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Effect of SHP2 on proliferation and B-cell receptor signaling in a chronic lymphocytic leukemia cell model

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Affidavit

Effect of SHP2 on proliferation and B-cell receptor signaling in a chronic lymphocytic leukemia cell model

written by: Mrs. Sarah Naomi Bolz

I hereby declare, on oath, that I have written the present bachelor thesis with the above-mentioned theme on my own and have not used other than the acknowledged resources and aids.

Date: Signature: _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

Sarah Naomi Bolz

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Abstract

In chronic lymphocytic leukemia (CLL) B-cell receptor (BCR) signaling plays a crucial role for the survival and proliferation of the malignant cells. This molecular system is greatly built up of kinases and phosphatases that use sarcoma (SRC)-homology 2 (SH2) domains to pursue their functions. An SH2-domain screening of 34 CLL patients was performed to find critical participants in pathologic BCR signaling and revealed a correlation between a long time to first treatment and a high binding activity of SH2-domain containing tyrosine phosphatase 2 (SHP2). To identify cellular mechanisms by which SHP2 binding influences BCR signaling in CLL, SHP2 wild type and a phosphatase inactive mutant were overexpressed in the CLL cell line MEC1. Additionally, a SHP2 directed knockdown in MEC1 was generated by small hairpin RNA (shRNA). Overexpression of SHP2 wild type, but not the phosphatase disabled mutant, negatively influenced proliferation and induced anergic characteristics in MEC1: Basal Ca^{2+} and mitogen activated protein kinase signaling were enhanced, while surface immunoglobulin M levels and BCR responsiveness were downregulated. Moreover, ibrutinib synergized with SHP2 overexpression in anti-proliferative effects. Together with the results of SH2-domain screening, these data suggest that SHP2 promotes the cellular state of anergy and thus an indolent clinical course in CLL. Notably, the results identify SHP2 as the previously unknown molecular link between the major anergic hallmarks.

Introduction

A short overview of chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder characterized by the accumulation of non-functional usually monoclonal in origin cluster of differentiation (CD)5^+ B-lymphocytes in blood, bone marrow, lymph nodes and spleen. CLL patients eventually develop lymphocytosis, lymphadenopathy, lymphocyte marrow invasion or splenomegaly. CLL belongs to the indolent lymphomas, meaning that it is slowly progressing. Nevertheless, the disease remains incurable.

Diagnosis of CLL is based on a complete and differential blood count. Criteria are high absolute B lymphocyte count and the typical CLL immunophenotype, which is determined by flow cytometry. CLL cells present B-cell related antigens, including CD19, CD20 and CD23, but also the T-cell associated antigen CD5 on their surface (Hallek *et al.*, 2008; Eichhorst *et al.*, 2015). Immunoglobulin (Ig) molecules are expressed at low levels in CLL cells (Kipps *et al.*, 1989; Fais *et al.*, 1998; Hallek *et al.*, 2008; Eichhorst *et al.*, 2015). Initially, most patients do not have any symptoms and are diagnosed with CLL following a routine blood count.

CLL is one of the most frequent leukemias in western countries, representing about 32 % of all new leukemia cases in the US in 2017 (Bethesda; Sant *et al.*, 2010). 191,000 global cases were reported in 2015 (Fitzmaurice *et al.*, 2017). However, incident rates differ between races and sexes. Whites are more commonly affected than Blacks or Asians (Bethesda; Dores *et al.*, 2007). In addition, CLL is more frequent in men than in women (Bethesda; Dores *et al.*, 2007; Sant *et al.*, 2010; Fitzmaurice *et al.*, 2017; Siegel, Miller and Jemal, 2017). The global age-standardized male/female incidence rate ratio was 1.5:1 in 2015 (Fitzmaurice *et al.*, 2017). CLL is termed a disease of the elderly as the median age at diagnosis is 70 years. Over 80 % of patients survive 5 years or longer after diagnosis (Bethesda).

Racial differences in incident rates suggest hereditary factors that predispose to CLL. In fact, relatives of CLL patients have a considerably higher risk to develop the disease (Lichtenstein *et al.*, 2000; Goldin *et al.*, 2004; Brown *et al.*, 2008; Cerhan and Slager, 2015). Among first-degree relatives, the frequency of CLL cases is 8.5-fold increased (Cerhan and Slager, 2015). This observation might be explained by single nucleotide polymorphisms (SNPs) in loci that are associated with familial CLL. Nearly 30 of such loci have already been identified (Calin *et al.*, 2005; Di Bernardo *et al.*, 2008; Crowther-Swanepoel *et al.*, 2010; Slager *et al.*, 2011; Berndt *et al.*, 2013, 2016; Speedy *et al.*, 2013; Law *et al.*, 2017). For instance, Calin et al. found a familial CLL associated SNP in the miR-15a-miR-16-1 primary precursor resulting in a decreased expression of miR-15a and miR-16-1 (Calin *et al.*, 2005). These microRNAs downregulate the anti-apoptotic gene BCL2 (Cimmino *et al.*, 2005) and loss of their expression is linked to increased levels of zeta-chain-associated protein kinase 70 (ZAP-70), a T-cell related protein that enhances B-cell receptor (BCR) signaling in CLL (Chen *et al.*, 2002; Calin *et al.*, 2005). The expression of ZAP-70 is a prognostic biomarker in CLL. High levels of ZAP-70 are associated with a more aggressive course and an unfavorable outcome (Crespo *et al.*, 2003; Dürig *et al.*, 2003; Orchard *et al.*, 2004). Further independent prognostic factors have been identified, including genetic alterations (like del(13q), del(17p), del(11q) and TP53 mutation), serum β2-microglobulin, sex and age (Hallek *et al.*, 1996; Döhner *et al.*, 2000; Pflug *et al.*, 2014).

Most patients with asymptomatic CLL do not require treatment until they develop symptoms or signs of disease progression (Hallek *et al.*, 2008; Eichhorst *et al.*, 2015). In fact, the time to first treatment (TTFT) is an indicator of disease aggressiveness in CLL. When therapy becomes necessary, the treatment strategy is chosen considering the patient's fitness and TP53 status. First line therapy for CLL patients that do not exhibit a TP53 deletion or mutation is chemoimmunotherapy with fludarabine, cyclophosphamide and the anti-CD20 monoclonal antibody rituximab (FCR) (Eichhorst *et al.*, 2015, 2016).

Whereas the recommended treatment for patients harboring a TP53 alteration is the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib (Eichhorst *et al.*, 2015).

BCR signaling in CLL

CLL cells usually co-express IgM and IgD, which build functional BCRs and are likely to bind autoantigen (Hervé *et al.*, 2005; Mockridge *et al.*, 2007). Depending on the stage of somatic hypermutation in the Ig heavy-chain variable region gene (IGHV) CLL divides into two main subsets: IGHV-unmutated and IGHV-mutated CLL. Typically, the disease is more aggressive if CLL cells express an unmutated IGHV (Damle *et al.*, 1999; Hamblin *et al.*, 1999). Since the IGHV mutation status determines the course of the disease (Damle *et al.*, 1999; Hamblin *et al.*, 1999), BCR affinity and specificity seems to be an important factor in disease progression. Moreover, the CLL Ig repertoire appears to be restricted (Kipps *et al.*, 1989; Fais *et al.*, 1998) and a large proportion of patients even expresses Ig "stereotypes" (Agathangelidis *et al.*, 2012), indicating that particular BCR binding activity characteristics are crucial for disease pathogenesis. Clinical success achieved by BCR signaling inhibitors like ibrutinib confirms the central role of BCR signaling in CLL (Byrd *et al.*, 2014).

Signaling pathways induced by BCR engagement in normal B-cells

The BCR is the key regulator of normal B-cell maturation, survival, proliferation and migration. It consists of surface Ig (sIg) complexed with the transmembrane heterodimer of Igα and Igβ. The main pathways of BCR signaling are summarized in figure 1. SIg engagement cross-links BCRs and leads to mono- or dual-phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) in the intracellular tail of Igα and Igβ by BCR associated sarcoma (SRC)-family tyrosine kinases, namely FYN, BLK and LYN. Via its SRC homology 2 (SH2) domain, LYN can bind the phosphorylated sites

of the ITAMs, resulting in increased levels of activated LYN. Furthermore, dual-ITAM-phosphorylation (unlike mono-phosphorylation) allows the tyrosine kinase SYK to bind the ITAMs via its two SH2 domains, leading to SYK activation. Once activated, SYK phosphorylates the B-cell linker protein (BLNK), which links the BCR signal to various SH2 domain containing proteins. Among these, BTK is a key enzyme that activates the phospholipase 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2 (PLC-γ2). Meanwhile, the SRC-family-phosphorylated adaptor protein CD19 recruits the phosphoinositide 3-kinase (PI3K) to the plasma membrane. Both PI3K and PLC-γ2 are initial points of downstream signaling pathways.

PI3K phosphorylates the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) at the inositol ring to produce phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 in turn stabilizes BTK activation and recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1), which induces the activation of the protein kinase AKT. Subsequently, this leads to the inhibition of FOXO, a family of transcription factors that can initiate apoptosis.

PLC-γ2 hydrolyzes PIP_2 to produce the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) and thereby initiates three main signaling pathways. First, (1) IP_3 triggers the release of intracellular Ca^{2+} (iCa^{2+}) into the cytoplasm, which initiates the stimulation of the nuclear factor of activated T-cells (NFAT) via calmodulin and calcineurin activation. In addition, Ca^{2+} and DAG are both responsible for the activation of protein kinase C (PKC), which on one hand (2) stimulates the rat sarcoma (RAS) pathway and on the other hand (3) initiates the activation of the transcription factor nuclear factor-kappa B (NF-κB). Within the RAS pathway, extracellular signal-regulated kinase 1/2 (ERK1/2) is activated and induces the stimulation of transcription factors MYC and ETS domain-containing protein ELK1. The RAS signaling pathway, which is also stimulated by various growth factors and mitogen stimuli, is likely the best-studied mitogen activated protein kinase (MAPK) system. Finally, transcription factors modulated by these three pathways affect B-cell differentiation, survival, proliferation and migration.

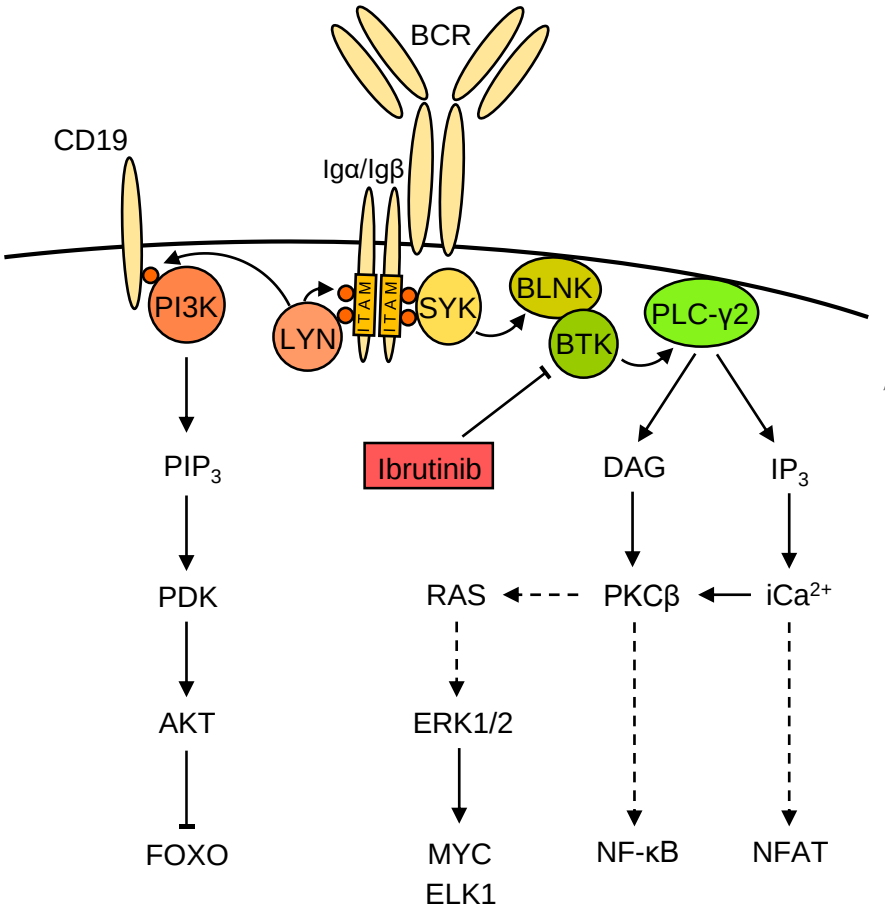

Figure 1: BCR signaling in healthy B-cells and targeted BCR signaling inhibition by ibrutinib. *The graph illustrates the four main pathways that are induced upon BCR cross-linking. PI3K and PLC-γ2 can be considered as the initial points of these pathways. BCR signaling pathways connect events of BCR engagement to several transcription factors that mediate cellular responses regarding B-cell differentiation, survival, proliferation and migration. Ibrutinib is able to inhibit BCR signaling, as it irreversibly binds BTK.*

In addition to the described activating events in BCR signaling, the pathways are also negatively regulated by inhibitory receptors, including CD22 and FcγRIIB, which exhibit ITAM analogous immunoreceptor tyrosine based inhibition motifs (ITIMs). LYN-dependent phosphorylation of the ITIMs recruits SH2 domain containing inhibitory phosphatases like the SH2 domain containing protein tyrosine phosphatase 1 (SHP1) and the SH2 domain containing inositol 5-phosphatase 1 (SHIP1). SHP1 and SHIP1

dephosphorylate adaptor proteins like BLNK and inhibit PI3K, respectively, thus attenuating BCR signaling.

Anergy

In CLL, the predominant BCR signaling response appears to be anergy (Packham *et al.*, 2014), a behavior that under certain conditions is also known from normal B-cells. It is defined as the inability to respond to BCR stimulation, which transfers cells into a state of lethargy with minimized proliferation rates (Cambier *et al.*, 2007). In normal B cells, permanent BCR engagement without the addition of considerable CD4 T-cell help leads to anergy, which eventually induces apoptosis, thus preventing B-cell autoimmunity (Fulcher and Basten, 1994; Gauld, Merrell and Cambier, 2006; Cambier *et al.*, 2007). Whereas anergic CLL cells seem to cause the indolent nature of the disease but somehow escape the death-bringing course.

To investigate anergic features various models have been developed (Cambier *et al.*, 2007). Among these, the hen egg lysozyme (HEL) double transgenic mouse model is one of the most popular. In this model, anergy is induced by the co-expression of a HEL-specific BCR and secreted HEL, which acts as the ligand and thereby arranges chronic BCR stimulation (Goodnow *et al.*, 1988). In addition, although less common, researchers were able to isolate naturally occurring anergic B-cells and analyzed their behavior (Cambier *et al.*, 2007).

Characteristics of anergy in B-cells most notably are attenuated BCR responsiveness (Cambier *et al.*, 2007), reduced sIgM levels (Goodnow *et al.*, 1988), increased basal ERK1/2 phosphorylation levels and raised basal iCa^{2+} levels (Healy *et al.*, 1997). The majority of CLL cells exhibits these anergic key hallmarks (Lanham *et al.*, 2003; Petlickovski *et al.*, 2005; Mockridge *et al.*, 2007; Muzio *et al.*, 2008; Minden *et al.*, 2012; Apollonio *et al.*, 2013). While three characteristics are direct parameters, BCR responsiveness asks for indirect determination by BCR responsiveness reflecting

components. For example, levels of iCa^{2+} upon BCR stimulation mirror the intensity of BCR response and are markedly reduced in anergic HEL-model B-cells in comparison to control B-cells (Cooke *et al.*, 1994). Reduction of mobilized iCa^{2+} has also been shown in primary CLL cells (Apollonio *et al.*, 2013).

Anergy is not an unchangeable but a reversible cellular state. In normal B-cells anergy has been shown to be dependent on continuous BCR engagement (Gauld *et al.*, 2005). Cultivated CLL cells restore their downregulated sIgM levels in vitro, as a result of the interrupted interaction with the microenvironment (Mockridge *et al.*, 2007).

The changes in BCR signaling pathways that cause and maintain the anergic image are poorly understood. Inhibitory signaling appears to play a significant role, since both the SHIP1 and SHP1 pathways are independently essential for the cellular state of anergy (Getahun *et al.*, 2016). SHIP1 is constitutively phosphorylated in anergic normal B-cells and anergic CLL cells (Gabelloni *et al.*, 2008; Yarkoni, Getahun and Cambier, 2010). As described above, this phosphatase is responsible for the inhibition of PI3K. Beside constitutive activation, another SHIP1 pathway stimulating mechanism, which has not been addressed in the context of CLL yet, is known to act in anergic B-cells: Chronic BCR engagement triggers ITAM mono-phosphorylation rather than dual-phosphorylation in anergic B-cells (O'Neill *et al.*, 2011). Since SYK exhibits two SH2 domains and does not bind mono-phosphorylated ITAMs, LYN activation is likely to be favored in this setting (Pao, Famiglietti and Cambier, 1998). In this case, positive BCR signals cannot be transferred to most downstream actors, while LYN pursues its key role in the negative regulation of BCR signaling. ITAM mono-phosphorylation has been shown to stimulate the SHIP1 pathway in anergic cells (O'Neill *et al.*, 2011). Although not proven, the same mechanism is likely to activate additional phosphatases downstream of LYN such as SHP1 (Getahun *et al.*, 2016). In CLL, SHP1 activation is associated with the overexpression and constitutive activation of LYN (Tibaldi *et al.*, 2011). Activated SHIP1 and SHP1 may act at both chronically engaged BCRs and remote receptors, including

sIg receptors and others like the CXC-chemokine receptor 4 (CXCR4) (Brauweiler *et al.*, 2007; Getahun *et al.*, 2016). This could, at least partly, explain the overall observed attenuated BCR responsiveness. However, the molecular events leading to reduced sIgM levels, increased basal ERK1/2 phosphorylation levels and raised basal iCa^{2+} levels as well as the way anergic characteristics relate to each other remain to be clarified.

Apoptosis is the fate of anergic normal B-cells. The BCL2-like protein 11 (commonly known as BIM) is a prominent apoptotic driver in anergic B-cells (Enders *et al.*, 2003; Oliver *et al.*, 2006). Since it is overexpressed in anergic normal B-Cells and in anergic CLL cells (Lesley *et al.*, 2004; Del Gaizo Moore *et al.*, 2007), both cell types should actually undergo apoptosis, but anergic CLL cells seem to ignore increased levels. It is thought that BCL2, which is overexpressed in CLL (Del Gaizo Moore *et al.*, 2007), might be the key to apoptosis resistance in anergic CLL cells. Supporting this statement, CLL cells react highly sensitive to BCL2 inhibitors like venetoclax (Souers *et al.*, 2013), which is currently tested in phase III clinical trials in combination therapies (Crombie and Davids, 2017). BCL2 prevents the death of CLL cells by sequestering BIM (Del Gaizo Moore *et al.*, 2007). In CLL, the overexpression of BCL2 is associated with the loss of miR-15a and miR-16-1 expression, induced by del(13q) or the before mentioned SNP in the miR-15a-miR-16-1 cluster. These microRNAs downregulate the expression of BCL2 (Pekarsky and Croce, 2015).

Anergic features are more frequently found in CLL cases that exhibit favorable prognostic markers linked to a more indolent course of the disease. In particular, mutated IGHV and the absence of ZAP-70 are associated with a strong tendency towards anergy in CLL (Packham *et al.*, 2014). For instance, constitutive ERK1/2 and SHIP1 phosphorylation is more evident in ZAP-70 negative CLL cases (Gabelloni *et al.*, 2008; Apollonio *et al.*, 2013). Less anergy could in turn explain the more aggressive course of the disease observed in CLL patients with an unmutated IGHV and elevated levels of ZAP-70 expression.

The concept of recirculation

Differences in BCR signaling behavior between individual cell populations of the same CLL clone led to the idea of recirculation (Packham *et al.*, 2014). Although the majority of CLL cells appears to be anergic, a proliferative fraction has been observed to be a feature of all CLL cases (Messmer *et al.*, 2005). This rather small amount of CLL cells, which positively responds to BCR engagement, is located in certain tissues like the lymph nodes, particularly in so-called proliferation centers (PCs) (Soma, Craig and Swerdlow, 2006; Herreros *et al.*, 2010; Herishanu *et al.*, 2011; Sachanas *et al.*, 2014). PCs are pale sites where CLL cells meet numerous prolymphocytes and paraimmunoblasts. In PCs, CLL cells are exposed to autoantigen, chemokines, integrins, cytokines as well as survival factors and possibly experience T-cell help, which together might induce proliferation (Kipps *et al.*, 2017). The CXC-chemokine ligand 12 (CXCL12)-specific receptor CXCR4 seems to be important for the migration of CLL cells into tissues (Han *et al.*, 2014). CXCL12 is secreted by stromal cells, which are part of the PCs.

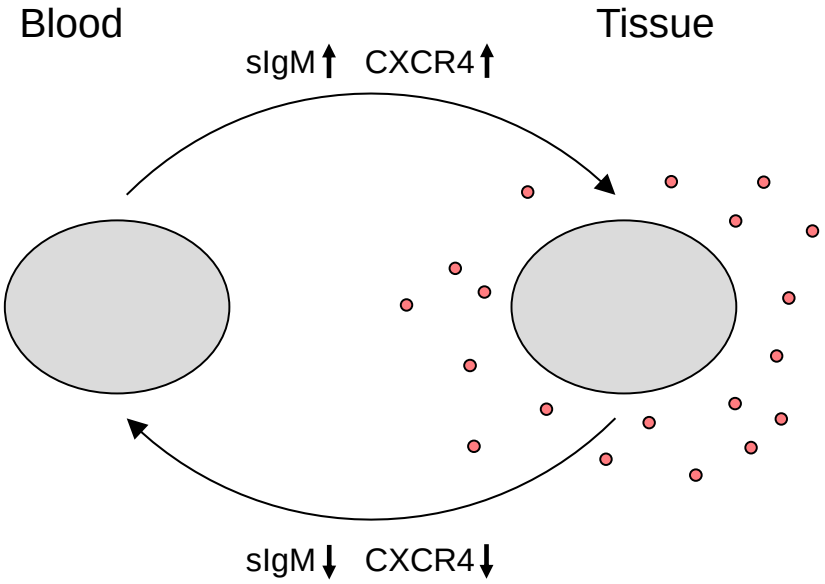

Figure 2: The model of cellular recirculation in CLL. *By circular up- and downregulation of sIgM and CXCR4 levels, cells are driven to migrate from blood into tissue and from tissue into blood. Red dots represent antigen and CXCR12.*

In the concept of CLL recirculation, which is based on anergic reversibility, continuous BCR stimulation and engagement of CXCR4 by CXCL12 in tissues leads to the reduction of sIgM and CXCR4 levels. This prompts CLL cells to exit into the blood, where they experience less stimulation. Consequently, they restore receptor levels and re-entry the tissues (Packham *et al.*, 2014) (Figure 2).

Evidence for this model is provided by intraclonal heterogeneity in sIgM, CXCR4 and Ki-67 levels, which has been found in CLL cells from blood. Ki-67 is a surface marker of proliferating cells (Coelho *et al.*, 2013). The theory is that circulating CLL cells carry a specific “imprint”, identifying them as early or late emigrants from tissues. Most recent emigrants exhibit low sIgM and CXCR4 levels and are Ki-67 positive or negative, while early emigrants already recovered sIgM and CXCR4 levels and are Ki-67 negative. Cell populations classified between these two extreme groups are Ki-67 negative and show increasing sIgM levels, which might reflect the process of receptor recovery. The sIgM levels of different CLL cell fractions correlate with their BCR responsiveness. Although not proliferating, the early emigrant fraction responds well to anti-IgM stimulation. Restored CXCR4 levels and adequate BCR responsiveness indicate that this subpopulation is about to re-enter the tissue and to proliferate (Packham *et al.*, 2014).

This circuit would include that circulating anergic CLL cells are potentially able to proliferate and that a small proportion in fact is driven to proliferation. On the other hand, proliferating CLL cells would at some point exit into the blood and adopt the cellular state of anergy.

MEC1 as a CLL cell model

The CLL cell line MEC1 was established from peripheral blood mononuclear cells (PBMCs) of a patient with CLL in prolymphocytic transformation. The cell line grew out of these PBMCs following incubation (Stacchini *et al.*, 1999).

Though this is rarely observed, CLL can as part of disease progression develop to more aggressive forms that clinically distinguish from CLL, such as prolymphocytic leukemia (PLL) (Foon and Gale, 1988). In contrast to CLL cells, PLL cells exhibit strong sIgM levels, are positive for the CD20 epitope FMC7 (Deans and Polyak, 2008) and do not express CD23 and CD5 (Collignon *et al.*, 2017). MEC1 has been shown to express CD23 and elevated levels of sIg, while FMC7 and CD5 are negative (Stacchini *et al.*, 1999). These markers suggest that MEC1 derived from CLL cells that already began but did not complete prolymphocytic transformation (Rasul *et al.*, 2014).

Usually primary CLL cells are unable to grow in vitro, but something prompted the MEC1 subset to proliferate under these conditions. MEC1 is positive for the Epstein-Barr virus (EBV) (Stacchini *et al.*, 1999). When this virus infects human cells, EBV-mediated proliferation, initiated by a virally encoded growth program, is repressed by the immune system (Klein, Nagy and Rasul, 2013). In cell culture immunological mechanisms are absent or modified, which allows EBV-induced proliferation (Bird, McLachlan and Britton, 1981). Thus EBV might also contribute to proliferation of MEC-1 cells. (Stacchini *et al.*, 1999; Rasul *et al.*, 2014).

MEC1 is classified into the more aggressive subset of CLL. Beside unmutated IGHV the cell line exhibits the chromosomal aberration del(17p). Furthermore, BCL2 is overexpressed in MEC1, which is likely a consequence of virus infection (Stacchini *et al.*, 1999).

MEC1 has been shown to be a suitable model to investigate CLL cell migration (Bailon *et al.*, 2014). In a xenograft mouse model MEC1 cells migrated into blood, lymph node, bone marrow and peritoneum (Bertilaccio *et al.*, 2010). Invasion might be available due to CXCR4 expression, which is present in MEC1 (Rasul *et al.*, 2014). The ability to migrate potentially qualifies MEC1 for recirculation investigations and thus for the analysis of induced anergy.

SHP2

The SH2 domain containing tyrosine phosphatase 2 (SHP2) is a 68 kDa protein structurally characterized by two adjacent SH2 domains at the N-terminus, one protein tyrosine phosphatase domain and a C-terminal hydrophobic tail that contains two tyrosine phosphorylation sites. It is encoded by the PTPN11 gene and ubiquitously expressed among vertebrates.

Basal phosphatase activity is kept low by an autoinhibitory mechanism. The SH2 domain at the outmost N-terminus binds the phosphatase domain via a hydrogen bond, which prevents phosphatase activities. Consistently, the presence of tyrosine phosphorylated SHP2 binding targets suffices to increase SHP2 activity, in that their binding releases the N-terminal SH2 domain of SHP2. Via its two SH2 domains, SHP2 can bind tyrosine phosphorylated receptors and docking proteins and is thereby recruited to the plasma membrane. For instance, the growth factor receptor-bound protein 2 (GRB) and the GRB-associated binder proteins (GABs) are known to bind and thus activate SHP2 (Ingham *et al.*, 1998, 2001; Maus *et al.*, 2009).

SHP2 has a prominent role in the promotion of MAPK signaling (Matozaki *et al.*, 2009). Mice that harbor a homozygous SHP2 inactivating mutation die as embryos, which is associated with decreased MAPK signaling downstream of the fibroblast growth factor receptor (Saxton *et al.*, 1997; Saxton and Pawson, 1999). Furthermore, the expression of catalytically inactive variants of SHP2 leads to extenuated insulin-induced MAPK signaling in mammalian cell culture (Milarski and Saltiel, 1994; Noguchi *et al.*, 1994; Yamauchi *et al.*, 1995). However, the exact mechanisms leading to MAPK stimulation remains to be elucidated.

In B-cells, the function of SHP2 is mainly unclear. Upon BCR stimulation, SHP2 has been shown to be recruited by GAB1 and GAB2 (Ingham *et al.*, 1998; Maus *et al.*, 2009). Furthermore, the co-ligation of several inhibitory receptors with the BCR recruits and activates SHP2 (Maeda *et al.*, 1999; Sármay *et al.*, 1999; Okazaki *et al.*, 2001; Wilkinson

et al., 2002; Ehrhardt *et al.*, 2003). Via these inhibitory receptors, the phosphatase is thought to mediate various inhibitory pathways in B-cells (Maeda *et al.*, 1999; Okazaki *et al.*, 2001; Wong, Hayball and Jackson, 2008; Neumann *et al.*, 2011; Sohn *et al.*, 2011).

Research aims and background

The central aim of this thesis was to investigate the impact of SHP2 on proliferation and B-cell receptor signaling in a CLL cell model.

The rationale for this study came from an SH2-domain screening, which served to identify additional players in the complex network of pathologic BCR signaling (performed by the Nollau research group, research institute Kinderkrebs-Zentrum Hamburg). PBMCs of 34 CLL patients were isolated and lysed. Using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western Blot technology, protein extracts were separated, blotted and subsequently incubated with SH2-domains of different proteins. Phosphotyrosine binding of the SH2-domains was detected by chemiluminescence. The signal intensity of this read-out directly indicated the binding activity of the respective protein. Unsupervised hierarchical cluster analysis revealed four different clusters of the 34 CLL samples. SHP2 binding was most predictive for the organization of the clusters and thereby particularly attracted notice. A high binding activity of SHP2 appeared to be advantageous for the patients, as it correlated with a longer TTFT.

To investigate potential cellular mechanisms by which SHP2 induces this benefit, SHP2 wild type and a phosphatase disabled mutant were overexpressed in the CLL cell line MEC1 and a SHP2 specific knockdown was generated by small hairpin RNA (shRNA). The wish was to answer the following questions: How does SHP2 binding activity influence CLL cell behavior? What kind of BCR responses are induced by SHP2 in CLL

cells? And since ibrutinib treatment targets BCR signaling in CLL cells, how does SHP2 act in ibrutinib treated CLL cells?

Material and Methods

Cell culture

Sequences encoding the wild type or the phosphatase inactive mutant C463S of SHP2 as well as a shRNA directed against SHP2 were cloned into Lentiviral Gene Ontology (LeGO)-iC2-Puro+ and LeGO-C/Zeo vectors (Weber *et al.*, 2010), respectively. Lentiviral transduction of these constructs into the MEC1 cell line was performed by Kristoffer Riecken. Thereby overexpression of wild type or phosphatase inactive SHP2 and a shRNA generated knockdown of SHP2 were produced in MEC1. At the same time, control MEC1 cell lines transduced with the respective empty vectors were established. Validation of the cell lines included Western Blot analysis (Figure 3) and sequencing of the target gene.

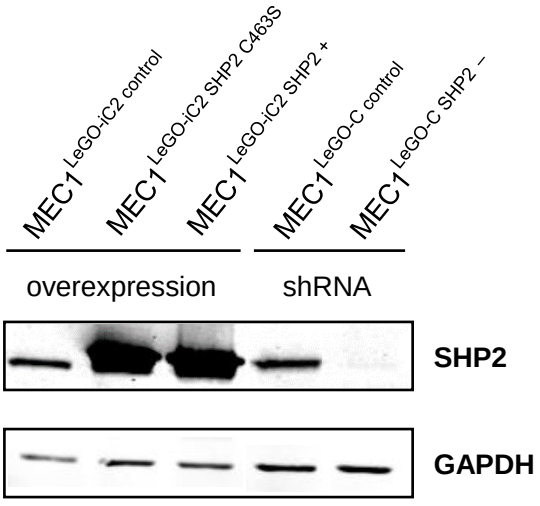

Figure 3: SHP2 expression, overexpression and knockdown in CLL cell line MEC1. *After lentiviral transduction, expression of SHP2 was analyzed via Western Blot analysis.*

MEC1 cell lines were cultured in the recommended growth medium Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific), which was supplemented by 10 % fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1 % Penicillin/Streptomycin (Sigma-Aldrich). To select LeGO-iC2-Puro or LeGO-C/Zeo transduced MEC1 cell clones, 2 µg

ml^{-1} Puromycin or 40 µg ml^{-1} Zeocin, respectively, was added to the medium. The cultivation was performed in a humified incubator at 5 % CO_2 and 37 °C. Viable cell numbers were determined via Cell Viability Analyzer Vi-Cell™ XR (Beckman Coulter). Concentrations were kept in a range of 10^5 to 2.5 x 10^6 viable cells ml^{-1} during continuous cultivation. A viable cell pellet was gained by centrifugation at 300 – 400 g for 5 min.

For cryoconservation a cell pellet of 10^7 to 10^8 cells was suspended in 1 ml freezing medium and transferred into a cryovial (Carl Roth). Freezing medium was made of 90 % FBS and 10 % dimethyl sulfoxide (Sigma-Aldrich). Vials were cooled down at -80 °C for minimum 12 h in a Cryo 1 °C Freezing Container (Thermo Fisher Scientific) filled with isopropanol. Afterwards they could be long-term stored at -80 °C. To thaw frozen cells, the cryovials were set on ice. Slightly unfrozen cells were suspended in 10 ml Dulbecco's Phosphate-Buffered Saline (DPBS) (Thermo Fisher Scientific) to unfreeze completely, pelletized and resuspended in the appropriate medium. On the next day, cells were washed for another time in medium.

Proliferation and cytotoxicity assay of lentivirally transduced MEC1 cells with ibrutinib

To determine half-maximum inhibitory concentrations of ibrutinib for lentivirally transduced MEC1 cells, triplicates of 5.5 x 10^4 MEC1 cells ml^{-1} were seeded in a 96-well plate and treated with a serial dilution of ibrutinib (Selleckchem). Ibrutinib concentrations of 20, 2, 0.2, 0.02 and 0 µM were tested. As a positive control, cells were treated with 0.66mmol/L oxaliplatinum (Sigma-Aldrich). The concentration of viable cells was determined using the Cell Counting Kit-8 (WST-8) (Sigma-Aldrich).

For the determination of differences in proliferation rates, 10^5 cells ml^{-1} were seeded in 10 ml of the appropriate medium and cultured in T25 suspension cell culture flasks for 120 h. Ibrutinib was added to a concentration of 0.5 µmol l^{-1}, when proliferation under ibrutinib treatment was studied. At the relevant time points, 500 µl of cell suspension

were taken to measure the viable cell count and replaced by 500 µl of the respective medium with or without ibrutinib. Each proliferation assay was prepared as a triplicate.

Flow cytometry

Flow cytometers FACSCalibur and LSRFortessa (BD Biosciences) were used to generate FACS Data. The cytometers were equipped with the standard filters. Sheath Fluid, Clean Solution and Rinse Solution were ordered readily prepared (BD Biosciences). Flowing Software 2.5 served as tool for data analysis.

Calcium Flux measurement

Washing and incubation steps were performed using PBS+. This solution was composed of DPBS with $MgCl_2$ and $CaCl_2$ (Sigma-Aldrich) supplemented by 1 mM sodium pyruvate (Biozol) and 25 mM HEPES (Thermo Fisher scientific). 10^6 cells were pelletized, washed in 10 ml PBS+ and resuspended in 850 µl PBS+. Afterwards 1 µl 5 mM FLUO-4 (Thermo Fisher Scientific) was added and the suspension was incubated for 45 min in a humified incubator at 5 % CO_2 and 37 °C. Following incubation, the cells were pelletized, washed in 10 ml PBS+ and resuspended in 500 µl PBS+. Another incubation of 30 min under the same conditions was carried out. The volume was divided on two FACS tubes. The fluorescence intensity measurement was performed using a FACSCalibur (BD Bioscience) and the BD CellQuest Pro Software. For BCR stimulation, approximately 40 µg ml^{-1} Goat anti-Human IgM Fc Secondary Antibody (Thermo Fisher Scientific) were added to cell suspension. Initially, fluorescence intensity was measured for about 15 - 30 s, then the cells were stimulated and the measurement was proceeded until the total measurement time reached 3 min.

Surface IgM staining

To investigate sIgM levels, 10^6 cells of each cell line were pelletized and washed in 4 ml PBS. For blocking, cells were resuspended in 2 ml PBS with 2 % BSA and incubated at room temperature for 30 min. After a centrifugation step, 5 µl anti-IgM-FITC or FITC-Isotype control (both from Beckman Coulter) were added to the barely dry cell pellet. The cells were incubated in the dark at 4 °C for 30 min. Subsequently, cells were washed in 4 ml PBS and resuspended in 250 µl PBS. The fluorescence intensity measurement was immediately performed using a FACSCalibur (BD Bioscience) and the BD CellQuest Pro Software.

BCR stimulation and Western Blot analysis

For BCR stimulation 10^7 cells were pelletized, resuspended in 10 ml PBS+ and incubated for 1 h in a humified incubator at 5 % CO_2 and 37 °C. Afterwards the cells were pelletized again and resuspended in 975 µl PBS+. After a 30 min incubation at 37 °C in a humified incubator with 5 % CO_2, the cells were stimulated by adding 25 µl Goat $F(ab')_2$ Anti-Human IgM-UNLB (Southern Biotech) to the suspension to achieve a final antibody concentration of 12.5 µg ml^{-1}. When the stimulation time was 5 min or longer, the cells were set into a humified incubator at 5 % CO_2 and 37 °C for that time. Following stimulation, the suspension was immediately centrifuged at 2000 g and 4 °C. The cells were washed in 900 µl ice-cold PBS and resuspended in 200 µl lysis buffer. For cell lysis, cells were incubated on ice for at least 30 min. The clear lysate was separated from cellular debris performing a 20 min centrifugation at 16,000 g and 4 °C. Lysates were stored at -80 °C.

The lysates as well as the molecular weight marker PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) were diluted 4:5 in self-prepared Lämmli loading buffer (Table 1) and boiled at 95 °C for 5 min. Lämmli buffer stocks were stored at -20 °C.

Table 1: Recipe for Lämmli sample buffer.

Component	Amount	Manufacturer, item number
Tris, 0.25 mol l^{-1}, pH 6.8	2.5 ml	Sigma-Aldrich, T1503
SDS	1.5 mg	Carl Roth, 2326
Glycerol	5 ml	Sigma-Aldrich, G7757
2-Mercaptoethanol	2.5 ml	Sigma-Aldrich, M6250
Bromophenol blue sodium salt	2 mg	Carl Roth, A512

SDS-PAGE was prepared and executed using the Mini-PROTEAN® Tetra Cell System (BIO-RAD). Self-casted 10 % SDS-polyacrylamide gels (Table 2) were directly used or stored in damp paper cloths at 4 °C.

Table 2: Recipe for two mini SDS-polyacrylamide gels containing 10 % acrylamide in the separating gel.

Component	Separating gel	Stacking gel	Manufacturer, item number
H_2O_{dd}	5.9 ml	2.7 ml	
Polyacrylamide, 30 %	5 ml	670 µl	AppliChem, A4983
Tris, 1.5 mol l^{-1}	3.8 ml (pH 8,8)	500 µl (pH 6,8)	Sigma-Aldrich, T1503
SDS, 10 %	150 µl	40 µl	Carl Roth, 2326
NH_4 persulfate, 10 %	150 µl	40 µl	Sigma-Aldrich, A3678
Temed	6 µl	4 µl	Carl Roth, 2367

Gel loading tips enabled clean loading of the sample volumes, which were in a range of 25 to 28 µl. Gel electrophoresis was run in TG buffer (Table 3), supplemented by 0.1 % SDS, at 120 V until the running front reached the lower end of the gel.

Table 3: Composition of the TG buffer. *Components were solved in distilled water.*

Component	Concentration	Manufacturer, item number
Tris	25 mM	Sigma-Aldrich, T1503
Glycin	192 mM	Carl Roth, 3908

Separated proteins were blotted on nitrocellulose (LI-COR) membranes by electrotransfer in a Mini Trans-Blot® Cell (BIO-RAD). The transfer buffer consisted of TG buffer supplemented with 20 % methanol. Nitrocellulose membranes were activated by gentle shaking in 4 °C cool transfer buffer for 15 min. Foam pads, Whatman® gel blotting papers (Sigma-Aldrich) and SDS gels were soaked in transfer buffer prior to their usage in the Western Blot sandwich. During installation of the sandwich, air bubbles were removed using a roller (BIO-RAD). The electrotransfer was run in transfer buffer at 35 V and 4 °C overnight.

Table 4: Composition of TBS-T. *Components were solved in distilled water.*

Component	Concentration	Manufacturer, item number
Sodium chloride	150 mM	Th. Geyer, 1367
Tris	50 mM	Sigma-Aldrich, T1503
Hydrochloric acid	Adjust pH 7.4	Carl Roth, N076
Tween	0.1 %	Sigma-Aldrich, P1379

Gentle shaking in 5 % bovine serum albumin (BSA) (Sigma-Aldrich) in TBS-T (Table 4) for 1 h was performed in order to block the membranes. Two 10 min washes in TBS-T followed. Membranes were incubated with primary antibodies (Table 5), diluted to the appropriate degree in TBS-T with 5 % BSA, at 4 °C overnight.

Table 5: Overview of primary antibodies used in Western Blot analyses.

Primary antibody	Applicated dilution	Manufacturer, item numer
ERK1/2	1:1000	Cell Signaling, 9107S
pERK1/2	1:1000	Cell Signaling, 9101S
GAPDH	1:200	Santa Cruz, sc-32233

3 washing steps of 10 min shaking in TBS-T were performed following primary antibody incubation. Fluorescently labeled secondary antibodies IRDye® 680RD Goat anti-Rabbit IgG and IRDye® 800CW Goat anti-Mouse IgG (both LI-COR) were diluted 1:7500 in TBS-T with 5 % BSA. After 1 h of secondary antibody incubation at room temperature, the membranes were washed another 3 x 10 min in TBS-T. The signal was read out using the Odyssey® CLx Imaging System (LI-COR).

Statistics

The LI-COR Image Studio software (version 2) was used for densitometric analysis of Western Blots.

In data presenting graphs, error bars illustrate the standard error of the mean. Data groups were compared by t-test or one-way analysis of variance (ANOVA) with subsequent Bonferroni post hoc test. A p-value ≤ 0.05 was accounted as statistically significant. Asterisks indicate the statistical significance, classified by the amount of the p-value ($p \leq 0.05 \triangleq *$; $p \leq 0.01 \triangleq **$; $p \leq 0.001 \triangleq ***$). When means differed from each other with a p-value > 0.05, the difference was either marked as not significant (n.s.) or not commented in the graphs. All statistics were performed using GraphPad Prism Software (version 5).

Other laboratory devices

The electrical devices that were used in daily laboratory practice, but have not been mentioned before, are listed in table 6.

Table 6: Devices used during daily laboratory practice.

Device	Manufacturer
Magnetic stirrer IKAMAG®RH	IKA-Werke
ThermoMixer C	Eppendorf
Vortex Genie 2	Bender und Hobein AG
Rotilabo®-mini-centrifuge	Carl Roth
Centrifuge 5417R	Eppendorf
Megafuge 1.0R, Rotor #2704	Heraeus
Water bath type 1012	GFL
PowerPac™ HC Power Supply	BIO-RAD
Safety workbench HS 12	Heraeus

Results

SHP2 overexpression negatively affects proliferation of MEC1

Since a high binding activity of SHP2 in SH2-domain screening of CLL patients correlates with a comparably long TTFT, SHP2 appears to have a favorable effect on the disease. To investigate the mechanisms behind this outcome, MEC1 cell lines overexpressing wild type or catalytically inactive mutant SHP2 and a MEC1 cell line harboring a shRNA generated SHP2 knockdown were chosen as models.

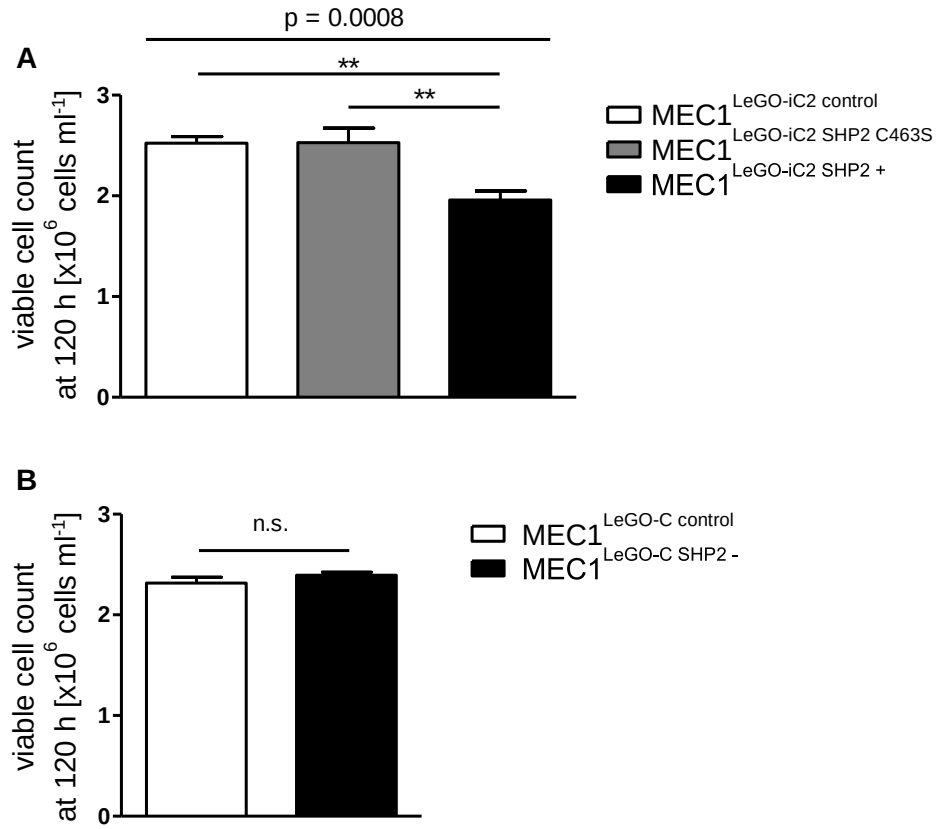

Figure 4: Proliferation analysis of lentivirally transduced MEC1 cells. *(A) Effect of SHP2 overexpression on proliferation of MEC1. Viable cell count was measured via cell viability analyzer 120 h after seeding. Groups were compared using one-way ANOVA followed by Bonferroni post hoc test (n = 9/group). (B) Effect of SHP2 knockdown on proliferation of MEC1. Viable cell count was measured via cell viability analyzer 120 h after seeding. Groups were compared by unpaired t-test (n = 12/group).*

In comparison to control MEC1 cells and to those overexpressing the phosphatase inactive mutant of SHP2, the wild type SHP2 overexpressing MEC1 cell line showed significantly reduced proliferation, identified by the viable cell count at 120 h in proliferation assays (Figure 4A). While the overexpression of SHP2 wild type influenced MEC1 proliferation, the knockdown of SHP2 had no effect (Figure 4B).

Levels of sIgM are reduced in SHP2 overexpressing MEC1 cells

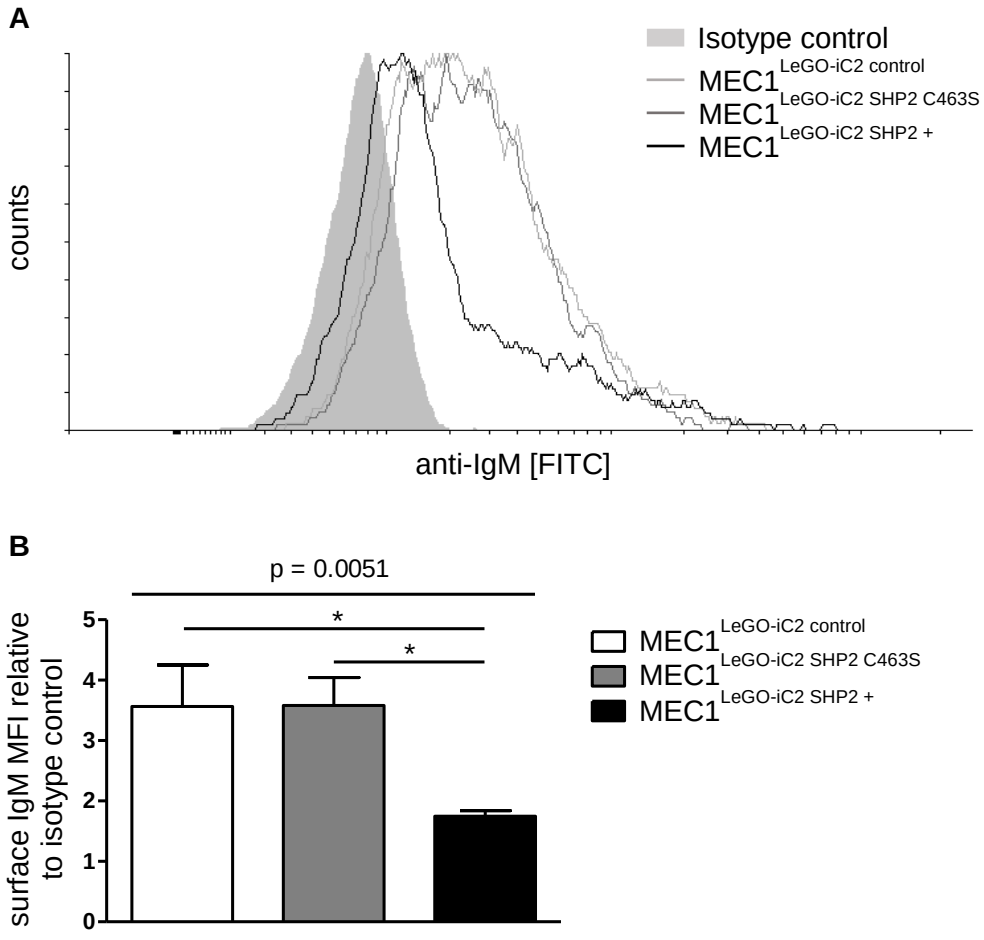

Figure 5: ***Flow cytometry analysis of MEC1 cell lines overexpressing wild type SHP2 or phosphatase disabled mutant SHP2 as well as control MEC1 cells regarding the sIgM levels in comparison to isotype control.*** *(A) A representative histogram is shown. (B) Summarized results are illustrated in a bar chart. Groups were compared using repeated measures one-way ANOVA followed by Bonferroni post hoc test (n = 6/group).*

Reduced proliferation rates indicate changes in BCR signaling, as these pathways are known to be crucial for B-cell proliferation. Hence, investigations regarding the initial point of BCR signaling, the BCR itself, are coherent. Using flow cytometry, sIgM levels on virally transduced MEC1 cell lines were studied. Investigations were not continued with the MEC1 cell line harboring a SHP2 specific knockdown, because no effect on proliferation was apparent.

All investigated MEC1 cell lines presented IgM on their cell surface. However, sIgM levels were significantly decreased in SHP2 wild type overexpressing MEC1 cells, as compared to the overexpression of the phosphatase inactive mutant of SHP2 and to the control (Figure 5).

Basal Ca^{2+} flux and ERK1/2 phosphorylation levels are elevated in MEC1 cells overexpressing SHP2

Downstream of the BCR, intracellular Ca^{2+} flux and ERK1/2 phosphorylation levels represent main signaling pathways and were investigated in control MEC1 cells as well as in those overexpressing wild type or phosphatase disabled SHP2.

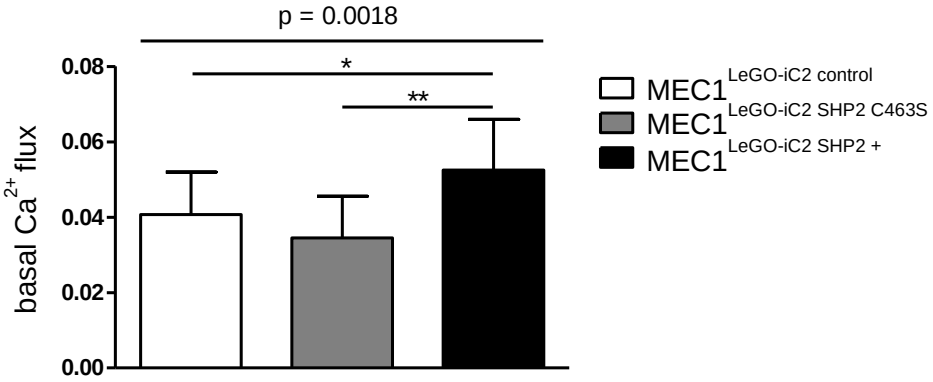

Figure 6: Basal Ca^{2+} levels in SHP2 overexpressing MEC1 cell lines and the control MEC1 cells measured via flow cytometry after FLUO-4 staining. *Groups were compared using repeated measures one-way ANOVA followed by Bonferroni post hoc test (n = 8/group).*

Flow cytometric analysis revealed significantly raised basal Ca^{2+} flux in SHP2 overexpressing MEC1 cells (Figure 6).

Western Blot analysis allowed insights into basal ERK1/2 phosphorylation levels. These were significantly increased in SHP2 wild type overexpressing MEC1 cells in comparison to the control cell line (Figure 7).

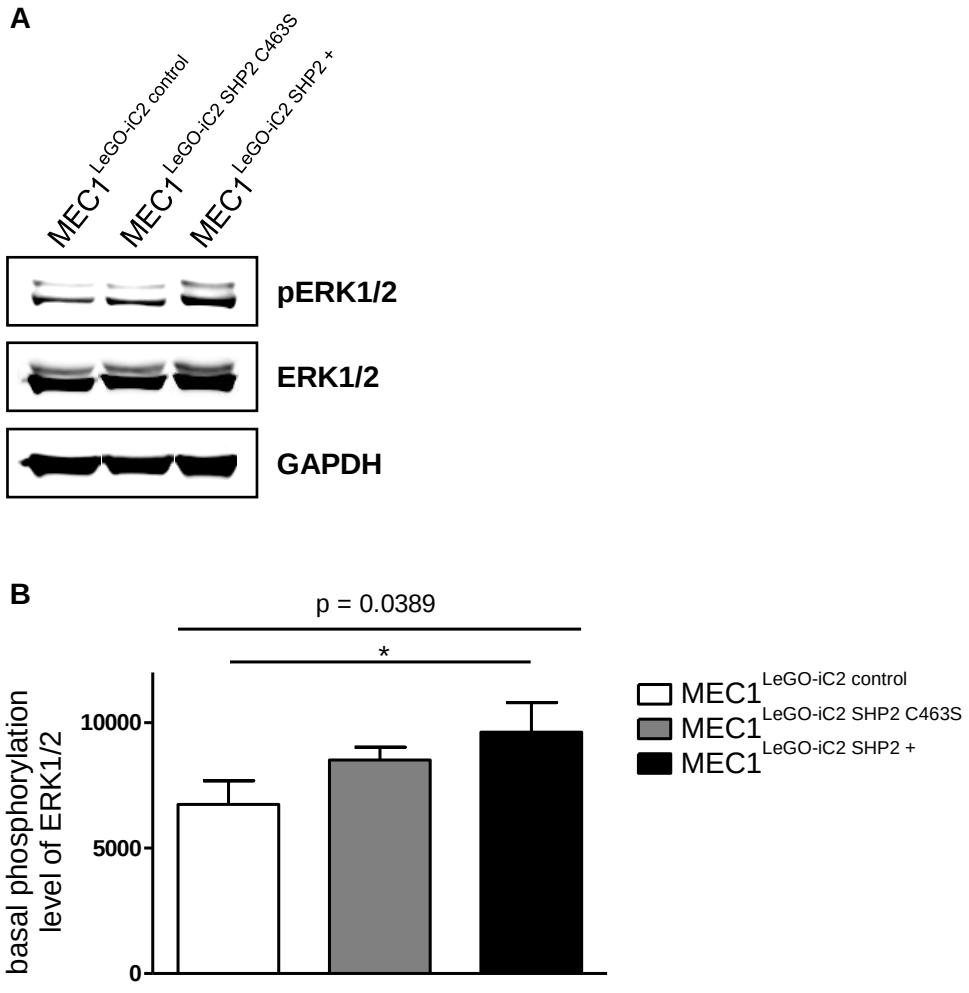

Figure 7: Basal ERK1/2 phosphorylation levels analyzed in SHP2 overexpressing MEC1 cell lines and control MEC1 cell line by Western Blot. *(A) One representative Western Blot is shown. (B) Summarized results are illustrated in a bar chart. Groups were compared using repeated measures one-way ANOVA followed by Bonferroni post hoc test (n = 6/group).*

SHP2 overexpression attenuates BCR responsiveness in MEC1

The same BCR signaling pathways were investigated upon anti-IgM stimulation to elucidate the SHP2 related BCR responsiveness of MEC1. Ca^{2+} mobilization reflects the increase in BCR signaling intensity, induced by BCR cross-linking. It could reproducibly be observed to be diminished in MEC1 cells overexpressing SHP2 wild type, whereas the phosphatase inactive variant of SHP2 did not influence Ca^{2+} mobilization in MEC1 (Figure 8).

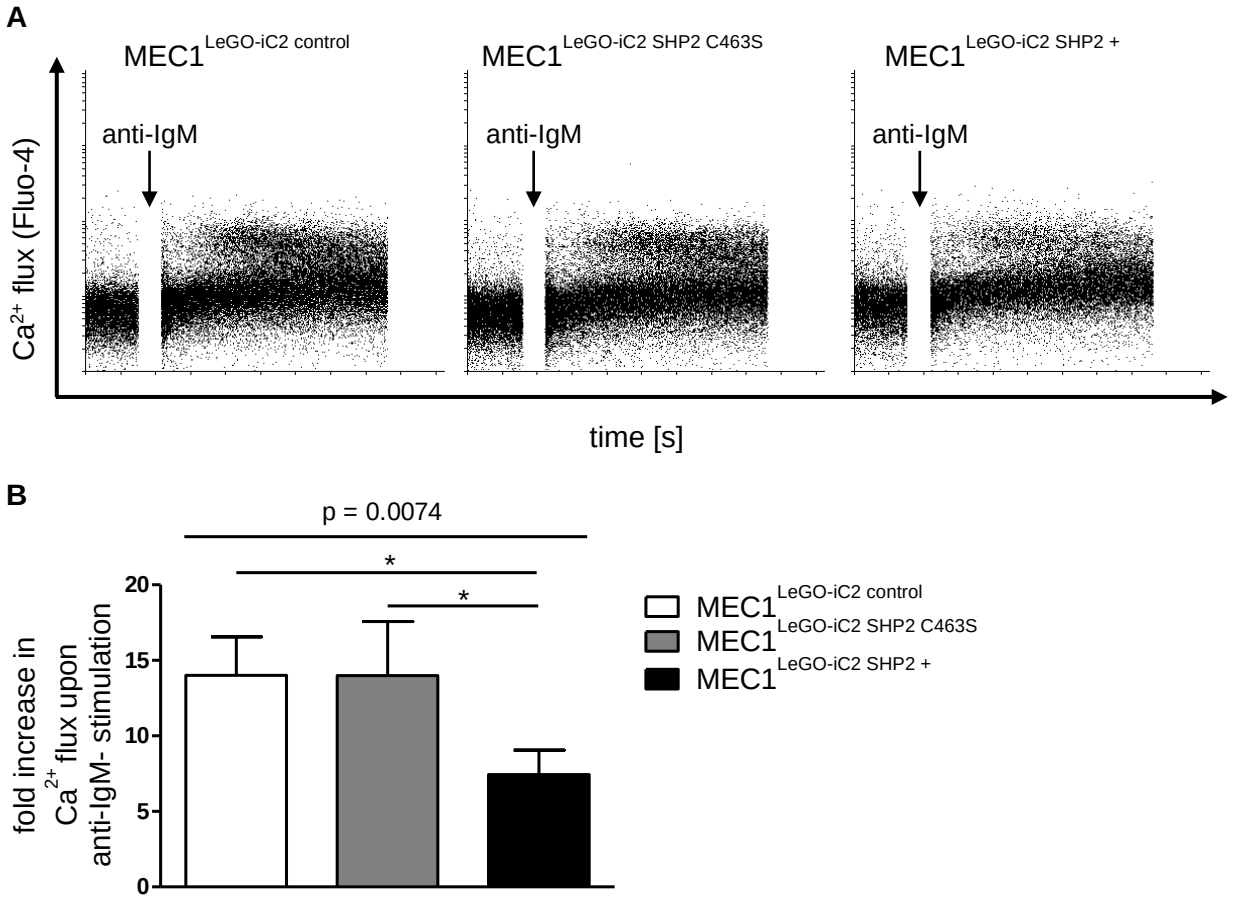

Figure 8: Ca^{2+} mobilization after FLUO-4 staining in response to anti-IgM stimulation in SHP2 overexpressing MEC1 cell lines and control MEC1 cell line determined by flow cytometry analysis. *(A) Representative flow cytometry dot plots that illustrate the Ca^{2+} mobilization in a time dependent manner. (B) Summarized results are illustrated in a bar chart. Groups were compared by repeated measures one-way ANOVA using Bonferroni post-hoc statistics (n = 5/group).*

Furthermore, dynamics of ERK1/2 phosphorylation level upon BCR stimulation were analyzed. Attenuated levels of ERK1/2 phosphorylation in response to anti-IgM stimulation were observed in SHP2 wildtype overexpressing cells in comparison to those overexpressing the phosphatase inactive variant of SHP2 and to the control MEC1 cells. This difference was significant at the analyzed timepoints 1 and 5 min (Figure 9).

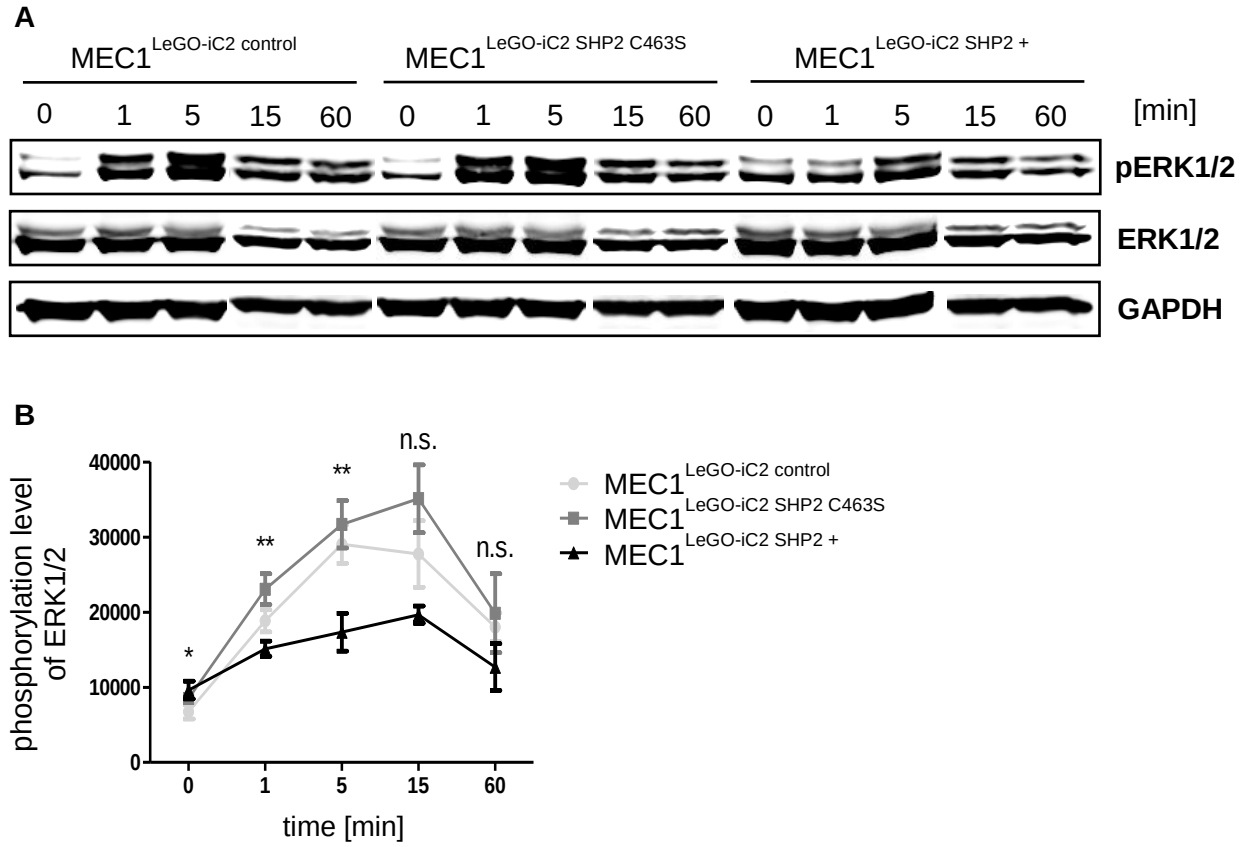

Figure 9: Anti-IgM stimulation induced ERK1/2 phosphorylation in SHP2 overexpressing MEC1 cell lines and control MEC1 cell line analyzed via Western Blot. *Cell lines were serum-starved for 2 hours and anti-IgM stimulated for 0, 1, 5, 15 or 60 min. Corresponding time points were always analyzed on the same blots. (A) Two representative Western Blots are shown that together constitute the results of one anti-IgM stimulation time course. (B) Summarized results of repeated anti-IgM stimulation are illustrated in a time dependent curve. For each time point, groups were compared using repeated measures one-way ANOVA (n = 2 – 6/group).*

Together reduced ERK1/2 and Ca^{2+} signaling upon anti-IgM stimulation reflect the restricted ability of MEC1 cells overexpressing SHP2 wild type to respond to BCR engagement.

SHP2 overexpression synergizes with ibrutinib in MEC1

In contrast to anti-IgM addition, ibrutinib treatment inhibits BCR signaling by irreversibly binding BTK. The half-maximal inhibitory concentration of ibrutinib for $MEC1^{LeGO\text{-}iC2\ control}$, which was determined via cytotoxicity assay, was 2 µM (Figure 10). Eventually, an ibrutinib concentration of 0.5 µM was chosen in proliferation assays to stay close at literature values.

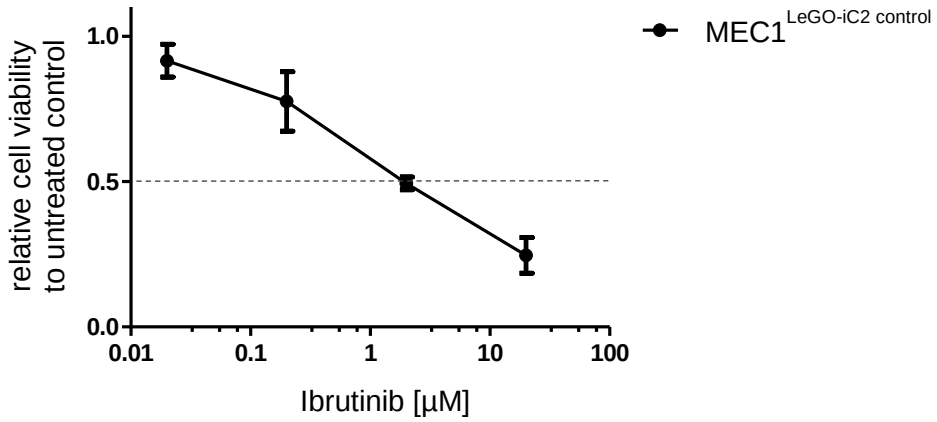

Figure 10: Determination of half-maximum inhibitory ibrutinib concentration in MEC1 cells. *Cells were seeded at equal concentrations and treated with a serial dilution of ibrutinib. Viable cell concentrations were determined using CCK-8.*

Ibrutinib inhibition led to decreased proliferation in all investigated MEC1 cell lines, as compared to untreated cells. Nonetheless, the degree of inhibition was higher in MEC1 cells overexpressing wild type SHP2, suggesting a synergy of SHP2 and ibrutinib in anti-proliferative effects (Figure 11).

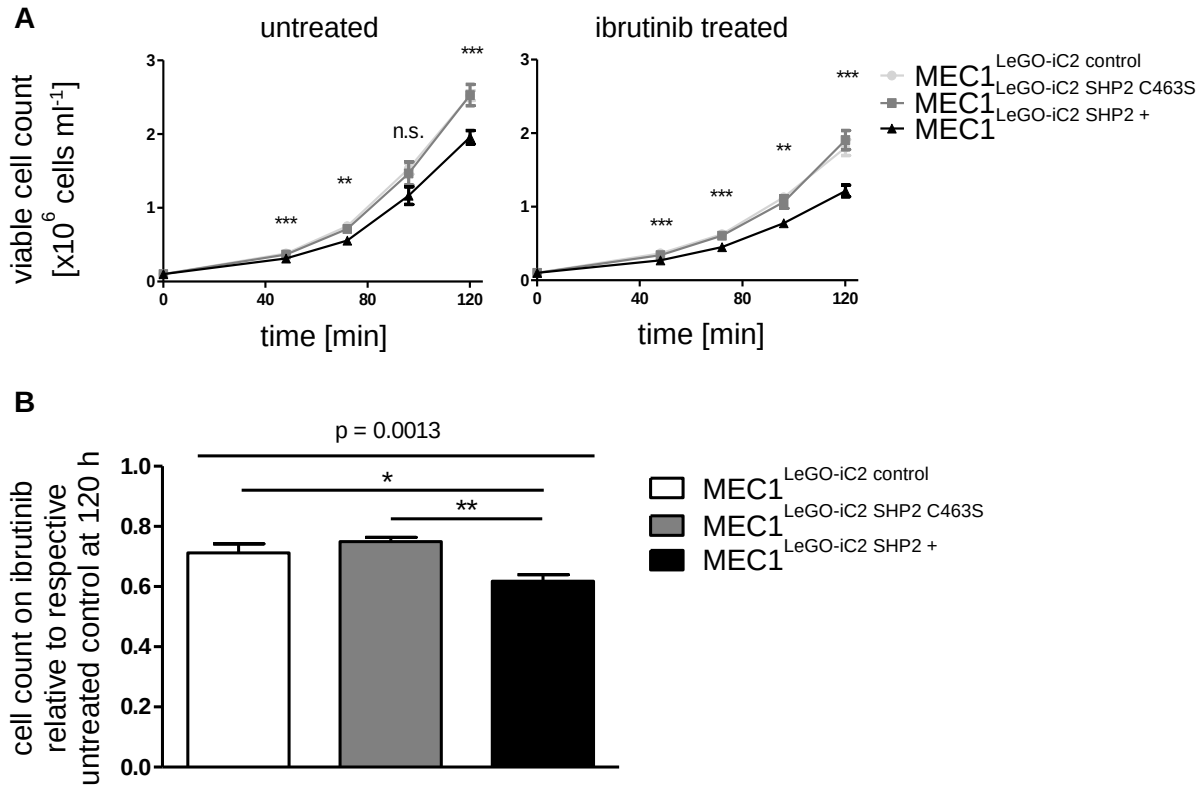

Figure 11: Antiproliferative effect of SHP2 overexpression in ibrutinib treated MEC1 cells. *SHP2 overexpressing MEC1 cell lines and control MEC1 cell line were seeded in medium with and without ibrutinib [0.5 µM]. (A) Viable cell count was measured via cell viability analyzer 48, 72, 96 and 120 h after seeding. For each time point, groups were compared using one-way ANOVA (n = 6 - 9/group). (B) Counts of ibrutinib treated cells at 120 h were normalized to respective untreated control cell counts at 120 h. Groups were compared using one-way ANOVA followed by Bonferroni post hoc test (n = 9/group)*

Discussion

BCR signaling plays a central role in the life circle of B-cells and has been shown to be crucial for the fate of CLL cells. For this reason, kinases and phosphatases that build a complex network to regulate BCR signaling are in the spotlight of CLL research. However, the role of SHP2 in pathologic BCR signaling of CLL cells was completely unknown so far. SH2-domain screening uncovered a correlation between long TTFT and high SHP2 binding activity and led to further studies regarding this phosphatase. The experiments presented in this study show that SHP2 wild type overexpressing MEC1 cells exhibit reduced proliferation rates, decreased sIgM levels, raised basal Ca^{2+} flux, elevated ERK1/2 phosphorylation levels and attenuated BCR responsiveness. These characteristics are well-known as the hallmarks of anergic B-cells (Packham *et al.*, 2014). Moreover, SHP2 overexpression synergizes with ibrutinib in terms of the anti-proliferative effect in MEC1 cells, thus enhancing the responsiveness to this drug.

Finding a suitable cell model to study high binding activities of SHP2 seems to be challenging. Overexpression elevates the level of diffusing SHP2 in cells, which markedly increases the probability that SHP2 meets binding partners, logically leading to an enhanced binding activity of SHP2. Hence, the overexpression of SHP2 in the major CLL cell line MEC1 appears to be a well-suited cell model to investigate the influence of high binding activities of SHP2 in CLL cells. Such high binding activities are accompanied by high SHP2 protein activities, because binding unblocks the autoinhibitory mechanism of SHP2. The opposite effect was achieved by knockdown of SHP2.

The results of this study identify SHP2 as the missing link between the different anergic hallmarks. The contrary functions of SHP2 in B-cells could explain how the phosphatase links these characteristics. In the absence of BCR stimulating components, SHP2 might primarily pursue its most prominent role in MAPK signaling (Matozaki *et al.*, 2009), as indicated by raised basal ERK1/2 signaling upon SHP2 overexpression in MEC1. Whereas upon anti-IgM stimulation, SHP2 is recruited to inhibitory receptors to mediate

diminishing signaling pathways (Maeda *et al.*, 1999; Okazaki *et al.*, 2001; Wong, Hayball and Jackson, 2008; Neumann *et al.*, 2011; Sohn *et al.*, 2011), which might be reflected by attenuated BCR responsiveness in MEC1 cells overexpressing SHP2. Additionally, reduced sIgM levels generated by SHP2 overexpression could be the reason for reduced BCR responsiveness. To perform its diverse functions in MEC1, SHP2 seems to be dependent on its phosphatase activity, as the overexpression of the phosphatase disabled mutant has neither effect on MEC1 proliferation nor BCR signaling. Likewise, the knockdown of SHP2 in MEC1 does not affect proliferation, indicating that the physiological level of SHP2 in MEC1 cells is at an already low level not influencing the biology in this model.

Factors that decide whether a CLL case is more anergic or proliferative are of high clinical interest, since there is a strong correlation between this decision and the aggressiveness of the disease. Anergy correlates with an indolent course and thus is the more favorable state in this setting (D'Avola *et al.*, 2016). Together, SH2-domain screening and overexpression of SHP2 in MEC1 (by reduced proliferation rates) indicate that a high binding activity of SHP2 causes a less aggressive course of CLL due to anti-proliferative effects as a consequence of anergy. Therefore, a high binding activity of SHP2 can be regarded as worthwhile. But according to the concept of recirculation even anergic harmless appearing CLL cells are potentially harmful, as they are generally able to reverse their anergic state and consequently re-enter the tissue (Packham *et al.*, 2014). To manipulate the balance between the anergic and proliferative compartment in the direction of anergy could be a promising concept for CLL therapy.

Ibrutinib treatment of IGHV-mutated CLL, which is considered more anergic, results in prolonged lymphocytosis and lower tissue cell death rate in comparison to less anergic IGHV-unmutated CLL (Byrd *et al.*, 2015; Woyach, Johnson and Byrd, 2016; Burger *et al.*, 2017). Hence, the efficacy of targeting BCR signaling in CLL therapy appears to be dependent on the degree of anergy with a decreased responsiveness in more anergic

cases. Quite conflicting, the anti-proliferative effect of ibrutinib is intensified in SHP2 overexpressing MEC1 cells, which exhibit anergic characteristics. The comparably high resistance to spontaneous apoptosis in anergic CLL cells (Apollonio *et al.*, 2013) possibly causes the reduced responsiveness to ibrutinib by protecting against ibrutinib-induced cell death. Meanwhile, anti-proliferative effects of ibrutinib may be enhanced in these cells. Since anergic CLL cells are likely to escape apoptosis largely by BCL2 overexpression, they are strongly dependent on this protein under ibrutinib treatment (Packham *et al.*, 2014; Deng *et al.*, 2017). A combination therapy of ibrutinib and the BCL2 inhibitor venetoclax thus could be particularly effective.

In CLL, about 1 % of the patients harbor activating mutations in PTPN11 (Puente *et al.*, 2015). So far, a high activity of SHP2 has been found to be rather cancer supportive than inhibiting. Mutations in PTPN11 that constitutively activate SHP2 are associated with the appearance of Noonan Syndrome and Leukemia. Such mutations are arranged at the sites of SHP2 autoinhibiton and thus block this mechanism. Furthermore, increased levels of SHP2 docking proteins, which in turn raise SHP2 activity, are linked to the pathogenesis of cancer (Matozaki *et al.*, 2009). In the context of BCR-ABL1-induced B-cell acute lymphoblastic leukemia, it has been shown that the binding of SHP2 to GAB2 is crucial for lymphoid transformation (Gu *et al.*, 2016). While the literature clearly identifies PTPN11 as proto-oncogene, the here presented results suggest a cancer inhibiting role of SHP2. Activating SHP2 mutations do possibly not contribute to disease progression and proliferation, but to malignant transformation.

In conclusion, SHP2 is identified as the previously unknown link between the major anergic hallmarks. A high binding activity of SHP2 not only causes the typical molecular signature of anergy in MEC1, but is also likely to promote a favorable clinical course due to the induction of anergy in CLL, which is indicated by the results of the SH2-domain screening. These findings broaden our understanding of pathologic BCR signaling in CLL and might give rise to new treatment strategies.

References

Agathangelidis, A. *et al.* (2012) 'Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies.', *Blood*. American Society of Hematology, 119(19), pp. 4467–75. doi: 10.1182/blood-2011-11-393694.

Apollonio, B. *et al.* (2013) 'Targeting B-cell anergy in chronic lymphocytic leukemia', *Blood*, 121(19), pp. 3879–3888. doi: 10.1182/blood-2012-12-474718.

Bailon, E. *et al.* (2014) 'Overexpression of progelatinase B/proMMP-9 affects migration regulatory pathways and impairs chronic lymphocytic leukemia cell homing to bone marrow and spleen', *Journal of Leukocyte Biology*, 96(2), pp. 185–199. doi: 10.1189/jlb.3HI0913-521R.

Di Bernardo, M. C. *et al.* (2008) 'A genome-wide association study identifies six susceptibility loci for chronic lymphocytic leukemia.', *Nature genetics*, 40(10), pp. 1204–10. doi: 10.1038/ng.219.

Berndt, S. I. *et al.* (2013) 'Genome-wide association study identifies multiple risk loci for chronic lymphocytic leukemia', *Nature Genetics*, 45(8), pp. 868–876. doi: 10.1038/ng.2652.

Berndt, S. I. *et al.* (2016) 'Meta-analysis of genome-wide association studies discovers multiple loci for chronic lymphocytic leukemia.', *Nature communications*. Nature Publishing Group, 7, p. 10933. doi: 10.1038/ncomms10933.

Bertilaccio, M. T. S. *et al.* (2010) 'A novel Rag2-/- c-/--xenograft model of human CLL', *Blood*, 115(8), pp. 1605–1609. doi: 10.1182/blood-2009-05-223586.

Bethesda, M. D. *SEER Cancer Stat Facts: Chronic Lymphocytic Leukemia. National Cancer Institute.* Available at: http://seer.cancer.gov/statfacts/html/clyl.html (Accessed: 24 May 2017).

Bird, A. G., McLachlan, S. M. and Britton, S. (1981) 'Cyclosporin A promotes spontaneous outgrowth in vitro of Epstein-Barr virus-induced B-cell lines.', *Nature*, 289(5795), pp. 300–1. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6256660 (Accessed: 6 June 2017).

Brauweiler, A. *et al.* (2007) 'Cutting Edge: Acute and Chronic Exposure of Immature B Cells to Antigen Leads to Impaired Homing and SHIP1-Dependent Reduction in Stromal Cell-Derived Factor-1 Responsiveness', *The Journal of Immunology*, 178(6). Available at: http://www.jimmunol.org/content/178/6/3353.long (Accessed: 4 June 2017).

Brown, J. R. *et al.* (2008) 'Prevalence of familial malignancy in a prospectively screened cohort of patients with lymphoproliferative disorders.', *British journal of haematology*, 143(3), pp. 361–8. doi: 10.1111/j.1365-2141.2008.07355.x.

Burger, J. A. *et al.* (2017) 'Leukemia cell proliferation and death in chronic lymphocytic leukemia patients on therapy with the BTK inhibitor ibrutinib', *JCI Insight*, 2(2), p. e89904. doi: 10.1172/jci.insight.89904.

Byrd, J. C. *et al.* (2014) 'Entering the Era of Targeted Therapy for Chronic Lymphocytic Leukemia: Impact on the Practicing Clinician', *Journal of Clinical Oncology*, 32(27), pp. 3039–3047. doi: 10.1200/JCO.2014.55.8262.

Byrd, J. C. *et al.* (2015) 'Three-year follow-up of treatment-naïve and previously treated patients with CLL and SLL receiving single-agent ibrutinib.', *Blood*, 125(16), pp. 2497–506. doi: 10.1182/blood-2014-10-606038.

Calin, G. A. *et al.* (2005) 'A MicroRNA Signature Associated with Prognosis and Progression in Chronic Lymphocytic Leukemia', *New England Journal of Medicine*, 353(17), pp. 1793–1801. doi: 10.1056/NEJMoa050995.

Cambier, J. C. *et al.* (2007) 'B-cell anergy: from transgenic models to naturally occurring anergic B cells?', *Nature Reviews Immunology*, 7(8), pp. 633–643. doi:

10.1038/nri2133.

Cerhan, J. R. and Slager, S. L. (2015) ‘Familial predisposition and genetic risk factors for lymphoma.’, *Blood*. American Society of Hematology, 126(20), pp. 2265–73. doi: 10.1182/blood-2015-04-537498.

Chen, L. *et al.* (2002) ‘Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia’, *Blood*, 100(13), pp. 4609–4614. doi: 10.1182/blood-2002-06-1683.

Cimmino, A. *et al.* (2005) ‘miR-15 and miR-16 induce apoptosis by targeting BCL2’, *Proceedings of the National Academy of Sciences*, 102(39), pp. 13944–13949. doi: 10.1073/pnas.0506654102.

Coelho, V. *et al.* (2013) ‘Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function.’, *Blood*, 122(15), pp. 2664–72. doi: 10.1182/blood-2013-02-485425.

Collignon, A. *et al.* (2017) ‘Prolymphocytic Leukemia: New Insights in Diagnosis and in Treatment’, *Current Oncology Reports*, 19(4), p. 29. doi: 10.1007/s11912-017-0581-x.

Cooke, M. P. *et al.* (1994) ‘Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells.’, *The Journal of experimental medicine*, 179(2), pp. 425–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8294858 (Accessed: 4 June 2017).

Crespo, M. *et al.* (2003) ‘ZAP-70 Expression as a Surrogate for Immunoglobulin-Variable-Region Mutations in Chronic Lymphocytic Leukemia’, *New England Journal of Medicine*, 348(18), pp. 1764–1775. doi: 10.1056/NEJMoa023143.

Crombie, J. and Davids, M. S. (2017) ‘Venetoclax for the treatment of patients with chronic lymphocytic leukemia’, *Future Oncology*, p. fon-2017-0031. doi: 10.2217/fon-2017-0031.

Crowther-Swanepoel, D. *et al.* (2010) 'Common variants at 2q37.3, 8q24.21, 15q21.3 and 16q24.1 influence chronic lymphocytic leukemia risk.', *Nature genetics*. Europe PMC Funders, 42(2), pp. 132–6. doi: 10.1038/ng.510.

D'Avola, A. *et al.* (2016) 'Surface IgM expression and function are associated with clinical behavior, genetic abnormalities, and DNA methylation in CLL.', *Blood*, 128(6), pp. 816–26. doi: 10.1182/blood-2016-03-707786.

Damle, R. N. *et al.* (1999) 'Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia.', *Blood*, 94(6), pp. 1840–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10477712 (Accessed: 24 May 2017).

Deans, J. P. and Polyak, M. J. (2008) 'FMC7 is an epitope of CD20', *Blood*, 111(4). Available at: http://www.bloodjournal.org/content/111/4/2492?sso-checked=true (Accessed: 7 June 2017).

Deng, J. *et al.* (2017) 'Bruton's tyrosine kinase inhibition increases BCL-2 dependence and enhances sensitivity to venetoclax in chronic lymphocytic leukemia.', *Leukemia*. doi: 10.1038/leu.2017.32.

Döhner, H. *et al.* (2000) 'Genomic Aberrations and Survival in Chronic Lymphocytic Leukemia', *New England Journal of Medicine*, 343(26), pp. 1910–1916. doi: 10.1056/NEJM200012283432602.

Dores, G. M. *et al.* (2007) 'Chronic lymphocytic leukaemia and small lymphocytic lymphoma: overview of the descriptive epidemiology.', *British journal of haematology*, 139(5), pp. 809–19. doi: 10.1111/j.1365-2141.2007.06856.x.

Dürig, J. *et al.* (2003) 'ZAP-70 expression is a prognostic factor in chronic lymphocytic leukemia', *Leukemia*, 17(12), pp. 2426–2434. doi: 10.1038/sj.leu.2403147.

Ehrhardt, G. R. A. *et al.* (2003) 'The inhibitory potential of Fc receptor homolog 4 on memory B cells.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(23), pp. 13489–94. doi:

10.1073/pnas.1935944100.

Eichhorst, B. *et al.* (2015) 'Chronic lymphocytic leukaemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up', *Annals of Oncology*. Oxford University Press, 26(suppl 5), pp. v78–v84. doi: 10.1093/annonc/mdv303.

Eichhorst, B. *et al.* (2016) 'First-line chemoimmunotherapy with bendamustine and rituximab versus fludarabine, cyclophosphamide, and rituximab in patients with advanced chronic lymphocytic leukaemia (CLL10): an international, open-label, randomised, phase 3, non-inferiority trial.', *The Lancet. Oncology*, 17(7), pp. 928–42. doi: 10.1016/S1470-2045(16)30051-1.

Enders, A. *et al.* (2003) 'Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells.', *The Journal of experimental medicine*. The Rockefeller University Press, 198(7), pp. 1119–26. doi: 10.1084/jem.20030411.

Fais, F. *et al.* (1998) 'Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 102(8), pp. 1515–25. doi: 10.1172/JCI3009.

Fitzmaurice, C. *et al.* (2017) 'Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015', *JAMA Oncology*, 3(4), p. 524. doi: 10.1001/jamaoncol.2016.5688.

Foon, K. A. and Gale, R. P. (1988) 'Clinical transformation of chronic lymphocytic leukemia.', *Nouvelle revue francaise d'hematologie*, 30(5–6), pp. 385–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3065735 (Accessed: 6 June 2017).

Fulcher, D. A. and Basten, A. (1994) 'Reduced life span of anergic self-reactive B cells in a double-transgenic model.', *The Journal of experimental medicine*, 179(1), pp. 125–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8270860 (Accessed: 4 June

2017).

Gabelloni, M. L. *et al.* (2008) 'SHIP-1 protein level and phosphorylation status differs between CLL cells segregated by ZAP-70 expression.', *British journal of haematology*, 140(1), pp. 117–9. doi: 10.1111/j.1365-2141.2007.06891.x.

Del Gaizo Moore, V. *et al.* (2007) 'Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 117(1), pp. 112–21. doi: 10.1172/JCI28281.

Gauld, S. B. *et al.* (2005) 'Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling.', *Nature immunology*, 6(11), pp. 1160–7. doi: 10.1038/ni1256.

Gauld, S. B., Merrell, K. T. and Cambier, J. C. (2006) 'Silencing of autoreactive B cells by anergy: a fresh perspective.', *Current opinion in immunology*, 18(3), pp. 292–7. doi: 10.1016/j.coi.2006.03.015.

Getahun, A. *et al.* (2016) 'Continuous inhibitory signaling by both SHP-1 and SHIP-1 pathways is required to maintain unresponsiveness of anergic B cells.', *The Journal of experimental medicine*, 213(5), pp. 751–69. doi: 10.1084/jem.20150537.

Goldin, L. R. *et al.* (2004) 'Familial risk of lymphoproliferative tumors in families of patients with chronic lymphocytic leukemia: results from the Swedish Family-Cancer Database.', *Blood*, 104(6), pp. 1850–4. doi: 10.1182/blood-2004-01-0341.

Goodnow, C. C. *et al.* (1988) 'Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice', *Nature*, 334(6184), pp. 676–682. doi: 10.1038/334676a0.

Gu, S. *et al.* (2016) 'Distinct GAB2 signaling pathways are essential for myeloid and lymphoid transformation and leukemogenesis by BCR-ABL1.', *Blood*. American Society of Hematology, 127(14), pp. 1803–13. doi: 10.1182/blood-2015-06-653006.

Hallek, M. *et al.* (1996) 'Serum β 2 -Microglobulin and Serum Thymidine Kinase are Independent Predictors of Progression-Free Survival in Chronic Lymphocytic Leukemia and Immunocytoma', *Leukemia & Lymphoma*, 22(5–6), pp. 439–447. doi: 10.3109/10428199609054782.

Hallek, M. *et al.* (2008) 'Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines', *Blood*, 111(12), pp. 5446–5456. doi: 10.1182/blood-2007-06-093906.

Hamblin, T. J. *et al.* (1999) 'Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia.', *Blood*, 94(6), pp. 1848–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10477713 (Accessed: 24 May 2017).

Han, T.-T. *et al.* (2014) 'Role of chemokines and their receptors in chronic lymphocytic leukemia: function in microenvironment and targeted therapy.', *Cancer biology & therapy*. Taylor & Francis, 15(1), pp. 3–9. doi: 10.4161/cbt.26607.

Healy, J. I. *et al.* (1997) 'Different nuclear signals are activated by the B cell receptor during positive versus negative signaling.', *Immunity*, 6(4), pp. 419–28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9133421 (Accessed: 4 June 2017).

Herishanu, Y. *et al.* (2011) 'The lymph node microenvironment promotes B-cell receptor signaling, NF- B activation, and tumor proliferation in chronic lymphocytic leukemia', *Blood*, 117(2), pp. 563–574. doi: 10.1182/blood-2010-05-284984.

Herreros, B. *et al.* (2010) 'Proliferation centers in chronic lymphocytic leukemia: the niche where NF-κB activation takes place', *Leukemia*. Nature Publishing Group, 24(4), pp. 872–876. doi: 10.1038/leu.2009.285.

Hervé, M. *et al.* (2005) 'Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity.', *The Journal of clinical investigation*, 115(6), pp. 1636–43. doi: 10.1172/JCI24387.

Ingham, R. J. *et al.* (1998) 'The Gab1 protein is a docking site for multiple proteins involved in signaling by the B cell antigen receptor.', *The Journal of biological chemistry*, 273(46), pp. 30630–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9804835 (Accessed: 8 June 2017).

Ingham, R. J. *et al.* (2001) 'The Gab1 Docking Protein Links the B Cell Antigen Receptor to the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway and to the SHP2 Tyrosine Phosphatase', *Journal of Biological Chemistry*, 276(15), pp. 12257–12265. doi: 10.1074/jbc.M010590200.

Kipps, T. J. *et al.* (1989) 'Developmentally restricted immunoglobulin heavy chain variable region gene expressed at high frequency in chronic lymphocytic leukemia.', *Proceedings of the National Academy of Sciences of the United States of America*, 86(15), pp. 5913–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2503826 (Accessed: 26 May 2017).

Kipps, T. J. *et al.* (2017) 'Chronic lymphocytic leukaemia', *Nature Reviews Disease Primers*, 3, p. 17008. doi: 10.1038/nrdp.2017.8.

Klein, E., Nagy, N. and Rasul, A. E. (2013) 'EBV genome carrying B lymphocytes that express the nuclear protein EBNA-2 but not LMP-1: Type IIb latency.', *Oncoimmunology*. Taylor & Francis, 2(2), p. e23035. doi: 10.4161/onci.23035.

Lanham, S. *et al.* (2003) 'Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia.', *Blood*, 101(3), pp. 1087–93. doi: 10.1182/blood-2002-06-1822.

Law, P. J. *et al.* (2017) 'Genome-wide association analysis implicates dysregulation of immunity genes in chronic lymphocytic leukaemia.', *Nature communications*. Nature Publishing Group, 8, p. 14175. doi: 10.1038/ncomms14175.

Lesley, R. *et al.* (2004) 'Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF.', *Immunity*, 20(4), pp. 441–53. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/15084273 (Accessed: 5 June 2017).

Lichtenstein, P. *et al.* (2000) 'Environmental and Heritable Factors in the Causation of Cancer — Analyses of Cohorts of Twins from Sweden, Denmark, and Finland', *New England Journal of Medicine*, 343(2), pp. 78–85. doi: 10.1056/NEJM200007133430201.

Maeda, A. *et al.* (1999) 'Paired immunoglobulin-like receptor B (PIR-B) inhibits BCR-induced activation of Syk and Btk by SHP-1', *Oncogene*, 18(14), pp. 2291–2297. doi: 10.1038/sj.onc.1202552.

Matozaki, T. *et al.* (2009) 'Protein tyrosine phosphatase SHP-2: A proto-oncogene product that promotes Ras activation', *Cancer Science*, 100(10), pp. 1786–1793. doi: 10.1111/j.1349-7006.2009.01257.x.

Maus, M. *et al.* (2009) 'Grb2 associated binder 2 couples B-cell receptor to cell survival', *Cellular Signalling*, 21(2), pp. 220–227. doi: 10.1016/j.cellsig.2008.10.002.

Messmer, B. T. *et al.* (2005) 'In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells.', *The Journal of clinical investigation*, 115(3), pp. 755–64. doi: 10.1172/JCI23409.

Milarski, K. L. and Saltiel, A. R. (1994) 'Expression of catalytically inactive Syp phosphatase in 3T3 cells blocks stimulation of mitogen-activated protein kinase by insulin.', *The Journal of biological chemistry*, 269(33), pp. 21239–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8063746 (Accessed: 27 July 2017).

Minden, M. D. *et al.* (2012) 'Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling', *Nature*, 489(7415), pp. 309–312. doi: 10.1038/nature11309.

Mockridge, C. I. *et al.* (2007) 'Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status.', *Blood*, 109(10), pp. 4424–31. doi: 10.1182/blood-2006-11-056648.

Muzio, M. *et al.* (2008) 'Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy', *Blood*, 112(1), pp. 188–195. doi: 10.1182/blood-2007-09-111344.

Neumann, K. *et al.* (2011) 'Fc gamma receptor IIb modulates the molecular Grb2 interaction network in activated B cells.', *Cellular signalling*, 23(5), pp. 893–900. doi: 10.1016/j.cellsig.2011.01.015.

Noguchi, T. *et al.* (1994) 'Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation.', *Molecular and cellular biology*, 14(10), pp. 6674–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7935386 (Accessed: 27 July 2017).

O'Neill, S. K. *et al.* (2011) 'Monophosphorylation of CD79a and CD79b ITAM motifs initiates a SHIP-1 phosphatase-mediated inhibitory signaling cascade required for B cell anergy.', *Immunity*, 35(5), pp. 746–56. doi: 10.1016/j.immuni.2011.10.011.

Okazaki, T. *et al.* (2001) 'PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine.', *Proceedings of the National Academy of Sciences of the United States of America*, 98(24), pp. 13866–71. doi: 10.1073/pnas.231486598.

Oliver, P. M. *et al.* (2006) 'Loss of the proapoptotic protein, Bim, breaks B cell anergy.', *The Journal of experimental medicine*, 203(3), pp. 731–41. doi: 10.1084/jem.20051407.

Orchard, J. A. *et al.* (2004) 'ZAP-70 expression and prognosis in chronic lymphocytic leukaemia', *The Lancet*, 363(9403), pp. 105–111. doi: 10.1016/S0140-6736(03)15260-9.

Packham, G. *et al.* (2014) 'The outcome of B-cell receptor signaling in chronic lymphocytic leukemia: proliferation or anergy.', *Haematologica*. Ferrata Storti Foundation, 99(7), pp. 1138–48. doi: 10.3324/haematol.2013.098384.

Pao, L. I., Famiglietti, S. J. and Cambier, J. C. (1998) 'Asymmetrical phosphorylation

and function of immunoreceptor tyrosine-based activation motif tyrosines in B cell antigen receptor signal transduction.', *Journal of immunology (Baltimore, Md. : 1950)*, 160(7), pp. 3305–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9531288 (Accessed: 4 June 2017).

Pekarsky, Y. and Croce, C. M. (2015) 'Role of miR-15/16 in CLL.', *Cell death and differentiation*. Nature Publishing Group, 22(1), pp. 6–11. doi: 10.1038/cdd.2014.87.

Petlickovski, A. *et al.* (2005) 'Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells.', *Blood*, 105(12), pp. 4820–7. doi: 10.1182/blood-2004-07-2669.

Pflug, N. *et al.* (2014) 'Development of a comprehensive prognostic index for patients with chronic lymphocytic leukemia', *Blood*, 124(1), pp. 49–62. doi: 10.1182/blood-2014-02-556399.

Puente, X. S. *et al.* (2015) 'Non-coding recurrent mutations in chronic lymphocytic leukaemia.', *Nature*, 526(7574), pp. 519–24. doi: 10.1038/nature14666.

Rasul, E. *et al.* (2014) 'The MEC1 and MEC2 Lines Represent Two CLL Subclones in Different Stages of Progression towards Prolymphocytic Leukemia', *PLoS ONE*. Edited by J. S. Pagano, 9(8), p. e106008. doi: 10.1371/journal.pone.0106008.

Sachanas, S. *et al.* (2014) 'Apoptotic and proliferative characteristics of proliferation centers in lymph node sections of patients with chronic lymphocytic leukemia', *Leukemia & Lymphoma*, 55(3), pp. 571–582. doi: 10.3109/10428194.2013.806802.

Sant, M. *et al.* (2010) 'Incidence of hematologic malignancies in Europe by morphologic subtype: results of the HAEMACARE project', *Blood*, 116(19), pp. 3724–3734. doi: 10.1182/blood-2010-05-282632.

Sármay, G. *et al.* (1999) 'Cooperation between SHP-2, phosphatidyl inositol 3-kinase and phosphoinositol 5-phosphatase in the Fc gamma RIIb mediated B cell regulation.', *Immunology letters*, 68(1), pp. 25–34. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/10397152 (Accessed: 8 June 2017).

Saxton, T. M. *et al.* (1997) 'Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2.', *The EMBO journal*, 16(9), pp. 2352–64. doi: 10.1093/emboj/16.9.2352.

Saxton, T. M. and Pawson, T. (1999) 'Morphogenetic movements at gastrulation require the SH2 tyrosine phosphatase Shp2.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(7), pp. 3790–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10097116 (Accessed: 27 July 2017).

Siegel, R. L., Miller, K. D. and Jemal, A. (2017) 'Cancer statistics, 2017', *CA: A Cancer Journal for Clinicians*, 67(1), pp. 7–30. doi: 10.3322/caac.21387.

Slager, S. L. *et al.* (2011) 'Genome-wide association study identifies a novel susceptibility locus at 6p21.3 among familial CLL.', *Blood*, 117(6), pp. 1911–6. doi: 10.1182/blood-2010-09-308205.

Sohn, H. W. *et al.* (2011) 'FcRL4 acts as an adaptive to innate molecular switch dampening BCR signaling and enhancing TLR signaling.', *Blood*. American Society of Hematology, 118(24), pp. 6332–41. doi: 10.1182/blood-2011-05-353102.

Soma, L. A., Craig, F. E. and Swerdlow, S. H. (2006) 'The proliferation center microenvironment and prognostic markers in chronic lymphocytic leukemia/small lymphocytic lymphoma', *Human Pathology*, 37(2), pp. 152–159. doi: 10.1016/j.humpath.2005.09.029.

Souers, A. J. *et al.* (2013) 'ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets.', *Nature medicine*, 19(2), pp. 202–8. doi: 10.1038/nm.3048.

Speedy, H. E. *et al.* (2013) 'A genome-wide association study identifies multiple susceptibility loci for chronic lymphocytic leukemia', *Nature Genetics*, 46(1), pp. 56–60. doi: 10.1038/ng.2843.

Stacchini, A. *et al.* (1999) 'MEC1 and MEC2: two new cell lines derived from B-chronic lymphocytic leukaemia in prolymphocytoid transformation.', *Leukemia research*, 23(2), pp. 127–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10071128 (Accessed: 6 June 2017).

Tibaldi, E. *et al.* (2011) 'Lyn-mediated SHP-1 recruitment to CD5 contributes to resistance to apoptosis of B-cell chronic lymphocytic leukemia cells', *Leukemia*, 25(11), pp. 1768–1781. doi: 10.1038/leu.2011.152.

Weber, K. *et al.* (2010) 'Lentiviral gene ontology (LeGO) vectors equipped with novel drug-selectable fluorescent proteins: new building blocks for cell marking and multi-gene analysis', *Gene Therapy*, 17(4), pp. 511–520. doi: 10.1038/gt.2009.149.

Wilkinson, R. *et al.* (2002) 'Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) acts as a regulator of B-cell development, B-cell antigen receptor (BCR)-mediated activation, and autoimmune disease', *Blood*, 100(1), pp. 184–193. doi: 10.1182/blood-2002-01-0027.

Wong, M.-X., Hayball, J. D. and Jackson, D. E. (2008) 'PECAM-1-regulated signalling thresholds control tolerance in anergic transgenic B-cells', *Molecular Immunology*, 45(6), pp. 1767–1781. doi: 10.1016/j.molimm.2007.09.024.

Woyach, J. A., Johnson, A. J. and Byrd, J. C. (2016) 'Chronic lymphocytic leukemia: recent progress and current challenges', *Seminars in Oncology*, 43(2), pp. 199–200. doi: 10.1053/j.seminoncol.2016.02.001.

Yamauchi, K. *et al.* (1995) 'Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling.', *Proceedings of the National Academy of Sciences of the United States of America*, 92(3), pp. 664–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7531337 (Accessed: 27 July 2017).

Yarkoni, Y., Getahun, A. and Cambier, J. C. (2010) 'Molecular underpinning of B-cell anergy.', *Immunological reviews*. NIH Public Access, 237(1), pp. 249–63. doi:

10.1111/j.1600-065X.2010.00936.x.

Abbreviations

B	
BCR	B-cell receptor
BIM	BCL2-like protein 11
BLNK	B-cell linker protein
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase

C	
CLL	Chronic lymphocytic leukemia
CD	Cluster of differentiation
CXCR4	CXC-chemokine receptor 4
CXCR12	CXC-chemokine ligand 12

D	
DAG	Diacylglycerol
DPBS	Dulbecco's Phosphate Buffered Saline

E	
EBV	Epstein-Barr virus
ERK1/2	Extracellular signal-regulated kinase 1/2

F	
FBS	Fetal bovine serum
FCR	Fludarabine, cyclophosphamide, rituximab

G	
GAB	GRB-associated binder protein
GRB	Growth factor receptor-bound protein 2

I	
iCa^{2+}	Intracellular Ca^{2+}
Ig	Immunoglobulin
IGHV	Heavy-chain variable region gene
IMDM	Iscove's Modified Dulbecco's Medium
IP_3	Inositol-1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif

L	
LeGO	Lentiviral Gene Ontology

M	
MAPK	Mitogen activated protein kinase
N	
NF-κB	Nuclear factor-kappa B
NFAT	Nuclear factor of activated T-cells
n.s.	Not significant
P	
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PC	Proliferation center
PDK1	3-Phosphoinositide-dependent protein kinase 1
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKC	Protein kinase C
PLC-γ2	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2
PLL	Prolymphocytic leukemia
R	
RAS	Rat sarcoma
S	
SDS	Sodium dodecyl sulfate
SH2	SRC-homology 2
SHIP1	SH2 domain containing inositol 5-phosphatase 1
SHP1	SH2 domain containing protein tyrosine phosphatase 1
SHP2	SH2 domain containing protein tyrosine phosphatase 2
sIg	Surface immunoglobulin
SNP	Single nucleotide polymorphism
SRC	Sarcoma
T	
TTFT	Time to first treatment
Z	
ZAP-70	Zeta-chain-associated protein kinase 70

Figures

Tables