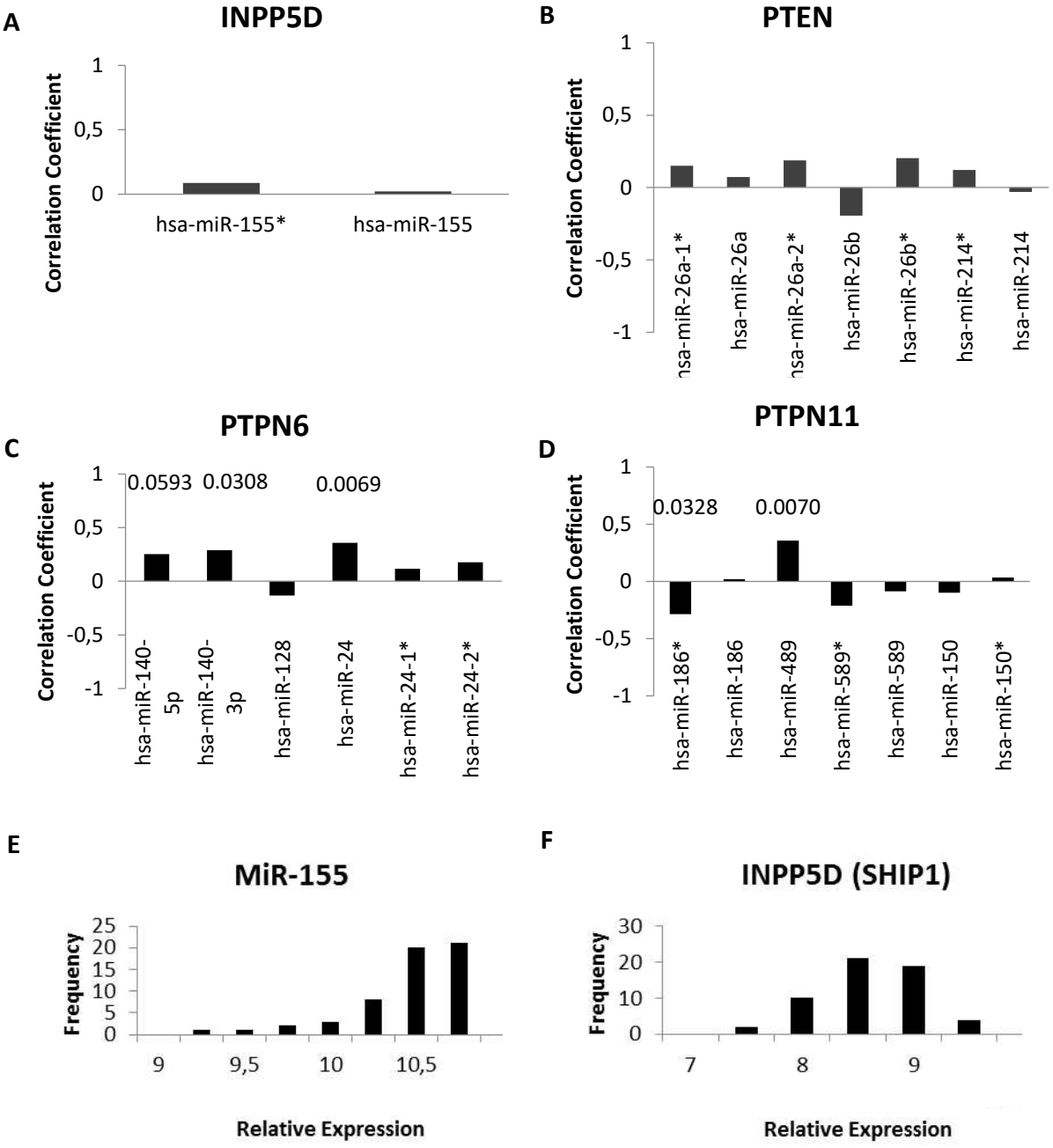


Figure 8 Phosphatases in the BCR signaling pathway and their predicted and reported targeting miRNAs. *(A) INPP5D is the coding gene for SHIP1, the transcript is targeted by miR-155; (B) PTEN-targeting, (C) PTPN6 or SHP1-targeting, and (D) PTPN11 or SHP2-targeting miRNAs expression; (E) distribution of miR-155 expression among CLL patients, only CLL cells; (F) distribution of INPP5D transcript levels among CLL patients, only CLL cells.*

4.2.2 SHIP1 and PTEN regulate BCR signalling in distinct patterns

As SHIP1 in contrast to PTEN is not directly inhibited by H_2O_2 treatment[260–262], I sought to determine the additional effect of the knock-down of the respective phosphatases on key signalling components. First, I discovered that SHIP1 levels differed between CLL- and other B-cell-derived cell lines while PTEN levels appeared to be consistent (Figure 9A). The knock-down through siRNA electroporation was successful but could not eliminate PTEN levels completely (Figure 9B). Interestingly, the knock-down of PTEN resulted in reduced electroporation-induced apoptosis compared to PBS-electroporated control (Figure 9C,D). Measuring phospho-AKT and phospho-PLCγ2 levels before and after the beforementioned H_2O_2-assisted stimulation in cells with SHIP1 or PTEN or no knock-down, I determined the impact of these phosphatases on stimulation-dependent BCR signalling. Treatment of cells with the PI3K inhibitor idelalisib resulted in decreased AKT and PLCγ2 phosphorylation upon stimulation and served as control. The knock-down of SHIP1 could increase stimulation-induced BCR signalling consistently while PTEN knock-down had no significant effect on phosphorylation levels (Figure 9E,F).

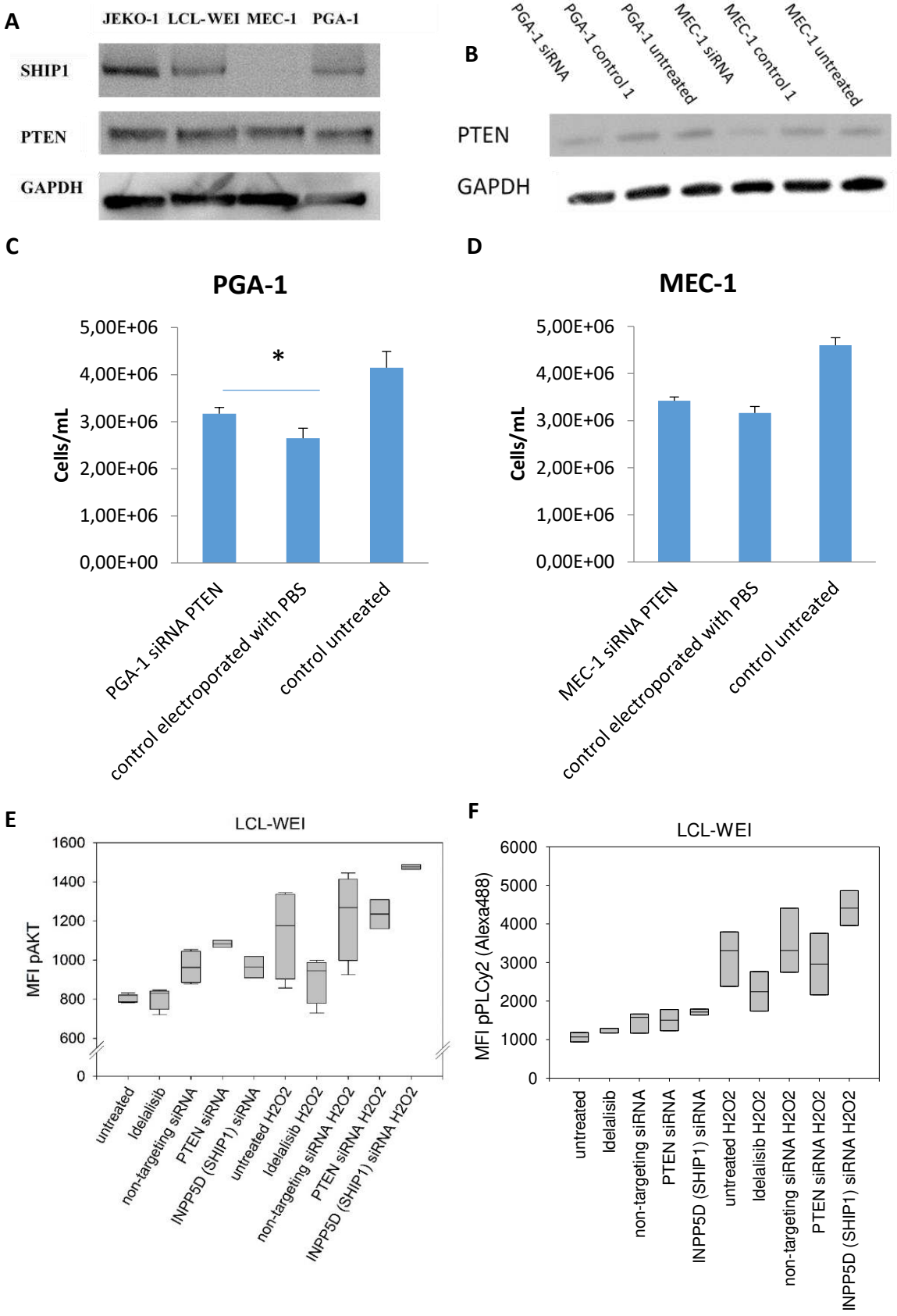


Figure 9 SiRNA knock-down of PTEN and SHIP1. *(A) B cell-/CLL-derived cell lines exhibit different levels of endogeneous SHIP1 while PTEN levels did not differ between cell lines (representative image); (B) siRNA could reduce but not eliminate PTEN protein levels; (C) PGA-1 and (D) MEC-1) CLL cell line viability after PTEN knock-down; (E) MFI Alexa647 pAKT and (F) MFI Alexa488-pPLCy2 in LCL-WEI cells.*

During the last two decades, SHIP1 came into the focus as both tumour suppressor and oncogene[263]. The research focus has led to the screening for potential inhibitors[179] and activators[147,148,264,265]. The inhibitor 3AC[179] and the agonist AQX-MN100[184] are well characterised for the use in experimental practice. AQX-MN100 was reported to reduce CLL cell viability. In the CLL cell line HG3 the reduction of cell viability could be observed at higher concentrations (Figure **10**A). Strikingly, AQX-MN100 was able to reduce phosphorylation levels upon stimulation of several BCR components that were found independent in the BCR signalling model[10]. The inhibition of SHIP1 activity by 3AC led to dose-dependent increased phosphorylation of AKT and PLCy2. When 3AC was combined with idelalisib, the increase in AKT and PLCy2 phosphorylation was nearly abolished. The abolished phosphorylation increase indicated that the SHIP1 inhibition effects were dependent on the PI3K pathway activation. The combination of AQX-MN100 and idelalisib reduced the AKT and PLCy2 phosphorylation levels to the levels of the unstimulated normalisation control (Figure 10B).

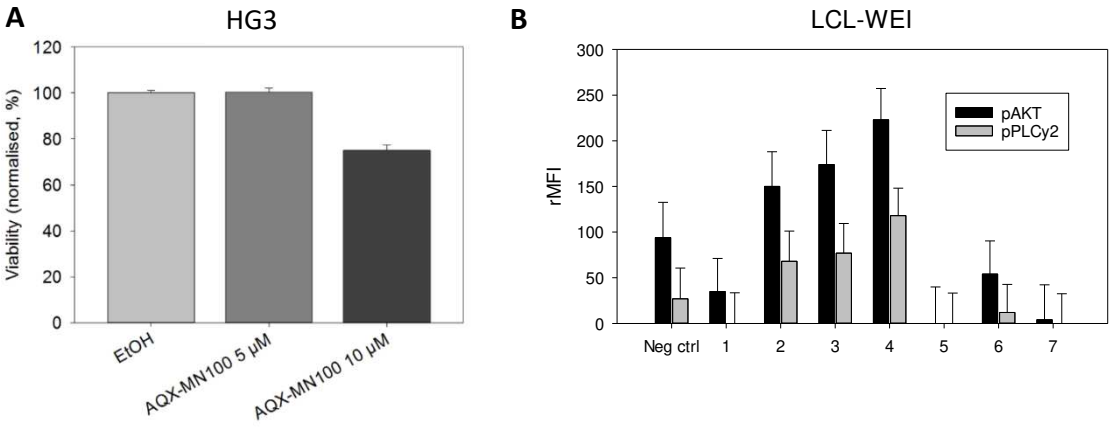


Figure 10 SHIP1 activator and inhibitor treatment, *(A) Viability measured by 7-AAD and Annexin V staining in flow cytometry, (B) reative MFIs antibodies against of phospho-AKT and phospho-PLCy2 under different activator and inhibitor treatments: neg ctrl: 0.3% EtOH; 1 – 30 µM AQX-MN100; 2 – 1 µM 3AC; 3 – 5 µM 3AC; 4 – 10 µM 3AC; 5 – 10 µM AQX-MN100 + 1 µM idelalisib; 6 – 5 µM 3AC + 1 µM idelalisib; 7 – 1 µM idelalisib.*

4.3 CLL cells adhere to stromal cells under shear stress conditions

The BM and lymphoid tissue protect CLL cells from apoptosis and promote CLL proliferation[266]. Whether CLL proliferation is partially driven by leukemic stem cells or B-cell malignancies generally consist of uniform, mature clones is yet under debate. To shed light onto the re-circulating capacity of CLL cells, the ability of PB CLL cells to adhere to stromal cells or coated surfaces under physiological shear stress conditions was tested. Physiological shear stress conditions in a practical

experimental set-up were reached by modifying an available pump system[267,268]. In this assay, a layer of stromal cells was seeded into a microscopy slide chamber. This slide was connected to tubing of defined length and diameter. PKH26-stained lymphocytes were injected directly onto the layer of stromal cells in the channel slide. After an initial adhesion phase, increasing shear stress was applied over time and during constant microscopy imaging. Cell adhesion was measured as a function of image area occupied by lymphocytes and normalised to the initial area occupied before either drug administration or start of shear stress. Two of these systems could run in parallel imaging. Despite its low throughput character, subsequent image analysis was performed on thousands of time-lapse images delivering high content data and allowed downstream assays to distinguish between adherent and non-adherent cell populations. In the system, previously stained stromal cell line HS-5 or human mesenchymal stem cells (hMSCs) were seeded into microscopy channel slides (µ-slides). After adherence and approximately 50% confluence, differently stained CLL cells were injected into the channel. CLL cells were allowed to adhere and located in close proximity to HS-5 cells or hMSCs (Figure 11A). The adhesion of CLL cells to different standard cell culture coatings was tested in comparison to the adhesion onto stromal cells. Similar as all other tested coatings, standard cell culture coating (ibiTreat®) resulted in very low attachment of CLL cells under shear stress. CLL cells covered an almost 10-fold larger area on stromal cells seeded on ibiTreat, compared to the coated, stromal cell-free control (Figure **11**B). Thus, a fraction of PB CLL cells had the ability to adhere onto stromal cells while the standard coating of adherent cell culture did not result in CLL cell retention or re-adherence. The proximity and the effect of the shear stress on stromal and CLL cells was assed using independent cell stainings (Figure 11C,D).

The small size (250 mm^2) of the channel slides and the technical set-up challenged downstream approaches with low cell numbers after detachment from the channel slide. An optimised flow cytometry protocol allowed to compare adherent from non-adherent fractions. Strikingly, in the CLL cell line HG3, the adherent cell fraction was composed of significantly fewer SHIP1+ cells than the non-adherent fraction. This difference increased over time under shear stress. Thus, only SHIP1 low or negative cells were able to remain attached to stromal cells under long and high exposure to shear stress (Figure 11E). While this observation was not significant in primary CLL samples, the paired comparisons showed a tendency of samples with larger SHIP1+ fractions to split into SHIP1+ non-adherent and SHIP1- adherent populations (Figure 11F). The adherent, non-adherent and not shear stress-challenged cell populations were further observed to express different levels of PTEN. Strikingly, the adherent cell population expressed the highest PTEN levels. PTEN levels were lowest in the non-adherent CLL cells. This difference that adherent CLL cells

express higher PTEN levels was also observed when the BCR signalling was inhibited by the PI3K inhibitor idelalisib (Figure 12). To summarise, the two phosphatases PTEN and SHIP1 showed reciprocal expression patterns in adherent versus non-adherent fractions of CLL cells that were previously extracted from PB.

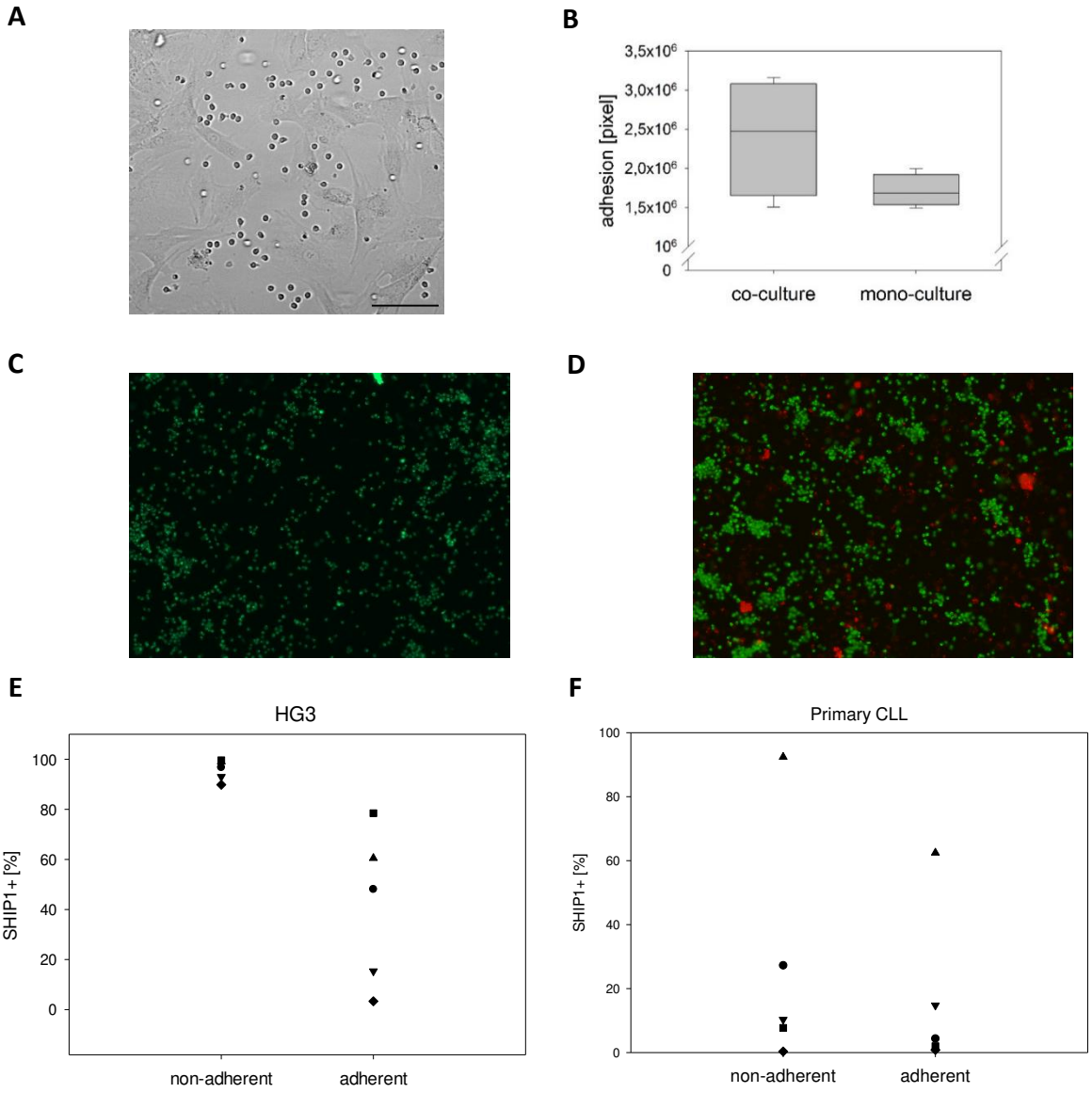


Figure 11 CLL cell adhesion under shear stress; *(A) CLL cells co-localise with MSCs and remain attached under flow conditions; (B) CLL cell attachment compared between co-culture with HS-5 cells and mono-culture; (C) staining of CLL cells for quantification; (D) merge with HS-5 cell staining.*

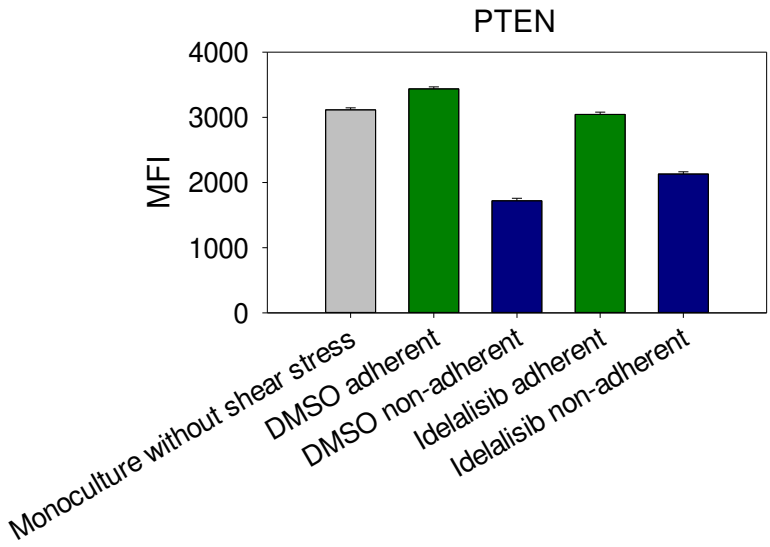


Figure 12 PTEN levels in CLL populations under flow.

4.4 The BCR signalling is associated to CLL cell adhesion onto stromal cells

To elucidate the mechanisms involved in CLL adhesion, the adherent and non-adherent fractions of HG-3 cells after six hours of increasing shear stress were separated and stained for phospho-flow of SYK, AKT and BTK. The phosphorylation levels of SYK, AKT and BTK were increased in the adherent fraction compared to the non-adherent fraction (Figure 13A). Thus, adherent CLL cells displayed a higher level of BCR activation. Next, primary CLL cells were pre-treated with anti-IgM antibodies to stimulate BCR signalling and compared to solvent control (Figure 13B,C). Interestingly, a larger M-CLL cell fraction adhered to stromal cells under shear stress above 0.15 dyn/cm^2 when pre-treated with a-IgM (Figure 13B). In contrast, U-CLL cells did not differ in the number of cells adherent to stromal cells when treated with a-IgM (Figure 13C). During no shear stress or shear stress below 0.15 dyn/cm^2, more CLL cells of both U-CLL and M-CLL (re-)adhered to stromal cells than detached from stromal cells. This observation was independent of the a-IgM treatment (Figure 13). U-CLL and M-CLL also differed in the adhesion response to PI3K inhibition. To test the impact of the PI3K inhibitor idelalisib on CLL adhesion, CLL cells were injected without prior inhibitor treatment into the pump system. After 20 min of low shear stress, idelalisib or solvent control was injected into the flow media. For U-CLL cells, PI3K treatment resulted in a minor reduction in adherent cell numbers (Figure 13D).

Similar to PI3K inhibition, BTK inhibition by ibrutinib resulted in a reduction of adherent cells in M-CLL (Figure 14A) but caused almost no additional loss of adherence under shear stress in U-CLL (Figure 14B). The effect of ibrutinib was observable within 20 min after drug application when

0.2 dyn/cm^2 shear stress was applied. When high shear stress of 0.8 dyn/cm^2 was applied, more cells lost adhesion but a fraction of CLL cells remained attached to stromal cells (Figure 14A,B). The effect of ibrutinib on CLL adhesion was stronger when the cells were incubated with ibrutinib before shear stress was applied but after initial co-culture incubation (Figure 14C). To confirm the reproducibility of observations within and between individual experiments, the correlation of replicates was determined and compared. Most samples and most technical replicates showed a strong correlation in the time-resolved change of adherent cell numbers. The correlation between technical and biological replicates proved the reproducibility of measurements inside the individual channel slides and between different channel slides (Figure 14D).

SHIP1 is an antagonist of PI3K[175]. Inhibitors and activators of SHIP1 have entered clinical research that also includes B-cell malignancies[179,181,264]. To test the role of SHIP1 activity in CLL cell adhesion, the SHIP1 inhibitor 3AC and the SHIP1 agonist AQX-MN100 were tested in the adhesion assay. The inhibition of SHIP1 by 3AC led to no loss of adherence under shear stress below 0.2 dyn/cm^2 and an increased retention of CLL cells on stromal cells under higher shear stress levels (Figure 15A). The activation of SHIP1 activity by AQX-MN100 reduced the number of CLL cells adherent under shear stress. The loss of adherence was observed from the lowest shear stress of 0.1 dyn/cm^2 and became more pronounced with 0.15 dyn/cm^2 and 0.2 dyn/cm^2 while higher shear stress did not increase the difference in cellular adhesion between treated and untreated samples. After more than 24 h of high shear stress, less than 10% of initially attached AQX-MN100-treated CLL cells remained bound to the stromal cells while more than 10% remained attached in the solvent control. Both control and AQX-MN100 treated conditions led to a continuous loss of adhesion under high shear stress without detaching all CLL cells in the measured time range. The reduced cell numbers were also observed in U-CLL samples (Figure 15B). Thus, the BCR in general and SHIP1 in particular impact the ability of CLL cells to adhere onto cells of their microenvironment.

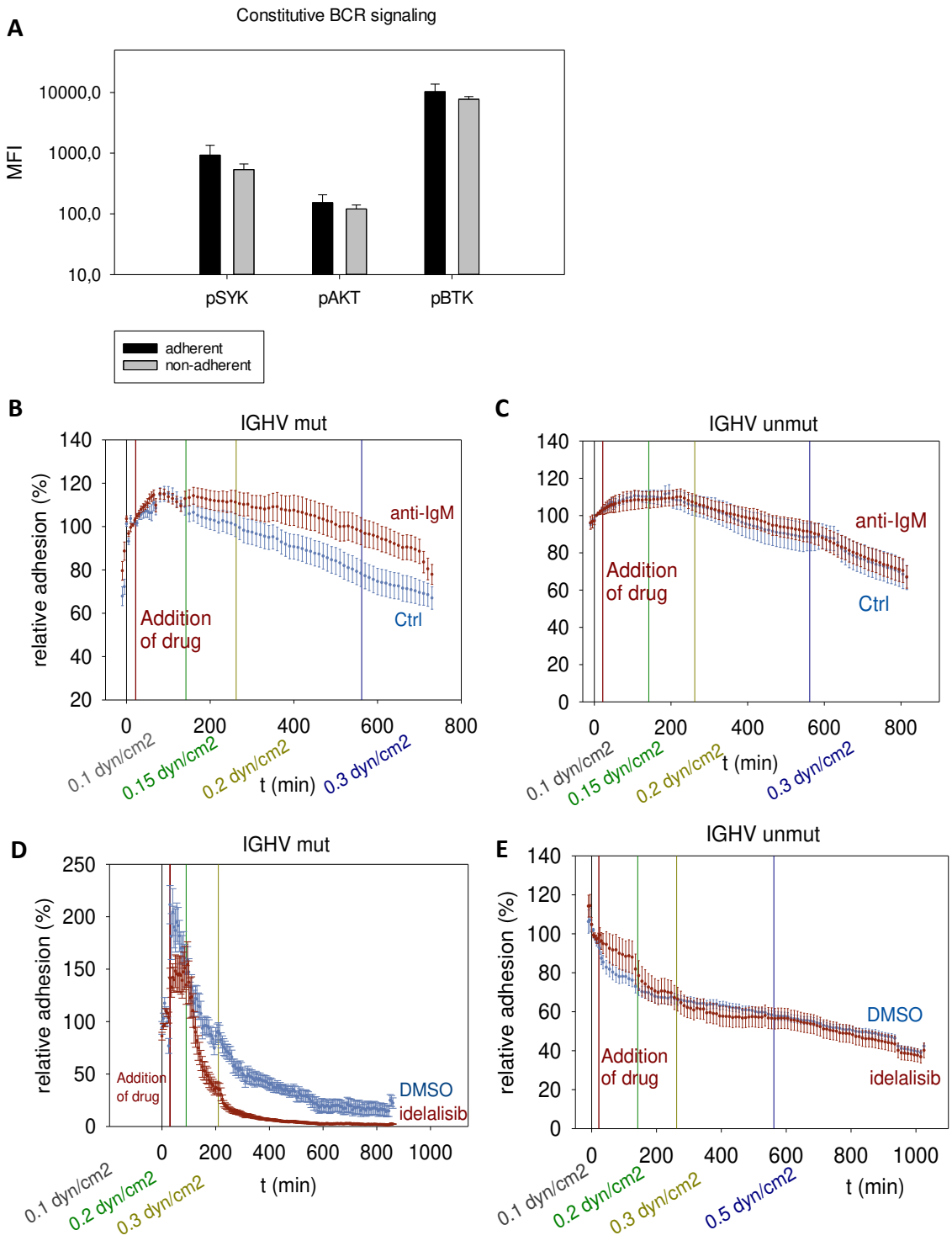


Figure 13 BCR signalling components affect CLL cell adherence capacity*; (A) phosphorylation levels of BCR components in adherent and non-adherent fractions of CLL; (B) adherent and non-adherent CLL cells were measured for phosphorylation of BCR signalling pathway components by flow cytometry; (B) M-CLL and (C) U-CLL cells were treated with anti-IgM during shear stress application; (D) M-CLL and (E) U-CLL cells were treated with the PI3K inhibitor idelalisib; the time of the addition of drug is indicated by a red vertical line, increases in shear stress by colour-coded vertical lines.*

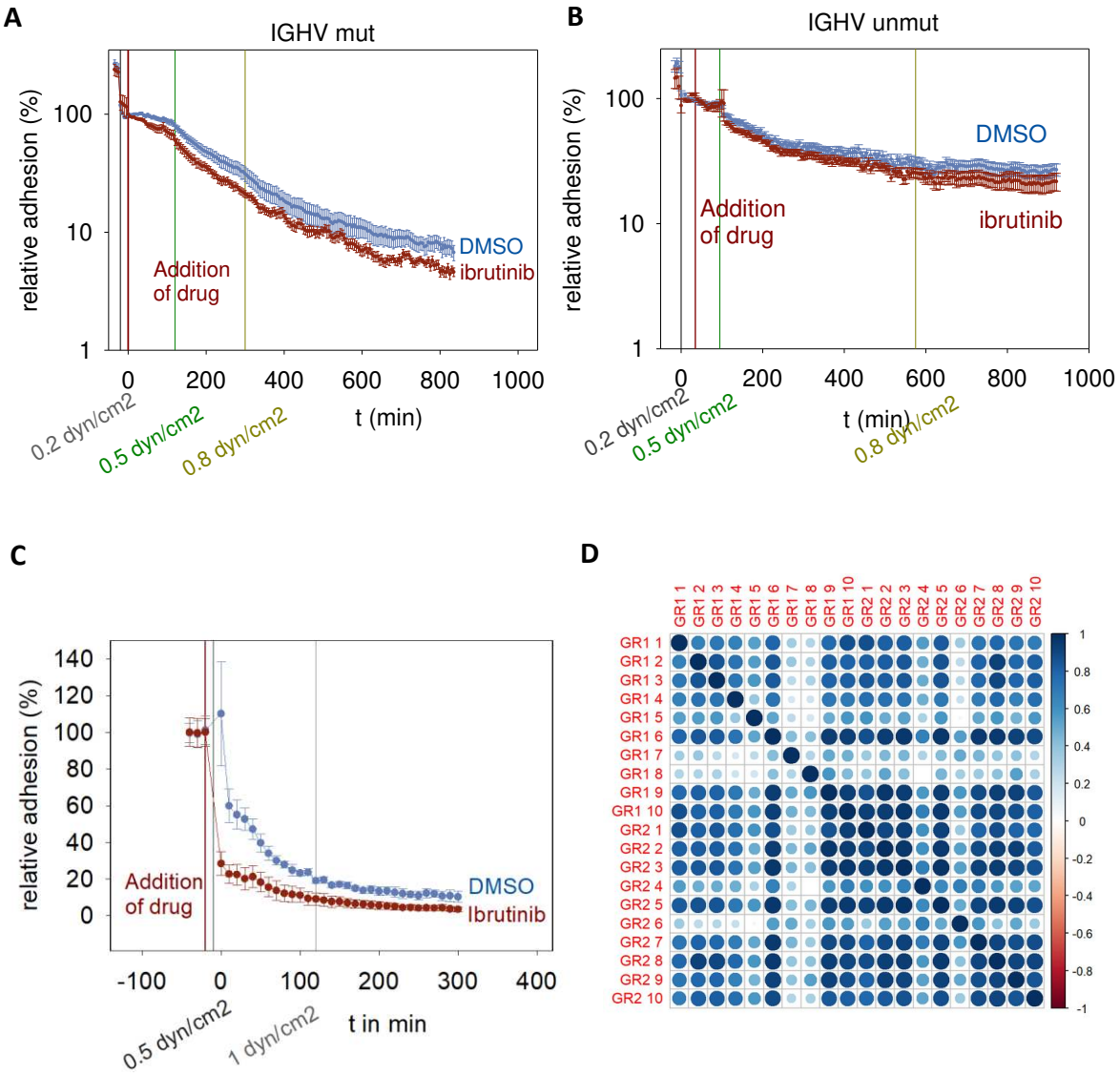


Figure 14 Validation of BCR involvement in CLL cell attachment*; (A) M-CLL and (B) U-CLL cells were treated with ibrutinib during shear stress application; (C) M-CLL cells were treated with ibrutinib before start of flow; (D) intra-assay validation through correlation of technical replicates, graph D contains joint data analysis with Dominic Edelmann.*

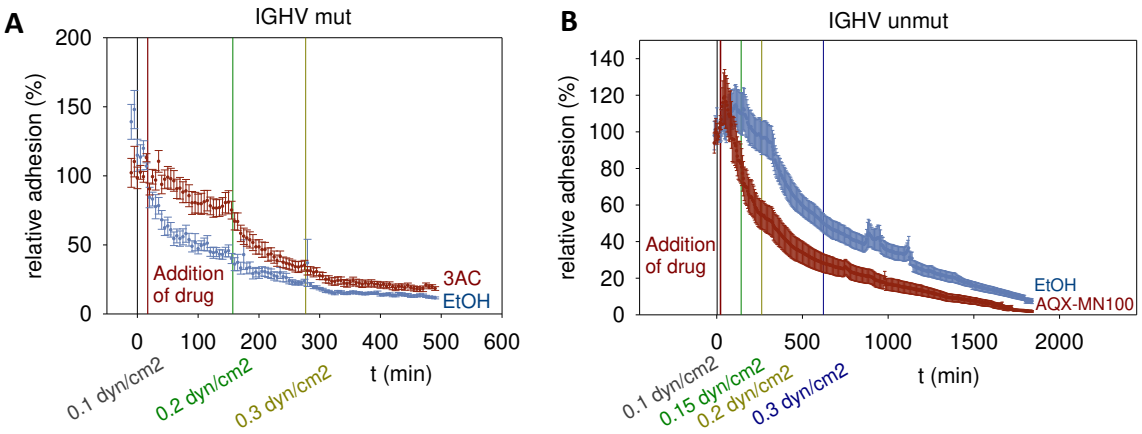


***Figure 15 SHIP1 activity determines adherence of M-CLL and U-CLL cells**; (A) M-CLL treated with SHIP1 inhibitor 3AC; (B) U-CLL cells treated with SHIP1 agonist.*

4.5 VHL bridges BCR signalling to adhesion molecules

To complete the picture of BCR-dependent adhesion of CLL cells onto cells of their microenvironment, a set of inhibitors and blocking antibodies were used to determine the link between the intracellular signalling machinery and the surface-bound adhesion-mediating receptors, integrins or other molecules. First, the role of CXCR4 was investigated. CXCR4 was reported to mediate CLL-retention in lymphoid tissue[242]. In the co-culture system, in the shear stress range from 0.5 dyn/cm^2 to 0.7 dyn/cm^2 (HG3; Figure 16A) or 0.1 dyn/cm^2 to 0.3 dyn/cm^2 (primary CLL cells; Figure 16B), there was no impact of CXCR4 inhibition observed (Figure 16). Among the candidates to be involved in CLL cell interaction with the lymphoid microenvironment was the vHL protein. Three knock-out cell lines were received from Michaela Reichenzeller, University Hospital of Ulm. The complete knock-out lines D3 and D5 as well as the knock-out of the long isoform only, D7. Complete (Figure 17A,B) as well as the knock-out of the long isoform (Figure 17C) reduced the adherence of CLL cells to stromal cells under shear stress. The D3 cell lines showed an increased ability to adhere onto stromal cells under low shear stress of 0.1 dyn/cm^2 with a similar adhesion loss under higher shear stress of 0.2 dyn/cm^2 compared to wildtype HG3 cells (Figure 17A). The full knock-out cell line D5 had similar adhesion capabilities as wild type under low shear stress but continued to increase cellular adhesion under higher shear stress while wild type cells lost adhesion or remained stably attached (Figure 17B). The vHL long isoform knock-out differed from wild type cells in the reduced loss of adhesion under higher shear stress (Figure 17C).

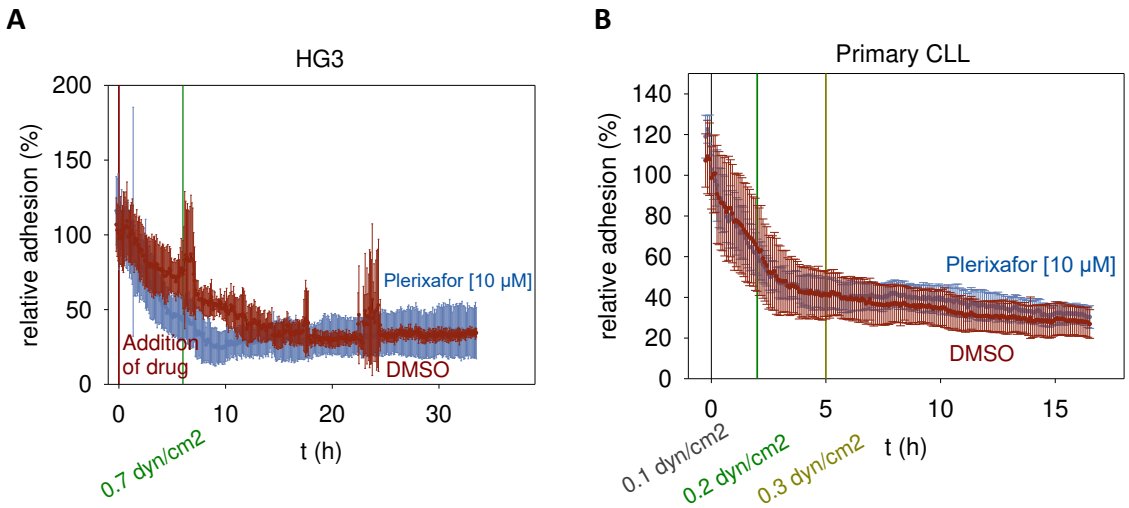


Figure 16 Plerixafor as inhibitor of CXCR4*; (A) HG3 cells and (B) primary CLL cells treated with plerixafor.*

All in all, the link between BCR signalling and CLL cell adhesion remains insufficiently characterised and appeared to be connected to the vHL protein, thus the HIF1a/VEGF pathway. SHIP1 plays an important role in inhibiting the adhesion of B lymphocytes to cells of the supportive microenvironment and is reduced and/or inactivated in adherent CLL cells. The established co-culture adhesion under flow assay could determine the effect of signaling factors and/or (small) molecules on CLL cell adhesion to cells of the lymphoid or BM microenvironment.

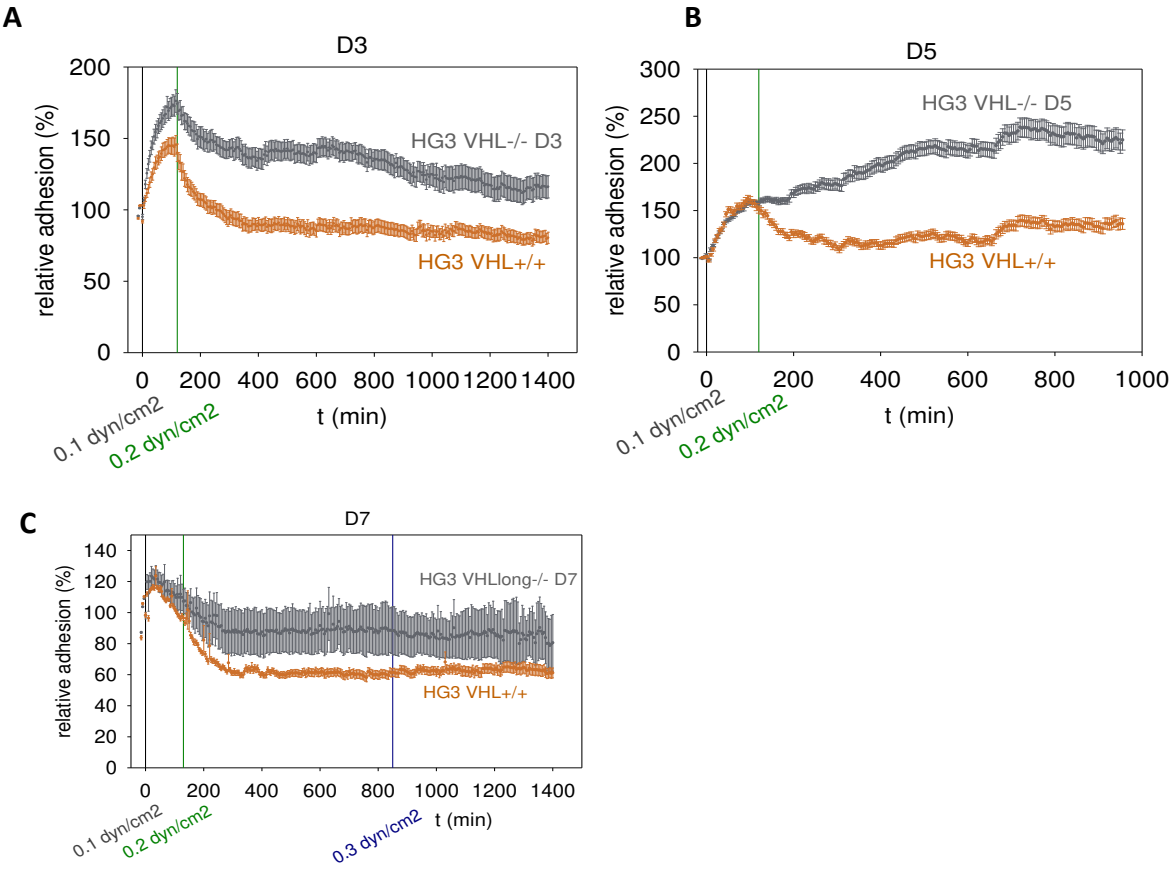


***Figure 17 The impact of vHL on CLL adherence**; (A) full knock-out clone D3; (B) full knock-out clone D5; (C) long isoform knock-out D7.*

5 Discussion

The dysregulation of signalling pathways was early on identified as a hallmark of cancer[1]. In cancer, the cellular response is neither adequate to nor independent of the respective external stimuli. The phosphorylation of signalling effectors can be hyperactive or hyper-reactive through dysregulated kinase activity. The serial events following the hyperactive signalling pathway lead to the translation of oncogenic target genes by the respective transcription factors. In B-cells, the BCR signalling pathway regulates differentiation, survival, proliferation, and antibody production[115,269,270]. The hyperactive BCR signalling in malignant B-cells through PI3K and AKT leads to dysregulated expression of NFATC and NF-KB target genes[125,127,271]. During the last decade, BCR signalling pathway-targeting therapies have revolutionised CLL treatment[108,136]. Strikingly, increased lymphocytosis is associated to treatment success[240]. This lymphocytosis was seen to derive from the loss of BCR-dependent adhesion of CLL cells to stromal cells[242]. Still, the link between BCR signalling and adhesion remains poorly understood. In the light of the recent advancements in targeting SHIP1[181,264], the antagonist of PI3K signalling[175], SHIP1 might complete the therapy regimen against BCR-driven CLL.

The role of inositol phosphatases in BCR signalling, CLL pathogenesis and ultimately, in CLL cell adhesion onto cells of the protective niche was investigated thoroughly in the present work. The in-depth quantitative and qualitative characterisation of BCR signalling components under stimulation[10] paved the way for the analysis of the contribution of SHIP1. SHIP1 negatively regulated the BCR signalling and likewise SHIP1 activity negatively impacted on CLL cell adhesion. The complete connection between BCR/SHIP1 and responsible adhesion molecules still has to be drawn. The clinical need for novel therapies[272] should drive further research on CLL adhesion mechanisms and SHIP1 targeting.

5.1 The BCR is the key activating receptor in CLL

With anti-IgM and H_2O_2 treatment the activation of the BCR signalling could be mimicked in the cultured CLL cells (Figure 2). The BCR activation caused changes in the phosphorylation landscape of signalling factors going beyond the presence of H_2O_2 in the cells or culture media or cytosol[273]. The alteration of the phosphorylation landscape indicated that the BCR activation changed the overall activation state of CLL cells. In healthy B-cells, BCR signalling leads to differentiation,

supports survival and proliferation, and regulates antibody production[115,269,270]. In CLL cells, active BCR signalling is essential for the malignant pathogenesis[133,134,274]. Though, the initial malignant transformation can rarely be associated with BCR signalling pathway mutations[19]. The BCR signalling inhibition reduces the differences in prognosis between IGHV-mutation status-dependent patient subgroups[136,137]. Though in the phospho-flow experiments, the BCR signalling activity patterns differed between IGHV subgroups[10].

When detecting co-dependencies between signalling factors, SHIP1 expression was associated with lower phosphorylation levels of PLCγ2 and BTK. BTK and PLCγ2 phosphorylation seemed not to be dependent on PTEN levels (Figure 4A-D). The observations of co-dependencies between BCR signalling factors and PTEN and SHIP1 expression was based on H_2O_2 and anti-IgM stimulation. The PIP_3 measurements indicated that H_2O_2 stimulation alone was not causing PIP_3 levels to rise (Figure 7). PIP_3 levels rose when anti-IgM was applied to CLL cells. Still, H_2O_2 alone was able to increase the phosphorylation of BCR signalling factors (Figure 3). Interestingly, a low degree of sensitivity to H_2O_2-dependent stimulation had been associated to more aggressive disease[275].

5.2 Both SHIP1 and PTEN control BCR signalling but under different stimulation states

As mentioned, SHIP1 but not PTEN expression was associated to lower BTK and PLCγ2 phosphorylation upon stimulation in single-cell resolved phospho-flow (Figure 4A-D). That association underlined the role of SHIP1 to antagonise activation-dependent phosphorylation[175,276,277]. PTEN but not SHIP1 was reported to be reversibly inhibited by H_2O_2. Thus, in an H_2O_2-dependent stimulation, the PTEN inhibition may play an important role in the control of BCR signalling[275,278]. The knock-down experiments confirmed the role of PTEN in constitutive BCR signalling but could not show an effect on stimulatability (Figure 9). Taken into consideration that knock-down experiments are generally and were also here conducted between 48 h to 72 h post nucleofection[279], the long-term effects of PTEN deficiency might not have been observed. PTEN deficiency was reported to be associated with loss of BCR expression. PTEN upregulation was associated with increased BCR selectivity through IgD BCR formation[280]. Thus, these results indicate the crucial role of PTEN in overall BCR expression and may explain the underrepresentation of PTEN mutations in CLL compared to other malignant entities[162,163]. The lack of significant SHIP1 mutations in the genetic CLL landscape might be explained by the crucial role of its PIP_3 degradation product $PI(3,4)P_2$ in the complete membrane-recruitment and

activation of AKT[177]. Through the degradation of PIP_3, SHIP1 regulates the amplitude of BCR signalling. By overlaying SHIP1 expression in combined pools of stimulated cells, it became evident that SHIP1 antagonised the activation-dependent phosphorylation (Figure 4). The high variability in regards to SHIP1 expression between CLL patients and the association of low SHIP1 expression to worse clinical parameters confirm the regulating role of SHIP1 in BCR signalling (Figure 11 and [281]). To further investigate the mechanisms of SHIP1 dysfunction or dysregulated expression in-depth transcriptional analysis will be necessary. The miRNAs that were predicted to target PTEN or SHIP1 showed neither positive nor negative correlation between the miRNA and phosphatase transcript quantifications (Figure 8). This missing correlation directs to the findings that PTEN and/or SHIP1 are regulated on protein level, *e.g.* by phosphorylation[281–285] or subcellular localisation[286–288]. The post-translational modifications of PTEN and SHIP1 are dysregulated in CLL cells and thus, the role of BCR control is reduced or lost[164,281]. Apart from translational and post-translational dysregulation, also transcriptional dysfunction at the PTEN locus was found to interfere with BCR control through loss of PTEN expression[162]. It is thus evident that the dysregulation of PTEN and/or SHIP1 lead to loss of BCR signalling control, either in constitutive or stimulation-dependent signalling, respectively.

5.3 CLL cells remain activated during adhesion to cells of the microenvironment

It is widely accepted that the TME plays a crucial role in CLL pathogenesis[187,188,289,290]. The microenvironmental conditions can directly impact on treatment outcome. *E.g.* T-cell dysfunction was generally observed in CLL[202] but anti-PD-1/anti-PD-L1 treatment was found to not be beneficial in refractory or relapsed CLL[291]. Thus, in refractory or relapsed CLL, an immunosuppressive TME likely causes the treatment failure[292]. During CLL pathogenesis, a crosstalk between malignant cells and cells of the microenvironment lead to changes in the cell signalling activity and the surface molecule composition[187,289,290,293]. The surface molecule composition decides about the quality and duration of direct contact between CLL and cells of the microenvironment. Direct interaction between stromal cells and CLL cells is indispensable for CLL cell survival and proliferation[294,295]. The survival and proliferation signals are triggered upon binding of CLL cells to integrins, to the B-cell activating factor (BAFF), and to a proliferation-inducing ligand (APRIL)[293]. Besides, cancer-reprogrammed stromal cells, including endothelial cells and fibroblasts, co-evolve with CLL. This co-evolution leads to promoting survival and proliferation and produces a *cold* TME[289]. The spatial organisation, the immune subset functionality within the

TME and other mechanisms induced or supported by stromal cells influence B-cell malignancy progression and aggressiveness[289,296].

In the LN, the main TME of CLL, lymphocytes generally enter through afferent lymphatics of lymphatic endothelial cells or through peripheral circulation along high endothelial venues. The homing is dependent on integrin and adhesion molecule surface expression as well as chemokines and chemokine receptor levels[289]. Furthermore, the LN stromal cells play an immunomodulatory role, *e.g.* by priming T cells[297], BM stromal cells also assist in B-cell differentiation[298,299]. The LN as TME of CLL is characterised by differential composition and expression patterns compared to healthy LNs[296,300]. In CLL LNs, *e.g.* higher ratios of αSMA^+ mesenchymal cells were detected compared to control LNs[300] and the level of angiogenesis, directed by an angiogenic switch in stromal cells[301], has a prognostic value in CLL diagnosis[302–304].

Also in a CLL mouse model, the co-development of progressive CLL and supporting stromal cells was described[305]. CLL cells secrete extracellular vesicles that change the phenotype of stromal cells into the cancer-associated fibroblasts phenotype[306,307].

By disrupting the contact between CLL cells and stromal cells, therapeutic options arise, *e.g.* in rituximab resistance CLL cases, blocking VLA-4, a crucial CLL cell integrin, restores vulnerability to treatment[196]. This therapeutic option and others targeting the stroma niche are currently under investigation[196,308–310].

The importance of adhesion in CLL cell survival is underlined by the lymphocytosis upon BCR inhibition treatment being a positive prognostic marker[240,241]. As both PTEN and SHIP1 activity have direct impact on BCR signalling (Figure 9), the question for their function in CLL cell adhesion arose. The role of PTEN and SHIP1 in the control of leukocyte adhesion and migration was extensively described for neutrophils. There, SHIP1 expression was found to be associated with reduced adhesion[311]. In the adhesion under flow experiments, SHIP1 inhibition led to a larger fraction of CLL cells adhering to stromal cells under shear stress. Thus, SHIP1 activity is present in at least a fraction of peripheral CLL cells limiting their capacity to home to the lymphoid tissue. Another fraction of peripheral CLL cells has already regained adhesive capacity and is able to remain attached on stromal cells even under physiological shear stress. This fraction represents the CLL cells at the state before migrating from the periphery to the lymphoid tissue. In contrast, inducing the activity of SHIP1 led to reduced adhesion of CLL cells on stromal cells. This reduced adhesion indicated that SHIP1 was expressed in the adherent cells but inactive. Thus, not expression levels but post-translational modifications and localisation regulate SHIP1 activity. This observation, by using the agonist AQX-MN100, opened the possibility for therapeutical

intervention using SHIP1 as a future target. As the differences in adherent fractions remained under different shear stress levels, the density of surface adhesion molecules may play an additional role in determining the adhesive capacity of CLL cell fractions.

5.4 Integrins of the HIF1a/VHL axis mediate adhesion but not CXCR4

Integrins mediate the direct interactions and adhesion between CLL cells and stromal cells. The subset of ß1- and ß2-integrins were found to mediate spontaneous and drug-induced apoptosis evasion by CLL cells through physical contact to endothelial stroma cells[312]. Besides integrins, chemokine receptors were also reported to play a role in lymphocyte adhesion[313–315]. *E.g.* CCR7 was observed to induce lymphoma cell homing[315]. Interestingly, Plerixafor, a CXCR4 inhibitor, inhibited adhesion in MCL[316] but not in my CLL adhesion model (Figure 16).

Through gene expression profiling comparing mono- and stromal co-cultured CLL cells, VHL was found downregulated in the stroma co-cultured fraction[317]. As VHL was previously shown to influence adhesive capacity of cancer cells under selective substrate and surface molecule compositions[318–320]. Interestingly, VHL and SHIP1 were both reported to be targeted by miR-155[259,321].

The adhesion experiments indicated that VHL plays an important role in regulating CLL adhesion. The knock-out of VHL increased the adhesive capacity of HG3 cells to stromal and among HG3 cells. The connection between VHL and SHIP1 needs to be further elucidated by addressing signalling linking factors and the target integrins and surface molecules as well as influences on transcriptional patterns.

5.5 SHIP1 as a target in CLL therapy

The development of small molecules targeting phosphatases is still far behind of the multitude of kinase inhibitors[322,323]. A SHIP1 inhibitor, 3AC, was first shown to upregulate the immunoregulatory capacity of myeloid cells[179]. While the adhesion assay indicated SHIP1 activation to be beneficial for CLL treatment through loss of adhesion, SHIP1 inhibition was also reported to induce apoptosis of cancer or haematological malignant cells[169,179,324]. With the sea sponge extract-derived pelorol, it was first shown that SHIP1 could be enzymatically activated[147].

The further synthesis and pre-clinical testing of SHIP1-activating compounds[147,148,182] led to first clinical trials[181,325] and further focus on SHIP1 in CLL treatment[185]. The current developments are especially interesting in the light of potential combination treatments to reduce potential paths of resistance development. Through the scientific analysis of the circuit topology of the BCR signalling pathway with modelled interconnectivity between the signalling factors, I provided a tool to understand potential paths for the success of combination treatments[10]. Thus, SHIP1 is a promising target to be evaluated in future combination treatments in CLL therapy.

5.6 Conclusion

The described work aimed to comprehensively elaborate the stimulation-dependent regulation of the BCR signalling pathway by PTEN and SHIP1 and link the activity of PTEN and SHIP1 to the adhesion capacity of CLL cells. For this reason, an optimised flow assay was set up and adhesion under shear stress measured in combination with flow cytometry analysis of signalling factors. The central finding is the involvement of SHIP1 in the inhibition of BCR-dependent adhesion of CLL cells to cells of the TME. Through distinct PIP_3 degradation, PTEN and SHIP1 impact on BCR regulation through different pathways, thereby being responsible for distinct control mechanisms.

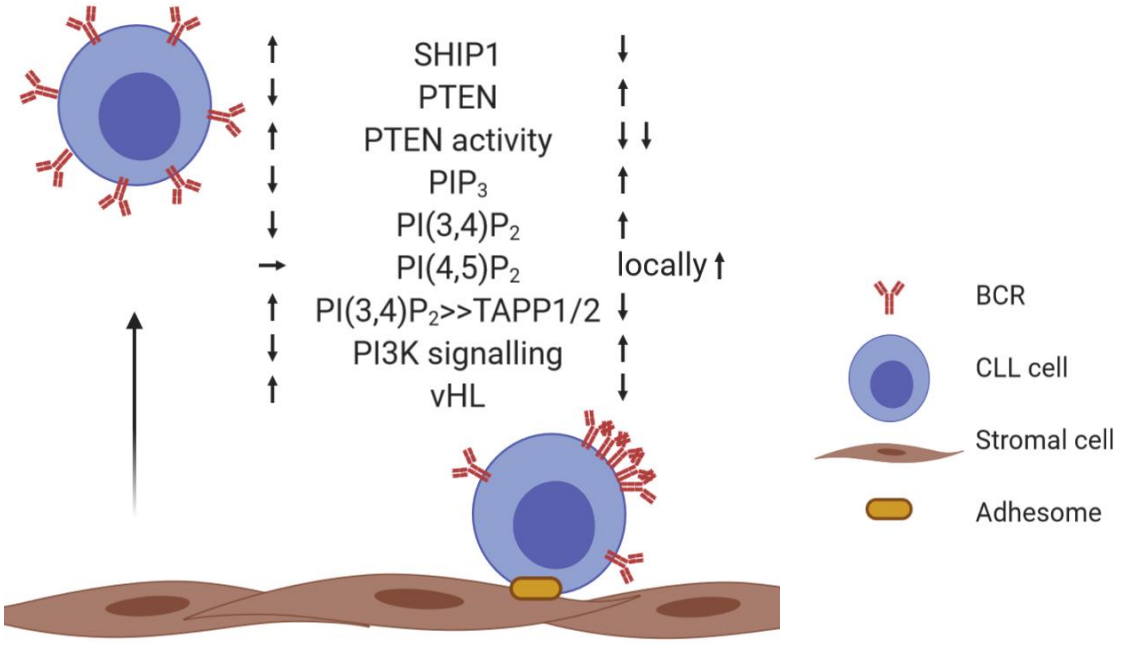


Figure 18 Signalling factors involved in CLL adherence.

Thus, the next steps include the further analysis of surface molecules of adherent and non-adherent CLL fractions and subsequent inhibition or blocking approaches. The knowledge gained from this project expands the pool of potential targets (Figure 18) for future combination treatments, especially in the light of SHIP1 being the first inositol phosphatase targeted by a biologically active agonist which has already been tested in clinical trials.

6 List of publications

Wolf, C.*, Maus, C.*, **Persicke, M.R.***, Filarsky, K., Tausch, E., Schneider, C., Döhner, H., Stilgenbauer, S., Lichter, P., Höfer, T. & Mertens, D. (2022) Modeling the B-cell receptor signaling on single cell level reveals a stable network circuit topology between non-malignant B-cells and chronic lymphocytic leukemia cells and between untreated cells and cells treated with kinase inhibitors. International Journal of Cancer.

*These authors contributed equally

7 References

1. Hanahan D, Weinberg RA. The Hallmarks of Cancer. *Cell* 2000;100:57–70.

2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.

3. Sever R, Brugge JS. Signal Transduction in Cancer. *Cold Spring Harb Perspect Med* 2015;5:a006098.

4. Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nature medicine* 2005;11:1188.

5. He Y, Sun MM, Zhang GG, Yang J, Chen KS, Xu WW, Li B. Targeting PI3K/Akt signal transduction for cancer therapy. *Signal Transduct Target Ther* 2021;6:425.

6. Lawrence MS, Stojanov P, Mermel CH, Garraway LA, Golub TR, Meyerson M, Gabriel SB, Lander ES, Getz G. Discovery and saturation analysis of cancer genes across 21 tumor types. *Nature* 2014;505:495.

7. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT Pathway for Cancer Drug Discovery. *Nat Rev Drug Discov* 2005;4:988–1004.

8. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* 2009;9:550–62.

9. Hallek M, Al-Sawaf O. Chronic lymphocytic leukemia: 2022 update on diagnostic and therapeutic procedures. *Am J Hematol* 2021;

10. Wolf C, Maus C, Persicke MR, Filarsky K, Tausch E, Schneider C, Döhner H, Stilgenbauer S, Lichter P, Höfer T, Mertens D. Modeling the B-cell receptor signaling on single cell level reveals a stable network circuit topology between non-malignant B cells and chronic lymphocytic leukemia cells and between untreated cells and cells treated with kinase inhibitors. *International Journal of Cancer* 2022;

11. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012;21:309–22.

12. Flaberg E, Markasz L, Petranyi G, Stuber G, Dicso F, Alchihabi N, Oláh È, Csízy I, Józsa T, Andrén O, Johansson J-E, Andersson S-O, et al. High-throughput live-cell imaging reveals differential inhibition of tumor cell proliferation by human fibroblasts. *Int J Cancer* 2011;128:2793–802.

13. Spaeth EL, Dembinski JL, Sasser AK, Watson K, Klopp A, Hall B, Andreeff M, Marini F. Mesenchymal Stem Cell Transition to Tumor-Associated Fibroblasts Contributes to Fibrovascular Network Expansion and Tumor Progression. *PLoS One* 2009;4:e4992.

14. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal Fibroblasts Present in Invasive Human Breast Carcinomas Promote Tumor Growth and Angiogenesis through Elevated SDF-1/CXCL12 Secretion. *Cell* 2005;121:335–48.

15. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. *Cancer Cell* 2010;17:135–47.

16. Kipps TJ, Stevenson FK, Wu CJ, Croce CM, Packham G, Wierda WG, O'Brien S, Gribben J, Rai K. Chronic lymphocytic leukaemia. *Nat Rev Dis Primers* 2017;3:1–22.

17. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer Statistics, 2007. *CA: A Cancer Journal for Clinicians* 2007;57:43–66.

18. Watson L, Wyld P, Catovsky D. Disease burden of chronic lymphocytic leukaemia within the European Union. *European Journal of Haematology* 2008;81:253–8.

19. Landau DA, Tausch E, Taylor-Weiner AN, Stewart C, Reiter JG, Bahlo J, Kluth S, Bozic I, Lawrence M, Böttcher S, Carter SL, Cibulskis K, et al. Mutations driving CLL and their evolution in progression and relapse. *Nature* 2015;526:525–30.

20. Rawstron AC, Bennett FL, O'Connor SJM, Kwok M, Fenton JAL, Plummer M, de Tute R, Owen RG, Richards SJ, Jack AS, Hillmen P. Monoclonal B-Cell Lymphocytosis and Chronic Lymphocytic Leukemia. *N Engl J Med* 2008;359:575–83.

21. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, Advani R, Ghielmini M, Salles GA, Zelenetz AD, Jaffe ES. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* 2016;127:2375–90.

22. Matutes E, Owusu-Ankomah K, Morilla R, Garcia Marco J, Houlihan A, Que TH, Catovsky D. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia* 1994;8:1640–5.

23. Ginaldi L, De Martinis M, Matutes E, Farahat N, Morilla R, Catovsky D. Levels of expression of CD19 and CD20 in chronic B cell leukaemias. *J Clin Pathol* 1998;51:364–9.

24. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, Hillmen P, Keating M, Montserrat E, Chiorazzi N, Stilgenbauer S, Rai KR, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. *Blood* 2018;131:2745–60.

25. Binet JL, Auquier A, Dighiero G, Chastang C, Piguet H, Goasguen J, Vaugier G, Potron G, Colona P, Oberling F, Thomas M, Tchernia G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer* 1981;48:198–206.

26. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. *Blood* 1975;46:219–34.

27. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, Buchbinder A, Budman D, Dittmar K, Kolitz J, Lichtman SM, Schulman P, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 1999;94:1840–7.

28. Orchard JA, Ibbotson RE, Davis Z, Wiestner A, Rosenwald A, Thomas PW, Hamblin TJ, Staudt LM, Oscier DG. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. *Lancet* 2004;363:105–11.

29. Rassenti LZ, Huynh L, Toy TL, Chen L, Keating MJ, Gribben JG, Neuberg DS, Flinn IW, Rai KR, Byrd JC, Kay NE, Greaves A, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* 2004;351:893–901.

30. Shanafelt TD, Geyer SM, Bone ND, Tschumper RC, Witzig TE, Nowakowski GS, Zent CS, Call TG, Laplant B, Dewald GW, Jelinek DF, Kay NE. CD49d expression is an independent predictor of overall survival in patients with chronic lymphocytic leukaemia: a prognostic parameter with therapeutic potential. *Br J Haematol* 2008;140:537–46.

31. 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;94:1848–54.

32. Davi F, Langerak AW, de Septenville AL, Kolijn PM, Hengeveld PJ, Chatzidimitriou A, Bonfiglio S, Sutton L-A, Rosenquist R, Ghia P, Stamatopoulos K, ERIC, the European Research Initiative on CLL, and the EuroClonality-NGS Working Group. Immunoglobulin gene analysis in chronic lymphocytic leukemia in the era of next generation sequencing. *Leukemia* 2020;34:2545–51.

33. Allsup DJ, Kamiguti AS, Lin K, Sherrington PD, Matrai Z, Slupsky JR, Cawley JC, Zuzel M. B-cell receptor translocation to lipid rafts and associated signaling differ between prognostically important subgroups of chronic lymphocytic leukemia. *Cancer Res* 2005;65:7328–37.

34. Hervé M, Xu K, Ng Y-S, Wardemann H, Albesiano E, Messmer BT, Chiorazzi N, Meffre E. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J Clin Invest* 2005;115:1636–43.

35. Kulis M, Heath S, Bibikova M, Queirós AC, Navarro A, Clot G, Martínez-Trillos A, Castellano G, Brun-Heath I, Pinyol M, Barberán-Soler S, Papasaikas P, et al. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nat Genet* 2012;44:1236–42.

36. Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, Dürig J, Küppers R. Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J Exp Med* 2012;209:2183–98.

37. ten Hacken E, Burger JA. Microenvironment interactions and B-cell receptor signaling in Chronic Lymphocytic Leukemia: implications for disease pathogenesis and treatment. *Biochim Biophys Acta* 2016;1863:401–13.

38. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 1997;91:661–72.

39. Miyazaki K, Miyazaki M, Murre C. The Establishment of B versus T Cell Identity. *Trends Immunol* 2014;35:205–10.

40. Inlay MA, Bhattacharya D, Sahoo D, Serwold T, Seita J, Karsunky H, Plevritis SK, Dill DL, Weissman IL. Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development. *Genes Dev* 2009;23:2376–81.

41. Alt FW, Blackwell TK, DePinho RA, Reth MG, Yancopoulos GD. Regulation of genome rearrangement events during lymphocyte differentiation. *Immunol Rev* 1986;89:5–30.

42. Brack C, Hirama M, Lenhard-Schuller R, Tonegawa S. A complete immunoglobulin gene is created by somatic recombination. *Cell* 1978;15:1–14.

43. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: molecular biology and regulation. *Annu Rev Immunol* 1992;10:359–83.

44. Grandien A, Fucs R, Nobrega A, Andersson J, Coutinho A. Negative selection of multireactive B cell clones in normal adult mice. *Eur J Immunol* 1994;24:1345–52.

45. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science* 2003;301:1374–7.

46. Gay D, Saunders T, Camper S, Weigert M. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J Exp Med* 1993;177:999–1008.

47. Tiegs S, Russell D, Nemazee D. Receptor editing in self-reactive bone marrow B cells. *J Exp Med* 1993;177:1009–20.

48. Noorchashm H, Bui A, Li HL, Eaton A, Mandik-Nayak L, Sokol C, Potts KM, Puré E, Erikson J. Characterization of anergic anti-DNA B cells: B cell anergy is a T cell-independent and potentially reversible process. *Int Immunol* 1999;11:765–76.

49. Rathmell JC, Cooke MP, Ho WY, Grein J, Townsend SE, Davis MM, Goodnow CC. CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature* 1995;376:181–4.

50. Foote LC, Marshak-Rothstein A, Rothstein TL. Tolerant B Lymphocytes Acquire Resistance to Fas-mediated Apoptosis after Treatment with Interleukin 4 but Not after Treatment with Specific Antigen Unless a Surface Immunoglobulin Threshold Is Exceeded. *J Exp Med* 1998;187:847–53.

51. Cyster JG, Hartley SB, Goodnow CC. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature* 1994;371:389–95.

52. Blink EJ, Light A, Kallies A, Nutt SL, Hodgkin PD, Tarlinton DM. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J Exp Med* 2005;201:545–54.

53. De Silva NS, Klein U. Dynamics of B cells in germinal centres. *Nat Rev Immunol* 2015;15:137–48.

54. Allen CDC, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, Cyster JG. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* 2004;5:943–52.

55. Shapiro-Shelef M, Lin K-I, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 2003;19:607–20.

56. Phan TG, Paus D, Chan TD, Turner ML, Nutt SL, Basten A, Brink R. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J Exp Med* 2006;203:2419–24.

57. Methot SP, Di Noia JM. Molecular Mechanisms of Somatic Hypermutation and Class Switch Recombination. *Adv Immunol* 2017;133:37–87.

58. Wang Y, Liu J, Burrows PD, Wang J-Y. B Cell Development and Maturation [Internet]. In: Wang J-Y, ed. B Cells in Immunity and Tolerance. Singapore: Springer, 2020 [cited 2022 Sep 5]. 1–22.Available from: https://doi.org/10.1007/978-981-15-3532-1_1

59. Baumgarth N. A Hard(y) look at B-1 cell development and function*. *Journal of immunology (Baltimore, Md : 1950)* 2017;199:3387.

60. Forconi F, Potter KN, Wheatley I, Darzentas N, Sozzi E, Stamatopoulos K, Mockridge CI, Packham G, Stevenson FK. The normal IGHV1-69-derived B-cell repertoire contains stereotypic patterns characteristic of unmutated CLL. *Blood* 2010;115:71–7.

61. Chiorazzi N, Ferrarini M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. *Blood* 2011;117:1781–91.

62. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic leukaemia. *Nat Rev Cancer* 2016;16:145–62.

63. Kikushige Y, Ishikawa F, Miyamoto T, Shima T, Urata S, Yoshimoto G, Mori Y, Iino T, Yamauchi T, Eto T, Niiro H, Iwasaki H, et al. Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia. *Cancer Cell* 2011;20:246–59.

64. Damm F, Mylonas E, Cosson A, Yoshida K, Della Valle V, Mouly E, Diop M, Scourzic L, Shiraishi Y, Chiba K, Tanaka H, Miyano S, et al. Acquired initiating mutations in early hematopoietic cells of CLL patients. *Cancer Discov* 2014;4:1088–101.

65. Caligaris-Cappio F. B-Chronic Lymphocytic Leukemia: A Malignancy of Anti-Self B Cells. *Blood* 1996;87:2615–20.

66. Chiorazzi N, Ferrarini M. B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor. *Annu Rev Immunol* 2003;21:841–94.

67. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, Freedman A, Inghirami G, Cro L, Baldini L, Neri A, Califano A, et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med* 2001;194:1625–38.

68. Tangye SG, Liu Y-J, Aversa G, Phillips JH, Vries JE de. Identification of Functional Human Splenic Memory B Cells by Expression of CD148 and CD27. *The Journal of Experimental Medicine* 1998;188:1691.

69. Colombo M, Cutrona G, Reverberi D, Bruno S, Ghiotto F, Tenca C, Stamatopoulos K, Hadzidimitriou A, Ceccarelli J, Salvi S, Boccardo S, Calevo MG, et al. Expression of Immunoglobulin Receptors with Distinctive Features Indicating Antigen Selection by Marginal Zone B Cells from Human Spleen. *Molecular Medicine* 2013;19:294.

70. Kraal G, Mebius R. New Insights into the Cell Biology of the Marginal Zone of the Spleen. *International Review of Cytology* 2006;250:175.

71. Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, Plebani A, Kumararatne DS, Bonnet D, Tournilhac O, Tchernia G, Steiniger B, et al. Human blood IgM “memory” B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* 2004;104:3647.

72. Weill J-C, Weller S, Reynaud C-A. Human marginal zone B cells. *Annu Rev Immunol* 2009;27:267–85.

73. Weller S, Mamani-Matsuda M, Picard C, Cordier C, Lecoeuche D, Gauthier F, Weill J-C, Reynaud C-A. Somatic diversification in the absence of antigen-driven responses is the hallmark of the IgM+IgD+CD27+ B cell repertoire in infants. *The Journal of Experimental Medicine* 2008;205:1331.

74. William J, Euler C, Christensen S, Shlomchik MJ. Evolution of Autoantibody Responses via Somatic Hypermutation Outside of Germinal Centers. *Science* 2002;297:2066–70.

75. Oakes CC, Seifert M, Assenov Y, Gu L, Przekopowitz M, Ruppert AS, Wang Q, Imbusch CD, Serva A, Koser SD, Brocks D, Lipka DB, et al. DNA methylation dynamics during B cell maturation underlie a continuum of disease phenotypes in chronic lymphocytic leukemia. *Nat Genet* 2016;48:253–64.

76. Vardi A, Agathangelidis A, Sutton L-A, Chatzouli M, Scarfò L, Mansouri L, Douka V, Anagnostopoulos A, Darzentas N, Rosenquist R, Ghia P, Belessi C, et al. IgG-Switched CLL Has a Distinct Immunogenetic Signature from the Common MD Variant: Ontogenetic Implications. *Clin Cancer Res* 2014;20:323–30.

77. Stamatopoulos K, Agathangelidis A, Rosenquist R, Ghia P. Antigen receptor stereotypy in chronic lymphocytic leukemia. *Leukemia* 2017;31:282–91.

78. Messmer BT, Albesiano E, Efremov DG, Ghiotto F, Allen SL, Kolitz J, Foa R, Damle RN, Fais F, Messmer D, Rai KR, Ferrarini M, et al. Multiple Distinct Sets of Stereotyped Antigen Receptors Indicate a Role for Antigen in Promoting Chronic Lymphocytic Leukemia. *The Journal of Experimental Medicine* 2004;200:519.

79. Ghiotto F, Fais F, Valetto A, Albesiano E, Hashimoto S, Dono M, Ikematsu H, Allen SL, Kolitz J, Rai KR, Nardini M, Tramontano A, et al. Remarkably similar antigen receptors among a subset of patients with chronic lymphocytic leukemia. *J Clin Invest* 2004;113:1008–16.

80. Binder M, Léchenne B, Ummanni R, Scharf C, Balabanov S, Trusch M, Schlüter H, Braren I, Spillner E, Trepel M. Stereotypical chronic lymphocytic leukemia B-cell receptors recognize survival promoting antigens on stromal cells. *PLoS ONE* 2010;5:e15992.

81. Crowther-Swanepoel D, Houlston RS. Genetic variation and risk of chronic lymphocytic leukaemia. *Semin Cancer Biol* 2010;20:363–9.

82. Slager SL, Skibola CF, Bernardo MCD, Conde L, Broderick P, McDonnell SK, Goldin LR, Croft N, Holroyd A, Harris S, Riby J, Serie DJ, et al. Common variation at 6p21.31 (BAK1) influences the risk of chronic lymphocytic leukemia. *Blood* 2012;120:843.

83. Speedy HE, Di Bernardo MC, Sava GP, Dyer MJS, Holroyd A, Wang Y, Sunter NJ, Mansouri L, Juliusson G, Smedby KE, Roos G, Jayne S, et al. A genome-wide association study identifies multiple susceptibility loci for chronic lymphocytic leukemia. *Nat Genet* 2014;46:56–60.

84. Puente XS, Pinyol M, Quesada V, Conde L, Ordóñez GR, Villamor N, Escaramis G, Jares P, Beà S, González-Díaz M, Bassaganyas L, Baumann T, et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* 2011;475:101–5.

85. Puente XS, Beà S, Valdés-Mas R, Villamor N, Gutiérrez-Abril J, Martín-Subero JI, Munar M, Rubio-Pérez C, Jares P, Aymerich M, Baumann T, Beekman R, et al. Non-coding recurrent mutations in chronic lymphocytic leukaemia. *Nature* 2015;526:519–24.

86. Döhner H, Stilgenbauer S, Benner A, Leupolt E, Kröber A, Bullinger L, Döhner K, Bentz M, Lichter P. Genomic Aberrations and Survival in Chronic Lymphocytic Leukemia. *N Engl J Med* 2000;343:1910–6.

87. Zenz T, Mertens D, Küppers R, Döhner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nat Rev Cancer* 2010;10:37–50.

88. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* 2002;99:15524–9.

89. Klein U, Lia M, Crespo M, Siegel R, Shen Q, Mo T, Ambesi-Impiombato A, Califano A, Migliazza A, Bhagat G, Dalla-Favera R. The DLEU2/miR-15a/16-1 Cluster Controls B Cell Proliferation and Its Deletion Leads to Chronic Lymphocytic Leukemia. *Cancer Cell* 2010;17:28–40.

90. Schaffner C, Stilgenbauer S, Rappold GA, Döhner H, Lichter P. Somatic ATM Mutations Indicate a Pathogenic Role of ATM in B-Cell Chronic Lymphocytic Leukemia. *Blood* 1999;94:748–53.

91. Foucar K, Rydell RE. Richter's syndrome in chronic lymphocytic leukemia. *Cancer* 1980;46:118–34.

92. Richter MN. Generalized Reticular Cell Sarcoma of Lymph Nodes Associated with Lymphatic Leukemia. *Am J Pathol* 1928;4:285-292.7.

93. Chigrinova E, Rinaldi A, Kwee I, Rossi D, Rancoita PMV, Strefford JC, Oscier D, Stamatopoulos K, Papadaki T, Berger F, Young KH, Murray F, et al. Two main genetic pathways lead to the transformation of chronic lymphocytic leukemia to Richter syndrome. *Blood* 2013;122:2673–82.

94. Fabbri G, Khiabanian H, Holmes AB, Wang J, Messina M, Mullighan CG, Pasqualucci L, Rabadan R, Dalla-Favera R. Genetic lesions associated with chronic lymphocytic leukemia transformation to Richter syndrome. *The Journal of Experimental Medicine* 2013;210:2273.

95. Quesada V, Conde L, Villamor N, Ordóñez GR, Jares P, Bassaganyas L, Ramsay AJ, Beà S, Pinyol M, Martínez-Trillos A, López-Guerra M, Colomer D, et al. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nat Genet* 2011;44:47–52.

96. Baliakas P, Mattsson M, Stamatopoulos K, Rosenquist R. Prognostic indices in chronic lymphocytic leukaemia: where do we stand how do we proceed? *J Intern Med* 2016;279:347–57.

97. Ahn IE, Tian X, Ipe D, Cheng M, Albitar M, Tsao LC, Zhang L, Ma W, Herman SEM, Gaglione EM, Soto S, Dean JP, et al. Prediction of Outcome in Patients With Chronic

Lymphocytic Leukemia Treated With Ibrutinib: Development and Validation of a Four-Factor Prognostic Model. *Journal of Clinical Oncology* 2021;39:576.

98. Gribben JG, Zahrieh D, Stephans K, Bartlett-Pandite L, Alyea EP, Fisher DC, Freedman AS, Mauch P, Schlossman R, Sequist LV, Soiffer RJ, Marshall B, et al. Autologous and allogeneic stem cell transplantations for poor-risk chronic lymphocytic leukemia. *Blood* 2005;106:4389.

99. Dreger P, Döhner H, Ritgen M, Böttcher S, Busch R, Dietrich S, Bunjes D, Cohen S, Schubert J, Hegenbart U, Beelen D, Zeis M, et al. Allogeneic stem cell transplantation provides durable disease control in poor-risk chronic lymphocytic leukemia: long-term clinical and MRD results of the German CLL Study Group CLL3X trial. *Blood* 2010;116:2438–47.

100. Chemotherapeutic options in chronic lymphocytic leukemia: a meta-analysis of the randomized trials. CLL Trialists' Collaborative Group. *J Natl Cancer Inst* 1999;91:861–8.

101. Rai KR, Peterson BL, Appelbaum FR, Kolitz J, Elias L, Shepherd L, Hines J, Threatte GA, Larson RA, Cheson BD, Schiffer CA. Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. *N Engl J Med* 2000;343:1750–7.

102. Huhn D, von Schilling C, Wilhelm M, Ho AD, Hallek M, Kuse R, Knauf W, Riedel U, Hinke A, Srock S, Serke S, Peschel C, et al. Rituximab therapy of patients with B-cell chronic lymphocytic leukemia. *Blood* 2001;98:1326–31.

103. Hagemeister F. Rituximab for the treatment of non-Hodgkin's lymphoma and chronic lymphocytic leukaemia. *Drugs* 2010;70:261–72.

104. Cragg MS, Walshe CA, Ivanov AO, Glennie MJ. The biology of CD20 and its potential as a target for mAb therapy. *Curr Dir Autoimmun* 2005;8:140–74.

105. Fischer K, Bahlo J, Fink AM, Goede V, Herling CD, Cramer P, Langerbeins P, von Tresckow J, Engelke A, Maurer C, Kovacs G, Herling M, et al. Long-term remissions after FCR chemoimmunotherapy in previously untreated patients with CLL: updated results of the CLL8 trial. *Blood* 2016;127:208–15.

106. Yosifov D, Wolf C, Stilgenbauer S, Mertens D. From Biology to Therapy. *Hemasphere [Internet]* 2019 [cited 2019 Mar 11];Publish Ahead of Print. Available from: insights.ovid.com

107. Advani RH, Buggy JJ, Sharman JP, Smith SM, Boyd TE, Grant B, Kolibaba KS, Furman RR, Rodriguez S, Chang BY, Sukbuntherng J, Izumi R, et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J Clin Oncol* 2013;31:88–94.

108. Brown JR, Byrd JC, Coutre SE, Benson DM, Flinn IW, Wagner-Johnston ND, Spurgeon SE, Kahl BS, Bello C, Webb HK, Johnson DM, Peterman S, et al. Idelalisib,

an inhibitor of phosphatidylinositol 3-kinase p110δ, for relapsed/refractory chronic lymphocytic leukemia. *Blood* 2014;123:3390–7.

109. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, Dayton BD, Ding H, Enschede SH, Fairbrother WJ, Huang DCS, Hymowitz SG, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med* 2013;19:202–8.

110. Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD, Gerecitano JF, Kipps TJ, Anderson MA, Brown JR, Gressick L, Wong S, Dunbar M, et al. Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. *N Engl J Med* 2016;374:311–22.

111. Stilgenbauer S, Eichhorst B, Schetelig J, Coutre S, Seymour JF, Munir T, Puvvada SD, Wendtner C-M, Roberts AW, Jurczak W, Mulligan SP, Böttcher S, et al. Venetoclax in relapsed or refractory chronic lymphocytic leukaemia with 17p deletion: a multicentre, open-label, phase 2 study. *The Lancet Oncology* 2016;17:768–78.

112. Rezazadeh H, Astaneh M, Tehrani M, Hossein-Nataj H, Zaboli E, Shekarriz R, Asgarian-Omran H. Blockade of PD-1 and TIM-3 immune checkpoints fails to restore the function of exhausted CD8+ T cells in early clinical stages of chronic lymphocytic leukemia. *Immunol Res* 2020;68:269–79.

113. Porter DL, Hwang W-T, Frey NV, Lacey SF, Shaw PA, Loren AW, Bagg A, Marcucci KT, Shen A, Gonzalez V, Ambrose D, Grupp SA, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Sci Transl Med* 2015;7:303ra139.

114. Turtle CJ, Hay KA, Hanafi L-A, Li D, Cherian S, Chen X, Wood B, Lozanski A, Byrd JC, Heimfeld S, Riddell SR, Maloney DG. Durable Molecular Remissions in Chronic Lymphocytic Leukemia Treated With CD19-Specific Chimeric Antigen Receptor-Modified T Cells After Failure of Ibrutinib. *J Clin Oncol* 2017;35:3010–20.

115. Casola S, Otipoby KL, Alimzhanov M, Humme S, Uyttersprot N, Kutok JL, Carroll MC, Rajewsky K. B cell receptor signal strength determines B cell fate. *Nat Immunol* 2004;5:317–27.

116. Gold MR, Matsuuchi L, Kelly RB, DeFranco AL. Tyrosine phosphorylation of components of the B-cell antigen receptors following receptor crosslinking. *Proc Natl Acad Sci USA* 1991;88:3436–40.

117. Mansueto MS, Reens A, Rakhilina L, Chi A, Pan B-S, Miller JR. A reevaluation of the spleen tyrosine kinase (SYK) activation mechanism. *J Biol Chem* 2019;

118. Tsang E, Giannetti AM, Shaw D, Dinh M, Tse JKY, Gandhi S, Ho H, Wang S, Papp E, Bradshaw JM. Molecular mechanism of the Syk activation switch. *J Biol Chem* 2008;283:32650–9.

119. Liu W, Meckel T, Tolar P, Won Sohn H, Pierce SK. Intrinsic Properties of immunoglobulin IgG1 Isotype-Switched B Cell Receptors Promote Microclustering and the Initiation of Signaling. *Immunity* 2010;32:778–89.

120. Humphries LA, Dangelmaier C, Sommer K, Kipp K, Kato RM, Griffith N, Bakman I, Turk CW, Daniel JL, Rawlings DJ. Tec Kinases Mediate Sustained Calcium Influx via Site-specific Tyrosine Phosphorylation of the Phospholipase Cγ Src Homology 2-Src Homology 3 Linker. *J Biol Chem* 2004;279:37651–61.

121. Gold MR, Scheid MP, Santos L, Dang-Lawson M, Roth RA, Matsuuchi L, Duronio V, Krebs DL. The B cell antigen receptor activates the Akt (protein kinase B)/glycogen synthase kinase-3 signaling pathway via phosphatidylinositol 3-kinase. *J Immunol* 1999;163:1894–905.

122. Lindvall J, Islam TC. Interaction of Btk and Akt in B cell signaling. *Biochemical and Biophysical Research Communications* 2002;293:1319–26.

123. Afar DE, Park H, Howell BW, Rawlings DJ, Cooper J, Witte ON. Regulation of Btk by Src family tyrosine kinases. *Molecular and Cellular Biology* 1996;16:3465–71.

124. 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;191:1745–54.

125. Antony P, Petro JB, Carlesso G, Shinners NP, Lowe J, Khan WN. B cell receptor directs the activation of NFAT and NF-kappaB via distinct molecular mechanisms. *Exp Cell Res* 2003;291:11–24.

126. Han A, Saijo K, Mecklenbräuker I, Tarakhovsky A, Nussenzweig MC. Bam32 links the B cell receptor to ERK and JNK and mediates B cell proliferation but not survival. *Immunity* 2003;19:621–32.

127. Herishanu Y, Pérez-Galán P, Liu D, Biancotto A, Pittaluga S, Vire B, Gibellini F, Njuguna N, Lee E, Stennett L, Raghavachari N, Liu P, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-κB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* 2011;117:563–74.

128. Herndon TM, Chen S-S, Saba NS, Valdez J, Emson C, Gatmaitan M, Tian X, Hughes TE, Sun C, Arthur DC, Stetler-Stevenson M, Yuan CM, et al. Direct in vivo evidence for increased proliferation of CLL cells in lymph nodes compared to bone marrow and peripheral blood. *Leukemia* 2017;31:1340–7.

129. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, Yang L, Pickeral OK, Rassenti LZ, Powell J, Botstein D, Byrd JC, et al. Relation of Gene Expression Phenotype to Immunoglobulin Mutation Genotype in B Cell Chronic Lymphocytic Leukemia. *J Exp Med* 2001;194:1639–48.

130. Burger JA, Chiorazzi N. B cell receptor signaling in chronic lymphocytic leukemia. *Trends Immunol* 2013;34:592–601.

131. Agathangelidis A, Darzentas N, Hadzidimitriou A, Brochet X, Murray F, Yan X-J, Davis Z, van Gastel-Mol EJ, Tresoldi C, Chu CC, Cahill N, Giudicelli V, et al. Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood* 2012;119:4467.

132. Murray F, Darzentas N, Hadzidimitriou A, Tobin G, Boudjogra M, Scielzo C, Laoutaris N, Karlsson K, Baran-Marzsak F, Tsaftaris A, Moreno C, Anagnostopoulos A, et al. Stereotyped patterns of somatic hypermutation in subsets of patients with chronic lymphocytic leukemia: implications for the role of antigen selection in leukemogenesis. *Blood* 2008;111:1524–33.

133. Ringshausen I, Schneller F, Bogner C, Hipp S, Duyster J, Peschel C, Decker T. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. *Blood* 2002;100:3741–8.

134. Gobessi S, Laurenti L, Longo PG, Carsetti L, Berno V, Sica S, Leone G, Efremov DG. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia* 2009;23:686–97.

135. Hallek M. Signaling the end of chronic lymphocytic leukemia: new frontline treatment strategies. *Blood* 2013;122:3723–34.

136. Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, Grant B, Sharman JP, Coleman M, Wierda WG, Jones JA, Zhao W, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2013;369:32–42.

137. Veldurthy A, Patz M, Hagist S, Pallasch CP, Wendtner C-M, Hallek M, Krause G. The kinase inhibitor dasatinib induces apoptosis in chronic lymphocytic leukemia cells in vitro with preference for a subgroup of patients with unmutated IgVH genes. *Blood* 2008;112:1443–52.

138. Currie KS, Kropf JE, Lee T, Blomgren P, Xu J, Zhao Z, Gallion S, Whitney JA, Maclin D, Lansdon EB, Maciejewski P, Rossi AM, et al. Discovery of GS-9973, a selective and orally efficacious inhibitor of spleen tyrosine kinase. *J Med Chem* 2014;57:3856–73.

139. Friedberg JW, Sharman J, Sweetenham J, Johnston PB, Vose JM, Lacasce A, Schaefer-Cutillo J, De Vos S, Sinha R, Leonard JP, Cripe LD, Gregory SA, et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* 2010;115:2578–85.

140. Woyach JA, Bojnik E, Ruppert AS, Stefanovski MR, Goettl VM, Smucker KA, Smith LL, Dubovsky JA, Towns WH, MacMurray J, Harrington BK, Davis ME, et al. Bruton's tyrosine kinase (BTK) function is important to the development and expansion of chronic lymphocytic leukemia (CLL). *Blood* 2014;123:1207–13.

141. Ponader S, Chen S-S, Buggy JJ, Balakrishnan K, Gandhi V, Wierda WG, Keating MJ, O'Brien S, Chiorazzi N, Burger JA. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood* 2012;119:1182–9.

142. Chen S-S, Chang BY, Chang S, Tong T, Ham S, Sherry B, Burger JA, Rai KR, Chiorazzi N. BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia. *Leukemia* 2016;30:833–43.

143. Jayappa KD, Portell CA, Gordon VL, Capaldo BJ, Bekiranov S, Axelrod MJ, Brett LK, Wulfkuhle JD, Gallagher RI, Petricoin EF, Bender TP, Williams ME, et al. Microenvironmental agonists generate de novo phenotypic resistance to combined ibrutinib plus venetoclax in CLL and MCL. *Blood Adv* 2017;1:933–46.

144. Kaur V, Swami A. Ibrutinib in CLL: a focus on adverse events, resistance, and novel approaches beyond ibrutinib. *Ann Hematol* 2017;96:1175–84.

145. Ahn IE, Underbayev C, Albitar A, Herman SEM, Tian X, Maric I, Arthur DC, Wake L, Pittaluga S, Yuan CM, Stetler-Stevenson M, Soto S, et al. Clonal evolution leading to ibrutinib resistance in chronic lymphocytic leukemia. *Blood* 2017;129:1469–79.

146. Woyach JA, Ruppert AS, Guinn D, Lehman A, Blachly JS, Lozanski A, Heerema NA, Zhao W, Coleman J, Jones D, Abruzzo L, Gordon A, et al. BTKC481S-Mediated Resistance to Ibrutinib in Chronic Lymphocytic Leukemia. *JCO* 2017;JCO.2016.70.2282.

147. Meimetis LG, Nodwell M, Yang L, Wang X, Wu J, Harwig C, Stenton GR, Mackenzie LF, MacRury T, Patrick BO, Ming-Lum A, Ong CJ, et al. Synthesis of SHIP1-Activating Analogs of the Sponge Meroterpenoid Pelorol. *European Journal of Organic Chemistry* 2012;2012:5195–207.

148. Stenton GR, Mackenzie LF, Tam P, Cross JL, Harwig C, Raymond J, Toews J, Wu J, Ogden N, MacRury T, Szabo C. Characterization of AQX-1125, a small-molecule SHIP1 activator. *Br J Pharmacol* 2013;168:1506–18.

149. Wan Z, Xu C, Chen X, Xie H, Li Z, Wang J, Ji X, Chen H, Ji Q, Shaheen S, Xu Y, Wang F, et al. PI(4,5)P2 determines the threshold of mechanical force-induced B cell activation. *J Cell Biol* 2018;217:2565–82.

150. Xu C, Wan Z, Shaheen S, Wang J, Yang Z, Liu W. A PI(4,5)P2-derived "gasoline engine model" for the sustained B cell receptor activation. *Immunological Reviews* 2019;291:75–90.

151. Lee J-O, Yang H, Georgescu M-M, Di Cristofano A, Maehama T, Shi Y, Dixon JE, Pandolfi P, Pavletich NP. Crystal Structure of the PTEN Tumor Suppressor: Implications for Its Phosphoinositide Phosphatase Activity and Membrane Association. *Cell* 1999;99:323–34.

152. Ono M, Okada H, Bolland S, Yanagi S, Kurosaki T, Ravetch JV. Deletion of SHIP or SHP-1 Reveals Two Distinct Pathways for Inhibitory Signaling. *Cell* 1997;90:293–301.

153. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998;273:13375–8.

154. Damen JE, Liu L, Rosten P, Humphries RK, Jefferson AB, Majerus PW, Krystal G. The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. *PNAS* 1996;93:1689–93.

155. Lioubin MN, Algate PA, Tsai S, Carlberg K, Aebersold A, Rohrschneider LR. p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev* 1996;10:1084–95.

156. Scharenberg AM, El-Hillal O, Fruman DA, Beitz LO, Li Z, Lin S, Gout I, Cantley LC, Rawlings DJ, Kinet JP. Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *EMBO J* 1998;17:1961–72.

157. Park WS, Heo WD, Whalen JH, O'Rourke NA, Bryan HM, Meyer T, Teruel MN. Comprehensive identification of PIP3-regulated PH domains from C. elegans to H. sapiens by model prediction and live imaging. *Mol Cell* 2008;30:381–92.

158. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998;95:29–39.

159. Cairns P, Okami K, Halachmi S, Halachmi N, Esteller M, Herman JG, Jen J, Isaacs WB, Bova GS, Sidransky D. Frequent Inactivation of PTEN/MMAC1 in Primary Prostate Cancer. *Cancer Research* 1997;57:4997–5000.

160. Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI, Li J, Parsons R, Ellenson LH. Mutations in PTEN Are Frequent in Endometrial Carcinoma But Rare in Other Common Gynecological Malignancies1. *Cancer Research* 1997;57:3935–40.

161. Simpson L, Parsons R. PTEN: life as a tumor suppressor. *Exp Cell Res* 2001;264:29–41.

162. Leupin N, Cenni B, Novak U, Hügli B, Graber HU, Tobler A, Fey MF. Disparate expression of the PTEN gene: a novel finding in B-cell chronic lymphocytic leukaemia (B-CLL). *British Journal of Haematology* 2003;121:97–100.

163. Zou Z-J, Fan L, Wang L, Xu J, Zhang R, Tian T, Li J-Y, Xu W. miR-26a and miR-214 down-regulate expression of the PTEN gene in chronic lymphocytic leukemia, but not PTEN mutation or promoter methylation. *Oncotarget* 2015;6:1276–85.

164. Carrà G, Panuzzo C, Torti D, Parvis G, Crivellaro S, Familiari U, Volante M, Morena D, Lingua MF, Brancaccio M, Guerrasio A, Pandolfi PP, et al. Therapeutic inhibition of USP7-PTEN network in chronic lymphocytic leukemia: a strategy to overcome TP53 mutated/deleted clones. *Oncotarget* 2017;

165. Zou Z-J, Zhang R, Fan L, Wang L, Fang C, Zhang L-N, Yang S, Li Y-Y, Li J-Y, Xu W. Low expression level of phosphatase and tensin homolog deleted on chromosome ten predicts poor prognosis in chronic lymphocytic leukemia. *Leuk Lymphoma* 2013;54:1159–64.

166. Maehama T, Dixon JE. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends in Cell Biology* 1999;9:125–8.

167. Lee S-R, Yang K-S, Kwon J, Lee C, Jeong W, Rhee SG. Reversible inactivation of the tumor suppressor PTEN by H2O2. *J Biol Chem* 2002;277:20336–42.

168. Pauls SD, Marshall AJ. Regulation of immune cell signaling by SHIP1: A phosphatase, scaffold protein, and potential therapeutic target. *Eur J Immunol* 2017;

169. Helgason CD, Kalberer CP, Damen JE, Chappel SM, Pineault N, Krystal G, Humphries RK. A Dual Role for Src Homology 2 Domain–Containing Inositol-5-Phosphatase (Ship) in Immunity. *Journal of Experimental Medicine* 2000;191:781–94.

170. Brauweiler A, Tamir I, Dal Porto J, Benschop RJ, Helgason CD, Humphries RK, Freed JH, Cambier JC. Differential regulation of B cell development, activation, and death by the src homology 2 domain-containing 5’ inositol phosphatase (SHIP). *J Exp Med* 2000;191:1545–54.

171. Miletic AV, Anzelon-Mills AN, Mills DM, Omori SA, Pedersen IM, Shin D-M, Ravetch JV, Bolland S, Morse HC, Rickert RC. Coordinate suppression of B cell lymphoma by PTEN and SHIP phosphatases. *Journal of Experimental Medicine* 2010;jem.20091962.

172. Bolland S, Pearse RN, Kurosaki T, Ravetch JV. SHIP Modulates Immune Receptor Responses by Regulating Membrane Association of Btk. *Immunity* 1998;8:509–16.

173. Li T, Tsukada S, Satterthwaite A, Havlik MH, Park H, Takatsu K, Witte ON. Activation of bruton’s tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. *Immunity* 1995;2:451–60.

174. Carver DJ, Aman MJ, Ravichandran KS. SHIP inhibits Akt activation in B cells through regulation of Akt membrane localization. *Blood* 2000;96:1449–56.

175. Aman MJ, Lamkin TD, Okada H, Kurosaki T, Ravichandran KS. The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J Biol Chem* 1998;273:33922–8.

176. Brauer H, Strauss J, Wegner W, Müller-Tidow C, Horstmann M, Jücker M. Leukemia-associated mutations in SHIP1 inhibit its enzymatic activity, interaction

with the GM-CSF receptor and Grb2, and its ability to inactivate PI3K/AKT signaling. *Cell Signal* 2012;24:2095–101.

177. Ma K, Cheung SM, Marshall AJ, Duronio V. PI(3,4,5)P3 and PI(3,4)P2 levels correlate with PKB/akt phosphorylation at Thr308 and Ser473, respectively; PI(3,4)P2 levels determine PKB activity. *Cellular Signalling* 2008;20:684–94.

178. Franke TF, Kaplan DR, Cantley LC, Toker A. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 1997;275:665–8.

179. Brooks R, Fuhler GM, Iyer S, Smith MJ, Park M-Y, Paraiso KHT, Engelman RW, Kerr WG. SHIP1 Inhibition Increases Immunoregulatory Capacity and Triggers Apoptosis of Hematopoietic Cancer Cells. *J Immunol* 2010;184:3582–9.

180. Kennah M, Yau TY, Nodwell M, Krystal G, Andersen RJ, Ong CJ, Mui AL-F. Activation of SHIP via a small molecule agonist kills multiple myeloma cells. *Experimental Hematology* 2009;37:1274–83.

181. Nickel JC, Egerdie B, Davis E, Evans R, Mackenzie L, Shrewsbury SB. A Phase II Study of the Efficacy and Safety of the Novel Oral SHIP1 Activator AQX-1125 in Subjects with Moderate to Severe Interstitial Cystitis/Bladder Pain Syndrome. *J Urol* 2016;196:747–54.

182. Cross J, Stenton GR, Harwig C, Szabo C, Genovese T, Di Paola R, Esposito E, Cuzzocrea S, Mackenzie LF. AQX-1125, a small molecule SHIP1 activator, inhibits bleomycin-induced pulmonary fibrosis. *Br J Pharmacol* 2017;

183. Choi Y, Zhang J, Murga C, Yu H, Koller E, Monia BP, Gutkind JS, Li W. PTEN, but not SHIP and SHIP2, suppresses the PI3K/Akt pathway and induces growth inhibition and apoptosis of myeloma cells. *Oncogene* 2002;21:5289–300.

184. Ong CJ, Ming-Lum A, Nodwell M, Ghanipour A, Yang L, Williams DE, Kim J, Demirjian L, Qasimi P, Ruschmann J, Cao L-P, Ma K, et al. Small-molecule agonists of SHIP1 inhibit the phosphoinositide 3-kinase pathway in hematopoietic cells. *Blood* 2007;110:1942–9.

185. Lemm EA, Valle-Argos B, Smith LD, Richter J, Gebreselassie Y, Carter MJ, Karolova J, Svaton M, Helman K, Weston-Bell NJ, Karydis L, Williamson CT, et al. Preclinical evaluation of a novel SHIP1 phosphatase activator for inhibition of PI3K signaling in malignant B-cells. *Clin Cancer Res [Internet]* 2019 [cited 2020 Jan 8];Available from: https://clincancerres.aacrjournals.org/content/early/2019/12/12/1078-0432.CCR-19-2202

186. Ryan DH, Nuccie BL, Abboud CN, Liesveld JL. Maturation-dependent adhesion of human B cell precursors to the bone marrow microenvironment. *J Immunol* 1990;145:477–84.

187. Ghia P, Chiorazzi N, Stamatopoulos K. Microenvironmental influences in chronic lymphocytic leukaemia: the role of antigen stimulation. *J Intern Med* 2008;264:549–62.

188. Burger JA. Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program* 2011;2011:96–103.

189. Kurtova AV, Balakrishnan K, Chen R, Ding W, Schnabl S, Quiroga MP, Sivina M, Wierda WG, Estrov Z, Keating MJ, Shehata M, Jäger U, et al. Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance. *Blood* 2009;114:4441–50.

190. Panayiotidis P, Jones D, Ganeshaguru K, Foroni L, Hoffbrand AV. Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. *Br J Haematol* 1996;92:97–103.

191. Lagneaux L, Delforge A, De Bruyn C, Bernier M, Bron D. Adhesion to bone marrow stroma inhibits apoptosis of chronic lymphocytic leukemia cells. *Leuk Lymphoma* 1999;35:445–53.

192. Gehrke I, Gandhirajan RK, Poll-Wolbeck SJ, Hallek M, Kreuzer K-A. Bone marrow stromal cell-derived vascular endothelial growth factor (VEGF) rather than chronic lymphocytic leukemia (CLL) cell-derived VEGF is essential for the apoptotic resistance of cultured CLL cells. *Mol Med* 2011;17:619–27.

193. Redondo-Muñoz J, Escobar-Díaz E, Samaniego R, Terol MJ, García-Marco JA, García-Pardo A. MMP-9 in B-cell chronic lymphocytic leukemia is up-regulated by alpha4beta1 integrin or CXCR4 engagement via distinct signaling pathways, localizes to podosomes, and is involved in cell invasion and migration. *Blood* 2006;108:3143–51.

194. Ringshausen I, Dechow T, Schneller F, Weick K, Oelsner M, Peschel C, Decker T. Constitutive activation of the MAPkinase p38 is critical for MMP-9 production and survival of B-CLL cells on bone marrow stromal cells. *Leukemia* 2004;18:1964–70.

195. 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;94:3658–67.

196. Mraz M, Zent CS, Church AK, Jelinek DF, Wu X, Pospisilova S, Ansell SM, Novak AJ, Kay NE, Witzig TE, Nowakowski GS. Bone Marrow Stromal Cells Protect Lymphoma B-cells from Rituximab-Induced Apoptosis and Targeting Integrin alfa-4-beta-1 (VLA-4) with Natalizumab can Overcome this Resistance. *Br J Haematol* 2011;155:53–64.

197. Herbst SA, Stolarczyk M, Becirovic T, Czernilofsky F, Liu Y, Kolb C, Knoll M, Herling M, Müller-Tidow C, Dietrich S. Phagocytosis by stroma confounds coculture studies. *iScience [Internet]* 2021 [cited 2022 Jun 27];24. Available from: https://www.cell.com/iscience/abstract/S2589-0042(21)01030-0

198. Burger JA, Quiroga MP, Hartmann E, Bürkle A, Wierda WG, Keating MJ, Rosenwald A. 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;113:3050–8.

199. Ding W, Nowakowski GS, Knox TR, Boysen JC, Maas ML, Schwager SM, Wu W, Wellik LE, Dietz AB, Ghosh AK, Secreto CR, Medina KL, et al. Bi-directional activation between mesenchymal stem cells and CLL B-cells: implication for CLL disease progression. *Br J Haematol* 2009;147:471–83.

200. Messmer BT, Messmer D, Allen SL, Kolitz JE, Kudalkar P, Cesar D, Murphy EJ, Koduru P, Ferrarini M, Zupo S, Cutrona G, Damle RN, et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J Clin Invest* 2005;115:755–64.

201. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell–derived factor-1. *Blood* 2000;96:2655–63.

202. Brusa D, Serra S, Coscia M, Rossi D, D'Arena G, Laurenti L, Jaksic O, Fedele G, Inghirami G, Gaidano G, Malavasi F, Deaglio S. The PD-1/PD-L1 axis contributes to T-cell dysfunction in chronic lymphocytic leukemia. *Haematologica* 2013;98:953–63.

203. Fayad L, Keating MJ, Reuben JM, O'Brien S, Lee BN, Lerner S, Kurzrock R. Interleukin-6 and interleukin-10 levels in chronic lymphocytic leukemia: correlation with phenotypic characteristics and outcome. *Blood* 2001;97:256–63.

204. Ramsay AG, Clear AJ, Fatah R, Gribben JG. Multiple inhibitory ligands induce impaired T-cell immunologic synapse function in chronic lymphocytic leukemia that can be blocked with lenalidomide: establishing a reversible immune evasion mechanism in human cancer. *Blood* 2012;120:1412–21.

205. Sidhaye VK, Schweitzer KS, Caterina MJ, Shimoda L, King LS. Shear stress regulates aquaporin-5 and airway epithelial barrier function. *Proc Natl Acad Sci U S A* 2008;105:3345–50.

206. Malek AM, Izumo S. Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress. *J Cell Sci* 1996;109 (Pt 4):713–26.

207. Sweet DT, Hall JD, Welsh J, Kahn ML, Jiménez JM. Investigating Effects of Fluid Shear Stress on Lymphatic Endothelial Cells. *Methods Mol Biol* 2018;1846:213–27.

208. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. *JAMA* 1999;282:2035–42.

209. Cheng CP, Herfkens RJ, Taylor CA. Inferior vena caval hemodynamics quantified in vivo at rest and during cycling exercise using magnetic resonance imaging. *American Journal of Physiology-Heart and Circulatory Physiology* 2003;284:H1161–7.

210. Andrews AM, Jaron D, Buerk DG, Kirby PL, Barbee KA. Direct, real-time measurement of shear stress-induced nitric oxide produced from endothelial cells in vitro. *Nitric Oxide* 2010;23:335–42.

211. Hampton HR, Chtanova T. Lymphatic Migration of Immune Cells. *Frontiers in Immunology [Internet]* 2019 [cited 2022 Jul 5];10. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01168

212. Torcellan T, Hampton HR, Bailey J, Tomura M, Brink R, Chtanova T. In vivo photolabeling of tumor-infiltrating cells reveals highly regulated egress of T-cell subsets from tumors. *Proceedings of the National Academy of Sciences* 2017;114:5677–82.

213. Karikoski M, Irjala H, Maksimow M, Miiluniemi M, Granfors K, Hernesniemi S, Elima K, Moldenhauer G, Schledzewski K, Kzhyshkowska J, Goerdt S, Salmi M, et al. Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. *European Journal of Immunology* 2009;39:3477–87.

214. Gashev AA, Davis MJ, Delp MD, Zawieja DC. Regional variations of contractile activity in isolated rat lymphatics. *Microcirculation* 2004;11:477–92.

215. Jafarnejad M, Cromer WE, Kaunas RR, Zhang SL, Zawieja DC, Moore JE. Measurement of shear stress-mediated intracellular calcium dynamics in human dermal lymphatic endothelial cells. *American Journal of Physiology-Heart and Circulatory Physiology* 2015;308:H697–706.

216. Dixon JB, Greiner ST, Gashev AA, Cote GL, MOORE Jr. JE, Zawieja DC. Lymph Flow, Shear Stress, and Lymphocyte Velocity in Rat Mesenteric Prenodal Lymphatics. *Microcirculation* 2006;13:597–610.

217. Koller A, Mizuno R, Kaley G. Flow reduces the amplitude and increases the frequency of lymphatic vasomotion: role of endothelial prostanoids. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 1999;277:R1683–9.

218. Kawai Y, Yokoyama Y, Kaidoh M, Ohhashi T. Shear stress-induced ATP-mediated endothelial constitutive nitric oxide synthase expression in human lymphatic endothelial cells. *American Journal of Physiology-Cell Physiology* 2010;298:C647–55.

219. Miteva DO, Rutkowski JM, Dixon JB, Kilarski W, Shields JD, Swartz MA. Transmural flow modulates cell and fluid transport functions of lymphatic endothelium. *Circ Res* 2010;106:920–31.

220. Marschel P, Schmid-Schönbein GW. Control of fluid shear response in circulating leukocytes by integrins. *Ann Biomed Eng* 2002;30:333–43.

221. Shin HY, Fukuda S, Schmid-Schönbein GW. Fluid shear stress-mediated mechanotransduction in circulating leukocytes and its defect in microvascular dysfunction. *Journal of Biomechanics* 2021;120:110394.

222. Zucchetto A, Benedetti D, Tripodo C, Bomben R, Dal Bo M, Marconi D, Bossi F, Lorenzon D, Degan M, Rossi FM, Rossi D, Bulian P, et al. CD38/CD31, the CCL3 and CCL4 Chemokines, and CD49d/Vascular Cell Adhesion Molecule-1 Are Interchained by Sequential Events Sustaining Chronic Lymphocytic Leukemia Cell Survival. *Cancer Research* 2009;69:4001–9.

223. Rose DM, Han J, Ginsberg MH. Alpha4 integrins and the immune response. *Immunol Rev* 2002;186:118–24.

224. Walsby E, Buggins A, Devereux S, Jones C, Pratt G, Brennan P, Fegan C, Pepper C. Development and characterization of a physiologically relevant model of lymphocyte migration in chronic lymphocytic leukemia. *Blood* 2014;123:3607–17.

225. de la Fuente MT, Casanova B, Moyano JV, Garcia-Gila M, Sanz L, Garcia-Marco J, Silva A, Garcia-Pardo A. Engagement of α4β1 integrin by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. *Journal of Leukocyte Biology* 2002;71:495–502.

226. Alon R, Chen S, Puri KD, Finger EB, Springer TA. The kinetics of L-selectin tethers and the mechanics of selectin-mediated rolling. *J Cell Biol* 1997;138:1169–80.

227. Burger M, Hartmann T, Krome M, Rawluk J, Tamamura H, Fujii N, Kipps TJ, Burger JA. Small peptide inhibitors of the CXCR4 chemokine receptor (CD184) antagonize the activation, migration, and antiapoptotic responses of CXCL12 in chronic lymphocytic leukemia B cells. *Blood* 2005;106:1824–30.

228. Stamatopoulos B, Haibe-Kains B, Equeter C, Meuleman N, Sorée A, De Bruyn C, Hanosset D, Bron D, Martiat P, Lagneaux L. Gene expression profiling reveals differences in microenvironment interaction between patients with chronic lymphocytic leukemia expressing high versus low ZAP70 mRNA. *Haematologica* 2009;94:790–9.

229. Härzschel A, Zucchetto A, Gattei V, Hartmann TN. VLA-4 Expression and Activation in B Cell Malignancies: Functional and Clinical Aspects. *International Journal of Molecular Sciences* 2020;21:2206.

230. Trimarco V, Ave E, Facco M, Chiodin G, Frezzato F, Martini V, Gattazzo C, Lessi F, Giorgi CA, Visentin A, Castelli M, Severin F, et al. Cross-talk between chronic lymphocytic leukemia (CLL) tumor B cells and mesenchymal stromal cells (MSCs): implications for neoplastic cell survival. *Oncotarget* 2015;6:42130–49.

231. Pedersen IM, Kitada S, Leoni LM, Zapata JM, Karras JG, Tsukada N, Kipps TJ, Choi YS, Bennett F, Reed JC. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. *Blood* 2002;100:1795–801.

232. Balakrishnan K, Burger JA, Wierda WG, Gandhi V. AT-101 induces apoptosis in CLL B cells and overcomes stromal cell–mediated Mcl-1 induction and drug resistance. *Blood* 2009;113:149–53.

233. Amigo-Jimenez I, Bailon E, Aguilera-Montilla N, Jose Terol M, Garcia-Marco JA, Garcia-Pardo A. Bone marrow stroma-induced resistance of chronic lymphocytic leukemia cells to arsenic trioxide involves Mcl-1 upregulation and is overcome by inhibiting the PI3K delta or PKC beta signaling pathways. *Oncotarget* 2015;6:44832–48.

234. Shehata M, Schnabl S, Demirtas D, Hilgarth M, Hubmann R, Ponath E, Badrnya S, Lehner C, Hoelbl A, Duechler M, Gaiger A, Zielinski C, et al. Reconstitution of PTEN activity by CK2 inhibitors and interference with the PI3-K/Akt cascade counteract the antiapoptotic effect of human stromal cells in chronic lymphocytic leukemia. *Blood* 2010;116:2513–21.

235. Lam P, Yoo SK, Green JM, Huttenlocher A. The SH2-domain-containing inositol 5-phosphatase (SHIP) limits the motility of neutrophils and their recruitment to wounds in zebrafish. *J Cell Sci* 2012;125:4973–8.

236. Nishio M, Watanabe K, Sasaki J, Taya C, Takasuga S, Iizuka R, Balla T, Yamazaki M, Watanabe H, Itoh R, Kuroda S, Horie Y, et al. Control of cell polarity and motility by the PtdIns(3,4,5)P3 phosphatase SHIP1. *Nat Cell Biol* 2007;9:36–44.

237. Suzuki A, Kaisho T, Ohishi M, Tsukio-Yamaguchi M, Tsubata T, Koni PA, Sasaki T, Mak TW, Nakano T. Critical Roles of Pten in B Cell Homeostasis and Immunoglobulin Class Switch Recombination. *J Exp Med* 2003;197:657–67.

238. Rey-Ladino JA, Huber M, Liu L, Damen JE, Krystal G, Takei F. The SH2-Containing Inositol-5'-Phosphatase Enhances LFA-1-Mediated Cell Adhesion and Defines Two Signaling Pathways for LFA-1 Activation. *The Journal of Immunology* 1999;162:5792–9.

239. Sattler M, Verma S, Pride YB, Salgia R, Rohrschneider LR, Griffin JD. SHIP1, an SH2 Domain Containing Polyinositol-5-phosphatase, Regulates Migration through Two Critical Tyrosine Residues and Forms a Novel Signaling Complex with DOK1 and CRKL. *J Biol Chem* 2001;276:2451–8.

240. Herman SEM, Niemann CU, Farooqui M, Jones J, Mustafa RZ, Lipsky A, Saba N, Martyr S, Soto S, Valdez J, Gyamfi JA, Maric I, et al. Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukemia: correlative analyses from a phase II study. *Leukemia* 2014;28:2188–96.

241. de Rooij MFM, 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;125:2306–9.

242. Montresor A, Toffali L, Rigo A, Ferrarini I, Vinante F, Laudanna C. CXCR4- and BCR-triggered integrin activation in B-cell chronic lymphocytic leukemia cells depends on JAK2-activated Bruton's tyrosine kinase. *Oncotarget* 2018;9:35123–40.

243. Lanemo Myhrinder A, Hellqvist E, Bergh A-C, Jansson M, Nilsson K, Hultman P, Jonasson J, Buhl AM, Bredo Pedersen L, Jurlander J, Klein E, Weit N, et al. Molecular characterization of neoplastic and normal "sister" lymphoblastoid B-cell lines from chronic lymphocytic leukemia. *Leuk Lymphoma* 2013;54:1769–79.

244. Roecklein BA, Torok-Storb B. Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. *Blood* 1995;85:997–1005.

245. Drexler HG, MacLeod RAF. Malignant hematopoietic cell lines: in vitro models for the study of mantle cell lymphoma. *Leuk Res* 2002;26:781–7.

246. Lewin N, Aman P, Mellstedt H, Zech L, Klein G. Direct outgrowth of in vivo Epstein-Barr virus (EBV)-infected chronic lymphocytic leukemia (CLL) cells into permanent lines. *Int J Cancer* 1988;41:892–5.

247. Fialkow PJ, Najfeld V, Reddy AL, Singer J, Steinmann L. Chronic lymphocytic leukaemia: Clonal origin in a committed B-lymphocyte progenitor. *Lancet* 1978;2:444–6.

248. Palomba ML, Piersanti K, Ziegler CGK, Decker H, Cotari JW, Bantilan K, Rijo I, Gardner JR, Heaney M, Bemis D, Balderas R, Malek SN, et al. Multidimensional single-cell analysis of BCR signaling reveals proximal activation defect as a hallmark of chronic lymphocytic leukemia B cells. *PLoS ONE* 2014;9:e79987.

249. Myhrvold IK, Cremaschi A, Hermansen JU, Tjønnfjord GE, Munthe LA, Taskén K, Skånland SS. Single cell profiling of phospho-protein levels in chronic lymphocytic leukemia. *Oncotarget* 2018;9:9273–84.

250. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A* 2003;55:61–70.

251. Lee J, Robinson ME, Ma N, Artadji D, Ahmed MA, Xiao G, Sadras T, Deb G, Winchester J, Cosgun KN, Geng H, Chan LN, et al. IFITM3 functions as PIP3-scaffold to amplify PI3K signaling in B-cells. *Nature* 2020;588:491–7.

252. Guillou H, Stephens LR, Hawkins PT. Quantitative Measurement of Phosphatidylinositol 3,4,5-trisphosphate [Internet]. In: Methods in Enzymology. Academic Press, 2007 [cited 2018 Oct 2]. 117–30.Available from: http://www.sciencedirect.com/science/article/pii/S007668790734007X

253. Sbrissa D, Ikonomov OC, Deeb R, Shisheva A. Phosphatidylinositol 5-phosphate biosynthesis is linked to PIKfyve and is involved in osmotic response pathway in mammalian cells. *J Biol Chem* 2002;277:47276–84.

254. Agarwal V, Bell GW, Nam J-W, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *eLife* 2015;4:e05005.

255. Yang H, Kong W, He L, Zhao J-J, O'Donnell JD, Wang J, Wenham RM, Coppola D, Kruk PA, Nicosia SV, Cheng JQ. MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res* 2008;68:425–33.

256. Cai Z, Hao X-Y, Liu F-X. MicroRNA-186 serves as a tumor suppressor in oral squamous cell carcinoma by negatively regulating the protein tyrosine phosphatase SHP2 expression. *Arch Oral Biol* 2018;89:20–5.

257. Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, Lund E, Dahlberg JE. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci USA* 2005;102:3627–32.

258. Costinean S, Sandhu SK, Pedersen IM, Tili E, Trotta R, Perrotti D, Ciarlariello D, Neviani P, Harb J, Kauffman LR, Shidham A, Croce CM. Src homology 2 domain–containing inositol-5-phosphatase and CCAAT enhancer-binding protein β are targeted by miR-155 in B cells of Eμ-*MiR-155* transgenic mice. *Blood* 2009;114:1374–82.

259. O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. *PNAS* 2009;106:7113–8.

260. Cheung SMS, Kornelson JC, Al-Alwan M, Marshall AJ. Regulation of phosphoinositide 3-kinase signaling by oxidants: hydrogen peroxide selectively enhances immunoreceptor-induced recruitment of phosphatidylinositol (3,4) bisphosphate-binding PH domain proteins. *Cell Signal* 2007;19:902–12.

261. Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A, Downes CP. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *EMBO J* 2003;22:5501–10.

262. Ross SH, Lindsay Y, Safrany ST, Lorenzo O, Villa F, Toth R, Clague MJ, Downes CP, Leslie NR. Differential redox regulation within the PTP superfamily. *Cell Signal* 2007;19:1521–30.

263. Kerr WG. Inhibitor and activator: dual functions for SHIP in immunity and cancer. *Ann N Y Acad Sci* 2011;1217:1–17.

264. Kerr WG, Pedicone C, Dormann S, Pacherille A, Chisholm JD. Small molecule targeting of SHIP1 and SHIP2. *Biochem Soc Trans* 2020;48:291–300.

265. Pedicone C, Meyer ST, Chisholm JD, Kerr WG. Targeting SHIP1 and SHIP2 in Cancer. *Cancers (Basel)* 2021;13.

266. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood* 2009;114:3367–75.

267. Sabine A, Bovay E, Demir CS, Kimura W, Jaquet M, Agalarov Y, Zangger N, Scallan JP, Graber W, Gulpinar E, Kwak BR, Mäkinen T, et al. FOXC2 and fluid shear stress stabilize postnatal lymphatic vasculature. *Journal of Clinical Investigation* 2015;125:3861–77.

268. Mack JJ, Mosqueiro TS, Archer BJ, Jones WM, Sunshine H, Faas GC, Briot A, Aragón RL, Su T, Romay MC, McDonald AI, Kuo C-H, et al. NOTCH1 is a mechanosensor in adult arteries. *Nat Commun* 2017;8:1620.

269. Srinivasan L, Sasaki Y, Calado DP, Zhang B, Paik JH, DePinho RA, Kutok JL, Kearney JF, Otipoby KL, Rajewsky K. PI3 kinase signals BCR-dependent mature B cell survival. *Cell* 2009;139:573–86.

270. Chen-Bettecken U, Wecker E, Schimpl A. IgM RNA switch from membrane to secretory form is prevented by adding antireceptor antibody to bacterial lipopolysaccharide-stimulated murine primary B-cell cultures. *Proc Natl Acad Sci U S A* 1985;82:7384–8.

271. Wolf C, Garding A, Filarsky K, Bahlo J, Robrecht S, Becker N, Zucknick M, Rouhi A, Weigel A, Claus R, Weichenhan D, Eichhorst B, et al. NFATC1 activation by DNA hypomethylation in chronic lymphocytic leukemia correlates with clinical staging and can be inhibited by ibrutinib. *Int J Cancer* 2018;142:322–33.

272. Weinstock DM, Dalla-Favera R, Gascoyne RD, Leonard JP, Levy R, Lossos IS, Melnick AM, Nowakowski GS, Press OW, Savage KJ, Shipp MA, Staudt LM. A roadmap for discovery and translation in lymphoma. *Blood* 2015;125:2175–7.

273. Lim JB, Langford TF, Huang BK, Deen WM, Sikes HD. A reaction-diffusion model of cytosolic hydrogen peroxide. *Free Radical Biology and Medicine* 2016;90:85–90.

274. Schmid VK, Khadour A, Ahmed N, Brandl C, Nitschke L, Rajewsky K, Jumaa H, Hobeika E. B cell antigen receptor expression and phosphatidylinositol 3-kinase signaling regulate genesis and maintenance of mouse chronic lymphocytic leukemia. *Haematologica* 2022;

275. Cavallini C, Chignola R, Dando I, Perbellini O, Mimiola E, Lovato O, Laudanna C, Pizzolo G, Donadelli M, Scupoli MT. Lowcatalaseexpression confers redox hypersensitivity and identifies an indolent clinical behavior in CLL. *Blood* 2018;

276. Liu Q, Oliveira-Dos-Santos AJ, Mariathasan S, Bouchard D, Jones J, Sarao R, Kozieradzki I, Ohashi PS, Penninger JM, Dumont DJ. The inositol polyphosphate 5-phosphatase ship is a crucial negative regulator of B cell antigen receptor signaling. *J Exp Med* 1998;188:1333–42.

277. Okada H, Bolland S, Hashimoto A, Kurosaki M, Kabuyama Y, Iino M, Ravetch JV, Kurosaki T. Role of the inositol phosphatase SHIP in B cell receptor-induced Ca2+ oscillatory response. *J Immunol* 1998;161:5129–32.

278. Cesano A, Perbellini O, Evensen E, Chu CC, Cioffi F, Ptacek J, Damle RN, Chignola R, Cordeiro J, Yan X, Hawtin RE, Nichele I, et al. Association between B-cell receptor responsiveness and disease progression in B-cell chronic lymphocytic leukemia: results from single cell network profiling studies. *Haematologica* 2013;98:626–34.

279. Shih T, De S, Barnes BJ. RNAi Transfection Optimized in Primary Naïve B Cells for the Targeted Analysis of Human Plasma Cell Differentiation. *Front Immunol* 2019;10:1652.

280. Setz CS, Khadour A, Renna V, Iype J, Gentner E, He X, Datta M, Young M, Nitschke L, Wienands J, Maity PC, Reth M, et al. Pten controls B-cell responsiveness and germinal center reaction by regulating the expression of IgD BCR. *EMBO J* 2019;38:e100249.

281. Gabelloni ML, Borge M, Galletti J, Cañones C, Calotti PF, Bezares RF, Ávalos JS, Giordano M, Gamberale R. SHIP-1 protein level and phosphorylation status differs between CLL cells segregated by ZAP-70 expression. *British Journal of Haematology* 2008;140:117–9.

282. Edimo WE, Janssens V, Waelkens E, Erneux C. Reversible Ser/Thr SHIP phosphorylation: a new paradigm in phosphoinositide signalling?: Targeting of SHIP1/2 phosphatases may be controlled by phosphorylation on Ser and Thr residues. *Bioessays* 2012;34:634–42.

283. Rahdar M, Inoue T, Meyer T, Zhang J, Vazquez F, Devreotes PN. A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. *Proc Natl Acad Sci USA* 2009;106:480–5.

284. Ross AH, Gericke A. Phosphorylation keeps PTEN phosphatase closed for business. *Proc Natl Acad Sci USA* 2009;106:1297–8.

285. Vazquez F, Grossman SR, Takahashi Y, Rokas MV, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J Biol Chem* 2001;276:48627–30.

286. Valiente M, Andrés-Pons A, Gomar B, Torres J, Gil A, Tapparel C, Antonarakis SE, Pulido R. Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. *J Biol Chem* 2005;280:28936–43.

287. Pauls SD, Ray A, Hou S, Vaughan AT, Cragg MS, Marshall AJ. FcγRIIB-Independent Mechanisms Controlling Membrane Localization of the Inhibitory Phosphatase SHIP in Human B Cells. *The Journal of Immunology* 2016;197:1587–96.

288. Wu X, Hepner K, Castelino-Prabhu S, Do D, Kaye MB, Yuan XJ, Wood J, Ross C, Sawyers CL, Whang YE. Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. *Proc Natl Acad Sci USA* 2000;97:4233–8.

289. Apollonio B, Ioannou N, Papazoglou D, Ramsay AG. Understanding the Immune-Stroma Microenvironment in B Cell Malignancies for Effective Immunotherapy. *Front Oncol* 2021;11:626818.

290. Gerousi M, Laidou S, Gemenetzi K, Stamatopoulos K, Chatzidimitriou A. Distinctive Signaling Profiles With Distinct Biological and Clinical Implications in Aggressive CLL Subsets With Stereotyped B-Cell Receptor Immunoglobulin. *Front Oncol [Internet]* 2021 [cited 2021 Nov 25];11. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8595110/

291. Ding W, LaPlant BR, Call TG, Parikh SA, Leis JF, He R, Shanafelt TD, Sinha S, Le-Rademacher J, Feldman AL, Habermann TM, Witzig TE, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. *Blood* 2017;129:3419–27.

292. Lee MJ, Park SY, Ko JH, Lee HJ, Ryu JS, Park JW, Khwarg SI, Yoon S-O, Oh JY. Mesenchymal stromal cells promote B-cell lymphoma in lacrimal glands by inducing immunosuppressive microenvironment. *Oncotarget* 2017;8:66281–92.

293. Cols M, Barra CM, He B, Puga I, Xu W, Chiu A, Tam W, Knowles DM, Dillon SR, Leonard JP, Furman RR, Chen K, et al. Stromal endothelial cells establish a bidirectional crosstalk with chronic lymphocytic leukemia cells through the TNF-related factors BAFF, APRIL, and CD40L. *J Immunol* 2012;188:6071–83.

294. Lutzny G, Kocher T, Schmidt-Supprian M, Rudelius M, Klein-Hitpass L, Finch AJ, Dürig J, Wagner M, Haferlach C, Kohlmann A, Schnittger S, Seifert M, et al. Protein kinase c-β-dependent activation of NF-κB in stromal cells is indispensable for the survival of chronic lymphocytic leukemia B cells in vivo. *Cancer Cell* 2013;23:77–92.

295. Heinig K, Gätjen M, Grau M, Stache V, Anagnostopoulos I, Gerlach K, Niesner RA, Cseresnyes Z, Hauser AE, Lenz P, Hehlgans T, Brink R, et al. Access to Follicular Dendritic Cells Is a Pivotal Step in Murine Chronic Lymphocytic Leukemia B-cell Activation and Proliferation. *Cancer Discov* 2014;4:1448–65.

296. Vega F, Medeiros LJ, Lang W-H, Mansoor A, Bueso-Ramos C, Jones D. The stromal composition of malignant lymphoid aggregates in bone marrow: variations in architecture and phenotype in different B-cell tumours. *Br J Haematol* 2002;117:569–76.

297. Vokali E, Yu SS, Hirosue S, Rinçon-Restrepo M, V Duraes F, Scherer S, Corthésy-Henrioud P, Kilarski WW, Mondino A, Zehn D, Hugues S, Swartz MA. Lymphatic endothelial cells prime naïve CD8+ T cells into memory cells under steady-state conditions. *Nat Commun* 2020;11:538.

298. Nombela-Arrieta C, Isringhausen S. The Role of the Bone Marrow Stromal Compartment in the Hematopoietic Response to Microbial Infections. *Front Immunol* 2016;7:689.

299. Tokoyoda K, Egawa T, Sugiyama T, Choi B-I, Nagasawa T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* 2004;20:707–18.

300. Ruan J, Hyjek E, Kermani P, Christos PJ, Hooper AT, Coleman M, Hempstead B, Leonard JP, Chadburn A, Rafii S. Magnitude of stromal hemangiogenesis correlates with histologic subtype of non-Hodgkin's lymphoma. *Clin Cancer Res* 2006;12:5622–31.

301. Ding W, Knox TR, Tschumper RC, Wu W, Schwager SM, Boysen JC, Jelinek DF, Kay NE. Platelet-derived growth factor (PDGF)–PDGF receptor interaction activates bone marrow–derived mesenchymal stromal cells derived from chronic lymphocytic leukemia: implications for an angiogenic switch. *Blood* 2010;116:2984–93.

302. Ibrahim S, Jilani I, O'Brien S, Rogers A, Manshouri T, Giles F, Faderl S, Thomas D, Kantarjian H, Keating M, Albitar M. Clinical relevance of the expression of the CD31 ligand for CD38 in patients with B-cell chronic lymphocytic leukemia. *Cancer* 2003;97:1914–9.

303. McCabe D, Bacon L, O'Regan K, Condron C, O'Donnell JR, Murphy PT. CD38 expression on B-cell chronic lymphocytic leukemic cells is strongly correlated with vascular endothelial growth factor expression. *Leukemia* 2004;18:649–50.

304. Molica S, Vacca A, Ribatti D, Cuneo A, Cavazzini F, Levato D, Vitelli G, Tucci L, Roccaro AM, Dammacco F. Prognostic value of enhanced bone marrow angiogenesis in early B-cell chronic lymphocytic leukemia. *Blood* 2002;100:3344–51.

305. Farinello D, Woźińska M, Lenti E, Genovese L, Bianchessi S, Migliori E, Sacchetti N, di Lillo A, Bertilaccio MTS, de Lalla C, Valsecchi R, Gleave SB, et al. A retinoic acid-dependent stroma-leukemia crosstalk promotes chronic lymphocytic leukemia progression. *Nat Commun [Internet]* 2018 [cited 2019 Jan 16];9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5934403/

306. Dörsam B, Bösl T, Reiners KS, Barnert S, Schubert R, Shatnyeva O, Zigrino P, Engert A, Hansen HP, von Strandmann EP. Hodgkin Lymphoma-Derived Extracellular Vesicles Change the Secretome of Fibroblasts Toward a CAF Phenotype. *Front Immunol* 2018;9:1358.

307. Paggetti J, Haderk F, Seiffert M, Janji B, Distler U, Ammerlaan W, Kim YJ, Adam J, Lichter P, Solary E, Berchem G, Moussay E. Exosomes released by chronic lymphocytic leukemia cells induce the transition of stromal cells into cancer-associated fibroblasts. *Blood* 2015;126:1106–17.

308. Jiang G-M, Xu W, Du J, Zhang K-S, Zhang Q-G, Wang X-W, Liu Z-G, Liu S-Q, Xie W-Y, Liu H-F, Liu J-S, Wu B-P. The application of the fibroblast activation protein α-targeted immunotherapy strategy. *Oncotarget* 2016;7:33472–82.

309. Bertolini F, Fusetti L, Mancuso P, Gobbi A, Corsini C, Ferrucci PF, Martinelli G, Pruneri G. Endostatin, an antiangiogenic drug, induces tumor stabilization after

chemotherapy or anti-CD20 therapy in a NOD/SCID mouse model of human high-grade non-Hodgkin lymphoma. *Blood* 2000;96:282–7.

310. Claus C, Ferrara C, Xu W, Sam J, Lang S, Uhlenbrock F, Albrecht R, Herter S, Schlenker R, Hüsser T, Diggelmann S, Challier J, et al. Tumor-targeted 4-1BB agonists for combination with T cell bispecific antibodies as off-the-shelf therapy. *Sci Transl Med* 2019;11:eaav5989.

311. Mondal S, Subramanian KK, Sakai J, Bajrami B, Luo HR. Phosphoinositide lipid phosphatase SHIP1 and PTEN coordinate to regulate cell migration and adhesion. *Mol Biol Cell* 2012;23:1219–30.

312. Maffei R, Fiorcari S, Bulgarelli J, Martinelli S, Castelli I, Deaglio S, Debbia G, Fontana M, Coluccio V, Bonacorsi G, Zucchini P, Narni F, et al. Physical contact with endothelial cells through β1- and β2- integrins rescues chronic lymphocytic leukemia cells from spontaneous and drug-induced apoptosis and induces a peculiar gene expression profile in leukemic cells. *Haematologica* 2012;97:952–60.

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

314. de Gorter DJJ, Beuling EA, Kersseboom R, Middendorp S, van Gils JM, Hendriks RW, Pals ST, Spaargaren M. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity* 2007;26:93–104.

315. Rehm A, Mensen A, Schradi K, Gerlach K, Wittstock S, Winter S, Büchner G, Dörken B, Lipp M, Höpken UE. Cooperative function of CCR7 and lymphotoxin in the formation of a lymphoma-permissive niche within murine secondary lymphoid organs. *Blood* 2011;118:1020–33.

316. 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;113:4604–13.

317. Yosifov DY, Idler I, Bhattacharya N, Reichenzeller M, Close V, Ezerina D, Scheffold A, Jebaraj BMC, Kugler S, Bloehdorn J, Bahlo J, Robrecht S, et al. Oxidative stress as candidate therapeutic target to overcome microenvironmental protection of CLL. *Leukemia* 2020;34:115–27.

318. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1α by the von Hippel-Lindau tumor suppressor protein. *The EMBO Journal* 2000;19:4298–309.

319. Davidowitz EJ, Schoenfeld AR, Burk RD. VHL induces renal cell differentiation and growth arrest through integration of cell-cell and cell-extracellular matrix signaling. *Mol Cell Biol* 2001;21:865–74.

320. Zhou Q, Chen T, Ibe JCF, Usha Raj J, Zhou G. Knockdown of von Hippel–Lindau protein decreases lung cancer cell proliferation and colonization. *FEBS Letters* 2012;586:1510–5.

321. Kong W, He L, Richards EJ, Challa S, Xu C-X, Permuth-Wey J, Lancaster JM, Coppola D, Sellers TA, Djeu JY, Cheng JQ. Upregulation of miRNA-155 promotes tumour angiogenesis by targeting VHL and is associated with poor prognosis and triple-negative breast cancer. *Oncogene* 2014;33:679–89.

322. Vintonyak VV, Waldmann H, Rauh D. Using small molecules to target protein phosphatases. *Bioorganic & Medicinal Chemistry* 2011;19:2145–55.

323. Mullard A. Phosphatases start shedding their stigma of undruggability. *Nature Reviews Drug Discovery* 2018;17:847–9.

324. Fuhler GM, Brooks R, Toms B, Iyer S, Gengo EA, Park M-Y, Gumbleton M, Viernes DR, Chisholm JD, Kerr WG. Therapeutic potential of SH2 domain-containing inositol-5'-phosphatase 1 (SHIP1) and SHIP2 inhibition in cancer. *Mol Med* 2012;18:65–75.

325. Imamura M, Scott NW, Wallace SA, Ogah JA, Ford AA, Dubos YA, Brazzelli M. Interventions for treating people with symptoms of bladder pain syndrome: a network meta-analysis. *Cochrane Database Syst Rev* 2020;2020:CD013325.

8 Acknowledgements

I would like to acknowledge and thank many people who have made this project and its conclusion possible and fruitful.

First of all, I want to express my heartedly gratitude to PD Dr Daniel Mertens, not only for his guidance as direct supervisor but also for continuous personal and professional support, simply for being an extraordinary group leader who I strongly believe will remain a friend. This project would not have been possible without his trust that I could thrive on an independent project, his openness and patience to try new paths and his ability to connect projects and people in the most fruitful and honest manner.

Not less gratitude do I feel for Dr Martina Seiffert who throughout the years provided constructive feedback, support and adopted me into the scientific and social activities of her research group. I felt at home from the first day thanks to her proactive and kind welcoming. She became a role model for scientific knowledge and standards as well as empathy and group leading.

It was a great pleasure to be part of the bridging group Mechanisms of Leukemogenesis, being a part of the B061 group at the DKFZ, the B06x division and at the same time of the Internal Medicine III at the University Hospital Ulm. Especially, the group members Viola Close, Michaela Reichenzeller, Deyan Yosifov, Sabrina Kugler, Karol Urbanek and Alena Ehrmann provided valuable feedback and assistance. I would also like to extent my gratitude to my intern student Coralie Crouzit who made me rethink and question the experimental approaches to improve and optimise.

Great gratitude I also want to express to the members of my Thesis Advisory Committee PD Dr Daniel Mertens, Prof Dr Stefan Wiemann, Prof Dr Jan Krönke and Prof Dr Peter Lichter as well as to Dr Martina Seiffert who accepted my invitation to attend, for providing constructive, honest and motivating feedback and guidance.

Further I want to thank all past and present members of the Tumour Microenvironment group, especially Dr Laura Llaó Cid, Verena Kalter, Sibylle Ohl, Dr Lavinia Arseni, Dr Lena Jassowicz, Dr Philipp Rößner, Dr Selcen Öztürk-Ince and Dr Ibrahim Sektioglu for providing valuable feedback and support.

Special thanks go to the people who I shared office with besides not working on related projects: Dr Lena Herbst and Prof Dr Harald Herrmann-Lerdon.

I would like to thank all members of the B06X division and subgroups for very valuable support. Especially Frauke Devens, Petra Schroeter and Magdalena Schlotter were always there to answer laboratory-related questions. Not less did I appreciate the support by Theresa Schmid who cared for a good lab atmosphere and lab environment.

I am thankful for the people from other research groups both at the DKFZ and at the University Hospital of Ulm that contributed their share to this project. These were Prof Dr Sascha Dietrich, Prof Dr Stephan Stilgenbauer, Dr Anna Pirona, Dr Alena Ivanova, Dr Felix Bestvater, Dr Manuela Brom, Dr Damir Krunic, Dr Stffen Schmidt, Dr Florian Blum and others from the FSP-B, the DKFZ core facilities and colleagues at TP3.

Also, I feel a great gratitude to my colleagues of the PhD students council: Dr Lucie Wolf, Dr Florian Köhler, Dr Gintvile Valinciute and Dr Maria Bonsack and also to all members of the Welcome Team 2017/18.

My thanks also go the secretaries in Heidelberg and Ulm, especially to Jasmin Müller and Michele Zimmerman.

Diese Doktorarbeit bedeutete für mein privates Umfeld eine große Herausforderung. Deshalb will ich denen, die im innersten Kern meines Herzens wohnen, ganz besonderen Dank für ihre Geduld, ihre bedingungslose Zuneigung und Liebe und ihre fortwährende Unterstützung aussprechen: Meinen Töchtern Emma und Amanda, meinen Eltern Anke und Klaus, meiner Oma Anni, Jule, Tante Ute, Onkel Axel, Tante Heike, Anja und Familie, Vinia und Familie, Christopher, Daniel und Heidi mit Felix, Robert und Jenny mit Milena, Abby und Thea.

With the greatest affection, I would like to express my thanks to the friends who I got to know throughout my PhD and who will always remain close to my heart: Dr Florian Köhler, Dr Laura Llaó Cid, Dr Ines Gräßer, Dr Markus Muckenhuber, Dr Umar Khalid, Dr Apurva Gopisetti, Dr Tobias Hein, Dr Ulvi Ahmadov, Dr Tanvi Sharma Saklavi, Dr Pooja Middha, Dr Tolga Lokumcu, Dr Umar Khalid, and Dr David Feuersinger.