B cell receptor signaling and its role in pathology and therapy of Chronic Lymphocytic Leukemia

Dissertation

zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät I – Biowissenschaften –

der Martin-Luther-Universität
Halle-Wittenberg,

vorgelegt

von Frau Lisa von Wenserski

geb. am 07.01.1991 in Hennigsdorf

Gutachter:	Prof. Dr. Stefan Hüttelmaier
	Prof. Dr. med. Mascha Binder
	Prof. Dr. Il-Kang Na
Datum der Verteidigung:	26. Oktober 2021

Table of contents

Abstract

Chronic lymphocytic leukemia (CLL) represents the most common leukemia in the western world and despite extensive research over the last decades it is still considered incurable. Affected patients show a highly heterogenic course and the mutational status of IGHV gene used by the respective B cell receptor expressed on the tumor cells has been established as the main factor to discriminate in terms of prognosis and therapy response. This underscores the importance of B cell receptor signaling for the pathogenesis of CLL that is also seen in the successful development of novel therapies that directly target components of this pathway. However, resistance remains a serious problem and novel therapeutic approaches are an urgent clinical need.

This thesis aims at further understanding the differences in B cell receptor signaling in CLL cells that result from their mutational status and to evaluate the effects of two routinely used therapy regimen on the healthy B cell repertoire and their implications on the immunocompetence of the patients during treatment.

The first question was addressed in two projects arising from a SH2 domain screening conducted with primary CLL samples to link known signaling molecules to CLL biology. Indeed, we were able to identify SHP2 and EAT2 as key players that might be decisive for a more indolent disease course.

Using cellular model systems, we could show that SHP2 is directly involved in anergic signaling in CLL tumor cells. Overexpression of SHP2 in the MEC-1 CLL cells led to a decreased expression of sIgM and increased basal calcium flux and MAPK signaling but mitigated responses to BCR crosslinking.

EAT2 is known to be involved in the activation of T and NK cells and a downstream modulator of receptors of the SLAM family. We could indeed show in a second cohort of CLL patients that a high expression level of SLAMF1 and SLAMF7 ($SLAMF^{high}$) was associated with a longer time to first treatment and a mutated BCR. Overexpression of SLAMF1 or SLAMF7 in MEC-1 CLL cells led to reduced proliferation and BCR signaling whereas it was necessary to knock out both of the receptors to elicit opposing effects. Given that EAT2 itself is inconsistently expressed in CLL and EAT2 expression and

SLAMF status did not correlate in our cohort, we conducted a biotinylation screen and found PHB2 as the putative downstream mediator of the observed effects. siRNA experiments confirmed its function in physiological BCR signaling and led us to hypothesize that SLAMF1 and SLAMF7 act by recruiting and thereby detaining PHB2 from that role. An additional aspect of the favorable clinical course of SLAMF^{high} CLL patients became evident when we performed co-culture experiments to explore the role of SLAMF receptors in NK cell mediated anti-CLL immunity. We observed an increased degranulation capacity in those NK cells that we isolated from SLAMF^{high} patients, independent of the SLAMF status of the target CLL cells, suggesting some kind of educational mechanism that improves anti CLL immunity in those patients.

In the third project we used next generation sequencing to compare the impact of standard chemoimmunotherapy and targeted inhibition of BCR signaling components on the healthy B cell repertoire. This question is important for the management and prophylaxis of opportunistic infections as a common side effect of CLL therapy. We collected matched samples before and after treatment and analyzed them for repertoire diversity and the fraction of antigen experienced B cell clones. While FCR treated patients experienced a repertoire renewal with mainly naïve B cells the treatment with ibrutinib resulted in stable repertoire diversity but impaired replenishment of new B cells.

Taken together our data greatly contributes to the deeper understanding of CLL biology and how B cell receptor signaling shapes the heterogeneity of this disease. Based on the findings described in this thesis the use of established therapies can be optimized and new concepts can be developed to maximize the benefit for patients of all prognostic groups.

Zusammenfassung

Die chronische lymphatische Leukämie (CLL) stellt die häufigste Leukämie in der westlichen Welt dar und gilt trotz umfangreicher Forschung in den letzten Jahrzehnten immer noch als unheilbar. Betroffene Patienten zeigen einen sehr heterogenen Verlauf und der Mutationsstatus des jeweiligen B-Zell-Rezeptors, der von den Tumorzellen exprimiert wird, hat sich als Hauptunterscheidungsmerkmal in Bezug auf Prognose und Therapieansprechen etabliert. Dies unterstreicht die Bedeutung des B-Zell-Rezeptor-signalings für die Pathogenese der CLL, die sich auch in der erfolgreichen Entwicklung neuartiger Therapien zeigt, die direkt auf Komponenten dieses Signalweges abzielen. Dennoch bleiben Resistenzen ein großes Problem und neuartige therapeutische Ansätze sind dringend gebraucht.

Diese Arbeit zielt darauf ab, die Unterschiede im B-Zell-Rezeptor-signaling in CLL-Zellen, die aus ihrem Mutationsstatus resultieren, besser zu verstehen und die Auswirkungen von zwei routinemäßig eingesetzten Therapieschemata auf das gesunde B-Zell-Repertoire und damit auf die Immunkompetenz der Patienten während der Behandlung zu untersuchen.

Die erste Frage wurde in zwei Projekten angegangen, die aus einem SH2-Domänen-Screening mit primären CLL-Proben hervorgingen, um bekannte Signalmoleküle mit der Biologie der CLL zu verknüpfen. Tatsächlich konnten wir SHP2 und EAT2 als Schlüsselmoleküle identifizieren, die für einen indolenteren Krankheitsverlauf entscheidend sein könnten.

Wir konnten zeigen, dass SHP2 direkt an der Etablierung eines anergen Phänotyps in CLL-Tumorzellen beteiligt ist. Die Überexpression von SHP2 im MEC-1 CLL-Zellmodell führte zu einer verminderten Expression von sIgM und einem erhöhten basalen Kalziumfluss und MAPK signaling, verringerte aber die Reaktionen auf BCR Vernetzung.

Von EAT2 ist bekannt, dass es an der Aktivierung von T- und NK-Zellen beteiligt und ein nachgeschalteter Modulator von Rezeptoren der SLAM-Familie ist. In der Tat konnten wir in einer zweiten Kohorte von CLL-Patienten zeigen, dass ein hohes Expressionsniveau von SLAMF1 und SLAMF7 ($SLAMF^{high}$) mit einer längeren Zeit bis zur ersten Behandlung und einem mutierten BCR assoziiert war.

Die Überexpression von SLAMF1 oder SLAMF7 allein in MEC 1 Zellen führte zu einer reduzierten Proliferation und BCR signaling, während ein knock out beider Rezeptoren notwendig war, um gegensätzliche Effekte hervorzurufen. Da EAT2 selbst in CLL Zellen inkonsistent exprimiert wird und EAT2-Expression und SLAMF-Status auch in unserer Kohorte nicht korrelierten, führten wir einen Biotinylierungs-Screen durch und identifizierten PHB2 als mutmaßlich verantwortlich für die beobachteten Effekte. siRNA-Experimente bestätigten seine Funktion in der physiologischen BCR-Signalgebung und führten uns zu der Hypothese, dass SLAMF1 und SLAMF7 PHB2 durch Rekrutierung von dieser Rolle abhalten. Ein weiterer Aspekt des günstigen klinischen Verlaufs von SLAMF^{high} CLL Patienten wurde deutlich, als wir Co-Kultur-Experimente durchführten, um die Rolle der SLAMF-Rezeptoren in der NK-Zell-vermittelten anti-CLL Immunität zu untersuchen. Wir beobachteten eine erhöhte Degranulationskapazität in den NK-Zellen, die wir von SLAMF^{high}-Patienten isolierten, unabhängig vom SLAMF-Status der Zielzellen, was auf einen edukativen Mechanismus hinweist.

Im dritten Projekt haben wir Next Generation Sequencing eingesetzt, um die Auswirkungen der Standard-Chemoimmuntherapie und der gezielten Inhibition von BCR-Signalkomponenten auf das gesunde B-Zell-Repertoire zu vergleichen. Diese Frage ist wichtig für das Management und die Prophylaxe von opportunistischen Infektionen als häufige Nebenwirkung der CLL-Therapie. Wir sammelten longitudinale Patientenproben vor und nach der Behandlung und analysierten sie auf die Repertoire-Diversität und den Anteil der Antigen-erfahrenen B-Zellklone. Während mit FCR behandelte Patienten eine Erneuerung ihres Repertoires mit hauptsächlich naiven B-Zellen zeigten, führte die Behandlung mit Ibrutinib zu einer stabilen Diversität des Repertoires, aber einer verminderten Neubildung von B-Zellen.

Zusammengenommen tragen unsere Daten wesentlich zum tieferen Verständnis der CLL-Biologie bei und zeigen, wie das B-Zell-Rezeptor signaling die Heterogenität dieser Krankheit prägt. Auf Basis der in dieser Arbeit beschriebenen Erkenntnisse kann der Einsatz etablierter Therapien optimiert und neue Konzepte entwickelt werden, um den Nutzen für Patienten aller prognostischen Gruppen zu maximieren.

1. Introduction

1.1. B cells in immunity

1.1.1. B cell receptor

B lineage cells are one of the main components of the adaptive immune system and drive the humoral immune response. The unique molecular identity and functionality of a B cell is defined by its B cell receptor. It is composed of a surface immunoglobulin (Ig) with a tetrameric structure build by two heavy and two light chains that are connected via disulfide bonds and form a Y-shaped molecule of about 150 kDa. Both light chains in one receptor are either of the kappa (κ) or lambda (λ) class. Whereas the C terminus of the molecule is termed constant region (C region) and differs only between the Ig isotypes IgM, IgD, IgG, IgA and IgE, the N terminus of the immunoglobulin is highly variable (V region) and contains the antigen binding site.

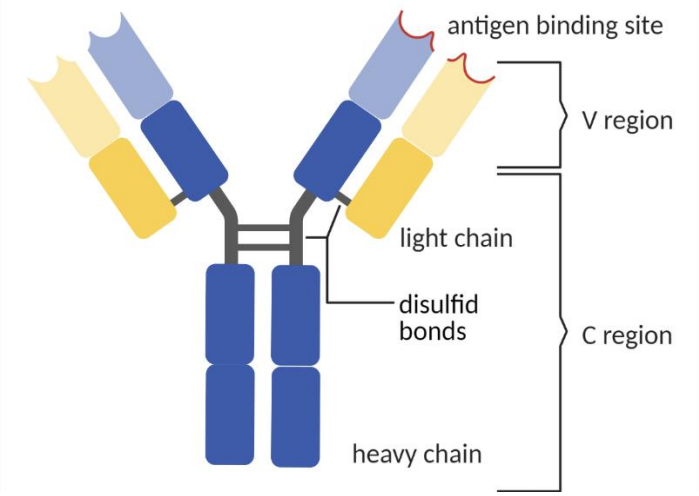


Figure 1: Structure of the B cell receptor. (Created with BioRender)

After contact with the respective antigen and with the help of T cells, B cells further differentiate to antibody producing plasma cells and memory B cells. The antibody secreted resembles the BCR in its structure but instead of being anchored to the cell membrane, the C region (and therefore the isotype) is responsible for the interaction with effector cells and molecules.[1]

The B cell repertoire, that Is the entirety of different immunoglobulins in one human, is estimated to consist of at least 10^{11} individual BCRs. To ensure this enormous diversity a tightly regulated multi-step

process has evolved. The sequence that determines the antibody specificity of one individual BCR is generated by the rearrangement of genomic DNA segments within the immunoglobulin gene loci in B lineage cells, the variable (V), diversity (D) and joining (J) genes. This process was termed somatic or VDJ recombination and occurs in a similar manner in T cells to generate the individual T cell receptors (TCRs).[2]

The V region of the light chain (L) consists of a V_L and a J_L gene segment whereas the V region of the heavy chain (H) contains an additional D_H gene segment besides the V_H and J_H segments. The specific combination of gene segments results in an individual V region sequence consisting of three (light chain) or four (heavy chain) framework (FR1-FR4) and three complementary determining (CDR1-CDR3) regions that form the antibody binding site in the readily assembled BCR.

All of the three immunoglobulin chains – heavy, kappa and lambda light chains – are organized in a similar fashion on different chromosomal loci and each of them contains a different number of V, D and J gene segments. The V genes can be further grouped into seven (V_H, V_κ) to eight (V_λ) different families that are characterized by at least 80 % DNA similarity. The total number of gene segments for each immunoglobulin chain (see table 1) and the arising possible combinations of a light and heavy chain is what determines a great proportion of the variability among the individual B cell receptors and large number of antigens recognized by them.[3-5]

Table 1: Number of gene segments in the respective immunoglobulin chain locus[1]

gene segment	**light chain**		**heavy chain**
	kappa	lambda	
V	34-38	29-33	38-46
D	0	0	23
J	5	4-5	6

The correct recombination site is guided by so called recombination signal sequences (RSS) flanking the V, D and J gene segments. The RSSs are conserved noncoding DNA sequences consisting of a hepta- and nonamer that are separated by either a 23 (V_H, V_λ, J_κ, J_H) or 12 (V_κ, J_λ, D_H) base pair long spacer sequence. According to the 12/23 rule, gene segments with a 12 bp spacer RSS are typically joined to such flanked by a 23 bp spacer RSS. The actual V(D)J recombination is carried out by an enzyme complex in which the recombination activating gene 1 and 2 (RAG1 and RAG2) are the main components. Their expression is strictly limited to the developmental stage of lymphocytes. They recognize the RSSs and induce the joining of the two gene segments via the induction of DNA double strand breaks that are further processed and ligated by additional, non-specific enzymes. During this process, random nucleotides are inserted in the joining region between the gene segments. The joints between the V_L and J_L segments or the V_H, D_H and J_H segments are located in the CDR3, further emphasizing the significance of this hypervariable region for the recognition of a great variety of antigens.[6,7]

A secondary mechanism of antibody diversification occurs after the naïve B cell encounters its specific antigen for the first time. The so called somatic hypermutation introduces point mutations in the V region of the light and heavy chain of the BCR that alter the affinity of the antibody to the respective antigen. This modification is initiated by the activation-induced cytidine deaminase (AID), an enzyme that is only expressed in activated B cells. It selectively deaminates cytidine residues and turns them into uracil. The resulting uracil-guanin mismatch in the nucleotide sequence is sensed by the DNA repair machinery via mismatch or base-excision repair mechanisms that introduce further mutations. Mutations that lead to an increased affinity for the antigen are positively selected for survival.[8] Somatic hypermutation takes places in secondary lymphoid organs where so called germinal centers (GCs) are formed after B cells have bound their respective antigen, internalized them and presented them via major histocompability complex (MHC) class II molecules on their surface. and are then further activated by helper T cells with the same specificity.[9]

1.1.2. BCR signaling

The B cell receptor as described above is capable of recognizing and binding antigens but not to induce signaling by itself. On the cell surface, it is invariably associated with two additional immunoglobulin chains, Igα and Igβ (CD79a and CD79b respectively). They are connected by a disulfide bond between their extracellular domains and coupled to the BCR via hydrophilic interactions between the transmembrane domains.[10] The intracellular domain of the Igα and Igβ chain each possesses an ITAM, an immunoreceptor tyrosine-based activation motif. Those sequences contain two tyrosines that are phosphorylated and bind SH2 domain containing proteins for further signal transduction. They also play a role in T cell receptor signaling pathways and several other immune cells.[11] Phosphorylation of the tyrosines is mediated by src family protein kinases, in B cells namely Lyn (Lck/Yes novel tyrosine kinase), Fyn and Blk (B lymphocyte kinase).[12] They are only weakly associated with the inactive receptor but upon antigen binding and cross-linking, they are activated. This in turn recruits spleen tyrosine kinase (Syk) via its two SH2 domains to the phospho sites and resulting in Syk activation that can be increased by autophosphorylation.[13,14] Activated Syk phosphorylates the scaffold protein BLNK (B cell linker protein). BLKN forms several distinct signaling complexes via its multiple phosphorylation sites that recruit a broad range of SH2 domain containing proteins.[15] One central pathway diverging from this node is the activation of phospholipase C gamma 2 (PLC-$\gamma 2$) with the help of Bruton's tyrosine kinase (BTK). BTK is not restricted to BCR signaling but also acts in other immune related pathways like chemokine induced signaling, Toll-like receptor signaling and Fc receptor signaling. Furthermore, it is also expressed in cells of the myeloid lineage and controls important processes in macrophages.[16] After BCR activation, BTK is recruited via its pleckstrin homology (PH) domain. PH domains bind PIP_3, a membrane lipid generated by conversion of PIP_2 through the phosphoinositide 3-kinases (PI3K). This enzyme is brought to the cell membrane by binding to Syk phosphorylated B cell adaptor protein (BCAP) and CD19, an important co-receptor of the BCR.[17] At the cell membrane BTK is activated by phosphorylation of its kinase domain by Syk that leads to autophosphorylation of the SH3 domain an increased stability.[18] Fully activated, BTK phosphorylates

PLC-γ2 which then generates diacylglycerol (DAG) and inositoltriphosphate (IP_3), two other important second messenger molecules that subsequently activate different pathways.[19] IP_3 activates calcium signaling in B cells by opening ion channels in the endoplasmatic reticulum. This activates calcineurin, a phosphatase responsible for the activation of the transcription factor NFAT (nuclear factor of activated T cells). Together with DAG, the increased calcium levels further activate protein kinase C (PKC) which then leads to NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells) signaling.[20] Further upstream, BTK is also involved in another signaling branch downstream of the BCR: it positively regulates the activation of Akt.[21] Akt induces pro survival programs mediated by FOXO transcription factors and NF-κB pathways.[22]

A negative feedback loop is needed to prevent the continuous signaling and uncontrolled proliferation in B cells after antigen exposure. Antagonistic signaling is mainly mediated by phosphatases like SHIP1, SHP1 and PTEN that are recruited to the inhibitory receptors CD22 and CD32B after the phosphorylation of their immunoreceptor tyrosine-based inhibitory motifs (ITIMs).[23] Once activated, they counteract BCR signaling by dephosphorylating several key enzymes like CD79 and Syk or opposing PLCγ2 activity via the removal of phosphate groups. Responsible for the phosphorylation of the ITIMs is Lyn.[24] Interestingly, it's role in negatively regulating BCR signaling seems to be essential while the activating function is redundant and can be compensated by other kinases of the src family.[25]

Due to the stochastic nature of BCR generation the production of autoreactive B cells (B cells that recognize self-antigens) is inevitable. It is estimated, that nearly 70% of newly formed B cells carry such autoreactive BCRs.[26] To prevent harmful autoimmune events, these cells are tightly controlled through various mechanisms that occur during the above described affinity maturation. Autoreactive signaling of immature B cells leads to receptor editing where the used light chain allele is replaced. The now harmless cell wanders into the periphery while continued autoreactivity leads to clonal deletion.[27-29]

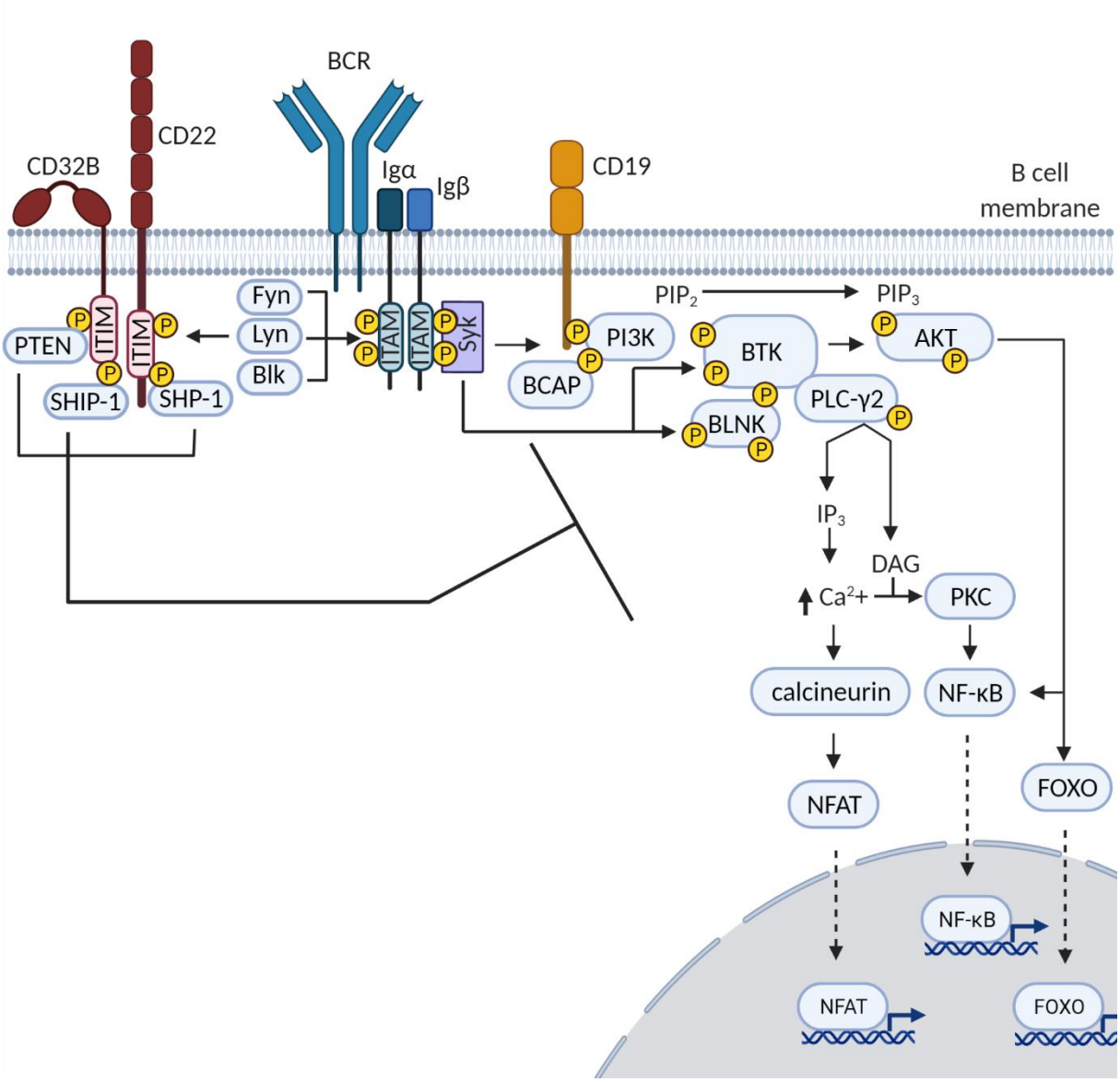


Figure 2: B cell receptor signaling. (Created with BioRender)

If the avidity, the functional affinity of a BCR to its antigen, is significantly strong but fails to induce these central tolerance mechanisms, peripheral tolerance sets in and the respective B cell becomes anergic.[30] Anergy is a functional state determined by a cell's hyporesponsiveness to antigen stimulation as seen in decreased calcium flux, insufficient upregulation of activation markers and failure to proliferate and differentiate.[31] Importantly, these effects are due to the chronic occupation of the BCRs by antigen without secondary activation signals by T cells (presumably because the respective autoreactive T cells are assumed to be deleted by T cell specific tolerance mechanisms in the thymus) and are therefore reversible.[32,33] They can also be overridden by contact with inflammatory cytokines and other activating factors.[34] An important role in the maintenance of anergic signaling is attributed

to Lyn causing monophosphorylation of CD79 ITAMs and phosphorylation of ITIMs on co receptors such as CD22 and CD32B. The phosphorylation of only one tyrosine in the CD79a/b ITAM prevents binding of Syk, that needs two phospho sites to be activated.[35] Increased Lyn activity on the other hand leads to enhanced activation of inhibitory pathways mediated by SHP1 and SHIP1.[36] Absence or mutation of at least one of these molecules is associated with auto immune diseases like Systemic lupus erythematosus (SLE) in mice and human and their depletion from anergic B cells in vivo leads to rapid proliferation and the production of auto antibodies.[37,38] Anergic B cells in the periphery show a rather short life span and are more prone to apoptosis than their normal counterparts (5 vs 40 days, respectively).[39] Increased apoptosis is linked to BIM expression, a pro-apoptotic regulator of the BCL-2 protein family.[40]

1.1.3 SLAMF receptors

Besides the B cell receptor and its co-receptors there are countless other molecules that modulate the immune response, one of them is the family of signaling lymphocytic activation molecules (SLAMF). It is formed by nine type I transmembrane receptors that are solely found on various cells of the immune system, including B cells.[41] They all consist of two Ig-like domains (or four in the case of SLAMF3), one variable and one constant domain that are located extracellular whereas the cytoplasmatic domain differs between the members of the family.[42,43] SLAMF1, SLAMF3, SLAMF4, SLAMF5, SLAMF6 and SLAMF7 carry a variable number of immunoreceptor tyrosine-based switch motifs (ITSMs), SLAMF8 and SLAMF9 miss most of their cytoplasmatic part.[44,45] The SLAMF receptors interact in an homophilic fashion with themselves except for SLAMF4 that pairs with SLAMF2 (CD48).[46,47] SLAMF2 is also atypical as it is anchored in the cell membrane via glycosyl-phosphatidylinositol and possesses no cytoplasmatic domain.[48,49]

Signaling downstream of the SLAMF receptors is best studied in T cells, natural killer (NK) cells and natural killer T (NKT) cells where they can either initiate activating or inhibitory cascades depending on their interaction partner.[50,51] Positive signaling is induced by binding of members of the SLAM

associated protein (SAP) family to the ITSMs. In humans this group of proteins is formed by SAP and Ewing sarcoma activated transcript 2 (EAT2) which are mainly composed of a SH2-domain mediating the binding to the SLAMF receptors.[52] In the absence of SAP and EAT2, the SLAMF receptors can also be occupied by SH2 domain containing phosphatases like SHP1, SHIP1 and SHP2. Binding of these proteins triggers inhibitory signaling.[53] A dysregulation of either SLAMF receptors or their adaptors is linked with autoimmunity and immunological disorders like SLE or X-linked lymphoproliferative disease.[54]

The role of SLAMF receptors in B cells has been of growing interest over the last couple of years but is still insufficiently understood. Over the course of B cell maturation and following activation, each SLAMF receptor is expressed in a unique pattern.[55] They seem to play an important role in the antibody response and survival mainly by mediating the interactions with T cells and other immune cells.[56] SLAMF related signaling in B cells appears to be largely independent of SAP and EAT2 since these adaptors are not or not consistently expressed.[52]

1.2. Chronic Lymphocytic Leukemia

1.2.1. General

The WHO classifies Chronic Lymphocytic Leukemia (CLL) as a mature B-cell neoplasm with leukemic course.[57] It is characterized by the accumulation of clonal CD5 positive ($CD5^+$) B lymphocytes in the blood and the lymphatic tissues.[58] CLL is the most common leukemia in the western countries and presents as a disease of the elderly with the median age at diagnosis being around 73 years. Men are more often affected than women, this reflects in the incidence as well as in mortality.[59,60]

Although the process of leukemogenic transformation that ultimately leads to CLL is still subject of intense investigation, increasing evidence suggests that it starts as early as at the hematopoietic stem cell (HSC) stage and involves large alterations of chromosomal material rather than single gene mutations that seem to occur later in CLL development.[61,62] Genetic profiling and comparison to physiological human subsets points to $CD5^+$ B cells that are believed to acquire additional mutations

during antigen-driven clonal selection and ultimately give rise to CLL as the cellular origin.[63] About 5 % of CLL patients are affected by a transformation into an clonally related aggressive diffuse large B cell lymphoma (DLBCL), a process called Richter's transformation.[64]

1.2.2. Diagnosis

Since CLL is often present without any symptoms it is commonly diagnosed during routine blood counts when white blood cells are elevated (leukocytosis).

According to the guidelines established by the international workshop on Chronic Lymphocytic Leukemia (iwCLL), CLL is diagnosed when the blood count shows more than $5x10^9$/l B lymphocytes for at least three months and clonal restriction of these cells is seen in flow cytometry for either kappa or lambda light chains. Less than $5x10^9$/l B cells are indicative of monoclonal B cell lymphocytosis (MBL), a relatively common premalignant condition that confers a 1-2 % risk for progressing to CLL per year.[65] CLL cells share a common immunophenotype that can be confirmed by flow cytometry. They express CD5 together with known B cell antigens as CD19 and CD23 and CD20, the latter at lower levels compared to physiological B lymphocytes.[66] This observation is also seen for surface immunoglobulins, of which the vast majority of CLLs express the IgM subtype.[67]

1.2.3. BCR signaling in CLL

The importance of BCR signaling in CLL is underscored by the observation that patients can be grouped into two main categories depending on the extend of somatic hypermutation the IGHV gene used by their tumor cells has undergone. If it shows ≥ 98% identity with the closest germline gene it is considered unmutated (U-CLL) whereas in CLL patients with a mutated IGHV gene (M-CLL) > 2% sequence variation can be found.[68] They differ substantially in their clinical and biological features but both subsets seem to derive from $CD5^+$ B cells with the difference between those using a mutated or unmutated BCR being the requirement for T-cell help and taking place of the GC reaction during maturation.[69] A common progenitor is also suggested by similar methylation patterns of the tumor genomes, regardless of the mutational status.[70]

The distribution of IGHV gene usage found among CLL patients is also not as random as might be expected. Several studies show an enrichment of almost identical BCRs sharing distinct motifs in their CDR3 region. These so-called stereotyped B-cell receptors are found in approximately one third of unrelated CLL patients and more often in those with an unmutated IGHV gene.[71,72] U-CLLs generally express low-affinity, poly- and self-reactive BCRs against autoantigens like DNA, cytoskeletal components (vimentin, myosin) or neo-autoantigens arising e.g. during apoptosis or glycosylation whereas M-CLLs tend to express oligo- and mono reactive antigen receptors directed against high-affinity autoantigens.[73,74]

Chronic BCR signaling is a hallmark of CLL pathogenesis, regardless of the mutational status. It is associated with the auto-reactivity of many CLL-BCRs and homotypic interactions between individual Ig receptors on the tumor cells.[75] Both subsets are downregulating sIgM compared to normal B cells and show increased overall tyrosine phosphorylation due to constitutively active Lyn.[76] However, M-CLL shows a more anergic signature and consistently, a high proportion of anergic cells correlates with an indolent clinical course in this disease.[77]

1.2.4. Staging

In daily practice, initial assessment of stage and prognosis is based on physical performance and blood counts according to two major systems: Rai and Binet.

The Rai System was developed in 1975 and initially defined 5 subgroups (0-IV).[78] It was later modified to using 3 subgroups with different prognostic outcome (low-, intermediate- and high-risk disease), analogous to the Binet system, that was established in 1981 and discriminated patients in Binet stadium A to C.[79,80] Both systems are equally simple and can be applied by physicians without the need for imaging, allowing for worldwide use. However, the Rai system is commonly used in the US whereas the Binet system is widely accepted in Europe.

Table 2: Rai staging criteria

stage	lympho-cytosis	enlarged Lymphnodes	enlarged liver/spleen	anemia/thrombo-cytopenia	risk
0	yes	no	no/no	no/no	low
I	yes	yes	no/no	no/no	intermediate
II	yes	yes	no/yes	no/no	
III	yes	yes	likely/yes	yes/no	high
IV	yes	yes	yes/yes	likely/yes	

Table 3: Binet staging criteria

stage	nr. of enlarged lymphoid tissues	anemia	thrombocytopenia	risk
A	< 3	no	no	low
B	≥ 3	no	no	intermediate
C	any	yes	yes	high

1.2.5. Prognostic factors

The staging systems described above are still valuable tools but lack of an update considering latest advances in research focusing on biological properties that can be used to refine the prognosis for CLL patients

The discrimination between U- and M-CLL also reflects differences in the expected clinical outcome, with the former having an inferior prognosis.[81,82] One main advantage of assessing the mutational status of the tumor cells is its stability over the disease course in contrast to chromosomal changes that constantly evolve.[83] Additionally, the use of stereotyped BCR subset influences the outcome, IGHV3-21/IGLV3-21 for example is part of the CLL subset #2 and associated with an unfavorable prognosis, irrespective of the mutational status.[84]

Whole exome sequencing studies revealed TP53, NOTCH1, MYD88, ATM and SFB31 as the most important genes that are found mutated in CLL, often associated with specific cytogenetic lesions.[61] More than 80% of CLL cases show such alterations, most commonly deletions in chromosomes 11, 13 or 17 as well as an additional chromosome 12 and they are all indicative of a distinct prognostic profile.[85]

A deletion on the long arm of chromosome 13 (del13q14) has been connected to the loss of micro RNAs miR-15 and miR-16-1 that are involved in cell cycle control and regulation of BCL2 expression, an anti-apoptotic protein.[86] Deletions in the locus carried by genetically engineered mice lead to the development of MBL and CLL like disorders.[87] It is found in more than half of the CLL patients and, if found as an isolated alteration, is a positive prognostic factor.[85]

Del11q is detected in approximately 35 % of patients but more frequently in advanced disease stages. It often involves 11q23 where the ATM gene is located, a DNA damage response kinase. Affected patients are rather rapidly progressing and have short overall survival.[88] However, it seems that some of these patients especially benefit from chemoimmunotherapy.[89]

Deletions on chromosome 17 are found in 5-8 % of CLL patients at diagnosis. These almost always affects the TP53 locus, encoding the well-known TP53 tumor suppressor and are associated with poor prognosis and resistance to chemotherapy. TP53 mutations are also frequently accompanied by a complex karyotype emphasizing the role of TP53 in DNA damage repair. More than 80 % of CLL patients with a del17p also show aberrations of TP53 in the remaining allele.[90]

Although trisomy 12 is observed in 10 to 20 % of CLL patients, its underlying genetic consequences are largely unknown. Recent studies show a correlation with secondary tumors and Richter's transformation as well as co-occurrence of NOTCH1 mutations.[91]

The expression of SLAMF receptors on CLL cells has also been evaluated in comparison to the healthy B cell compartment. While SLAMF1, SLAMF2 and SLAMF7 are rather downregulated on tumor cells, SLAMF3, SLAMF5 and SLAMF6 are upregulated.[92] However, the prognostic implications are largely unknown except for SLAMF1 where the reduced expression on CLL cells has been associated with an unmutated IGHV gene, a more aggressive disease course and reduced overall survival.[93]

1.2.6. Therapy

1.2.6.1. Treatment options

For decades monotherapy with the alkylating agent chlorambucil has been the standard first-line therapy in CLL, mostly because of its low toxicity, costs and convenient application as it can be administered orally. However, complete remission (CR) with chlorambucil is hardly achieved and extended use causes severe hematopoietic side effects.[94] The introduction of the purine analogues fludarabine and bendamustine as monotherapies improved remission rates but not overall survival (OS).[95,96] Finally, the combination of fludarabine with the alkylating agent cyclophosphamide (FC), proved to be superior to monotherapy in terms of CR, overall response (OR) and progression free survival (PSF).[97,98]

In 1998, the first monoclonal antibody against CD20 – rituximab – was developed and encouraging results in monotherapy led to the combination with the established FC regimen (FCR).[99] Large clinical trials demonstrated improved response and remission rates as well as progression free survival with only moderate increases in toxicity.[100,101] More in-depth analysis revealed that especially patients that carrya del(13q), trisomy 12 or del(11q) benefit from this combination.[102] Remarkably, in a fraction of patients with a mutated IGHV there was no relapse even more than ten years after initial treatment highlighting the curative potential for this regimen.[103]

Increased understanding of the importance of B cell receptor signaling for CLL cells led to the development of a new class of inhibitory drugs that target different kinases downstream of the BCR that gradually enters clinical practice.[104] Among them the most important one to date is ibrutinib, an

orally active small molecule inhibitor that covalently binds to BTK. It was first described in 2010 and FDA approved for the treatment of relapsed or refractory CLL in 2013 after it showed remarkably results even in highly pre-treated and high-risk patients.[105-107] Consequently, its usability as a first line therapy was tested. In high-risk patients not eligible for chemoimmunotherapy (CIT) ibrutinib could induce satisfying response rates and improved PFS.[108-110] In current clinical practice, ibrutinib is seen as the first line therapy of choice in high-risk patients with del(17p) and/or TP53 mutations and for fit patients with an unmutated IGHV gene. Convincing evidence for a survival benefit in IGHV mutated patients treated with ibrutinib compared to those treated with FCR is still lacking. For this subgroup of patients FCR remains the first-line therapy of choice especially considering the advantages of a limited duration of the treatment compared to the continuously application of ibrutinib and the possible long-term side effects that may occur.[94]

As in most targeted therapies, resistance remains a serious problem. Whole exome sequencing studies identified a cysteine at position 481 of BTK to be frequently exchanged by a serine, leading to a change in the binding site and only reversible inhibition by ibrutinib. Another mechanism of resistance are activating mutations in PLCγ2 downstream of BTK that result in autonomous BCR activity.[111] The mutations are always acquired during therapy and can often be detected before a clinical relapse is seen, this highlights their potential as early biomarkers.[112,113]

Other small molecules targeting components of the BCR pathway have also been extensively evaluated in mono- or combination therapy for CLL.

For example, the PI3K isoform PI3K-δ is only expressed in hematopoietic cells and the respective pathway is constitutively active in CLL cells. Idelalisib selectively inhibits this isoform, downregulating Akt and MAPK signaling in the tumor cells directly and also disturbing the interactions with the surrounding microenvironment.[114] Unfortunately it has an alarming toxicity profile especially in combination with monoclonal antibodies. It is therefore used only very selectively, especially for controlling high-risk disease harboring del(17p) and/or TP53 mutations where it leads to a survival benefit.[115,116]

A dysregulated apoptosis is another mechanism by which CLL cells acquire their tumorigenic potential, targeting this axis is therefore a promising approach for treating the disease. Venetoclax blocks the function of the anti-apoptotic Bcl2 protein.[117] It yielded convincing results in relapsed or refractory patients with high-risk features and as a second line therapy after kinase inhibitors as a monotherapy.[118,119] Results for the combination with monoclonal antibodies are also encouraging among all patient subgroups.[120,121] Recent studies are testing venetoclax in combination with ibrutinib in pretreated as well as untreated CLLs and preliminary results are showing high response and remission rates.[122,123]

When applying therapies that target signaling pathways it is important to keep in mind that CLL cells also depend on the cells in their microenvironment, mostly T cells, macrophages and dendritic cells. They support the survival of the lymphoma cells by providing chemokines, cytokines and stimuli for proliferation.[124] Many of the above-mentioned drugs also influence these cells and their communication with the tumor cells. This explains the clinical observation of an increase in lymphocyte blood counts that is often seen following treatment initiation due to a redistribution from the lymphoid compartments.[125]

Other approaches to treat CLL that are currently under investigation are monoclonal antibodies against programmed death 1 (PD-1) receptor and autologous chimeric antigen receptor T cells (CART cells) specific for CD19.[126,127] First small clinical trials with high-risk patients receiving CART cells after ibrutinib failure have shown very promising results and further studies are ongoing.[128]

1.2.6.2. Treatment indications

Therapy in low-risk CLL patients (Binet Stage A or Rai 0) is usually not indicated and common practice is so called “watchful waiting” where the patient is closely monitored for disease progression. Several studies have shown that early treatment with chemoimmunotherapy in asymptomatic patients does not result in survival benefits.[129] Whether this also applies for novel drug classes like small molecule inhibitors is currently under investigation but first results suggests that treatment-naïve early stage CLL patients could benefit from ibrutinib treatment but have to be monitored closely for adverse events.[130]

Patients with high-risk disease (Rai III and IV or Binet C) require immediate therapy whereas patients with an intermediate-risk (Rai I/II and Binet B) can be considered for watchful waiting until they show signs of active disease.

2. Publications

2.1. The phosphotyrosine phosphatase SHP2 promotes anergy in chronic lymphocytic leukemia

Authors

Simon Schliffke, Sophia Buhs, Sarah Bolz, Helwe Gerull, **Lisa von Wenserski**, Kristoffer Riecken, Boris Fehse, Peter Nollau, Mascha Binder.

Published in

Blood 2018 Apr 12;131(15):1755-1758. doi: 10.1182/blood-2017-06-788166. Epub 2018 Feb 23.

blood® Letters to *Blood*

TO THE EDITOR:

The phosphotyrosine phosphatase SHP2 promotes anergy in chronic lymphocytic leukemia

Simon Schliffke,[1] Sophia Buhs,[2] Sarah Bolz,[1] Helwe Gerull,[2] Lisa von Wenserski,[1] Kristoffer Riecken,[3] Boris Fehse,[3] Peter Nollau,[2] and Mascha Binder[1]

[1]Department of Oncology and Hematology, Bone Marrow Transplantation with Section Pneumology, Hubertus Wald Tumorzentrum–University Cancer Center Hamburg, [2]Research Institute Children's Cancer Center and Department of Pediatric Hematology and Oncology, and [3]Research Department Cell and Gene Therapy, Department of Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Anergy describes a state of B-cell receptor activation that leads to survival but not to proliferation in chronic lymphocytic leukemia (CLL) cells, and a high proportion of anergic cells correlates with an indolent clinical course. To gain deeper insights into anergic and proliferative CLL signaling, we performed Src homology 2 (SH2) domain profiling of phosphotyrosine-dependent signaling networks in a cohort of 34 untreated CLL patients by using the SH2 domains CRK, EAT2, GRB2, Pi3K, PLCγ, GAPN, SHIP2, and SHP2 (supplemental Figure 1A, available on the *Blood* Web site).[1] This analysis revealed 4 signaling clusters that were clinically relevant, because patients grouped in clusters 1 to 3 (58.8%) showed significantly longer time to first treatment (TTFT) than those in cluster 4 (26.5%) (supplemental Figure 1B). A third group (14.7%) was only distantly related and showed high EAT2 SH2 binding activity. Of the applied SH2 domains, the phosphatase SHP2 mainly accounted for differences between clusters 1 to 3 and cluster 4 with more SHP2 binding sites correlating with longer TTFT as a surrogate marker for an indolent clinical course (clusters 1 to 3 have an SHP2 SH2 high signature; supplemental Figure 1B-C). This clinical correlation was reproducible in a second cohort of 40 CLL patients using only the SH2 domain of SHP2. Taken together, patients with an SHP2 SH2 high signature had a significantly longer TTFT in the overall cohort (Figure 1A-B), suggesting that signaling pathways involving SHP2 might contribute to CLL pathobiology and disease progression.

SHP2 is a phosphotyrosine phosphatase (PTP) encoded by the *PTPN11* gene. Indeed, this PTP was expressed in virtually all CLL patients at varying levels of expression (supplemental Figure 2). However, SHP2 expression levels were not predictive for the SHP2 SH2 signature, suggesting that the activated phospho-tyrosyl bound forms rather than global protein levels (including the closed autoinhibitory conformation) were responsible for the clinical correlation. To substantiate this, we determined the phosphatase activity of SHP2 in primary CLL cells by using an assay based on 6,8 difluoro-4-methylumbelliferyl phosphate (supplemental Figure 3). Of note, only 4 of 20 available primary CLL samples showing the SHP2 SH2 high signature yielded sufficient SHP2 immunoprecipitate to be used in the phosphatase activity screen, but immunoprecipitations from 16 of 25 available samples showing the SHP2 SH2 low signature were successful. This result may be explained by significantly more competing binding sites for SHP2 in the SHP2 SH2 high cluster. In line with our hypothesis, the phosphatase activity screen of all available samples from the 2 clusters revealed a significantly increased phosphatase activity of SHP2 in CLL patients from the SHP2 SH2 high signature (Figure 1C-D).

To further decipher the molecular mechanisms underlying the SHP2 signature, we used an MEC-1 knockdown and overexpression model as well as an OSU-CLL overexpression model, assuming that the knockdown would mimic the SHP2 SH2 low signature whereas overexpression of this protein would increase the SHP2 activity thereby mimicking, at least in part, the SHP2 SH2 high signature. In line with our hypothesis, the knockdown of SHP2 did not result in any proliferative effects, because SHP2 levels were already low in the parental MEC-1 cell line (supplemental Figure 4A-B). In contrast, overexpression of SHP2, but not a phosphatase-dead SHP2 mutant, resulted in reduced proliferation (Figure 2A). We used PamGene kinase profiling as an unbiased approach to pin down potential signaling pathways in which SHP2 could be involved in CLL. Kinase profiling pointed at Ca^{2+} and MAPK signaling as differentially activated in SHP2 overexpressing versus control MEC-1 cells (supplemental Figure 5). This prompted us to analyze SHP2 involvement in pathological BCR signaling, since an increase in intracellular Ca^{2+} concentration and enhanced MAPK signaling is one of the major initial steps in B-cell activation upon BCR engagement. Interestingly, overexpression of functional SHP2 resulted in a subtle increase of basal Ca^{2+} (Figure 2B) and MAPK signaling (Figure 2C) and in a downregulation of surface BCR (Figure 2D-E). Moreover, SHP2 overexpression mitigated responses to anti-immunoglobulin M (anti-IgM) stimulation such as Ca^{2+} mobilization as well as activation of downstream MAPK signaling (Figure 2F-G). Antiproliferative effects of ibrutinib were enhanced in MEC-1 cells overexpressing SHP2 (Figure 2H). Confirming the observations in the MEC-1 cell line model, reduced proliferation and Ca^{2+} mobilization were also observed in OSU-CLL cells overexpressing SHP2 in contrast to a phosphatase-dead mutant SHP2 (supplemental Figure 6A-B). However, these effects were smaller in comparison with the MEC-1 cell line model, which was presumably due to the low level of overexpression achieved in OSU-CLL and comparable,

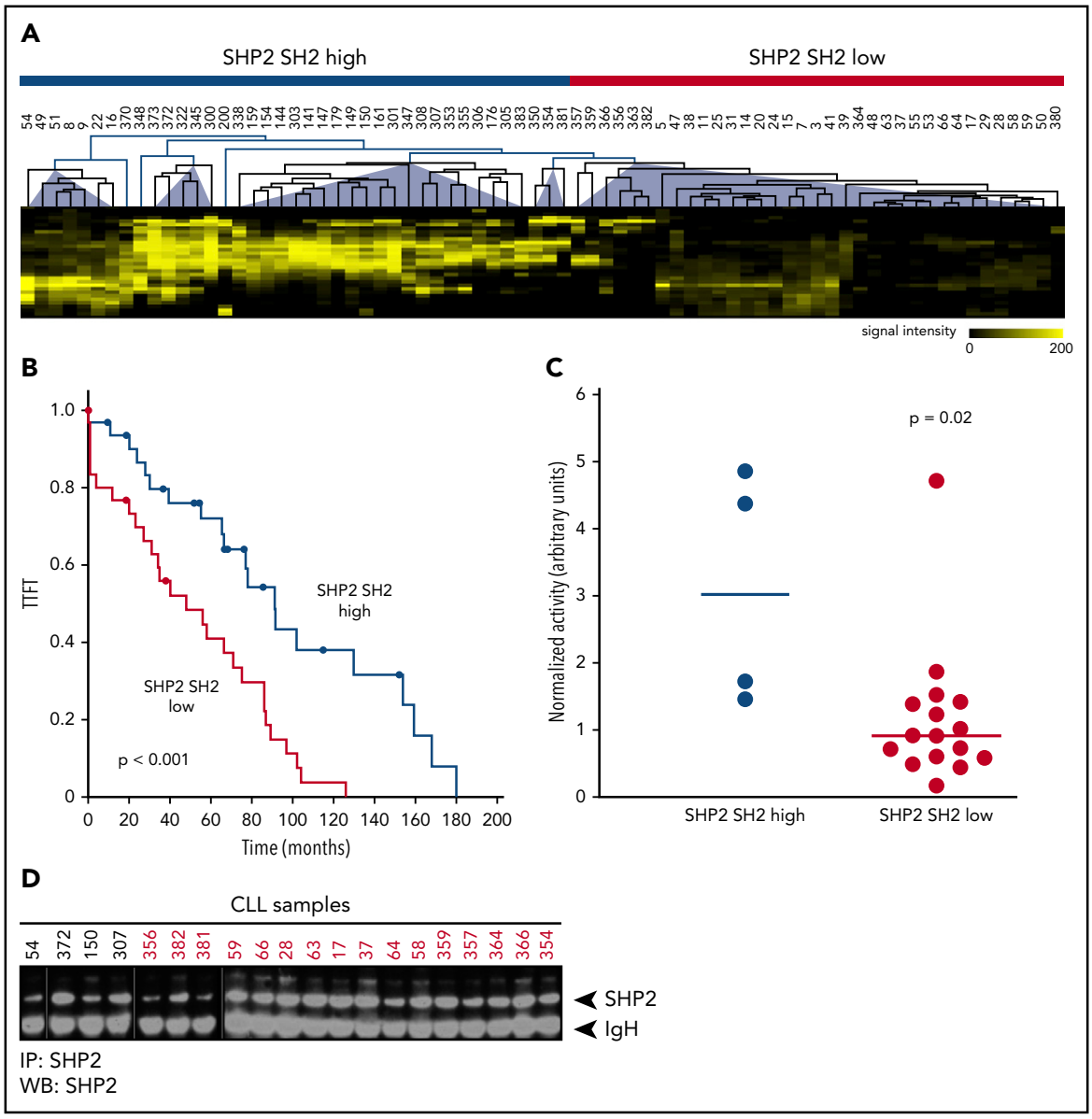


Figure 1. SH2 profiling of 74 primary CLL patients and clinical correlation. (A) Combined unsupervised cluster analysis of SH2 profiles of 74 primary CLL samples with the SHP2 SH2 domain. Far western blots (WBs) were scanned, images were digitalized, and lanes were horizontally subdivided into bins according to the molecular weight of the phosphoproteins. Hierarchical cluster analysis revealed 2 different clusters of low and high SHP2 SH2 binding. (B) TTFT Kaplan-Meier analysis according to SHP2 SH2 patient profiles (SHP2 SH2 high cluster: TTFT 96.2 ± 11.6 months; SHP2 SH2 low cluster: TTFT 50.9 ± 7.1 months; $P < .001$). (C) SHP2 activity determined after immunoprecipitation (IP) of SHP2 from 20 CLL samples of the high and low SHP2 SH2 clusters using 6,8 difluro-4-methylumbelliferyl phosphate as fluorogenic substrate. Fluorescence signals were normalized to SHP2 signals determined by immunoprecipitation and western blot analysis. Mean values of SHP2 activity are given as horizontal lines; level of significance was determined by the Wilcoxon rank-sum test. (D) SHP2 western blot analysis of the 20 CLL samples subsequent to SHP2 immunoprecipitation.

but very low, surface BCR expression between the different OSU-CLL sublines (supplemental Figure 6C-D).

The pattern of IgM downregulation with attenuated responses to BCR engagement along with enhanced basal MAPK signaling has been previously recognized as characteristic for anergic B cells.[2,3] Anergy is one of the most important physiological mechanisms to silence autoreactive B cells.[4] Whereas in normal B cells, this program leads to apoptosis in the long term,[5] in CLL cells, it seems to result in lethargic survival without proliferation as a result of widespread overexpression of the anti-apoptotic protein BCL-2 in this disease.[6,7] Conceptually, anergy may seem to be more desirable, and indeed a high proportion of anergic cells is correlated with an indolent clinical course in this disease.[8] Yet anergy describes a functional rather than an irreversible state of the cell, and therefore anergic cells are potentially harmful if they ultimately re-enter the proliferative cycle.[9,10] Such recirculating cells re-express high levels of surface IgM, show strong inducible anti-IgM responses, engage T-cell help, and ultimately proliferate in lymph node proliferation centers. The

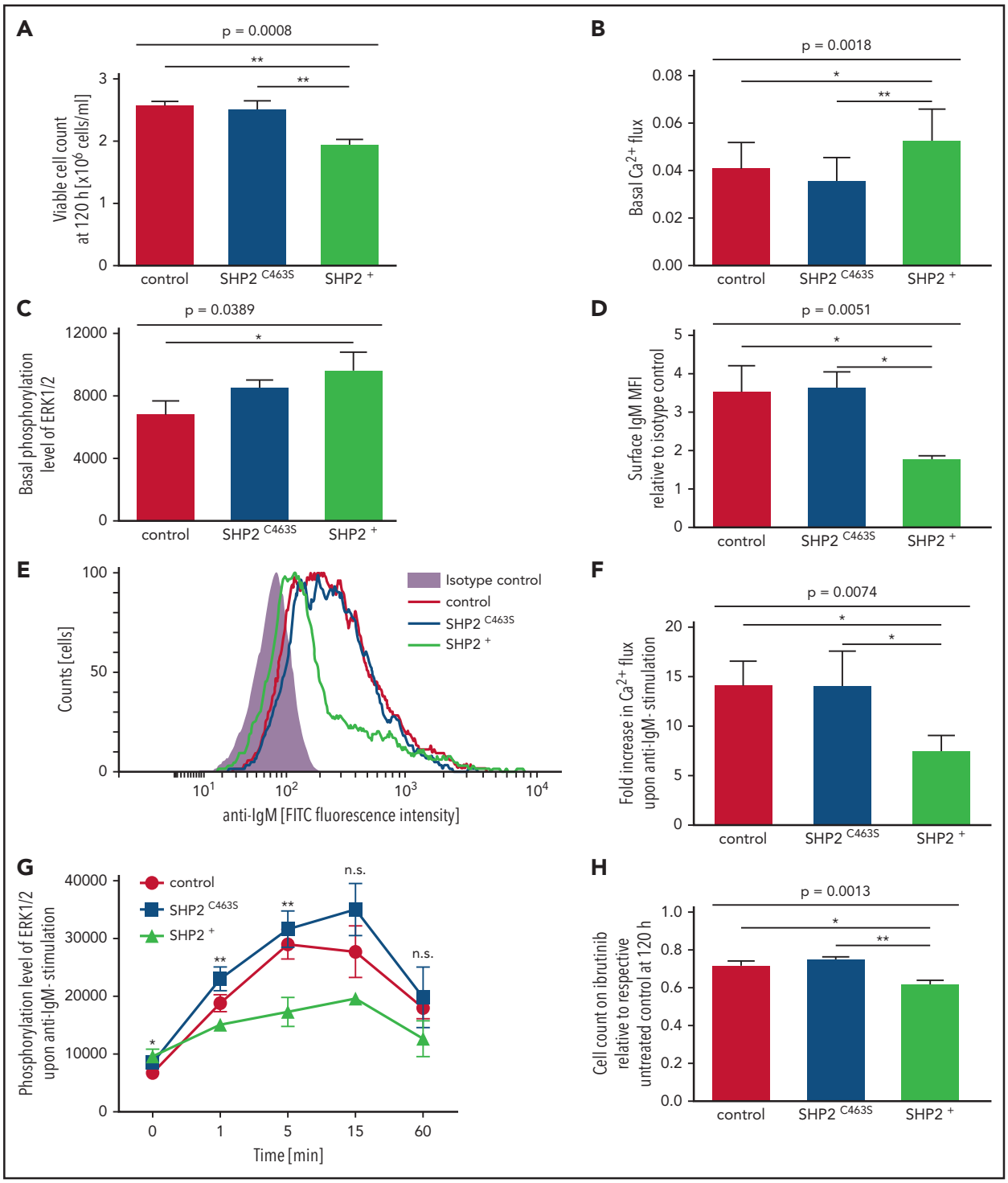


Figure 2. Anergic signature induced by SHP2 overexpression. Hallmarks of anergy were detectable in a transduced MEC-1 cell line overexpressing wild-type SHP2 or dysfunctional C463S-mutant SHP2 in comparison with MEC-1 cells transduced with empty vector control. Groups were compared by analysis of variance using Bonferroni post hoc statistics. (A) Effect of SHP2 overexpression on proliferation. Viable cell count was measured via cell viability analyzer 120 hours after seeding (n = 9 per group). (B) Basal Ca^{2+} levels were measured via flow cytometry after FLUO-4 staining (n = 8 per group). (C) Basal ERK1/2 phosphorylation levels were analyzed by densitometry of western blots (n = 6 per group). (D-E) Surface IgM expression in comparison with isotype control analyzed via flow cytometry (n = 6 per group). (F) Ca^{2+} flux after FLUO-4 staining in response to stimulation with soluble anti-IgM-F(ab')$_2$ determined by flow cytometry analysis (n = 5 per group). (G) Stimulation with soluble anti-IgM-F(ab')$_2$–induced ERK1/2 phosphorylation was analyzed by densitometry of western blots. Cell lines were serum starved for 2 hours and stimulated for 0, 1, 5, 15, or 60 minutes. Corresponding time points were always analyzed on the same blots (n = 2 to 6 per group). (H) Antiproliferative effect of SHP2 overexpression in ibrutinib-treated cells. Cell lines were seeded in medium with and without ibrutinib (0.5 μM). Viable cell count was measured via cell viability analyzer and normalized to the respective untreated control cell counts at 120 hours (n = 9 per group). (A-D,F,H) Error bars show the standard error of the mean; *$P < .05$; **$P < .001$. FITC, fluorescein isothiocyanate.

balance between positive signaling that leads to proliferation and negative signaling that leads to anergy is therefore of prognostic significance in CLL.[9,11]

Although the cellular hallmarks of anergy are well established, the molecular drivers of this signature have been insufficiently elucidated so far. Previous experiments showed that SHIP1 and SHP1 (2 downstream phosphatases of Lyn acting in parallel pathways) were found to be important for maintaining the anergic state in B cells.[12] One underlying mechanism could be the dephosphorylation of PIP3 by SHIP1 and PTEN that antagonizes PI3K signaling.[13] In line with this, a subset of anergic B cells seems to overexpress PTEN.[14] The SHP1 phosphatase plays a role in anergic feedback inhibition downstream of the BCR, but recent evidence suggests that it plays a much more complex role in CLL as part of the signalosome that orchestrates survival signals.[15] Although anergic CLL cells do show significantly enhanced ERK1/2 phosphorylation,[16] previous work failed to identify the link between BCR pathway attenuation and MAPK signaling. The data presented here indicate that SHP2 may serve as a molecular regulator of anergy in CLL and may be the missing link between attenuated responses to BCR stimulation and enhanced basal MAPK signaling as schematically shown in supplemental Figure 7. Importantly, our data clearly demonstrate that these effects are mediated by the phosphatase domain of SHP2, because a phosphatase-dead mutant of SHP2 did not induce an anergic phenotype. Taken together, our data broaden our understanding of pathological BCR signaling in CLL and may be exploited for therapeutic targeting.

Acknowledgments

The authors thank the FACS Core Facility of the University Medical Center Hamburg-Eppendorf for expert technical assistance.

This work was supported by grant BI 1711/4-1 from the Deutsche Forschungsgemeinschaft (M.B.) and by the Fördergemeinschaft Kinderkrebs-Zentrum Hamburg, Germany (S. Buhs, H.G., and P.N.). M.B. holds a professorship for immunological cancer research and therapy supported by the Hubertus Wald-Foundation, Hamburg, Germany.

Authorship

Contribution: M.B. designed the study, supervised the experiments, and wrote the manuscript; S.S. designed, performed, and interpreted the experiments and wrote the manuscript; S. Buhs and S. Bolz designed, performed, and interpreted experiments; H.G. performed experiments; L.v.W. analyzed data and wrote the manuscript; K.R. performed and interpreted experiments; B.F. critically revised the manuscript; and P.N. supervised the experiments, analyzed data, and critically revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Mascha Binder, Department of Oncology and Hematology, Bone Marrow Transplantation with Section Pneumology, Hubertus Wald Tumorzentrum–University Cancer Center Hamburg, University Medical Center Hamburg-Eppendorf, Martinistr 52, 22767 Hamburg, Germany; e-mail: m.binder@uke.de; and Peter Nollau, Research Institute Children's Cancer Center and Department of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Martinistr 52, Building N63, 2nd Floor, 20251 Hamburg, Germany; e-mail: nollau@uke.de.

Footnote

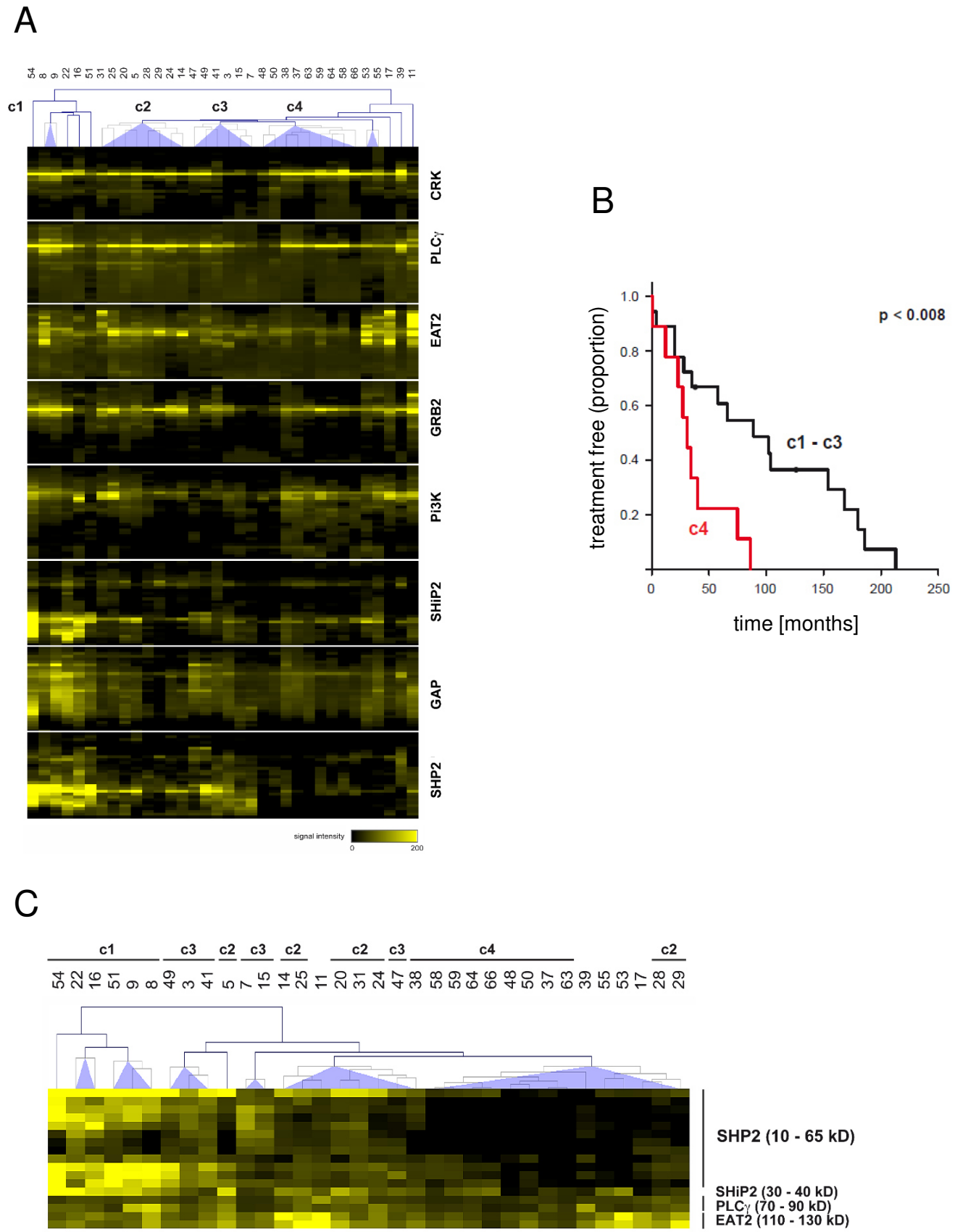
The online version of this article contains a data supplement.

REFERENCES

1. Nollau P, Mayer BJ. Profiling the global tyrosine phosphorylation state by Src homology 2 domain binding. *Proc Natl Acad Sci USA*. 2001;98(24):13531-13536.
2. Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol*. 2007;7(8):633-643.
3. Apollonio B, Scielzo C, Bertilaccio MT, et al. Targeting B-cell anergy in chronic lymphocytic leukemia. *Blood*. 2013;121(19):3879-3888.
4. Gauld SB, Merrell KT, Cambier JC. Silencing of autoreactive B cells by anergy: a fresh perspective. *Curr Opin Immunol*. 2006;18(3):292-297.
5. Fulcher DA, Basten A. Reduced life span of anergic self-reactive B cells in a double-transgenic model. *J Exp Med*. 1994;179(1):125-134.
6. Korz C, Pscherer A, Benner A, et al. Evidence for distinct pathomechanisms in B-cell chronic lymphocytic leukemia and mantle cell lymphoma by quantitative expression analysis of cell cycle and apoptosis-associated genes. *Blood*. 2002;99(12):4554-4561.
7. Scarfò L, Ghia P. Reprogramming cell death: BCL2 family inhibition in hematological malignancies. *Immunol Lett*. 2013;155(1-2):36-39.
8. D'Avola A, Drennan S, Tracy I, et al. Surface IgM expression and function are associated with clinical behavior, genetic abnormalities, and DNA methylation in CLL. *Blood*. 2016;128(6):816-826.
9. Mockridge CI, Potter KN, Wheatley I, Neville LA, Packham G, Stevenson FK. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood*. 2007;109(10):4424-4431.
10. Coelho V, Krysov S, Steele A, et al. Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function. *Blood*. 2013;122(15):2664-2672.
11. Packham G, Krysov S, Allen A, et al. The outcome of B-cell receptor signaling in chronic lymphocytic leukemia: proliferation or anergy. *Haematologica*. 2014;99(7):1138-1148.
12. 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(2):293-301.
13. Getahun A, Beavers NA, Larson SR, Shlomchik MJ, Cambier JC. Continuous inhibitory signaling by both SHP-1 and SHIP-1 pathways is required to maintain unresponsiveness of anergic B cells. *J. Exp. Med*. 2016;213(5):751-769.
14. Yarkoni Y, Getahun A, Cambier JC. Molecular underpinning of B-cell anergy. *Immunol Rev*. 2010;237(1):249-263.
15. Tibaldi E, Pagano MA, Frezzato F, et al. Targeted activation of the SHP-1/PP2A signaling axis elicits apoptosis of chronic lymphocytic leukemia cells. *Haematologica*. 2017;102(8):1401-1412.
16. Muzio M, Apollonio B, Scielzo C, et al. Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood*. 2008;112(1):188-195.

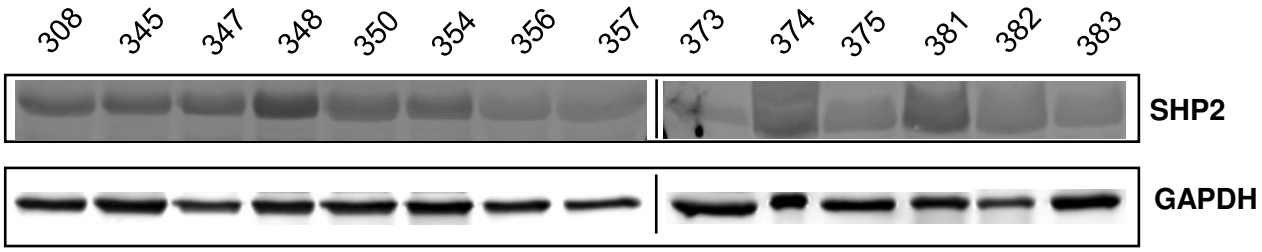
DOI 10.1182/blood-2017-06-788166

Supplementary Figure 1

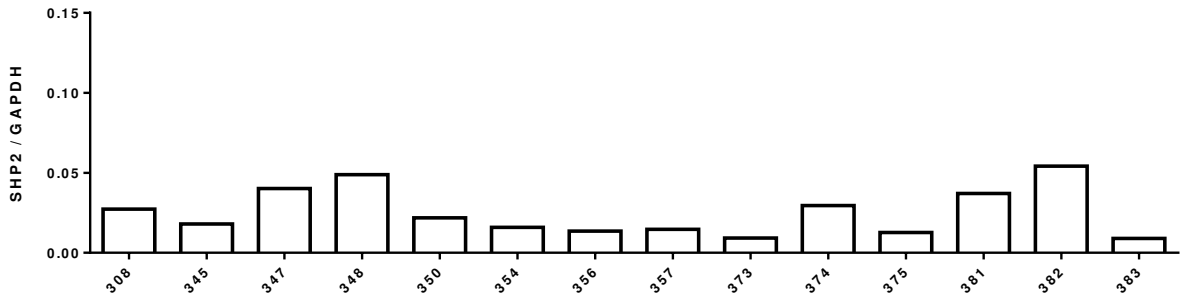


Supplementary Figure 2

A

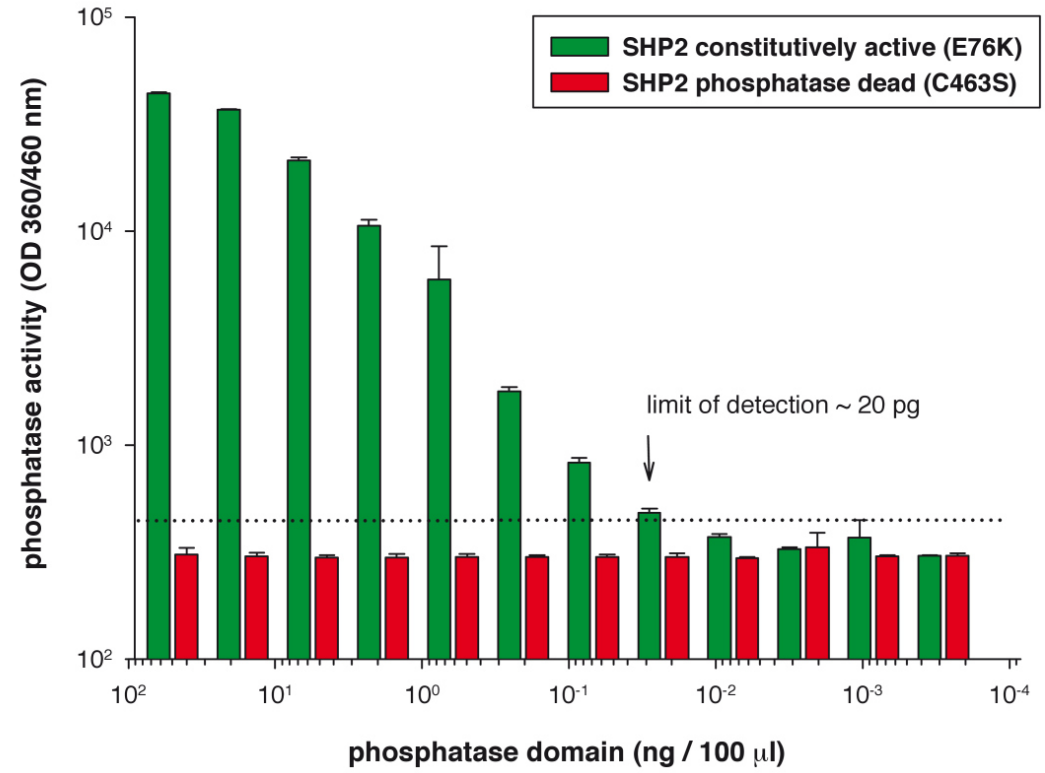


B

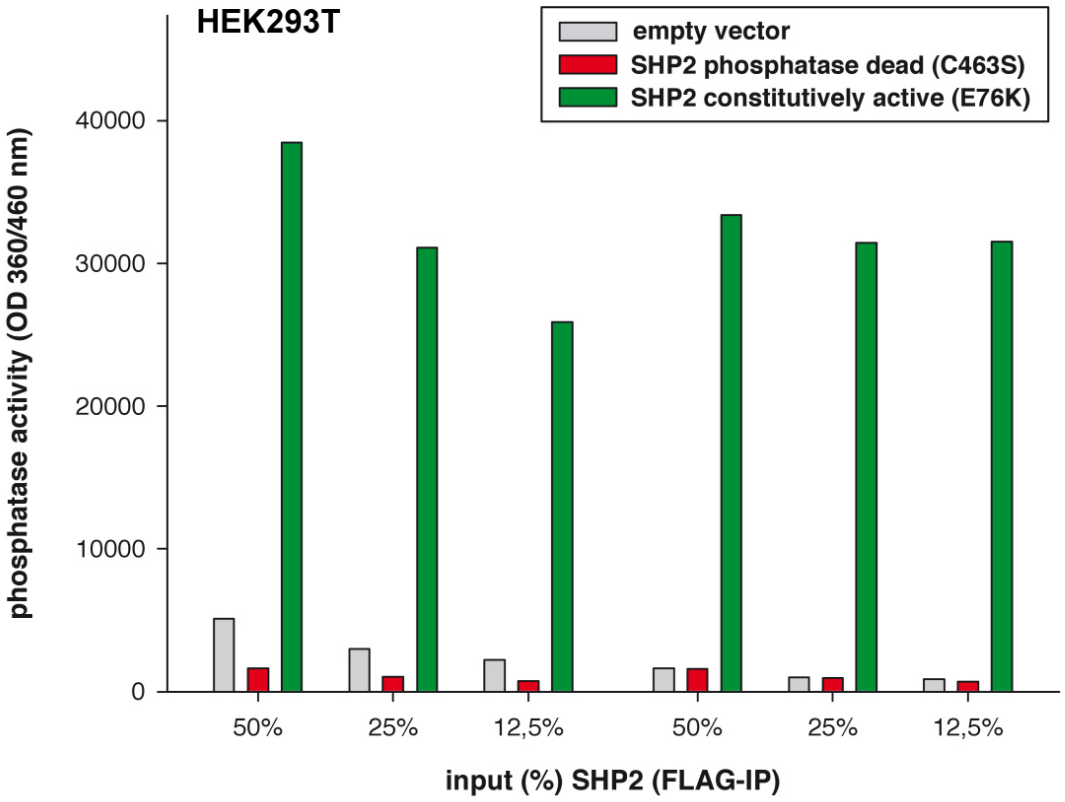


Supplementary Figure 3

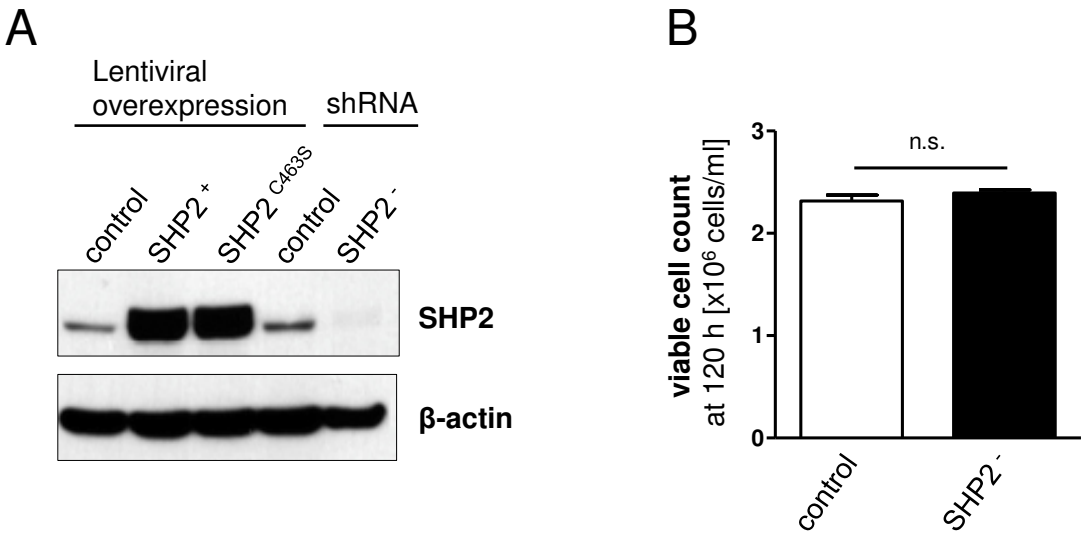
A



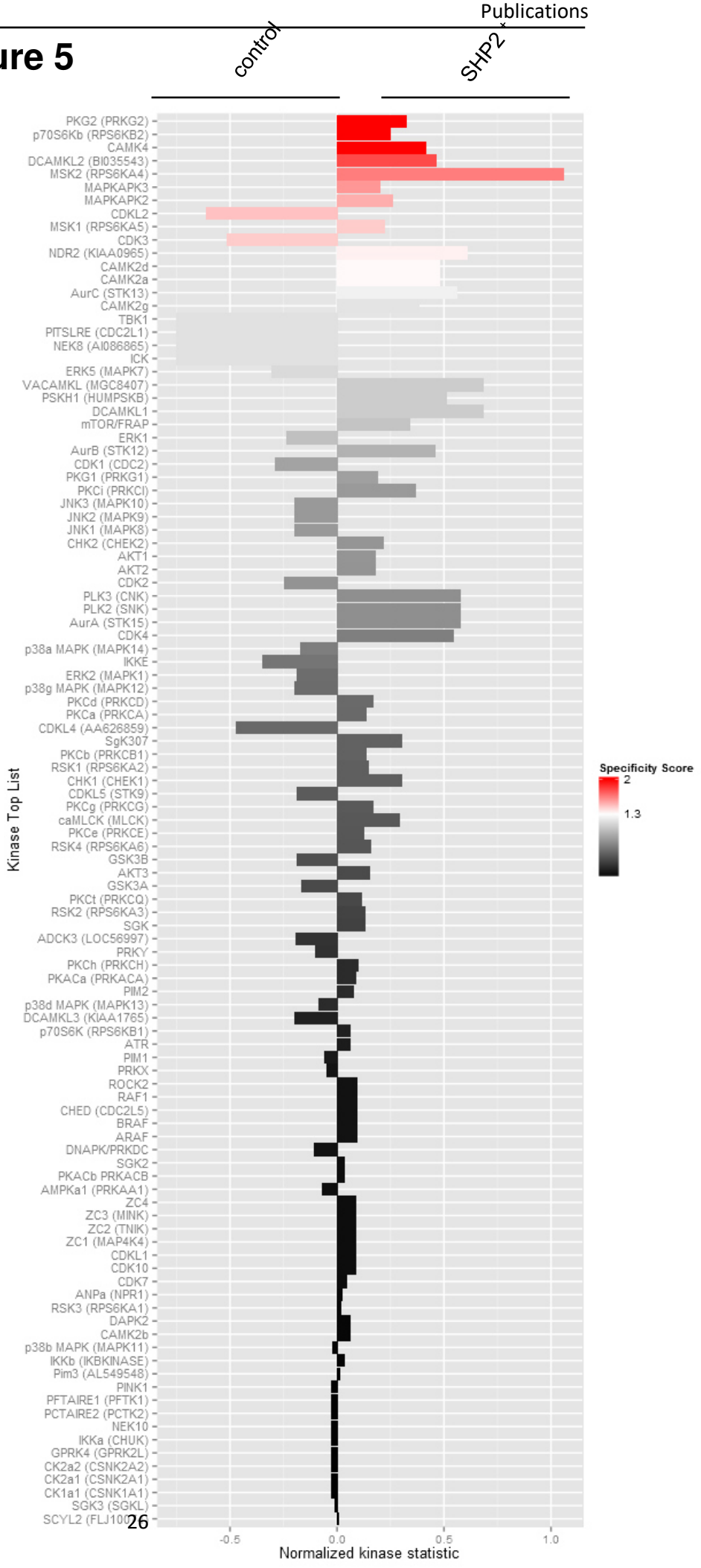
B



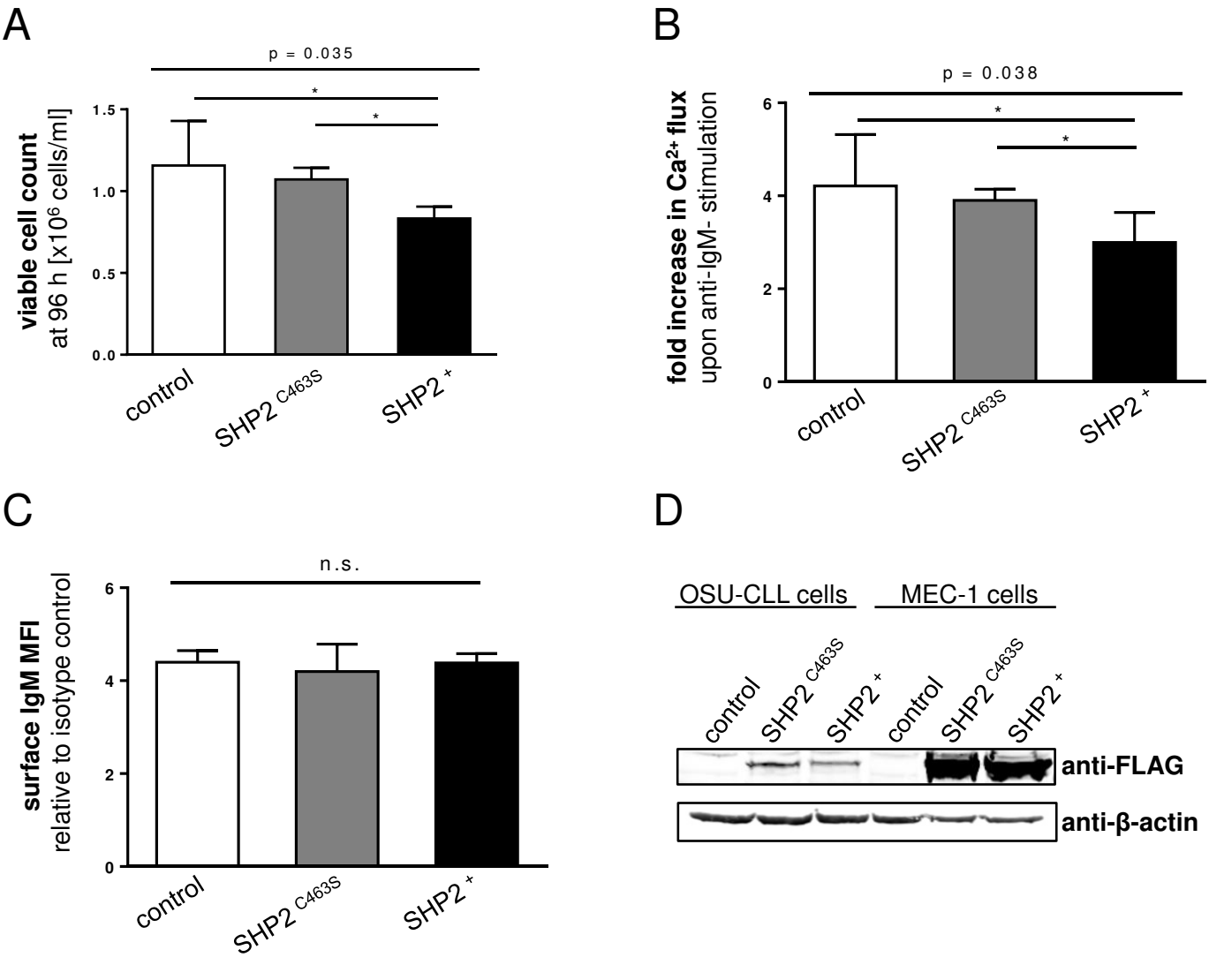
Supplementary Figure 4



Supplementary Figure 5

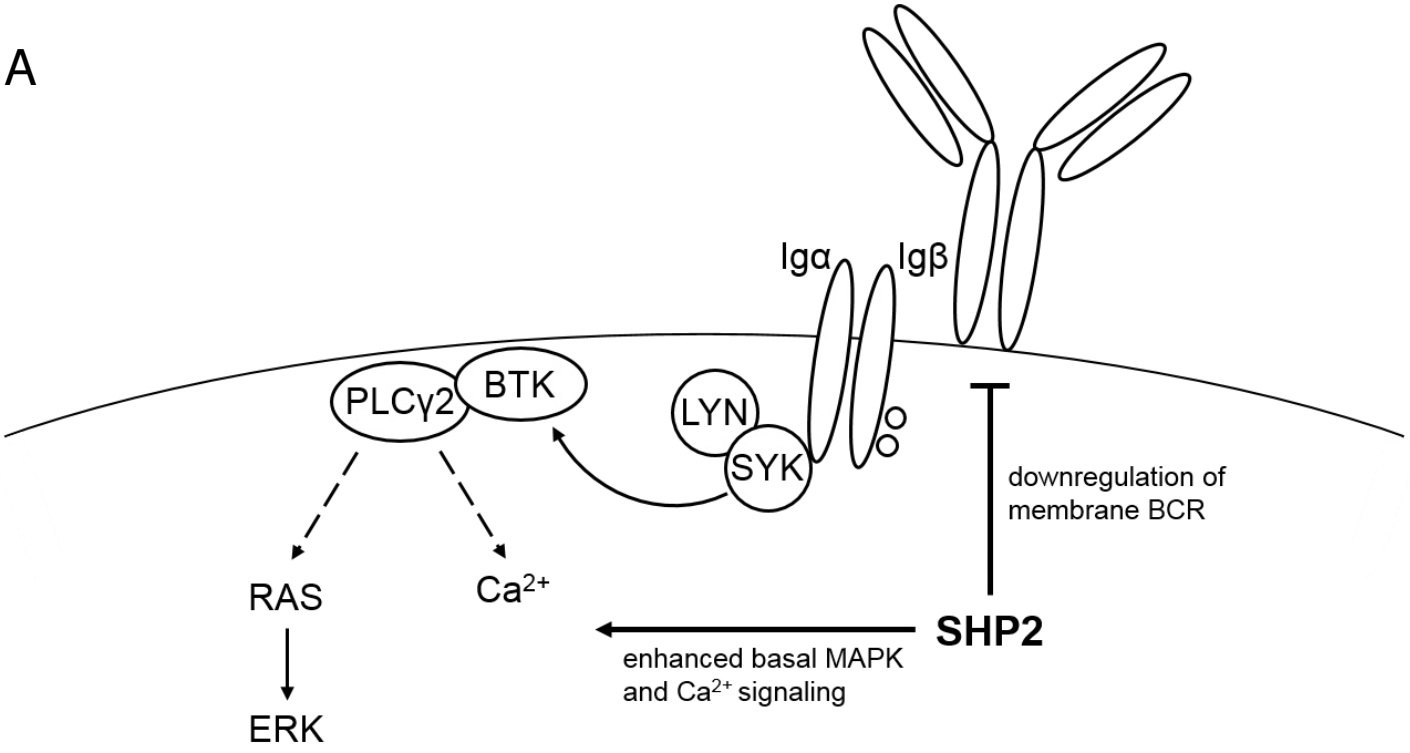


Supplementary Figure 6



Supplementary Figure 7

A



Supplementary Figure Legends

Supplementary Figure 1: SH2 profiling of 34 primary CLL cases and clinical correlation

(A) Unsupervised hierarchical cluster analysis of SH2 profiles of 34 primary CLL samples applying eight different SH2 domains. Far Western blots were scanned, images were digitalized and lanes were horizontally sub-divided in bins according to the molecular weight of the phosphoproteins. Hierarchical cluster analysis revealed four different clusters (c1 – c4); five (11, 17, 39, 53 and 55) of the 34 CLL samples, marked by strong EAT2 SH2 domain binding, were distantly related. (B) Time to first treatment (TTFT) Kaplan-Meier analysis according to SH2 profiles. (C) Determination of SH2 domains strongly predictive ($p < 0.01$) for cluster organization.

Supplementary Figure 2: SHP2 expression in primary CLL cells.

(A) Representative Western blot analyzing SHP2 expression levels in a cohort of 14 lysates from PBMCs of CLL patients (B) Expression level of SHP2 normalized to expression of GAPDH measured via densitometry of the Western blot signal.

Supplementary Figure 3: Measurement of SHP2 phosphatase activity using a 6,8 difluro-4-methylumbelliferyl phosphate (DiFMUP) based assay.

(A) Phosphatase activity determined in serial dilutions of constitutively activated SHP2 mutant E76K and the phosphatase-dead SHP2 mutant C463S. Proteins were expressed as bacterial GST fusion proteins and directly applied to the DiFMUP assay. (B) Phosphatase activity of constitutively activated SHP2 mutant E76K, phosphatase-dead SHP2 mutant C463S or vector control after transient expression in HEK293T-cells. FLAG-tagged SHP2 was immunoprecipitated, precipitates were split in different amounts (input) and SHP2 phosphatase activity was measured using DiFMUP; two independent experiments are shown.

Supplementary Figure 4: SHP2 overexpression and knockdown in the CLL cell line MEC-1

(A) SHP2 expression, overexpression and knockdown in the CLL cell line MEC-1. After lentiviral transduction, expression of SHP2 was analyzed via Western blot. (B) Effect of SHP2 knock-down on proliferation of MEC-1. Viable cell count was measured via cell viability analyzer 120 h after seeding. Groups were compared by unpaired T-Test (n = 12/group).

Supplementary Figure 5: Upstream analysis of differentially active kinases between SHP2 overexpressing and control MEC-1 cell line.

The analysis shows predicted differences in kinase activity between lysates of the SHP2 overexpressing and the control MEC-1 cell line. Based on the differentially phosphorylated peptides on the array, the PamApp for STK Upstream Kinase Analysis suggested the shown kinases with decreasing specificity from top to bottom to be differentially active.

Supplementary Figure 6: Effect of SHP2 overexpression in OSU-CLL.

Hallmarks of anergy were also detectable in a transduced OSU-CLL cell line overexpressing wild type SHP2 or dysfunctional C463S mutant SHP2 in comparison to cells transduced with empty vector control. Groups were compared by ANOVA using Bonferroni post-hoc statistics. (A) Effect of SHP2 overexpression on proliferation. Viable cell count was measured via cell viability analyzer 96 h after seeding (n = 6/group). (B) Ca^{2+} flux after FLUO-4 staining in response to stimulation with soluble anti-IgM-F(ab')$_2$ determined by flow cytometry analysis (n = 9/group). (C) Surface IgM expression in comparison to isotype control analyzed via flow cytometry (n = 6/group). (D) Verification of transgene expression in the established MEC-1 and OSU-CLL models after lentiviral transduction analyzed via western blot against FLAG-tag.

Supplementary Figure 7: Schematic overview of SHP2 effect on BCR signaling axis in CLL.

Material and Methods

Patient and sample characteristics

Blood samples of 74 randomly chosen untreated patients with clinical and laboratory features of CLL were collected after informed consent as approved by the ethics commissions of the Universities of Freiburg and Hamburg-Eppendorf (34 patients in primary cohort, 40 patients in validation cohort). Cells were purified by Ficoll separation. Only mononuclear cell preparations with >90% CLL content were used for this analysis.

SH2 Profiling

Far-Western blot analyses with biotinylated SH2 domain probes (CRK, EAT2, GRB2, Pi3K, PLCγ, GAPN, SHIP2 and SHP2) were applied to determine the tyrosine phosphorylation state of signaling proteins in cell lysates of 34 CLL patients of the primary cohort essentially as previously described.[1] 40 CLL patients were profiled using the SHP2 probe for the validation cohort.

Measurement of the phosphatase activity of SHP2 in primary CLL cells

Primary CLL cells ($5x10^6$) were lysed in 25 mM Tris HCl (pH 7.4), 150 mM NaCl, 5 mM Na_2EDTA (pH 8.0), 10% glycerol, 1% Triton X-100 in the presence of 1 mM DTT, 1 mM PMSF and aprotinin for 30 min on ice, lysate were cleared by centrifugation and SHP2 was immunoprecipitated from supernatants with a polyclonal rabbit SHP2 antibody (Bethyl) bound to protein G plus agarose (Santa Cruz) at 4°C overnight. Beads were washed twice with lysis buffer and phosphatase reaction buffer (50 mM Tris HCl (pH 7.4), 50 mM NaCl, 1 mM DTT, 0.05% Triton-X100), subsequently beads were resuspended in 50 µl phosphatase reaction buffer, 50 µl of DiFMUP diluted in reaction buffer (stock 10 mM in DMF, final concentration 200 µM; Thermofisher) were added and incubated for 30 min at room temperature in the dark. Subsequently, beads were separated by centrifugation, the supernatant was transferred to black colored, clear bottom microtiter plates and fluorescence was measured on a microplate reader (Tecan) at 360 nm (excitation) and 460 nm (emission) wavelenghts. For normalization and to control for immunoprecipitation, beads were boiled in gel loading buffer, subjected to Western blot analysis and probed with the polyclonal rabbit SHP2 antibody.

Overexpression and knock-down of SHP2 in MEC-1 cell line and overexpression of SHP2 in OSU-CLL cell line

Sequences encoding SHP2 and SHP2 C463S (defective phosphatase domain) were cloned into Lentiviral Gene Ontology (LeGO) vector LeGO-iC2-Puro. Small hairpin RNAs against SHP2 were cloned into vector LeGO-C/Zeo.[2] The MEC-1 cell line was cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% Penicillin/Streptomycin (Sigma-Aldrich). Lentivirally transduced MEC-1 cells were selected by 2µg/ml puromycin or 40µg/ml zeocin, respectively. The OSU-CLL cell line was cultured in Roswell Park Memorial Institute medium (RPMI 1640) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% Penicillin/Streptomycin (Sigma-Aldrich). Lentivirally transduced OSU-CLL cells were selected by 0.25µg/ml puromycin. Viable cell concentrations were measured by the Cell Viability Analyzer Vi Cell™ XR (Beckman Coulter).

Western blot analysis

Basal expression, overexpression and knock-down of SHP2 as well as expression of (p)ERK1/2 were controlled by western blot analysis using HRP-conjugated or fluorescently labelled secondary antibodies: (SHP-2 (D50F2), GAPDH, ERK1/2 and pERK1/2, Cell Signaling; β-Actin, Santa Cruz Biotechnologies; FLAG-tag, anti-rabbit IgG HRP-linked, Sigma Aldrich; IRDye® 680RD Goat anti-rabbit IgG, IRDye® 800CW Goat anti-Mouse IgG, LI-COR). When fluorescently labelled secondary antibodies were used, the signal was read out by the Odyssey CLx System (LI-COR).

PamGene serine/threonine kinase array analyses

The differential kinase activity between the MEC-1LeGO-iC2 SHP2 + and the MEC-1LeGO-iC2 control cell lines was estimated using a PamGene serine/threonine Chip according to the manufacturer's instructions. Briefly, 1 × 106 cells were lysed in M-PER Mammalian Extraction Buffer (Pierce) and subsequently treated as described previously.[3] Image analysis, signal quantification for kinomic analysis and data analysis were performed using the BioNavigator® software v. 6.2 (PamGene)

Proliferation and cytotoxicity assay of lentivirally transduced MEC-1 with ibrutinib

For time course inhibition analyses, lentivirally transduced MEC-1 cell lines seeded at 10^5 cells/ml were treated with 0,5 µmol/L of ibrutinib in triplicates in T25 flasks. After 120 hours, viable cell count was measured via cell viability analyzer.

Calcium Flux measurement

Transduced and untransduced cells were loaded with Fluo-4-AM as described by Schepers et al.[4] In short, cells were loaded with 5µM Fluo4-AM and resuspended in 1ml PBS with Ca^{2+}. Fluorescence intensity was measured using a FACSCalibur (BD Bioscience) and the BD CellQuest Pro Software. For BCR stimulation, 40 µg/ml goat-anti-human-Fab antibody (AbD-Serotec) was used. Analysis was done using FlowJo Software (Version 6.4.7, FlowJo).

Statistics

TTFT curves of the CLL cohort were plotted according to the Kaplan-Meier method using the SPSS software (version 15; SPSS, Inc., Chicago, Ill). Differences in distributions were studied with log-rank tests. Multivariate analyses were performed using ANOVA with adequate post-hoc tests.

Supplementary References

1. Dierck K, Machida K, Mayer BJ, Nollau P. Profiling the Tyrosine Phosphorylation State Using SH2 Domains. 2009;131–155.
2. Weber K, Mock U, Petrowitz B, Bartsch U, Fehse B. Lentiviral gene ontology (LeGO) vectors equipped with novel drug-selectable fluorescent proteins: new building blocks for cell marking and multi-gene analysis. *Gene Ther.* 2010;17(4):511–520.
3. Haetscher N, Feuermann Y, Wingert S, et al. STAT5-regulated microRNA-193b controls haematopoietic stem and progenitor cell expansion by modulating cytokine receptor signalling. *Nat. Commun.* 2015;6:8928.
4. Schepers E, Glorieux G, Dhondt A, Leybaert L, Vanholder R. Flow cytometric calcium flux assay: Evaluation of cytoplasmic calcium kinetics in whole blood leukocytes. *J. Immunol. Methods*. 2009;348(1–2):74–82.

Table S1: Clinical characteristics of the patient cohort.

#	Age	Binet Stage		#	Age	Binet Stage
3	62	B		**149**	50	B
5	71	B		**150**	63	A
7	64	C		**154**	68	A
8	61	B		**159**	72	A
9	56	B		**161**	53	n.e.
11	49	A		**176**	66	C
14	44	B		**179**	69	A
15	51	B		**200**	80	n.e.
16	47	C		**300**	58	A
17	70	A		**301**	70	C
20	45	A		**303**	74	C
22	58	A		**305**	64	A
24	72	C		**306**	58	B
25	74	A		**307**	52	A
28	64	A		**308**	54	A
29	62	B		**322**	49	B
31	53	A		**338**	61	C
37	78	n.e.		**345**	73	A
38	71	n.e.		**347**	61	A
39	39	B		**348**	66	B
41	69	A		**350**	73	A
47	64	A		**353**	58	A
48	68	A		**354**	70	A
49	41	A		**355**	87	n.e.
50	67	A		**356**	68	C
51	54	A		**357**	54	A
53	65	A		**359**	43	C
54	65	A		**363**	75	A
55	63	B		**364**	69	A
58	74	B		**366**	76	n.e.
59	71	B		**370**	58	A
63	49	B		**372**	62	C
64	58	B		**373**	66	A
66	73	n.e.		**380**	n.e.	n.e.
141	74	B		**381**	49	C
144	57	B		**382**	84	B
147	59	C		**383**	57	A

2.2. SLAMF receptors negatively regulate B cell receptor signaling in chronic lymphocytic leukemia via recruitment of prohibitin-2

Authors

Lisa von Wenserski, Christoph Schultheiß, Sarah Bolz, Simon Schliffke, Donjete Simnica, Edith Willscher, Helwe Gerull, Gerrit Wolters-Eisfeld, Kristoffer Riecken, Boris Fehse, Marcus Altfeld, Peter Nollau, Mascha Binder

Published in

Leukemia 2020 Aug 21. doi: 10.1038/s41375-020-01025-z. Online ahead of print.

Leukemia
https://doi.org/10.1038/s41375-020-01025-z

ARTICLE

Chronic lymphocytic leukemia

SLAMF receptors negatively regulate B cell receptor signaling in chronic lymphocytic leukemia via recruitment of prohibitin-2

Lisa von Wenserski[1] · Christoph Schultheiß[1] · Sarah Bolz[2] · Simon Schliffke[3] · Donjete Simnica[1] · Edith Willscher[1] · Helwe Gerull[4] · Gerrit Wolters-Eisfeld[4] · Kristoffer Riecken[5] · Boris Fehse[5] · Marcus Altfeld[6] · Peter Nollau[4] · Mascha Binder[1]

Received: 11 March 2020 / Revised: 30 July 2020 / Accepted: 7 August 2020

Abstract

We identified a subset of Chronic Lymphocytic Leukemia (CLL) patients with high Signaling Lymphocytic Activation Molecule Family (SLAMF) receptor-related signaling that showed an indolent clinical course. Since SLAMF receptors play a role in NK cell biology, we reasoned that these receptors may impact NK cell-mediated CLL immunity. Indeed, our experiments showed significantly decreased degranulation capacity of primary NK cells from CLL patients expressing low levels of SLAMF1 and SLAMF7. Since the SLAMF^{low} signature was strongly associated with an unmutated CLL immunoglobulin heavy chain (IGHV) status in large datasets, we investigated the impact of SLAMF1 and SLAMF7 on the B cell receptor (BCR) signaling axis. Overexpression of SLAMF1 or SLAMF7 in IGHV mutated CLL cell models resulted in reduced proliferation and impaired responses to BCR ligation, whereas the knockout of both receptors showed opposing effects and increased sensitivity toward inhibition of components of the BCR pathway. Detailed molecular analyzes showed that SLAMF1 and SLAMF7 receptors mediate their BCR pathway antagonistic effects via recruitment of prohibitin-2 (PHB2) thereby impairing its role in signal transduction downstream the IGHV-mutant IgM-BCR. Together, our data indicate that SLAMF receptors are important modulators of the BCR signaling axis and may improve immune control in CLL by interference with NK cells.

Introduction

B cell receptor (BCR) signaling plays a critical role in driving proliferation and survival of the malignant clone in chronic lymphocytic leukemia (CLL), supported by the clinical activity of inhibitors targeted toward BCR-associated kinases [1]. Encouraged by the results of clinical trials in relapsed/refractory CLL [2, 3], ibrutinib—an inhibitor of Bruton's tyrosine kinase (BTK) downstream the BCR—has been recently introduced as front-line treatment of CLL [4–6]. Interestingly, superior activity of BTK inhibition appears to be achievable in CLL with unmutated immunoglobulin heavy chain (IGHV) genes (U-CLL), which is currently deemed to be due to more growth-

These authors contributed equally: Mascha Binder, Peter Nollau

Supplementary information The online version of this article (https://doi.org/10.1038/s41375-020-01025-z) contains supplementary material, which is available to authorized users.

✉ Mascha Binder
Mascha.Binder@uk-halle.de

[1] Department of Internal Medicine IV Oncology/Hematology, Martin-Luther-University Halle-Wittenberg, Halle, Saale, Germany

[2] TU Dresden, Biotechnologisches Zentrum, Dresden, Germany

[3] Department of Oncology and Hematology, Bone Marrow Transplantation with Section Pneumology, Hubertus Wald Tumorzentrum-University Cancer Center Hamburg, Hamburg, Germany

[4] Research Institute Children's Cancer Center and Department of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

[5] Research Department Cell and Gene Therapy, Department of Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

[6] Research Department Virus Immunology, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany

Published online: 21 August 2020

promoting and less anergic BCR signaling in this subset compared to CLL with mutated IGHV genes (M-CLL) [7, 8]. Yet, it remains essentially unclear what exactly drives this differential sensitivity to BTK inhibition of U- and M-CLL on the molecular level. Also, clinical data on ibrutinib sensitivity of M-CLL suggest that this latter group may be heterogeneous in itself, but no molecular or genetic correlate for this observation has been identified so far [9]. Further insights into the modulation of BCR signaling in U- and M-CLL may therefore elucidate essential pathophysiological clues for more individualized targeting to achieve durable disease control in the majority of patients.

Signaling Lymphocytic Activation Molecule Family (SLAMF) receptors are a group of nine type I transmembrane receptors that are mainly expressed on a variety of immune cells. They are known to be involved in the regulation of NK and T cell responses, mostly by homotypic interactions except for SLAMF2 (CD48) and SLAMF4 that interact with each other [10]. In B cells, this class of receptors has been shown to be expressed in distinct patterns associated with development and activation [11]. A previous study demonstrated that SLAMF1, SLAMF2, and SLAMF7 receptors are rather downregulated on CLL cells as compared to their normal B cell counterpart [12], suggesting that high expression of these molecules may have detrimental (e.g., antiproliferative) effects in the CLL context.

In the work presented here, we provide compelling data that SLAMF1 and SLAMF7 receptors may not only enhance immune control of CLL but also negatively regulate BCR signaling and thereby impact sensitivity towards BTK inhibition in the substantial fraction of patients with SLAMF1 or SLAMF7 expressing M-CLL. This data opens up new perspectives on key pathophysiological mechanisms in this disease that may be exploited for biomarker development to guide treatment choices in CLL.

Methods

Patient and sample characteristics

Blood samples of 54 randomly chosen untreated patients with clinical and laboratory features of CLL were collected after informed consent as approved by the ethics commission of the Universities of Freiburg, Hamburg–Eppendorf and Halle (Saale). For the sample size calculation, a time to first treatment (TTFT) difference of 2400 days versus 1600 days in SLAMF1/7 receptor high versus low patients was estimated resulting in a minimum number of 50 patient samples to be included in this analysis to achieve a power of 80%. Cells were purified by Ficoll separation. Age, stage, immunoglobulin mutational status, and cytogenetics (FISH) were recorded (Table 1). In addition, 16 independent CLL samples were freshly used for NK cell experiments.

Peripheral mononuclear cells of patients were analyzed by flow cytometry for membrane SLAMF receptor expression with the following antibodies and respective isotype controls: SLAMF1-PE, SLAMF7-AF647, CD5-PC5.5, CD19-PC7, CD45-ECD. Analysis was performed on a Navios Flow Cytometer using the Kaluza software (Beckman Coulter, Brea, California).

Flow cytometry

All relevant antibodies used in flow cytometry are listed in Supplementary Table S1.

Generation of genetically engineered CLL sublines

The CLL cell lines MEC-1 and Hg3 were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany), JVM3 cells were a kind gift of Marco Herling. MEC-1 cells were maintained in IMDM medium, Hg3 and JVM3 in RPMI 1640 medium, both supplemented with 10% fetal bovine serum (Thermo Fisher, Waltham, MA) and 1% Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO).

Sequences encoding EAT2, SLAMF1 and SLAMF7 were cloned into Lentiviral Gene Ontology (LeGO) vector LeGO-iC2-Puro+ [13], which was used for CLL cell line transduction. Lentivirally-transduced cells were selected with 1 µg/ml puromycin containing medium. Successful overexpression of SLAMF1 and SLAMF7 was verified by flow cytometry and for EAT2 by western blotting.

gRNAs directed against SLAMF1 (CAGGGAGAG AAACAGCACGA) and SLAMF7 (ATGCAGCACGTAC TCCTGGG) were cloned into the lentiCRISPRv2 vector using the BsmBI restriction site as previously described [14]. Non-integrating lentiviruses were produced using the integrase defective packaging plasmid pCMVd8.74-D64.V [15]. After transduction, cells showing a complete knockout were sorted using a FACSAria Illu (BD Biosciences).

IgG switched MEC-1 cells were generated using CRISPR/Cas9 technology as described by Cheong et al. [16].

Proliferation and cytotoxicity assay of SLAMF receptor overexpressing or knockout CLL cell lines with Ibrutinib

For proliferation and inhibition analyzes, CLL sublines seeded at 0.1×10^6 cells/ml were treated with 1 µM of Ibrutinib (Selleckchem Chemicals, Houston, Texas) or left untreated. After 96 or 120 h, viable cell numbers were

Table 1 Clinical characteristics of the validation cohort.

#	Age	Stage	IgHV	MS	Cytogenetics
141	76	B	IGHV3-48	UM	trisomy 12, del13q
144	61	B	IGHV3-30	UM	del11q23, del13q
147	62	C	IGHV1-69	UM	del11q23, del13q
148	69	B	IGHV1-69	M	del13q
149	57	B	IGHV3-7	M	–
150	67	A	IGHV4-39	M	del13q
154	69	A	IGHV3-72	M	del11q23, del13q
159	80	A	IGHV1-69	M	del13q
160	62	B	IGHV3-30	UM	–
161	67	n.e.	IGHV1-2	UM	del13q, del17p
172	59	A	n.e.	n.e.	del13q
173	54	A	IGHV3-23	M	del13q
176	72	C	IGHV1-69	UM	del11q23, del13q
179	82	A	IGHV3-7	M	–
300	59	A	IGHV3-64	M	del13q, del17p
301	70	C	IGHV3-23	M	trisomy 12
303	75	C	IGHV3-33	UM	–
305	68	A	IGHV4-59	M	del13q
306	65	B	IGHV1-46	UM	del11q23, del13q
307	57	A	IGHV4-34	M	del13q
308	58	A	IGHV1-69	UM	–
322	58	B	IGHV3-11	UM	del13q
338	72	C	n.e.	n.e.	del13q
345	74	A	IGHV2-5	UM	del13q
347	65	A	IGHV7-4	UM	–
348	71	B	IGHV3-9	UM	–
350	76	A	IGHV1-69	UM	del13q, del17p
353	71	A	IGHV3-71	M	del13q
354	78	A	IGHV3-7	M	del13q
355	84	n.e.	IGHV3-30	UM	del13q, del17p
356	68	C	IGHV3-21	UM	del17p
357	54	A	IGHV4-55	M	n.e.
359	43	C	IGHV3-11	UM	del17p
360	66	A	IGHV1-69	M	del13q
362	67	n.e.	IGHV4-61	UM	–
363	75	A	IGHV3-23	M	n.e.
364	68	A	IGHV3-30	UM	n.e.
365	66	A	IGHV3-15	M	n.e.
366	81	n.e.	IGHV3-30	UM	–
368	64	C	IGHV4-55	UM	–
369	84	C	IGHV1-2	UM	–
370	62	A	IGHV3-11	UM	del11q23, del13q
372	78	C	n.e.	n.e.	del13q
373	69	A	IGHV3-30	UM	n.e.
374	63	C	IGHV3-48	M	–
375	77	A?	IGHV2-5	M	del13q
377	72	C	IGHV3-7	UM	–
378	49	C	IGHV1-69	UM	–
379	84	B	IGHV3-53	M	del13q, del17p
380	75	A?	IGHV1-69	M	–
381	49	B	IGHV1-69	UM	–
388	73	n.e.	IGHV4-34	M	n.e.
390	67	C	IGHV1-69	UM	del11q
391	56	A	IGHV4-30	UM	n.e.

n.e. not evaluated.

measured by trypan blue staining using the Cell Viability Analyzer Vi-Cell XR (Beckman Coulter).

Ca^{2+} flux measurement

Ca^{2+} flux in transduced cells was measured as described by Schepers et al. [17]. Briefly, cells were loaded with Fluo4-AM (Thermo Fisher) and resuspended in 1 ml PBS containing Ca^{2+}. For BCR crosslinking, 5 µg/ml goat-anti-human IgM or IgG Fc antibody (Thermo Fisher, H15000 or H10300) was used, respectively. Fluorescence intensity measurement was performed using a FACSCalibur and the BD CellQuest Pro Software (BD Biosciences).

RNAseq and pathway analysis

Normalized RNAseq data from 304 CLL patients (EGAS00001000374) was downloaded from the ICGC data portal and analyzed for SLAMF receptor expression levels.

Biotinylation screen

For the Biotinylation screen, the sequence of a promiscuous Biotin ligase [18] (BioID2) was cloned in frame to the C-terminus of SLAMF1 and SLAMF7 in the respective LeGO-vectors and cells were transduced with the resulting lentiviral particles at low multiplicity of infection. After puromycin selection, biotin was added to the media at a final concentration of 50 µM. After 24 h, cells were harvested, lysed, and subjected to either streptavidin pull down followed by gel electrophoresis and mass spectrometry or immunoblotting of whole cell lysates was performed for visualization of the differential biotinylation patterns.

Western blot analysis

All relevant primary antibodies used for immunoblotting are listed in Supplementary Table S2, secondary antibodies were: anti-mouse-HRP (HAF007, R&D Systems, Minneapolis, Minnesota) and anti-rabbit HRP (A0545, Sigma Aldrich). Biotinylation was visualized using HRP-Streptavidin (405210, Bio Legend). The signal was red out with the ImageQuant LAS 4000 (GE Healthcare, Chicago, IL).

Co-immunoprecipitation

Cells lysed in Buffer A (25 mM HEPES-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.3% SDS) were incubated with anti-PHB2 (12295-1-AP, Proteintech, Rosemont, Illinois), anti-SLAMF1 (MAB1642, R&D Systems) or anti-SLAMF7 (ab237730, Abcam, Cambridge, UK) loaded Protein G dynabeads (Thermo Fisher) o/n at

4 °C on a rotating wheel. Beads were magnetically separated, washed, and boiled in loading buffer followed by gelectrophoretic separation and western blotting using antibodies mentioned in Supplementary Table S2).

siRNA knock down

For siRNA transfection, we used the Amaxa Nucleofection system (Lonza, Basel, Switzerland). 2×10^6 cells were resuspended in solution V and transfected using program X-01. siRNAs were purchased from Qiagen, AllStars Negative Control siRNA was used as a transfection control and the final concentration was 0.5 µM.

NK cell assays

NK cell activity was evaluated in co-culture assays with the parental MEC-1 cell line using CD107a as a surrogate marker as described by Alter et al. [19]. NK cells from CLL patients were isolated from peripheral blood via negative selection using RosetteSep Human NK Cell Enrichment Cocktail (Stemcell Technologies, Vancouver, Canada). We assessed the purity of all NK cell fractions used for experiments via FC staining with CD56-PE. The obtained NK cells rested overnight in RPMI + 10% FCS containing 1 ng/ml IL15 (Peprotech, Rocky Hill, New Jersey). On the next day, the cells were mixed with the target cells at a 1:1 ratio in a 96-well plate in the presence of 1.2 µl CD107a-PE/Cy7, IL-15, and 5 µg/ml brefeldin A (Bio Legend, San Diego, California). The ratio was adjusted according to the abundance of residual CLL cells in the preparation. After 5 h, cells were washed, stained with CD3-FITC, and CD56-PE and measured on FACSCalibur using CellQuest software or LSR Fortessa with the FACS Diva Software (all BD Bioscience, Franklin Lakes, New Jersey).

Statistics

Survival curves were plotted according to the Kaplan–Meier method using PRISM8 (GraphPad, San Diego, CA). Multivariate analyzes were performed using ANOVA with adequate post-hoc tests or Cox regression using R software and the survival package [20].

Results

SH2-profiling of CLL samples reveals a distinct SLAMF receptor driven signaling cluster correlating with a favorable clinical course

We previously reported on an unsupervised hierarchical clustering analysis of signaling proteins using Scr-homolgy 2 (SH2) domains as probes [21] in a cohort of 34 patients with CLL. In addition to a signaling cluster essentially driven by the phosphotyrosine Src homology region 2 domain-containing phosphatase-2 (SHP2) [22], we identified a distantly related group of cases displaying high Ewing's sarcoma-associated transcript (EAT2) -SH2 domain binding. Cases within this latter cluster were characterized by an indolent clinical course with long TTFT as surrogate marker for the aggressiveness of the disease. Yet, the biological significance of the favorable EAT2 SH2 "high" signature remained unclear. EAT2 belongs to the family of SLAM-associated proteins (SAP), which are essential for the signal transduction of upstream SLAMF receptors [23] that modulate innate and adaptive immune responses in various immune cell types [24]. We therefore reasoned that patients in the EAT2 SH2 high cluster may be a subset with high SLAMF receptor levels and SLAMF-related signaling. To confirm our hypothesis, we performed flow cytometry for SLAMF receptors in a second independent CLL validation cohort consisting of 54 patients. These untreated patients encompassed patients with different risk profiles with 42% M- and 58% U-CLL cases (Table 1). From the nine characterized SLAMF receptors, we chose SLAMF1 and SLAMF7 that are expressed in CLL, but on average downregulated in comparison to normal B cells and that carry a cytoplasmic phosphorylation site able to bind to downstream signaling adapters [12]. Indeed, when correlating the expression with clinical data, we found significantly longer TTFT for patients with high expression of one of the SLAMF receptors (defined as less than upper boundary of standard deviation; $p = 0.0223$; Fig. 1a, b, Supplementary Fig. S1). While patients with high SLAMF1 or SLAMF7 receptor levels had a median TTFT of 2775 days ($N = 12$), patients with lower expressions ($N = 39$) had a median TTFT of only 1195 days. Interestingly, in the case of SLAMF7 where we observed a broad spectrum of membrane expression levels, we also found a linear correlation of SLAMF7 membrane positivity with TTFT (Fig. 1c, $p = 0.0158$, $r^2 = 0.1406$).

A functional link between the BCR and SLAMF receptor expression seemed plausible in that almost all patients from our cohort with high SLAMF receptor expression were M-CLL cases ($p < 0.0001$; Fig. 1d). Also, without using an arbitrary cut-off discriminating between SLAMF high and low CLL cases, we observed quantitative differences in patients with U- and M-CLL with U-CLL cases showing less SLAMF7 density on their CLL cells (Fig. 1e, $p = 0.0319$).

To further validate our findings, we used publicly available RNAseq data of CLL patients provided by the ICGC (EGAS00001000374). We used the same criteria ($SLAMF^{high}$ defined as normalized read counts greater than upper boundary of standard deviation) to divide the 304

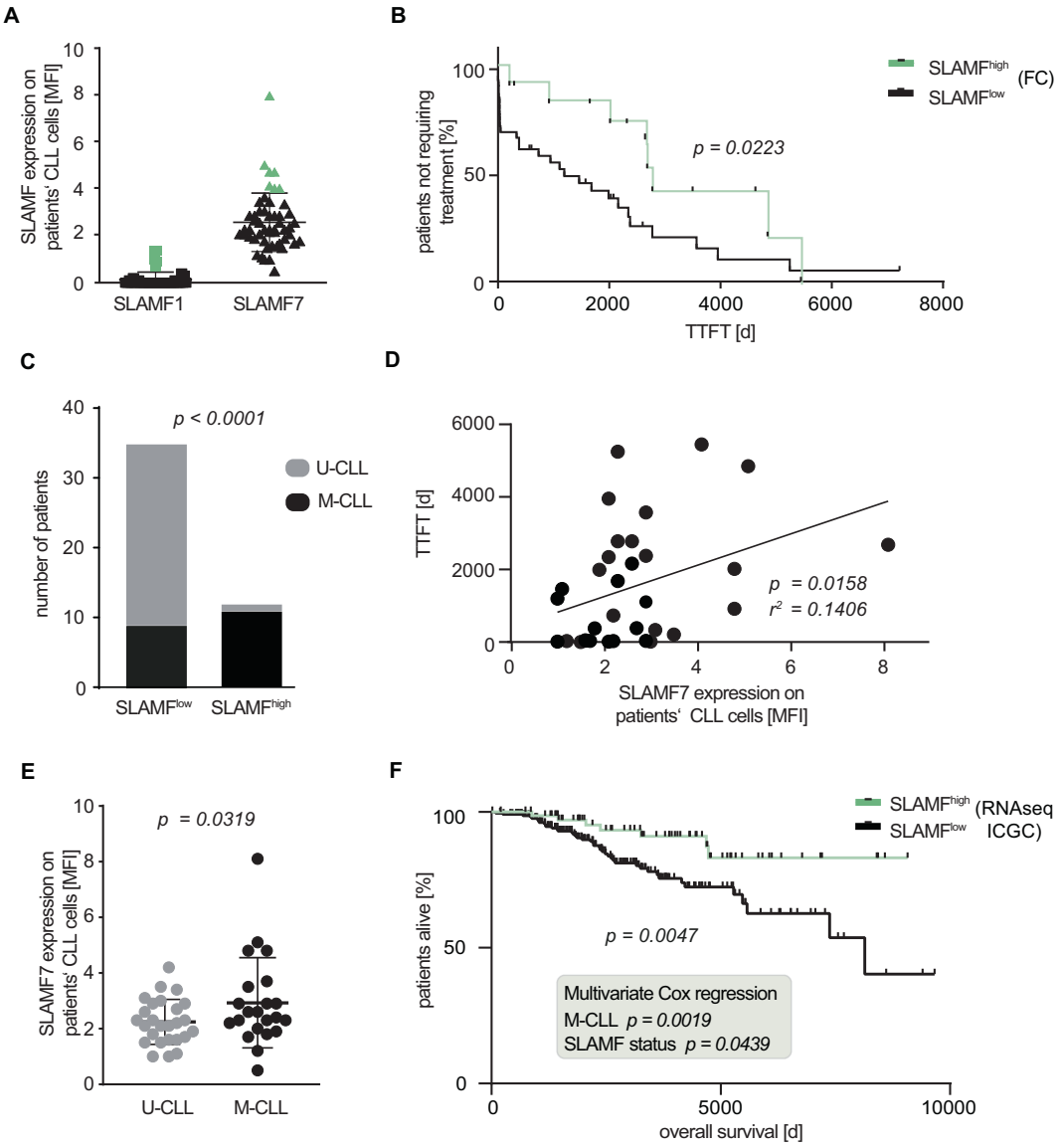


Fig. 1 SLAMF1 and SLAMF7 expression in CLL. a SLAMF1 and SLAMF7 levels on CLL patients' CLL cells measured via FC. Values above the upper boundary of SD were considered SLAMF^{high} (green symbols). $N = 52$. **b** TTFT-Kaplan–Meier analysis according to SLAMF status measured in **a**. TTFT-SLAMF$^{high} = 2775$ days, TTFT-SLAMF$^{low} = 1195$ days, $p = 0.0223$ calculated by log-rank test. (SLAMF$^{high} = 12$; SLAMF$^{low} = 39$) **c** Correlation between TTFT and SLAMF7 expression on patients' CLL cells. Pearsons correlation coefficient and statistical significance was calculated, $p = 0.0158$, $r^2 = 0.1406$. $N = 33$. **d** Association between IGHV mutational status and SLAMF expression. $p < 0.0001$, statistical significance was calculated using Fisher's exact test. **e** SLAMF7 expression on CLL cells of patients grouped by their IGHV mutational status. $p = 0.0319$, calculated with one-sided unpaired student's t test, error bars represent SD. **f** OS-Kaplan–Meyer analysis of ICGC dataset according to SLAMF1 and 7 expression on RNA level. $p = 0.0047$, calculated by log-rank-test. Multivariate Cox regression was performed to assess independency of variables IGHV and SLAMF status. $N = 304$ (SLAMF$^{high} = 75$; SLAMF$^{low} = 229$). FC flow cytometry, SD standard deviation, TTFT time to first treatment, IGHV immunoglobulin heavy chain, OS overall survival.

patients according to their SLAMF1 or SLAMF7 expression and investigated the subgroups' survival data. Indeed, the SLAMF^{high} group ($N = 75$) showed significantly longer overall survival than the SLAMF^{low} group ($N = 229$, $p = 0.0047$, Fig. 1f). Interestingly, a very small group of patients with both high SLAMF1 and high SLAMF7 receptor expression (10 of 304 patients) was identified and this small subset showed even superior overall survival (Supplementary Fig. S2A). Moreover, CLL cases with high SLAMF1/7 receptor expression showed a trend towards longer overall survival also within the M-CLL subset (SLAMF$^{high} = 36$, SLAMF$^{low} = 62$ patients; Supplementary Fig. S2B). Yet, due to the paucity of IGHV mutational status data in the ICGC dataset, only a Cox regression analysis over the full cohort could clearly show that the SLAMF receptor-related survival difference was not

confounded by the mutational status (Fig. 1f). This confirmed that also amongst the M-CLL cases, high SLAMF levels were an independent favorable prognostic marker.

Effect of overexpression or knockout of SLAMF1 and SLAMF7 on CLL proliferation

We reasoned that SLAMF receptors may directly influence proliferation in CLL, which could explain their prognostic role.

To test this, we used the M-CLL cell line MEC-1 that expresses SLAMF1 and SLAMF7 for overexpression and knockout experiments. In line with the previously established correlation between SLAMF receptor expression on primary CLL cells and an indolent clinical course, we observed lower proliferation rates of the SLAMF receptor overexpressing sublines MEC-1$^{\text{LeGO-SLAMF1+}}$ and MEC-1$^{\text{LeGO-SLAMF7+}}$ as compared to the control cells MEC-1$^{\text{LeGO-empty}}$ (Fig. 2a, b). Since individual knockouts of SLAMF1 or SLAMF7 did not result in significant changes in proliferation (Fig. 2c, d), we hypothesized that these receptors might share redundant functions and one receptor may substitute for the other. We therefore created a double knockout subline (MEC-1$^{\text{CRISPR-SLAMF1-/7-}}$) which showed a markedly increased proliferation compared to the control cell line MEC-1$^{\text{CRISPR-scr}}$ (Fig. 2e, f).

The proliferative consequences of SLAMF1 and SLAMF7 overexpression could be reproduced in the JVM3 cell line as a different M-CLL model that naturally expresses lower levels of SLAMF1 and SLAMF7 (Supplementary Fig. S3A, B).

Despite the fact that flow cytometry data from our clinical cohort suggested that only a negligible fraction of U-CLL cases shows high expression of SLAMF1 or SLAMF7 receptors (1 of 27 U-CLL cases, as shown in Fig. 1d), we sought to explore SLAMF1 and SLAMF7 overexpression in a U-CLL cellular context (Hg3). Interestingly, Hg3 cells were hard to transduce with SLAMF1/SLAMF7 receptor constructs and sublines resulting from continuous long-term selection pressure showed very low proliferation (Supplementary Fig. S3C, D). This, together with the clinical observation of only few U-CLL cases that highly express SLAMF1/7 receptors suggests that this subset of CLL relies on sufficient downregulation of SLAMF1 and SLAMF7 for survival.

Modulation of BCR signaling by SLAMF1 and SLAMF7 receptors

Next, we experimentally addressed the question if the antiproliferative effects of SLAMF1 and SLAMF7 receptors may consist in modulating BCR activity since low expression was closely associated with U-CLL. One of the initial steps in B cell activation after BCR engagement is Ca^{2+} flux which subsequently affects numerous cellular functions [25]. Indeed, SLAMF1 or SLAMF7 overexpressing cell lines showed considerably mitigated responses to anti-IgM stimulation (Fig. 3a and Supplementary Fig. S4A, C). Whereas the individual knockouts of SLAMF1 and SLAMF7 receptors did not show any effects on Ca^{2+} mobilization, we observed markedly increased responses to anti-IgM stimulation in MEC-1$^{\text{CRISPR-SLAMF1-/7-}}$ (Fig. 3b).

We reasoned that the inhibition of the BCR signaling axis may be less efficient in a cellular background of high SLAMF1 or SLAMF7 receptor expression since BCR signaling appears suppressed by these receptors. To test this, we treated our M-CLL MEC-1 sublines with the BTK inhibitor ibrutinib at half-maximal inhibitory doses. The only cell line showing significantly increased sensitivity toward ibrutinib compared to the respective control cell line was MEC-1$^{\text{CRISPR-SLAMF1-/7-}}$ (Fig. 3c, d). These data confirmed the inhibitory effect of SLAMF receptors on the BCR signaling axis. Ibrutinib sensitivity assays performed on our alternative U- and M-CLL models gave very similar results (Supplementary Fig. S4B, D). Of note, we observed not only a high ibrutinib responsiveness in the parental U-CLL Hg3 cell line—well in line with the clinical observation of high ibrutinib sensitivity in U-CLL—but also a markedly decreased sensitivity in the SLAMF1 and SLAMF7 overexpressing Hg3 sublines (Supplementary Fig. S4D).

Identification of BCR pathway inhibiting mediators downstream of SLAMF1 and SLAMF7 receptors

Based on these findings, we asked how SLAMF receptors modulate BCR signaling in CLL.

Since our screening platform uses SH2 domains provided by signaling molecules or adapters (EAT2, SHP2 etc.) to characterize activated signaling upstream thereof, the expression of the SH2-donating molecule itself in the target tissue is not required—even if the respective SH2 probe shows reactivity. Since SAP family proteins are not uniformly expressed in B cells [26, 27] and our MEC-1 cell line did also not express EAT2 (Fig. 4a), we hypothesized that EAT2 itself may not mediate the SLAMF receptor-related effects in our CLL cohort. To test for EAT2 expression, we randomly selected individual CLL cases with low or high SLAMF1 or SLAMF7 receptor expression levels and subjected these to western blot analysis for EAT2 using MEC-1 cells transduced to express EAT2 as positive control. In line with our assumption, we found no EAT2 expression in the majority of CLL samples (69%) and no correlation with SLAMF receptor status in the few samples positive for EAT2 supporting our hypothesis (Fig. 4a).

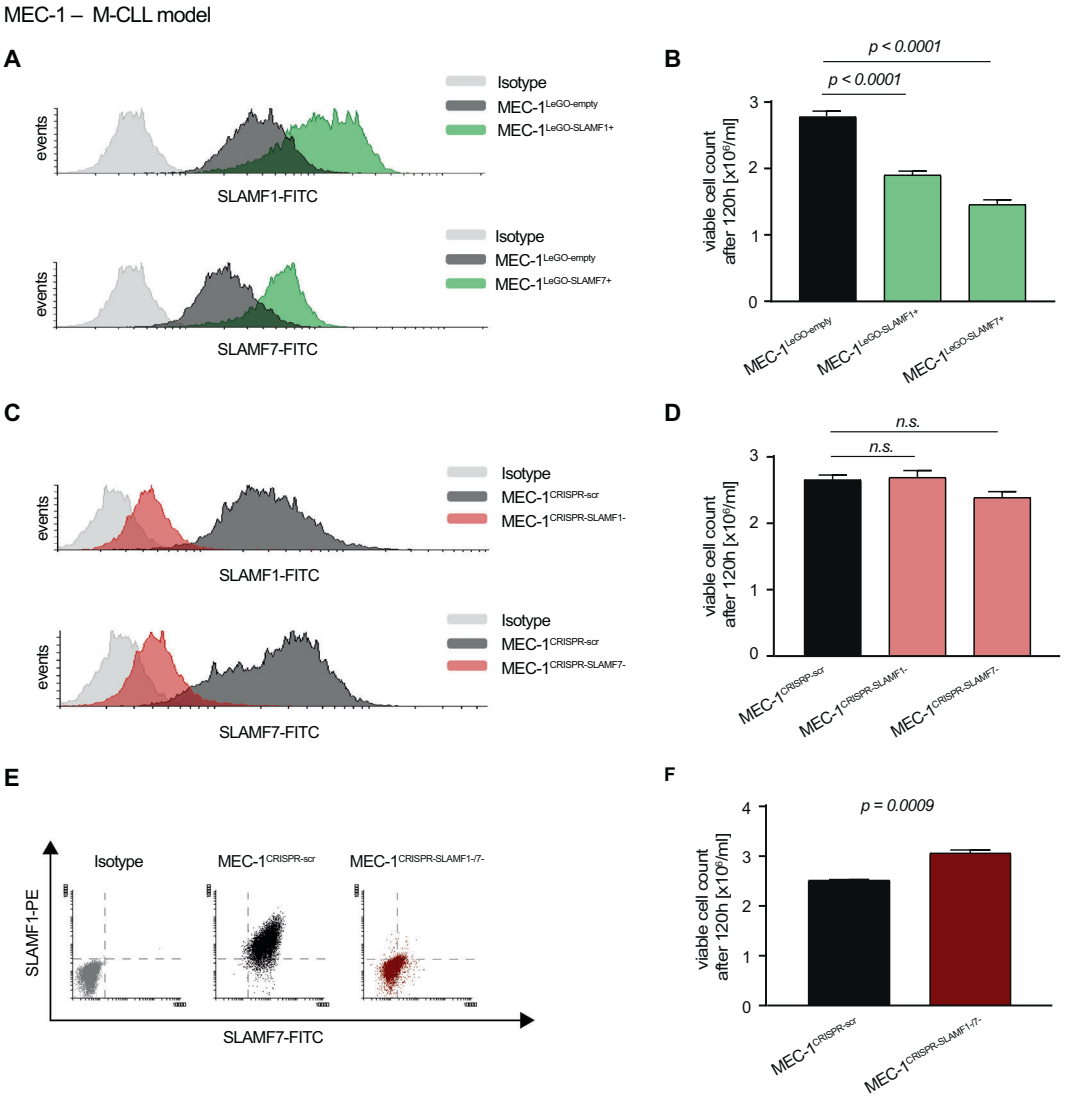


Fig. 2 SLAMF1 and SLAMF7 overexpression and knockout in the CLL cell line MEC-1. a FC analysis of MEC-1 cells transduced with lentiviral particles coding for SLAMF1 or SLAMF7. **b** Proliferation of SLAMF1 or 7 overexpressing MEC-1 cells after 120 h compared to control cell line transduced with empty vector. $N = 12$. **c** FC analysis of MEC-1 cells after knockout of SLAMF1 or 7 using CRISPR/Cas9 technology. **d** Proliferation of MEC-1cells depleted of SLAMF1 or 7 after 120 h compared to control cell line transduced with a non-targeting (scr) gRNA. $N = 9$. **e** FC analysis of MEC-1 cells after the subsequent knockout of both, SLAMF1 and SLAMF7. **f** Proliferation of MEC-1 cells after knockout of SLAMF1 and SLAMF7 after 120 h compared to control cell line. $N = 9$. Data from independent experiments are shown as mean, error bars represent SEM, statistical significance was calculated by one-way ANOVA and Bonferroni's post-hoc test. FC flow cytometry, SEM standard error of the mean.

RNAseq data of MEC-1 cells as well as immunoblotting of CLL samples could also rule out the other SAP family member SH2D1A as the downstream mediator of the SLAMF related effects in CLL as no expression could be detected (data not shown).

It was previously postulated, that SLAMF receptors can signal through inhibitory molecules such as SHP1/2 or SH2 domain containing inositol polyphosphate 5-phosphatase 1/2 (SHIP1/2) in the absence of SAP family proteins [10]. However, our SH2 screens performed using the respective SH2 domains did not show EAT2-like signatures. The only pattern potentially compatible with the EAT2 signature was that of SHP1, but knockdown of this target did not restore proliferation or Ca^{2+} signaling in MEC-1 overexpressing SLAMF receptors (data not shown).

To molecularly pin down downstream mediators, we finally conducted a biotinylation screen where we coupled a promiscuous biotin ligase (BioID2) at the C-terminus of SLAMF1 and SLAMF7 to selectively biotinylate and identify SLAMF receptor interaction partners (Fig. 4b) [28]. An ~35 kDa biotinylated protein band was visible both in SLAMF1-BioID2 and in SLAMF7-BioID2 overexpressing cells (Fig. 4c). Streptavidin pull down followed by mass spectrometry of excised proteins in the 30–40 kDa range identified

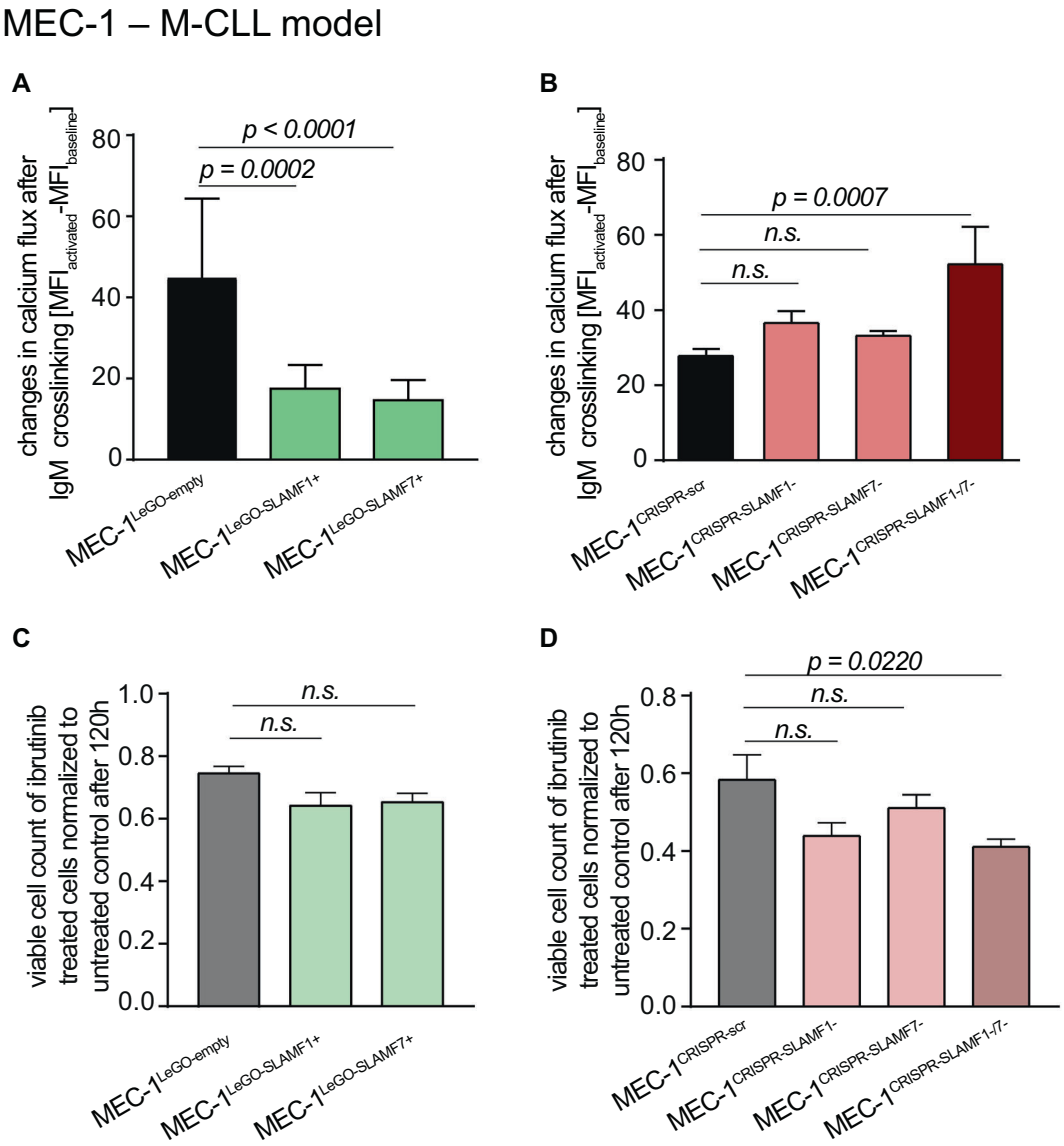


Fig. 3 Modulation of BCR signaling by SLAMF1 and SLAMF7 in MEC-1 cells. Cells were stained with FLUO4 and Ca^{2+} flux after stimulation with anti-IgM was assessed via FC in **a** SLAMF1 or 7 overexpressing MEC-1 cells, $N = 16$; **b** in MEC-1 cells after knockout of SLAMF1, SLAMF7 or both. $N = 8$. Cells were treated with 1 µM Ibrutinib and proliferation was measured after 120 h relative to untreated control in **c** MEC-1 cells transduced with empty vector control or overexpressing SLAMF1 or SLAMF7, $N = 12$ or **d** MEC-1 cells after knockout of SLAMF1, SLAMF7 or both, $N = 9$. Data from independent experiments are shown as mean, error bars represent SEM, statistical significance was calculated by one-way ANOVA and Bonferroni's post-hoc test. FC flow cytometry, SEM standard error of the mean.

prohibitin-2 (PHB2) as a binding partner of SLAMF1 and SLAMF7 receptors in CLL. This interaction was confirmed by co-immunoprecipitation with an antibody directed against SLAMF1 and SLAMF7 receptors (Fig. 4d). PHB2 was initially described as B-Cell Receptor Associated Protein BAP37. The fact that we found a direct interaction between SLAMF receptors and PHB2 strongly suggested to us that this protein was involved in the BCR pathway antagonistic effects produced by SLAMF1 or SLAMF7 overexpression. To address this experimentally, we transfected MEC-1 cells with siRNA specific for PHB2 (siPHB2) to explore the consequences of its knockdown on the SLAMF1/7 receptor induced BCR pathway antagonism. siPHB2 transfection resulted in partial loss of PHB2 expression in all MEC-1 sublines (Fig. 5a). Interestingly, MEC-$1^{LeGO\text{-}empty}$ control cells showed lower baseline calcium flux as well as decreased responses to IgM crosslinking after PHB2 knockdown compared to a transfection control (siRNActrl) indicating that PHB2 contributed to intact BCR signaling consistent with prior reports [29, 30]. The BCR antagonistic effects of the PHB2 knockdown were not observed in the SLAMF1/7 receptor overexpressing sublines (Fig. 5b, c). This led us to speculate that in CLL cells

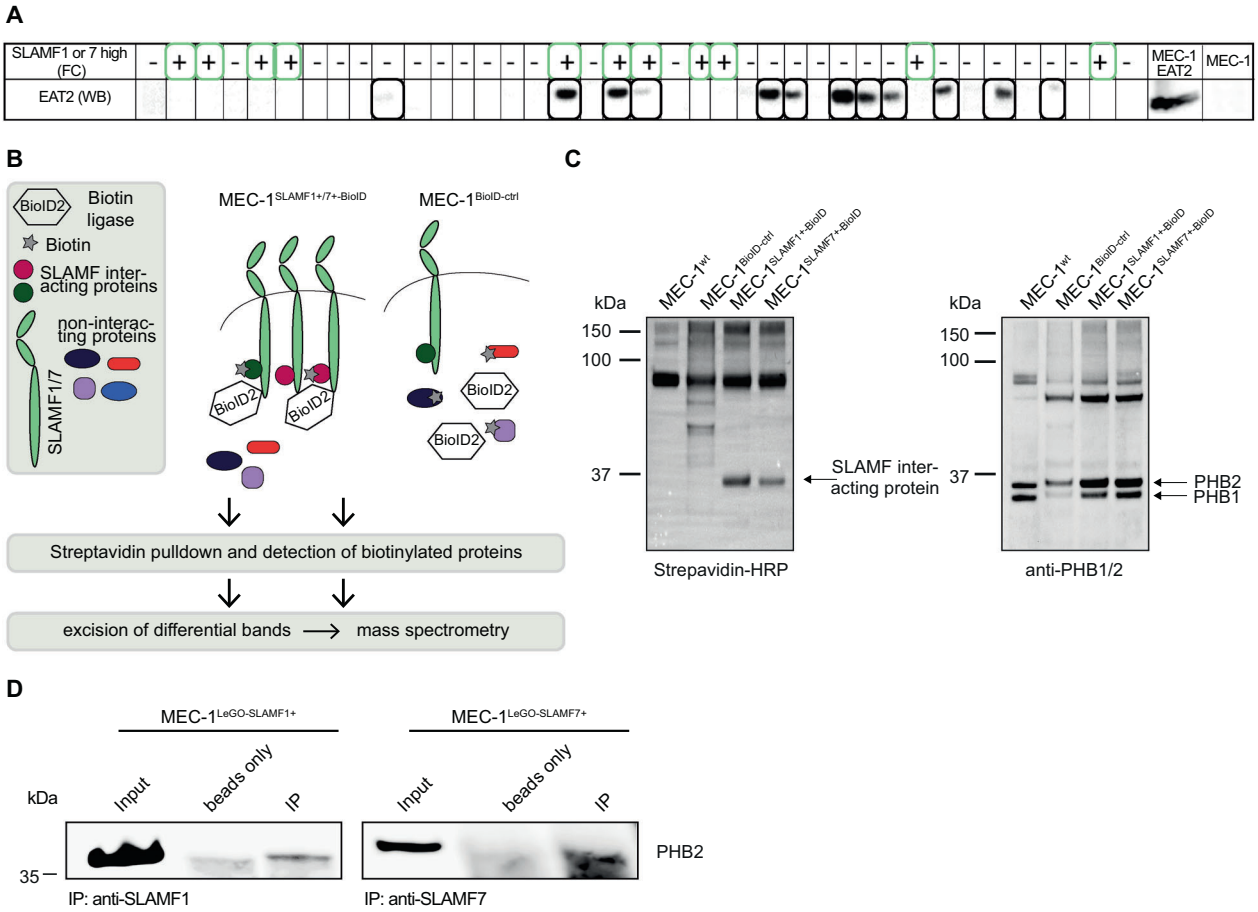


Fig. 4 Identification of downstream binding partners of SLAMF receptors in CLL. a WB analysis for EAT2 expression in CLL samples from the patient cohort and correlation with SLAMF status. MEC-1 cells overexpressing EAT2 were used as a positive control. **b** Experimental overview of the BioID screen to identify SLAMF1 and SLAMF7 receptor binding partners. **c** WB analysis of whole cell lysates from MEC-1 cells and sublines transduced with BioID2 constructs (either fused to SLAMF1 or 7 or uncoupled as control) incubated with Streptavidin-HRP or PHB1/2 antibodies. **d** Co-immunoprecipitation of SLAMF1 and SLAMF7 in MEC-1 cells. Immunoblotting was done using PHB2 antibody. WB western blot, BioID2 biotin ligase.

SLAMF1 and SLAMF7 receptors recruit PHB2 away from the IgM molecule thereby functionally inducing a PHB2 loss situation leading to impaired BCR signaling and the knockdown of PHB2 could not add up in this scenario. To more specifically study the role of PHB2 in SLAMF receptor-mediated effects on BCR signaling, we performed experiments using an Ig-switch model. Since PHB2 has been shown to associate specifically with the intracellular domain of the IgM-type BCR [31], we created an Ig-switched MEC-1 subline [16] that expresses IgG instead of IgM (Supplementary Fig. S5A). When PHB2 was immunoprecipitated from IgM versus IgG MEC-1 sublines, we found a much lesser amount of CD79a to be co-immunoprecipitated in IgG MEC-1 suggesting that PHB2 only weakly associates with IgG in our model thereby confirming previous work (Fig. 5d). In contrast to the IgM MEC-1 model, SLAMF1 or SLAMF7 overexpression in the IgG subline (successful overexpression shown in Supplementary Fig. S5B) neither impacted cellular proliferation, nor response to IgG ligation in terms of Ca^{2+} mobilization or AKT/ERK phosphorylation (Fig. 5e–g). Together, this suggested that SLAMF1 and SLAMF7 receptors mediate their IgM-BCR antagonism by recruiting PHB2 and thereby disturbing its function in BCR signal transduction.

SLAMF1 and SLAMF7 status of CLL patients impacts their NK cell degranulation

In addition to the effects of SLAMF receptors on proliferation and BCR signaling via PHB2, we reasoned that SLAMF receptors may promote CLL-directed immune control since these molecules are involved (mostly by homotypic stimulating interactions) in the NK/T cell axis [32]. To this end, we assessed if the levels of SLAMF receptor expression on the CLL patients' NK cells correlated with the levels on their CLL cells. NK cells derived from patients with CLL showed comparable membrane densities of SLAMF1 and SLAMF7 receptors as compared to healthy individuals and there was no difference in the

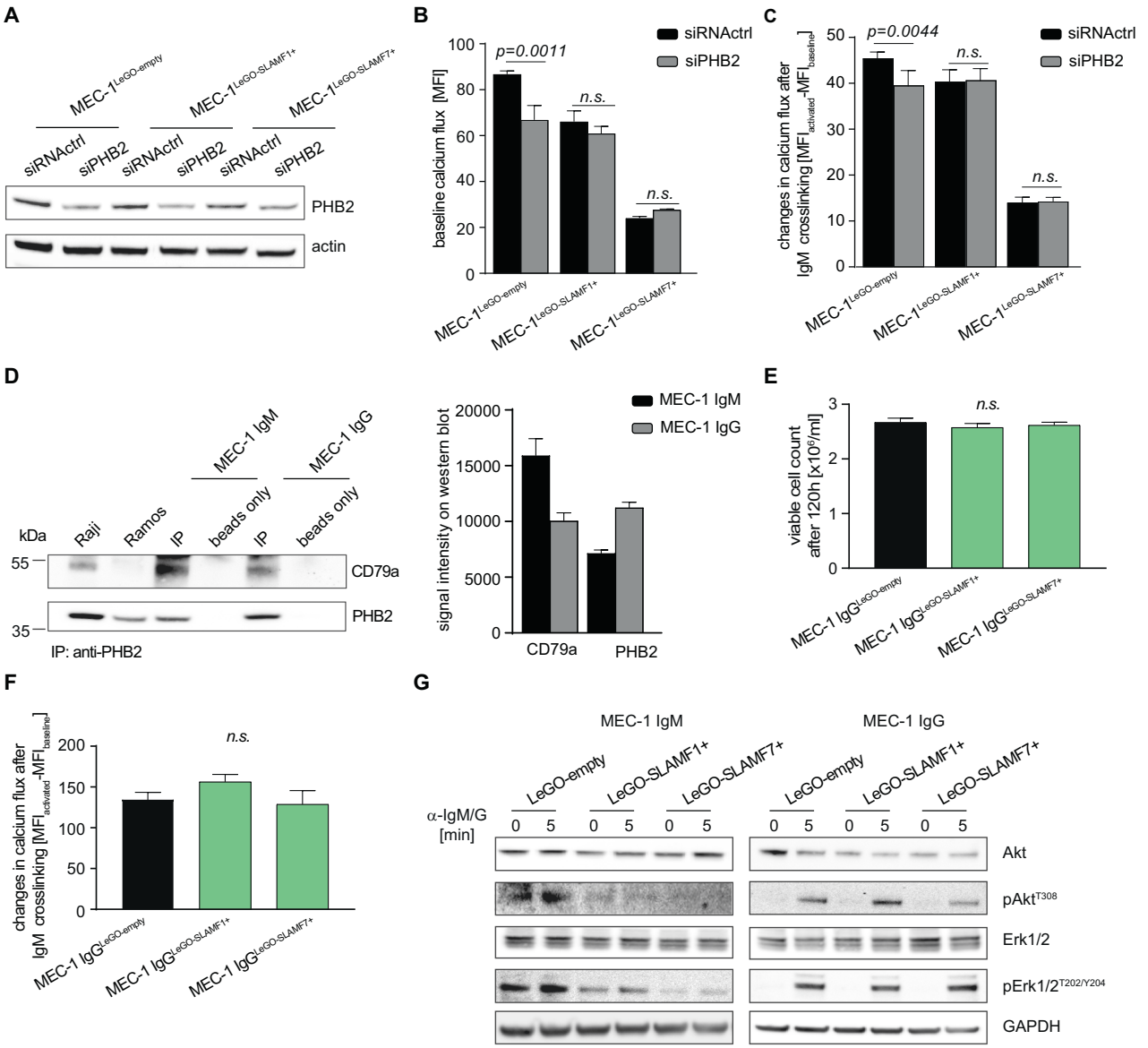


Fig. 5 The role of PHB2 in BCR pathway inhibitory effects of SLAMF1 and SLAMF7 receptors in CLL. a Representative WB analysis of PHB2 levels 96 h after siRNA transfection as indicated in the MEC-1 sublines. **b** Calcium flux was assessed 96 h after siRNA transfection of MEC-1 sublines baseline and **c** after IgM crosslinking. $N = 6$. **d** Co-immunoprecipitation of PHB2 in IgM and IgG-switched MEC-1 cells. Raji and Ramos cell lysates were used as a positive control for CD79a. For Western Blots, anti-CD79a and -PHB2 antibodies were used. Signal intensities of two independent western blots were quantified using ImageJ. **e** Proliferation was assessed in IgG-switched MEC-1 cells overexpressing SLAMF1 or SLAMF7 compared to empty vector control line after 120 h, $N = 9$. **f** Changes in calcium-flux after IgG crosslinking were assessed in IgG-switched MEC-1 cells overexpressing SLAMF1 or SLAMF7, $N = 6$. **g** Representative Western Blot analysis for Akt and Erk phosphorylation after stimulation with 10 µg/ml anti-IgM/IgG for 5 min in IgM or IgG-swiched MEC-1 cells overexpressing SLAMF1 or SLAMF7 compared to empty vector. Data from independent experiments are shown as mean, error bars represent SEM, statistical significance was calculated by one-way ANOVA and Bonferroni's post-hoc test. WB Western blot, SEM standard error of the mean, FC flow cytometry.

expression of SLAMF1 and SLAMF7 on the NK cells of CLL patients from the SLAMF^{high} or SLAMF^{low} group. This indicated that SLAMF receptor regulation only occurs in the neoplastic B cell, but not the NK cell compartment in CLL patients (Fig. 6a, b, SLAMF$^{high} = 4$, SLAMF$^{low} = 6$ patients). However, when comparing the degranulation capacity of CLL-derived NK cells by measuring CD107a expression we found a significant increase if the donating CLL patient was considered SLAMF^{high} (Fig. 6c, SLAMF$^{high} = 6$, SLAMF$^{low} = 8$ patients; $p = 0.0033$). Interestingly, there was no difference in the activity of NK cells from healthy donors when incubated with the genetically engineered MEC-1 sublines showing differential SLAMF receptor levels (Fig. 6d). In line with this,

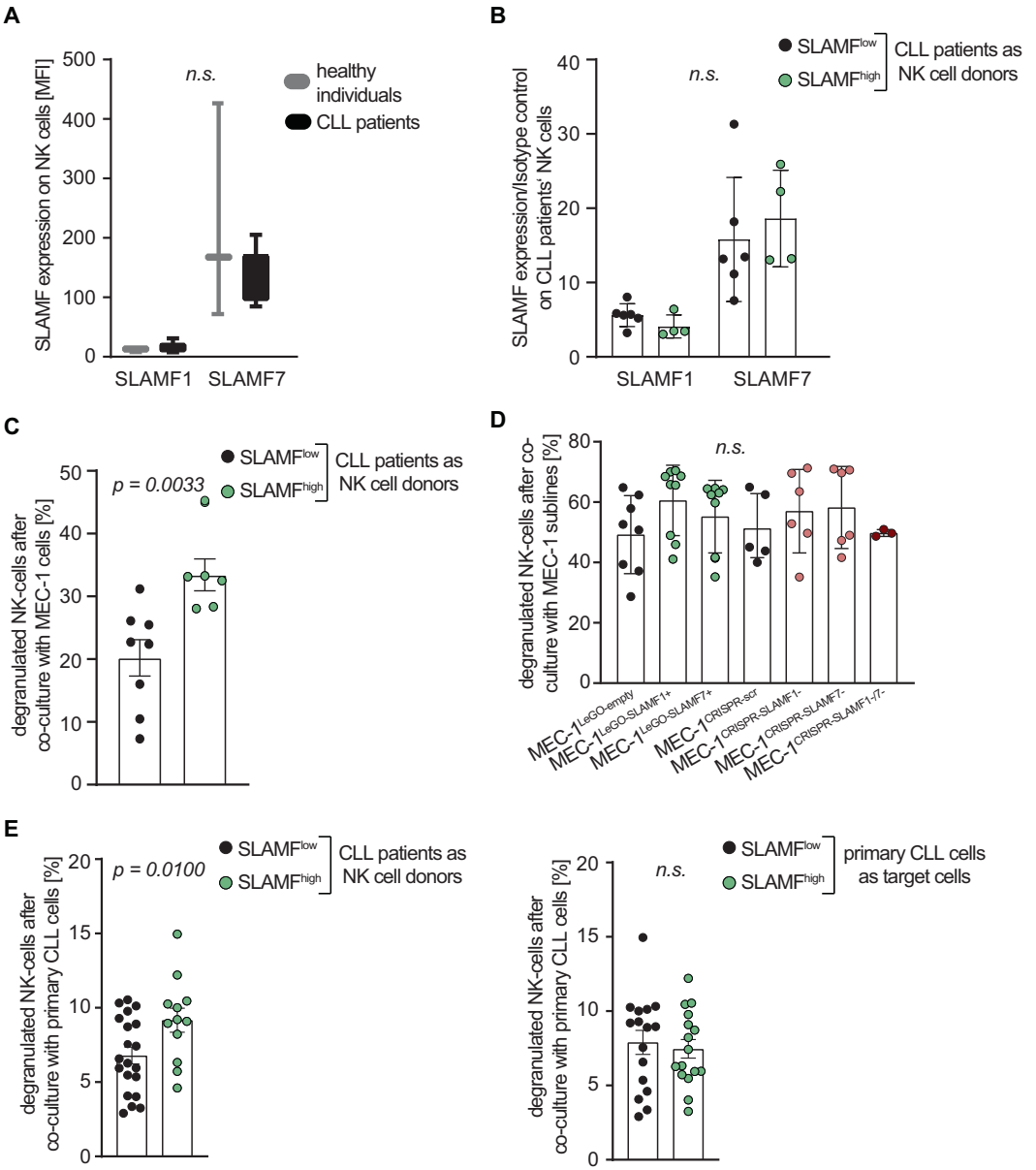


Fig. 6 SLAMF receptor expression in NK cell mediated immunity. **a** SLAMF1 and 7 expression on NK cells was measured in CLL patients and healthy individuals via flow cytometry, error bars represent SD. $N = 3$. **b** SLAMF1 and SLAMF7 on NK cells derived from SLAMF^{high} or SLAMF^{low} CLL patients measured by FC. Error bars represent SD, statistical significance was tested with student's t test. $N = 10$ (SLAMF$^{high} = 4$, SLAMF$^{low} = 6$). **c** NK cells from CLL patients were co-cultured with MEC-1 CLL cells; $p = 0.0033$. $N = 14$ (SLAMF$^{high} = 6$; SLAMF$^{low} = 8$). **d** NK cells from healthy donors were co-cultured with MEC-1 sublines as indicated. In **e** NK cells from CLL patients were co-cultured with primary CLL cells of patients being either categorized as SLAMF^{high} or SLAMF^{low}. $N = 8$ (SLAMF$^{high} = 3$; SLAMF$^{low} = 5$). The percentage of degranulated NK cells was measured by CD107a expression via FC. Data from independent experiments are shown as mean, error bars represent SEM, statistical significance was tested with one sided, unpaired student's t test or in **d** with one-way ANOVA and Bonferroni's post-hoc test. SD standard deviation, FC flow cytometry, SEM standard error of the mean.

degranulation of CLL-derived NK cells was unaffected by the SLAMF receptor levels of primary CLL cells that were used as target cells (Fig. 6e, SLAMF^{high} NK cell donors = 3; SLAMF^{low} NK cell donors = 5). Together, this indicated differential education of NK cells depending on the SLAMF1/7 receptor status of the respective CLL cells.

Discussion

Research from the last two decades showing that the BCR is a major driver in CLL has profoundly transformed our therapeutic landscape with the introduction of BCR pathway antagonists in essentially all treatment lines. Yet, we still need to define in which therapeutic sequence and with

which combination partners these drugs have to be used in order to achieve optimal clinical results in all patient subsets. These clinical questions require an increased understanding of how CLL cells are driven towards proliferation/survival e.g., by deciphering how BCR signaling—as a key mechanism in malignant CLL cells—is modulated in the different biological subsets of CLL. Also, due to the so far rather disappointing results of common immunotherapy principles in this disease (e.g., checkpoint inhibitors) [33], a novel understanding of CLL-specific immune evasion mechanisms is clearly warranted. These insights will be key for further therapeutic advances in this disease.

In the work reported here, we investigated the biological role of two SLAMF receptors found to have—if highly expressed—a favorable prognostic role in CLL that is independent of other known prognostic markers. We present compelling experimental evidence that high levels of SLAMF1 and SLAMF7 attenuate BCR signaling in the subset of IGHV mutated CLL. According to our data, this "internal" attenuation of BCR signaling may be relevant for ~50% of IGHV mutated cases and our experimental data indicate that it may lead to lesser therapeutic efficacy of the BCR pathway antagonist ibrutinib in this setting. Moreover, our data indicate that CLL cases with a lack of downregulation of SLAMF1 and/or SLAMF7 show more efficient NK cell mediated killing and thereby potentially more CLL immune control.

Previous investigations have already established SLAMF1 as prognostic marker in CLL and mechanistically this has been linked to modulation of autophagy [34–36]. Our data now contribute two independent mechanisms by which these receptors may impact both BCR signaling and NK-mediated CLL cell killing.

The mechanism how SLAMF1 and SLAMF7 attenuate BCR signaling in CLL was not evident for us at first glance. First of all, EAT2—one of the key downstream mediators of SLAMF receptor related effects in NK cells—was mostly found not expressed in CLL [37, 38]. In addition, none of the previously reported SLAMF receptor-interacting signaling molecules (SHP1/2, SHiP1/2) [10] could be confirmed to mediate the BCR pathway antagonistic effects in our CLL models. We therefore chose a biotinylation screen as a biochemical approach to pin down the SLAMF receptor downstream molecules relevant for BCR pathway interference in CLL. This analysis independently identified PHB2 as an interaction partner for both SLAMF1 and SLAMF7. PHB2 has been previously reported to be associated with the IgM BCR [31], but its role in BCR signaling has been largely unexplored to date. Our data now directly links SLAMF1/7 and PHB2 to the IgM-BCR via CD79a as a well-established part of the BCR signaling complex. If these interactions occur via sequential binding or as part of multi-protein complexes remains to be elucidated but co-immunoprecipitation experiments and our biotinylation screen point to a rather close proximity of the involved molecules. Moreover, our PHB2 knockdown experiments suggest that SLAMF1 and SLAMF7 receptors likely recruit PHB2 thereby detaching it from the BCR signaling machinery for which—at least in IGHV-mutant CLL IgM-BCR—this molecule seems to be of high importance. Of note, single versus double knockout and overexpression experiments clearly show that both receptors are able to recruit PHB2 and expression of only one of them is sufficient to induce the observed direct anti-proliferative effects. This aspect should be taken into account when considering diagnostic application. However, we found a very small subcohort (~2–3% of all CLL cases) to be highly positive for both SLAMF1 and SLAMF7. These patients show even better overall survival. In light of the data acquired for this manuscript, we believe that this additional survival benefit is not due to the BCR-related effects of SLAMF receptors reported here. Instead, we hypothesize that the increased overall survival of the double-high expressers could be due to BCR-unrelated effects, e.g., SLAMF1's role in autophagy [34].

Moreover, since we found the BCR signaling axis to be "internally" attenuated in cell lines with high expression of SLAMF1 or SLAMF7, the observation of relatively low inhibitory effects of BCR pathway antagonists in these lines was not surprising. This finding could also explain the clinical observation that in M-CLL (a subset in which about 50% of cases express high levels of SLAMF1 or SLAMF7), treatment with the BTK inhibitor ibrutinib results in prolonged lymphocytosis and lower tissue cell death rate in comparison to cases of U-CLL while sensitivity to chemotherapy is generally satisfactory [39]. It could imply that M-CLL cases with high SLAMF1 or SLAMF7 expression derive relatively lesser benefit from BTK inhibition as compared to U-CLL (that is predominantly SLAMF1/SLAMF7 low) or the ~50% of M-CLL cases that downregulate SLAMF1/SLAMF7. Future clinical trials should prospectively test this hypothesis since it may help to guide selection of M-CLL patients for upfront chemo(immuno)therapy versus BTK inhibition. Moreover, targeting PHB2 as a combinatorial approach with BTK inhibition may have the potential to deepen responses and should therefore be explored.

The other mechanism by which expression of SLAMF1 or SLAMF7 may impact the favorable outcome of this subset of patients, is their effect on the CLL–NK cell interaction. It is widely accepted that NK cells can recognize and kill CLL cells albeit with decreased efficacy [40–42]. Our own data show that NK cells derived from SLAMF^{high} CLL patients show increased degranulation capacity regardless of the SLAMF receptor expression levels of the target cell they are confronted with. This data suggests some kind of NK cell education rather than a

stoichiometric effect of high SLAMF receptor expression on the respective tumor cells in CLL patients with SLAMF^{high} status that leads to more efficient immune control. We recognize that these experiments have been conducted in an artificial co-culture system that lacks many of the immune cell populations present in the CLL lymph node or bone marrow environment. Despite this limitation, we postulate that the SLAMF receptor effect on NK cell killing may contribute to the clinical course of CLL expressing high levels of SLAMF1 or SLAMF7.

Taken together, we show that SLAMF receptors (and downstream PHB2) act as central regulators of BCR signaling and potentially also modulate NK-mediated immune control in CLL. Impact of SLAMF1 and SLAMF7 receptor expression on sensitivity toward BCR pathway inhibitors should trigger evaluation of these receptors as biomarkers of response in future clinical trials.

Acknowledgements We thank the staff of the diagnostic Flow cytometry lab of the University Medical Center Hamburg Eppendorf, the staff of the Cytometry and Sorting Core Unit of the University Medical Center Hamburg-Eppendorf and the core facility "Zellanalyse und Zellsortierung" of the University Medical Center Halle (Saale) for expert technical assistance. We are also grateful to the staff of "Hämatologisch-onkologische Gemeinschaftspraxis Spohn/Möller Halle" for the recruitment of additional CLL patients in the revision phase of the project. This work was supported by grant BI 1711/4-1 from the Deutsche Forschungsgemeinschaft (MB). Open access funding provided by Projekt DEAL.

Author contributions MB designed the study, supervised the experiments, and wrote the manuscript; LvW designed, performed, and interpreted the experiments and wrote the manuscript; CS, SS, and SB designed, performed, and interpreted experiments; DS and KR performed and interpreted experiments; HG and GW-E performed experiments; EW performed bioinformatics analysis; BF and MA critically revised the manuscript; PN supervised the experiments, analyzed data, and critically revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

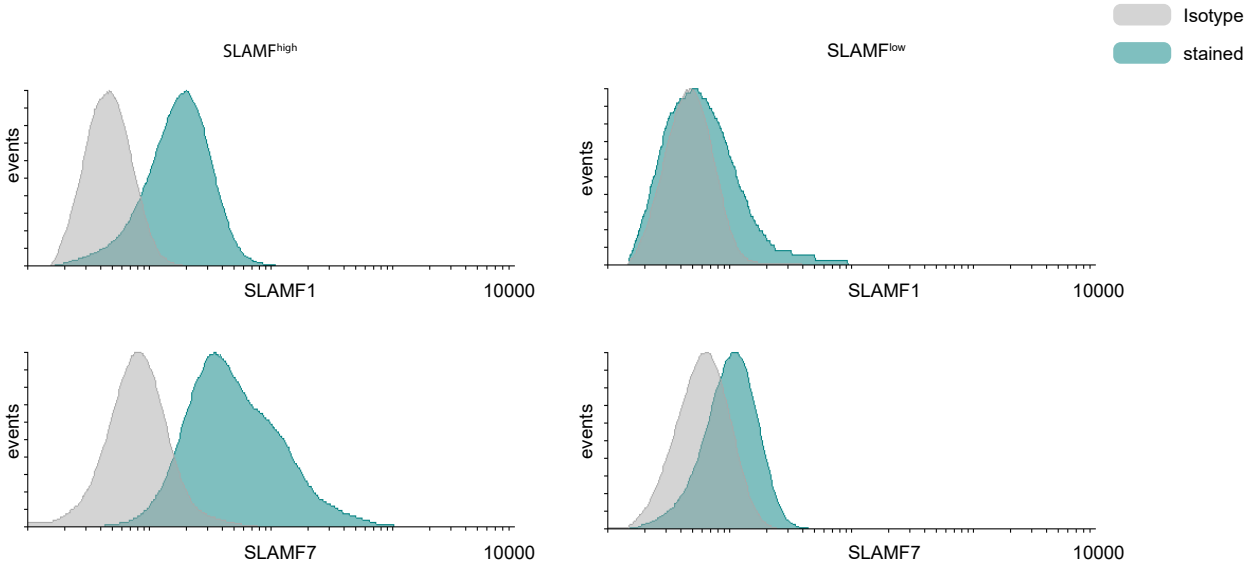
Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Jeyakumar D, O'Brien S. B cell receptor inhibition as a target for CLL therapy. Best Pract Res Clin Haematol. 2016;29:2–14.
2. Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. N Engl J Med. 2013;369:32–42.
3. Advani RH, Buggy JJ, Sharman JP, Smith SM, Boyd TE, Grant B, 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.
4. Burger JA, Tedeschi A, Barr PM, Robak T, Owen C, Ghia P, et al. Ibrutinib as initial therapy for patients with chronic lymphocytic leukemia. N Engl J Med. 2015;373:2425–37.
5. Woyach JA, Ruppert AS, Heerema NA, Zhao W, Booth AM, Ding W, et al. Ibrutinib regimens versus chemoimmunotherapy in older patients with untreated CLL. N Engl J Med. 2018;379:2517–28.
6. Shanafelt TD, Wang XV, Kay NE, Hanson CA, O'Brien S, Barrientos J, et al. Ibrutinib-rituximab or chemoimmunotherapy for chronic lymphocytic leukemia. N Engl J Med. 2019;381:432–43.
7. Ian Mockridge C, Potter KN, Wheatley I, Neville LA, Packham G, Stevenson FK. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. Blood. 2007;109:4424–31.
8. Guarini A, Chiaretti S, Tavolaro S, Maggio R, Peragine N, Citarella F, et al. BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgV H unmutated chronic lymphocytic leukemia (CLL) cells. Blood. 2008;112:782–92.
9. Shanafelt TD, Wang V, Kay NE, Hanson CA, O'Brien SM, Barrientos JC, et al. A randomized phase III study of ibrutinib (PCI-32765)-based therapy vs. standard fludarabine, cyclophosphamide, and rituximab (FCR) chemoimmunotherapy in untreated younger patients with Chronic Lymphocytic Leukemia (CLL): a trial of the ECOG-ACRIN cancer. Blood. 2018;132 (Suppl 1):LBA4.
10. Veillette A. SLAM-family receptors: immune regulators with or without SAP-family adaptors. Cold Spring Harb Perspect Biol. 2010;2:a002469.
11. Shachar I, Barak A, Lewinsky H, Sever L, Radomir L. SLAMF receptors on normal and malignant B cells. Clin Immunol. 2019;204:23–30.
12. Coma M, Tothova E, Guman T, Hajikova M, Giertlova M, Sarissky M. Altered expression pattern of SLAM family receptors on pathological B cells of patients with chronic lymphocytic leukemia. Leuk Lymphoma. 2017;58:1726–9.
13. Weber K, Bartsch U, Stocking C, Fehse B. A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. Mol Ther. 2008;16:698–706.
14. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelson T, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science. 2014;343:84–87.
15. Yáñez-Muñoz RJ, Balaggan KS, MacNeil A, Howe SJ, Schmidt M, Smith AJ, et al. Effective gene therapy with nonintegrating lentiviral vectors. Nat Med. 2006;12:348.
16. Cheong TC, Compagno M, Chiarle R. Editing of mouse and human immunoglobulin genes by CRISPR-Cas9 system. Nat Commun. 2016;7:10934.
17. Schepers E, Glorieux G, Dhondt A, Leybaert L, Vanholder R. Flow cytometric calcium flux assay: evaluation of cytoplasmic calcium kinetics in whole blood leukocytes. J Immunol Methods. 2009;348:74–82.

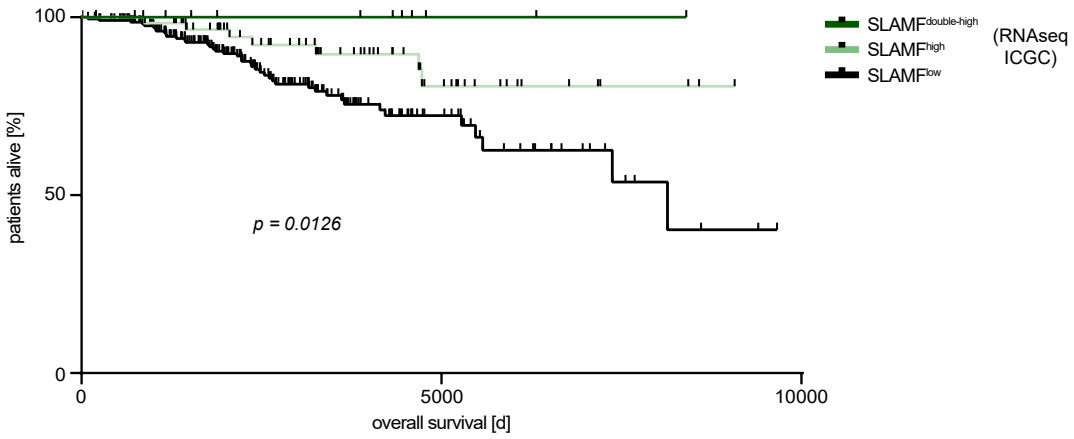
18. Kim DI, Jensen SC, Noble KA, Kc B, Roux KH, Motamedchaboki K, et al. An improved smaller biotin ligase for BioID proximity labeling. Mol Biol Cell. 2016;27:1188–96.
19. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods. 2004;294:15–22.
20. Therneau TM, Grambsch PM. Modeling survival data: extending the Cox model. Springer; 2000. p. 350.
21. Machida K, Thompson CM, Dierck K, Jablonowski K, Kärkkäinen S, Liu B, et al. High-throughput phosphotyrosine profiling using SH2 domains. Mol Cell. 2007;26:899–915.
22. Schliffke S, Buhs S, Bolz S, Gerull H, von Wenserski L, Riecken K, et al. The phosphotyrosine phosphatase SHP2 promotes anergy in chronic lymphocytic leukemia. Blood. 2018;131:1755–8.
23. Detre C, Keszei M, Romero X, Tsokos GC, Terhorst C. SLAM family receptors and the SLAM-associated protein (SAP) modulate T cell functions. Semin Immunopathol. 2010; 32:157–71.
24. Wu N, Veillette A. SLAM family receptors in normal immunity and immune pathologies. Curr Opin Immunol. 2016;38:45–51.
25. Baba Y, Kurosaki T. *Role of calcium signaling in B cell activation and biology.* Cham: Springer; 2015. p. 143–74.
26. Tangye SG, Weerdt BCMvd, Avery DT, Hodgkin PD. CD84 is up-regulated on a major population of human memory B cells and recruits the SH2 domain containing proteins SAP and EAT-2. Eur J Immunol. 2002;32:1640.
27. Veillette A. Immune regulation by SLAM family receptors and SAP-related adaptors. Nat Rev Immunol. 2006;6:56–66.
28. Roux KJ, Kim DI, Raida M, Burke B. A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. J Cell Biol. 2012;196:801–10.
29. Paris LL, Hu J, Galan J, Ong SS, Martin VA, Ma H, et al. Regulation of Syk by phosphorylation on serine in the linker insert. J Biol Chem. 2010;285:39844–54.
30. Lucas CR, Cordero-Nieves HM, Erbe RS, McAlees JW, Bhatia S, Hodes RJ, et al. Prohibitins and the cytoplasmic domain of CD86 cooperate to mediate CD86 signaling in B lymphocytes. J Immunol. 2013;190:723–36.
31. Terashima M, Kim KM, Adachi T, Nielsen PJ, Reth M, Kohler G, et al. The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. EMBO J. 1994;13:3782–92.
32. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol. 2008;9:495.
33. Ding W, LaPlant BR, Call TG, Parikh SA, Leis JF, He R, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. Blood. 2017;129:3419–27.
34. Bologna C, Buonincontri R, Serra S, Vaisitti T, Audrito V, Brusa D, et al. SLAMF1 regulation of chemotaxis and autophagy determines CLL patient response. J Clin Investig. 2016;126:181–94.
35. Schweighofer CD, Coombes KR, Barron LL, Diao L, Newman RJ, Ferrajoli A, et al. A two-gene signature, SKI and SLAMF1, predicts time-to-treatment in previously untreated patients with chronic lymphocytic leukemia. PloS One. 2011;6:e28277.
36. Bologna C, Buonincontri R, Serra S, Vaisitti T, Audrito V, Brusa D, et al. Slamf-1/CD150 is a signaling receptor expressed by a subset of chronic lymphocytic leukemia patients characterized by a favorable prognosis. Blood. 2012;120:1770.
37. Tassi I, Colonna M, Woollatt E, Sutherland GR, Lanier LL, Phillips JH. The cytotoxicity receptor CRACC (CS-1) recruits EAT-2 and activates the PI3K and phospholipase Cgamma signaling pathways in human NK cells. J Immunol. 2005;175: 7996–8002.
38. Binsky-Ehrenreich I, Marom A, Sobotta MC, Shvidel L, Berrebi A, Hazan-Halevy I, et al. CD84 is a survival receptor for CLL cells. Oncogene. 2014;33:1006–16.
39. Woyach JA, Smucker K, Smith LL, Lozanski A, Zhong Y, Ruppert AS, et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. Blood. 2014;123:1810–7.
40. Le Garff-Tavernier M, Decocq J, de Romeuf C, Parizot C, Dutertre CA, Chapiro E, et al. Analysis of CD16+CD56dim NK cells from CLL patients: evidence supporting a therapeutic strategy with optimized anti-CD20 monoclonal antibodies. Leukemia. 2011;25:101–9.
41. Pazina T, James AM, MacFarlane AW, Bezman NA, Henning KA, Bee C, et al. The anti-SLAMF7 antibody elotuzumab mediates NK cell activation through both CD16-dependent and -independent mechanisms. OncoImmunology. 2017;6:e1339853.
42. Sanchez-Martinez D, Lanuza PM, Gomez N, Muntasell A, Cisneros E, Moraru M, et al. Activated allogeneic NK cells preferentially kill poor prognosis B-cell chronic lymphocytic leukemia cells. Front Immunol. 2016;7:454.

Supplementary Figure 1

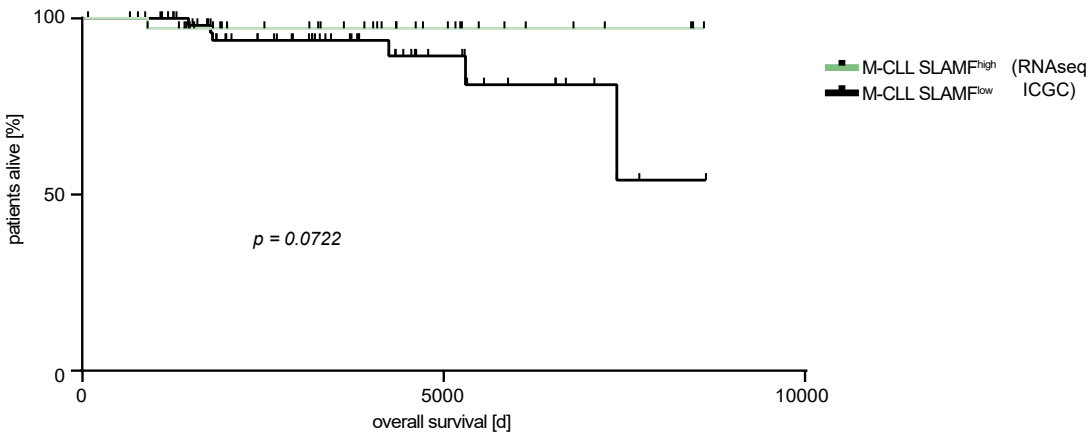


Supplementary Figure 2

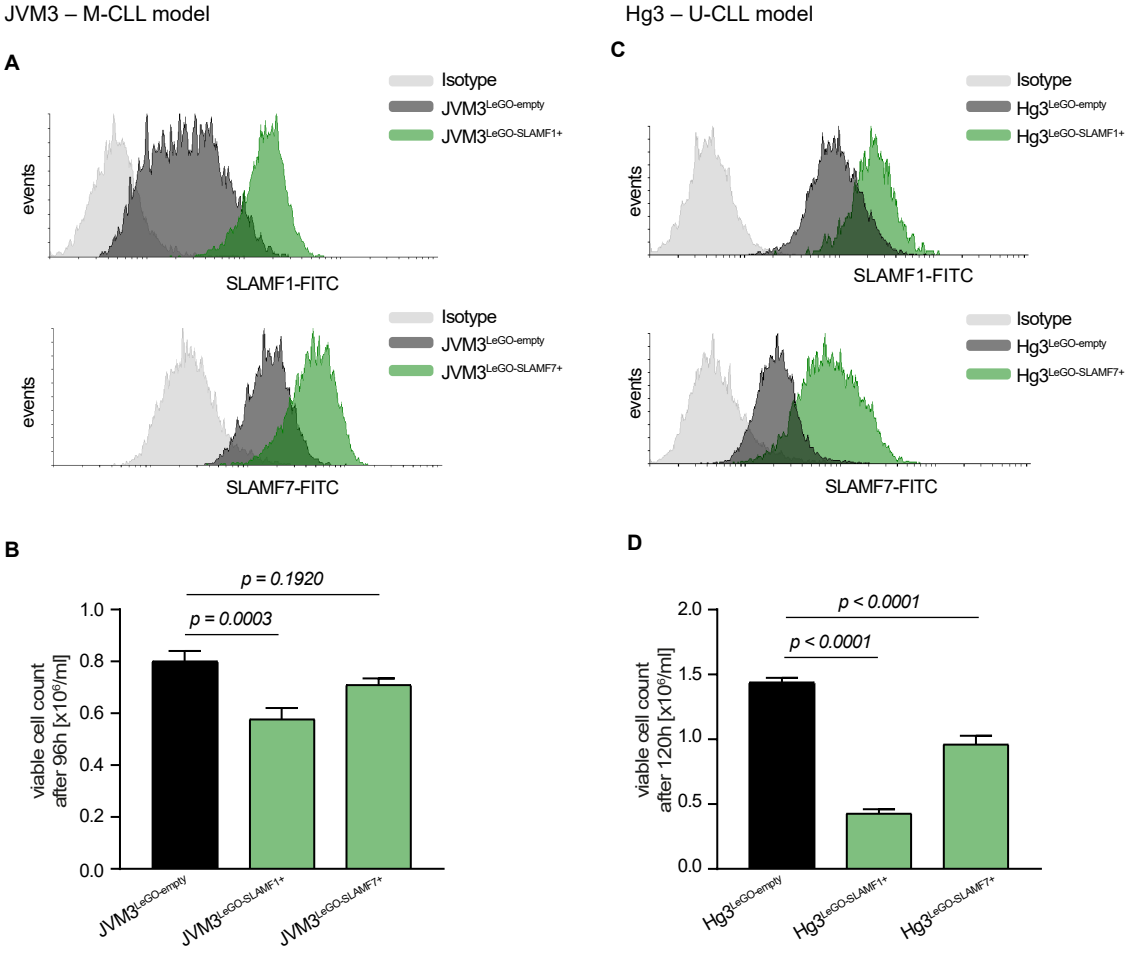
A



B



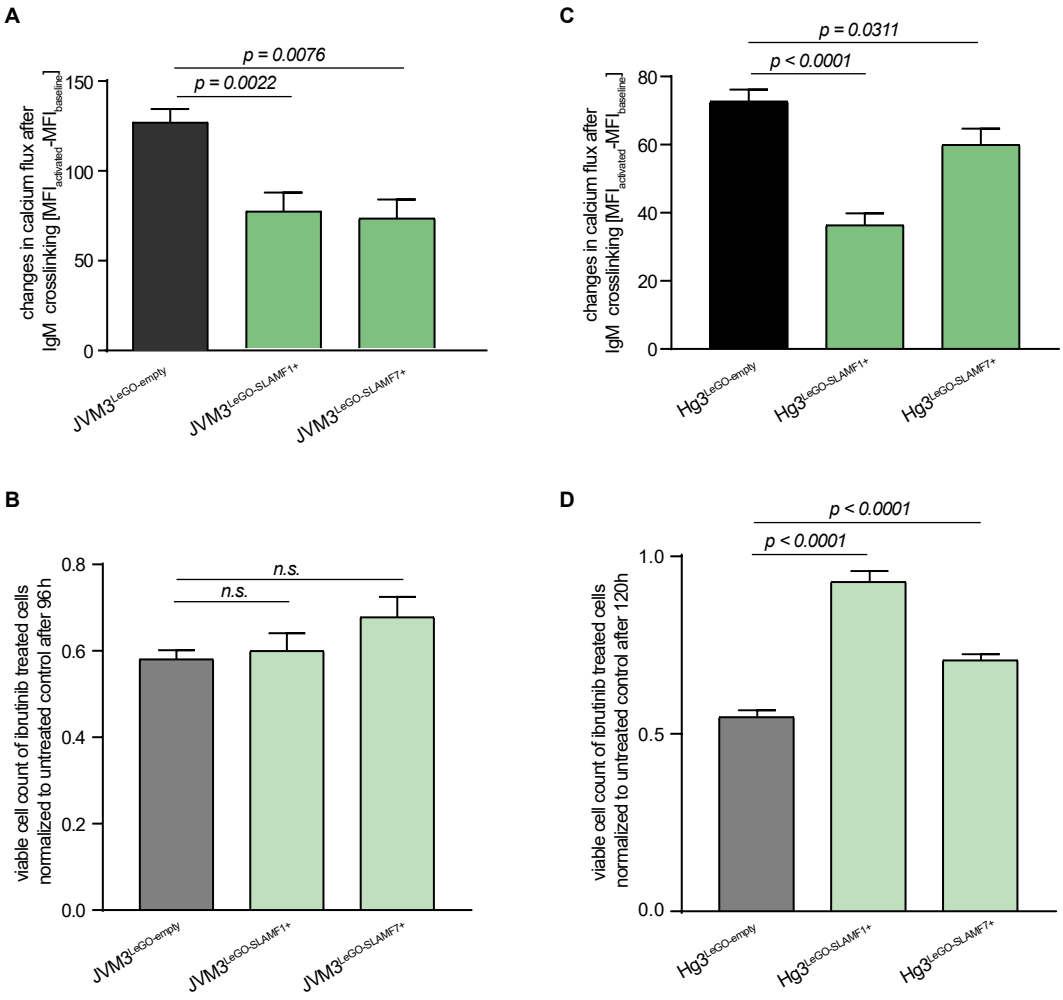
Supplementary Figure 3



Supplementary Figure 4

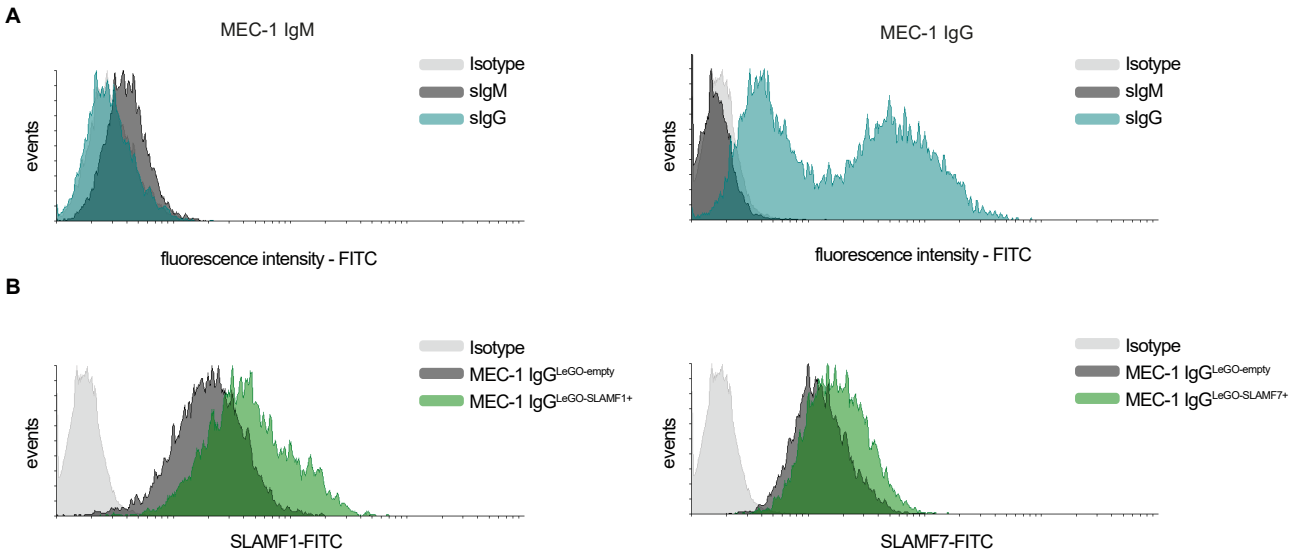
JVM3 – M-CLL model

Hg3 – U-CLL model



Supplementary Figure 5

MEC-1 – IgG class switch



Supplementary table 1: Antibodies used in flow cytometry

antigen-fluorochrome	manufacturer	clone	order number
SLAMF1-PE	BD Pharmingen	A12	559592
SLAMF7-AF647	BD Pharmingen	235614	564338
CD5-PC5.5	Beckman Coulter	BL1a	A70203
CD19-PC7	Beckman Coulter	J3-119	IM3628U
CD45-ECD	Beckman Coulter	J33	A07784
CD107a-PE/Cy7	Bio Legend	H4A3	328618
CD3-FITC	Beckman Coulter	UCHT1	IM2181U
CD56-PE	Beckman Coulter	N901	A07788
SLAMF1-FITC	Bio Legend	A-12 7D4	306306
SLAMF7-FITC	Bio Legend	162.1	331818
IgM-FITC	Beckman Coulter	SA-DA4	2040-02
IgG-FITC	Southern Biotech	polyclonal	B30655

Supplementary table 2: Antibodies used in immunoblotting

antigen	manufacturer	clone	order number
EAT2	abcam	polyclonal	ab67417
GAPDH	Santa Cruz	6C5	sc-32233
CD79a	abcam	EP3618	ab79414
PHB2	Santa Cruz	A-2	sc-133094
Akt	Cell Signaling	polyclonal	9272
pAkt (T308)	Cell Signaling	polyclonal	9275
Erk1/2	Cell Signaling	L34F12	4696
pErk1/2 (T202/Y204)	Cell Signaling	polyclonal	9101

Supplementary tables

Supplementary table 1: antibodies used in flow cytometry

Supplementary table 2: primary antibodies used in immunoblotting

Supplementary Figure Legends

Supplementary Figure 1: Representative histograms of CLL patient PBMCs stained for SLAMF1 and SLAMF7

Supplementary Figure 2: SLAMF1 and SLAMF7 expression and survival in CLL. (A) OS-Kaplan-Meier analysis of CLL patients from the ICGC dataset expressing both ($SLAMF^{double\text{-}high}$, N=10), only one ($SLAMF^{high}$, N=65) or none ($SLAMF^{low}$, N=229) of the SLAMF1 and SLAMF7 receptors as defined in Figure 1F. p= 0.0126. **(B)** OS-Kaplan-Meier analysis of M-CLL cases in the ICGC dataset according to their SLAMF status. p=0.0722. N=98; M-CLL-$SLAMF^{high}$=36, M-CLL-$SLAMF^{low}$=62. Statistical significance was calculated by using log-rank test.

OS – overall survival; M-CLL – CLL patients with a mutated immunoglobulin heavy chain gene

Supplementary Figure 3: SLAMF1 and SLAMF7 overexpression in the CLL cell lines JVM3 and Hg3. (A) JVM3 cells and **(C)** Hg3 cells were transduced with lentiviral particles encoding SLAMF1 and SLAMF7, overexpression was controlled via FC. Proliferation of SLAMF1 or 7 overexpressing **(B)** JVM3 and **(D)** Hg3 cells cells after 120h compared to control cell line transduced with empty vector. N=9. Data from independent experiments are shown as mean, error bars represent SEM, statistical significance was calculated using one-way Anova and Bonferroni's post-hoc tests.

FC – flow cytometry; SEM – standard error of the mean

Supplementary Figure 4: Modulation of BCR signaling by SLAMF1 and SLAMF7 in JVM3 and Hg3 CLL cells. (A) JVM3 cells were stained with FLUO4 and Ca^{2+} flux was assessed via FC after stimulation with anti-IgM in SLAMF1 or 7 overexpressing JVM3 cells, N=8. **(B)** Proliferation of JVM3 cells overexpressing SLAMF1 or SLAMF7 treated with 1µM Ibrutinb relative to untreated control after 96h. N=12. **(C)** Hg3 cells were stained with FLUO4 and Ca^{2+} flux was assessed via FC after stimulation with

anti-IgM in Hg3 cells after overexpression of SLAMF1 or SLAMF7. N=12. **(D)** Proliferation of Hg3 cells after overexpression of SLAMF1 or SLAMF7 treated with 1µM Ibrutinib relative to untreated control after 120h. N=12.
Data from independent experiments are shown as mean, error bars represent SEM, statistical significance was calculated by one-way ANOVA and Bonferroni's post-hoc test.

FC – flow cytometry; SEM – standard error of the mean

Supplementary Figure 5:MEC-1 Ig switch model (A) FC analysis of IgG switched MEC-1 cells. **(B**) FC analysis of IgG expressing MEC-1 cells transduced with lentiviral particles encoding for SLAMF1 or SLAMF7.

FC- flow cytometry

2.3. Dynamic changes of the normal B lymphocyte repertoire in CLL in response to ibrutinib or FCR chemo-immunotherapy

Authors

Simon Schliffke, Mariela Sivina, Ekaterina Kim, **Lisa von Wenserski**, Benjamin Thiele, Nuray Akyüz, Clemens Falker-Gieske, Donjete Statovci, Anna Oberle, Toni Thenhausen, Artus Krohn-Grimberghe, Carsten Bokemeyer, Nitin Jain, Zeev Estrov, Alessandra Ferrajoli, William Wierda, Michael Keating, Jan A Burger, Mascha Binder

Published in

Oncoimmunology 2018 Jan 15;7(4):e1417720. doi: 10.1080/2162402X.2017.1417720. eCollection 2018

ONCOIMMUNOLOGY
2018, VOL. 7, NO. 4, e1417720 (9 pages)
https://doi.org/10.1080/2162402X.2017.1417720

Taylor & Francis
Taylor & Francis Group

ORIGINAL RESEARCH

Dynamic changes of the normal B lymphocyte repertoire in CLL in response to ibrutinib or FCR chemo-immunotherapy

Simon Schliffke[a], Mariela Sivina[b], Ekaterina Kim[b], Lisa von Wenserski[a], Benjamin Thiele[a], Nuray Akyüz[a], Clemens Falker-Gieske[a], Donjete Statovci[a], Anna Oberle[a], Toni Thenhausen[c,d], Artus Krohn-Grimberghe[c,d], Carsten Bokemeyer[a], Nitin Jain[b], Zeev Estrov[b], Alessandra Ferrajoli[b], William Wierda[b], Michael Keating[b], Jan A. Burger[b,†], and Mascha Binder[a,†]

[a]Department of Oncology and Hematology, BMT with section Pneumology, Hubertus Wald Tumorzentrum / UCCH, University Medical Center Hamburg-Eppendorf, Martinistraße, Hamburg, Germany; [b]Department of Leukemia, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 0428, Houston, USA; [c]LYTIQ GmbH, Technologiepark, Paderborn, Germany; [d]Analytische Informationssysteme und Business Intelligence, Universität Paderborn, Warburger Straße, Paderborn, Germany

ABSTRACT

Using next-generation immunoglobulin (*IGH*) sequencing and flow cytometry, we characterized the composition, diversity and dynamics of non-malignant B cells in patients undergoing treatment with the Bruton tyrosine kinase (BTK) inhibitor ibrutinib or chemo-immunotherapy with fludarabine, cyclophosphamide, and rituximab (FCR). During ibrutinib therapy, non-malignant B cell numbers declined, but patients maintained stable *IGH* diversity and constant fractions of *IGH*-mutated B cells. This indicates partial preservation of antigen-experienced B cells during ibrutinib therapy, but impaired replenishment of the normal B cell pool with naïve B cells. In contrast, after FCR we noted a recovery of normal B cells with a marked predominance of B cells with unmutated *IGH*. This pattern is compatible with a deletion of pre-existing antigen-experienced B cells followed by repertoire renewal with antigen-naïve B cells. These opposite patterns in B cell dynamics may result in different responses towards neoantigens versus recall antigens, which need to be further defined.

ARTICLE HISTORY
Received 2 August 2017
Revised 24 November 2017
Accepted 12 December 2017

KEYWORDS
Ibrutinib; chronic lymphocytic leukemia
B lymphocyte repertoire; chemo-immunotherapy; Immunomonitoring

Introduction

Successful therapy of patients with chronic lymphocytic leukemia (CLL) depends upon effective disease control, but, equally important, the preservation of immunocompetence, given that common and opportunistic infections remain a leading cause of morbidity and mortality.[1] Disease-related immunodeficiency in CLL affects the innate and adaptive immune systems, and includes T cell defects, hypogammaglobulinemia, impaired complement activity, and neutrophil as well as natural killer cell dysfunction.[2] Despite an expansion of T lymphocytes in untreated CLL patients, these T cells are functionally defective, with impaired immunological synapse formation being a characteristic feature.[3-5] Normal B cell function also is impaired, resulting in hypogammaglobulinemia, which is part of the natural history of the disease.[6,7] Given these T and B cell deficiencies in CLL patients, antibody responses are often dampened, for example after prophylactic vaccination.[8,9] These disease-inherent immunodeficiencies are further modulated by CLL-directed therapy. Chemoimmunotherapy (CIT) with FCR, a standard first-line therapy regimen for younger patients with (*IGHV* mutated) low-risk disease,[10,11] and newer targeted agents, such as the BTK inhibitor ibrutinib, are highly effective treatments for CLL patients.[12-14] FCR, for example, effectively eliminates CLL cells in the majority of low-risk patients,[10,11] but it also causes a profound and prolonged depletion of T cells.[15] Therefore, infections that are attributed to neutropenia and/or T lymphopenia are relatively common after CIT.[16] In contrast, ibrutinib generally is not toxic to normal hematopoiesis, and particularly not to T cells.[12,17] Direct comparisons of infection rates between ibrutinib-based regimens and FCR are problematic due to lack of randomized data, but cross-trial comparisons suggest that the infectious risks in previously untreated CLL patients are lower with ibrutinib, at least during the first years on treatment.[10,12,18,19] Lack of myelo- and T lymphotoxicity, along with a change from Th2-biased immunity due to inhibition of interleukin-2-inducible T cell kinase (ITK) by ibrutinib may account for relatively low rates of infection,[20] together with generally stable immunoglobulin levels that may even improve during therapy (IgA).[21] However, in previously treated patients, infections remain a more common problem with ibrutinib; about one third of patients experience grade ≥3 infections during the first 12 months of ibrutinib treatment which is more comparable to infection rates in patients undergoing other salvage regimens.[14,22–24] In addition, atypical

CONTACT Mascha Binder MD m.binder@uke.de Department of Oncology and Hematology, BMT with section Pneumology, Hubertus Wald Tumorzentrum / UCCH, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 2067 Hamburg, Germany.

Supplemental data for this article can be accessed on the publisher's website.

[†]these authors share senior authorship.

Pneumocystis jirovecii pneumonia can also occur during ibrutinib treatment.[25] Due to the importance of a normal B cell compartment for immune function, we analyzed the non-malignant B cell repertoire in patients undergoing treatment with ibrutinib or FCR with immunosequencing technology to dissect composition, dynamics and diversity of this immune compartment.

Material and methods

Patient and sample characteristics

The complete blood counts, immunoglobulin levels, and infectious complications history were recorded and analyzed for a total of 40 patients treated at MD Anderson Cancer Center with either CIT (FCR) or ibrutinib on protocols that were approved by The University of Texas MD Anderson Cancer Center institutional review board and registered at clinicaltrials.gov (NCT00759798, NCT02007044). The clinical trial NCT00759798 is a single center phase II study testing FCR treatment in untreated or rituximab pretreated patients.[26] FCR treated patients received up to 6 courses of fludarabine 25 mg/m^2 given intravenously on days 2–4 of cycle 1 and days 1–3 of cycles 2 and beyond, cyclophosphamide 250 mg/m^2 given intravenously on days 2–4 of cycle 1 and days 1–3 of cycles 2 and beyond, and rituximab 375 mg/m^2 given intravenously on day 1 of course 1, and 500 mg/m^2 given intravenously on day 1 of subsequent cycles. The clinical trial NCT02007044 is a single center phase II study primarily testing ibrutinib or ibrutinib and rituximab (only samples from the ibrutinib arm were analyzed) in pretreated patients or untreated patients with 17p deletion or TP53 mutation. Ibrutinib (420 mg daily by mouth) was given continuously until disease progression or toxicities or complications precluded further therapy. Comparison of the patient characteristics between samples from both studies (Table 1), revealed that ibrutinib treated patients were more heavily pretreated, had lower white blood cell counts (WBC), lower Rai stage, and less favorable genetic risk factors. FCR treated patients completed a median of 6 cycles (n = 20, range: 3–6) and all ibrutinb treated patients included were on treatment in the analyzed period (n = 20). The prophylactic use of antimicrobials was at the treating physician's discretion. Informed consent for collection of research samples was obtained in accordance with institutional guidelines and the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) for next-generation sequencing (NGS) and flow cytometry analysis were collected from 10 representative patients from each cohort during study visits before and after treatment initiation with FCR (after 24 months) or ibrutinib (after 12 and 24 months of continuous treatment). Late follow-up samples after 42 months were analyzed from 4 FCR treated and 2 ibrutinib treated patients. In addition, material from 9 age-matched healthy donors and 30 previously published control patients without a hematological malignancy were analyzed.[27]

Multicolor flow cytometry for B cell phenotyping

As descriped previously,[27] within 2 hours of peripheral blood collection from control patients, erythrocyte lysis using a standard lysis buffer (ammonium chloride 8.29 g/l, EDTA 0.372 g/l, potassium hydrogen carbonate 1 g/l) was performed followed by flow cytometry using an 8-color flow cytometry panel (CD20-FITC, CD279(PD-1)-PE, CD38-ECD, IgM-PC-5.5, CD27-PC-7, CD19-APC – all purchased from Beckman Coulter). Measurements were performed on a Navios flow cytometer (Beckman Coulter, Krefeld, Germany). The gating strategy is shown in Figure S1.

Next-generation sequencing (NGS) of immunoglobulin heavy chain (IGH) immune repertoires

After isolation of genomic DNA from 5 × 10^6 – 3 × 10^7 PBMCs per sample using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Taufkirchen, Germany), the *IGH* gene locus containing the rearranged V_H, D_H and J_H segments was amplified by multiplex PCR (Supplementary Figure S2) using previously published primers.[17,28] Following purification with SPRIselect beads (Beckman Coulter, Krefeld,

Table 1. Patient characteristics*.

Characteristic	Ibrutinib (n = 20)	FCR (n = 20)	Healthy donor (n = 9)
Gender, male/female, n	15/5	13/7	4/5
Age, years (range)	65 (47 – 80)	59 (39 – 71)	60 (48 – 82)
Number of prior treatments, n (range)	2 (0 – 6)	0 (0 – 1)	—
Median time from last treatment, months (range)	15 (0 – 101)	0 (0 – 9)	—
Patients with prior FCR treatment, n	9	0	—
Median time from FCR treatment, months (range)	57 (29 – 105)	0	—
White blood cell count, 10^3/μl, median (range)	27,5 (8,8 – 224,7)	59,5 (23 – 219,4)	6,6 (5,9 – 8,2)
Hemoglobin, g/dl, median (range)	12 (10,2 – 16,1)	12,8 (8,9 – 16,4)	—
Platelet count 10^3/μl, median (range)	119,5 (31 – 368)	139,5 (67 – 254)	—
Rai stage, I/II/III/IV, n	3/8/2/6	1/8/7/4	—
Mutational status, unmutated/mutated, n	13/6	12/5	—
Cytogenetic abnormalities, n			
del(17p)	5	0	—
del(11q)	2	1	—
del(13q)	4	5	—
Trisomy 12	2	7	—
CD38, positive/negative, n	6/14	5/15	—
ZAP 70 positive/negative, n	11/6	11/8	—
Response to treatment, SD/PR/CR, n	1/16/3	0/1/19	—

*Data as absolute numbers or median (range). Material from 10 representative patients from each cohort was used for the NGS analysis.

Germany), amplicon extension with Illumina adapter sequences and unique barcodes was achieved through a second PCR reaction. Primers were purchased from Metabion (Martinsried, Germany) and PCRs were performed using Phusion HS II (Thermo Fisher Scientific Inc., Darmstadt, Germany) according to the supplier's instructions. Finally, amplicons with the expected size were purified after agarose gel electrophoresis using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). After amplicon quantification and quality control with a Qubit (QIAGEN, Hilden, Germany) and an Agilent 2100 Bioanalyzer (Agilent technologies, Böblingen, Germany) sequencing was performed on an Illumina MiSeq platform.

Immunoglobulin isotype specific NGS from pre- and post-treatment RNA samples.

After isolation of RNA from 5×10^6 – 3×10^7 PBMCs per sample using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), cDNA synthesis with the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia) allowed subsequent immunoglobulin isotype specific amplification as described previously.[29] Afterwards, preparation for NGS including amplicon extension with Illumina adapter sequences and unique barcodes was performed as described above.

Determination of absolute sizes of malignant and non-malignant peripheral blood B cell compartments by integration of flow cytometry and NGS data

The CD19+ subpopulation of previously frozen PBMCs was determined by flow cytometry (antibodies CD19-APC, CD5-FITC purchased from Beckman Coulter), which was performed on a FACS Calibur cytometer (BD Biosciences) and analyzed using Flowing Software (http://flowingsoftware.btk.fi). As visualized in Figure S3, for every sample and time point the number of granulocytes was subtracted from the white blood cell count (WBC), which equaled the concentration of PBMCs in the blood. This was multiplied with the relative amount of CD19+ cells to calculate the CD19+ cell count. Based on the CDR3 amino acid sequences determined by NGS, non-malignant B cells and CLL clones were defined. The fraction of non-malignant B cells was calculated by subtraction of sequences belonging to the CLL clone from the total B cell sequences. Afterwards, the absolute count of non-malignant B cells was determined by multiplication of the fraction of non-malignant B cells with the CD19+ cell count.

Illumina NGS, data analysis, bioinformatics and statistics.

NGS was performed using an Illumina MiSeq sequencer [500 or 600 cycle single indexed, paired-end runs (V2 or V3 chemistry)] with adapter-ligated spiked-in PhiX library to increase diversity. The MiSeq reporter performed demultiplexing. Subsequently, FASTQ data analysis was computed in an analysis pipeline, which is based on the MiXCR software.[30] FASTQ data are available from the Sequence Read Archive, accession number PRJEB23571. Summarized NGS data is available as supplemental table S1. *IGH* hypermutation was defined as less then 97% identity between the V_H-gene sequence and the corresponding germline gene. This definition was chosen based on correlation of the NGS results (pooled analysis of >360.000 aligned reads and >38.000 clonotypes) with flow cytometry measuring antigen-naïve and -experienced B cells in a cohort of 30 control patients (Figure S4 and S5). *IGH* clonotype diversity was quantified with the Shannon-Wiener and inverse Simpson diversity indices.[31,32] Shannon-Wiener is a diversity index of first order disproportionately sensitive to the rare species/clonotypes, inverse Simpson of second order disproportionately sensitive to the most common species/clonotypes.[31] Indices were calculated for each sample and time point to be able to monitor changes in immune repertoire diversity before and after treatment initiation. Only when both diversity indices increased after treatment, *IGH* diversification was assumed. Student's t-test (paired samples) and one-way ANOVA followed by suitable posthoc tests (multiple samples) was used to calculate statistical significance as described in detail in the figure legends.

Results

Ibrutinib quantitatively suppresses peripheral blood non-malignant B cell repertoires

We quantitatively analyzed B cell counts in a subset of ten representative patients treated with ibrutinib versus ten patients treated with FCR by flow cytometry and NGS of the *IGH* locus. While many of the patients in the ibrutinib cohort were substantially pre-treated, all patients treated with FCR received this as first-line therapy (median number of 2 versus 0 prior treatments). In addition, ibrutinib treated patients had a less favorable genetic risk (5 versus 0 patients with del(17p)). Pre-treatment CLL cell counts were higher in the FCR cohort compared to the ibrutinib cohort and significantly reduced 24 months after initiation of treatment in both cohorts (Figure 1A). The post-treatment reduction of the CLL cell count was more pronounced in the FCR cohort and complete remission of the disease was achieved in 9 of 10 patients after 24 months. Responses to ibrutinib were mostly partial remissions (Table 1), which is in agreement with previous studies.[12,14] Despite patients having received prior treatments in the ibrutinib cohort, the non-malignant B cell counts before treatment initiation were similar in both patient cohorts with relatively normal B cell counts (mean +/− SEM: FCR 268 +/− 83 cells/μl; ibrutinib 218 +/− 73 cells/μl). This is comparable to healthy elderly individuals in whom we measured B cell counts of 256 +/− 33 cells/μl (mean +/− SEM), which is in agreement with published values.[33] Ibrutinib treatment significantly decreased the non-malignant B cell count after 24 months of treatment (mean +/− SEM: 42 +/− 12 cells/μl, $p = 0.0201$, Fig. 1B), while B cell counts recovered back to baseline levels in the FCR cohort (mean +/− SEM: 344 +/− 166 cells/μl, Fig. 1B). After 42 months of ibrutinib treatment, B cell counts remained low in the two available follow-up samples (mean +/− SEM: 10 +/− 9 cells/μl, Fig. 1B). In four FCR treated patients with early recurrence of the disease, normal B cell counts were decreased nonsignificantly 42 months after treatment (mean +/− SEM: 96 +/− 48 cells/μl, Fig. 1B).

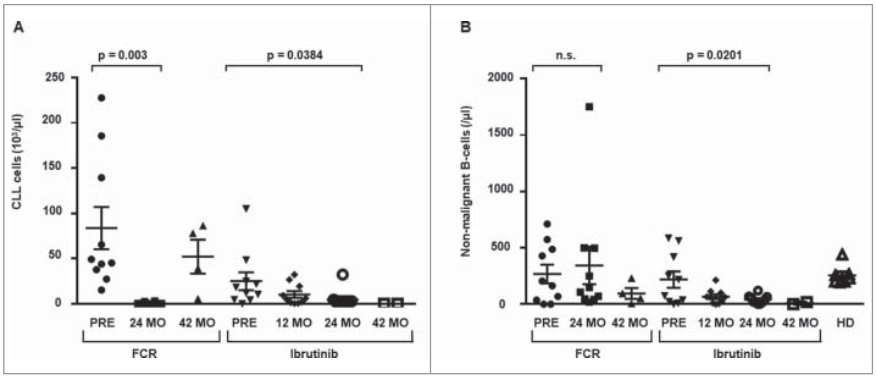


Figure 1. Global CLL and non-malignant B cell count in the peripheral blood. Changes in cell count were determined by flow cytometry and *IGH* next-generation sequencing prior, 24 months and 42 months after chemoimmuntherapy (FCR) or prior, 12 months, 24 months and 42 months after initiation of ibrutinib and from age-matched healthy donors (HD) A: Change in CLL cell count. B: Change in non-malignant B cell count. Horizontal lines show mean values and error bars show the SEM, n = 2-10 for each group. Statistical significance testing was performed using student's t-test.

Redistribution of IGH hypermutated, antigen-experienced non-malignant B cells over the course of treatment

Next, we determined the dynamics of non-malignant B cell immune repertoire composition before and after FCR or during continuous ibrutinib treatment. Based on the mutational status of the V_H gene, non-malignant B cells were classified as *IGH* hypermutated (<97% identity to the corresponding germline V_H gene, corresponding to antigen-experienced B cells) or *IGH* unmutated (≥97% identity to the corresponding germline V_H gene, corresponding to antigen-naïve B cells). This definition was experimentally determined in a control cohort of 30 individuals without hematological cancer through correlation of NGS immunosequencing with flow cytometry data from B cell immunophenotyping (Figure S4 and S5). Before treatment initiation, the mean percentage of antigen-experienced B cells was similar between the FCR cohort and age-matched healthy donors (HD) (mean +/− SEM of *IGH* mutated B cells: FCR 50 +/− 7%, HD 35 +/− 5%), while significantly less antigen-experienced B cells were measured in the ibrutinib cohort (mean +/− SEM of *IGH* mutated B cells: ibrutinib 22 +/− 6%, p = 0,0033). After 24 months, a significant decrease in antigen-experienced B cells was noted in the FCR cohort, while the ratio of antigen-experienced and antigen-naïve B cells remained unchanged in ibrutinib treated patients (mean +/− SEM of *IGH* mutated B cells: ibrutinib 34 +/− 7%, FCR 8 +/− 2%, p = 0.0001, Fig. 2). Confirming the time point at 24 months, stable distributions were determined in available samples after 42 months (mean +/− SEM of *IGH* mutated B cells: ibrutinib 39 +/− 13%, FCR 12 +/− 1%, p = 0.0001, Fig. 2) .Together, these data suggest a substantial deletion of the experienced B cell compartment, which is replaced with antigen-naïve B cells in the FCR cohort (exemplarily visualized in Fig. 3A). In contrast, ibrutinib treated patients had a preserved proportion of antigen-experienced B cells, while – quantitatively – the B cell repertoire was contracted (exemplarily visualized in Fig. 3B). We wished to confirm our results by *IGH* diversity analysis, assuming that the diversity of the regenerated FCR B cell repertoires should be highly increased, while ibrutinib B cell repertoires should show stable *IGH* diversity indices. Differences in *IGH* clonotype distributions were studied using the Shannon-Wiener diversity index, which is more sensitive to rare species/clonotypes, and the inverse Simpson diversity index, which is disproportionately sensitive to the most common species/clonotypes.[31] Indices were calculated for each sample and time point to be able to compare immune repertoire diversity before and after initiation of treatment. Before treatment initiation, the non-malignant *IGH* repertoire was composed of balanced numbers of antigen-experienced and antigen-naïve medium sized clones in both cohorts. In line with the *IGH* repertoire shift towards antigen-naïve B cells in FCR treated patients, the medium-sized clones disappeared after treatment, with large numbers of small-sized unmutated clones dominating after 24 months, resulting in increased repertoire diversity (Fig. 4A, p < 0.0001 and 4B, p = 0.0002). In ibrutinib treated patients, the B cell repertoire showed an unchanged distribution of antigen-experienced and antigen-naïve B cells, resulting in stable repertoire diversity (Fig. 4A and 4B). Immunoglobulin isotype specific NGS from RNA samples before and 24 months after FCR treatment confirmed that the diversification was driven by

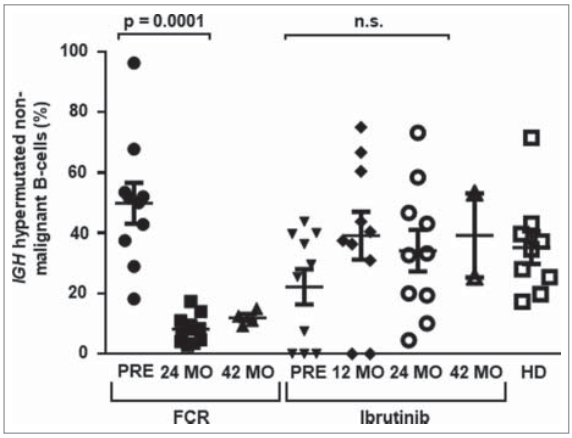


Figure 2. Distribution of experienced and naïve non-malignant B cells. Hypermutated antigen-experienced non-malignant B cells determined through NGS. Samples were analyzed prior, 24 months and 42 months after FCR or prior, 12 months, 24 months and 42 months after initiation of ibrutinib treatment and from age-matched healthy donors (HD). Horizontal lines show mean values and error bars show the SEM, n = 2-10 for each group. Statistical significance testing was performed using student's t-test.

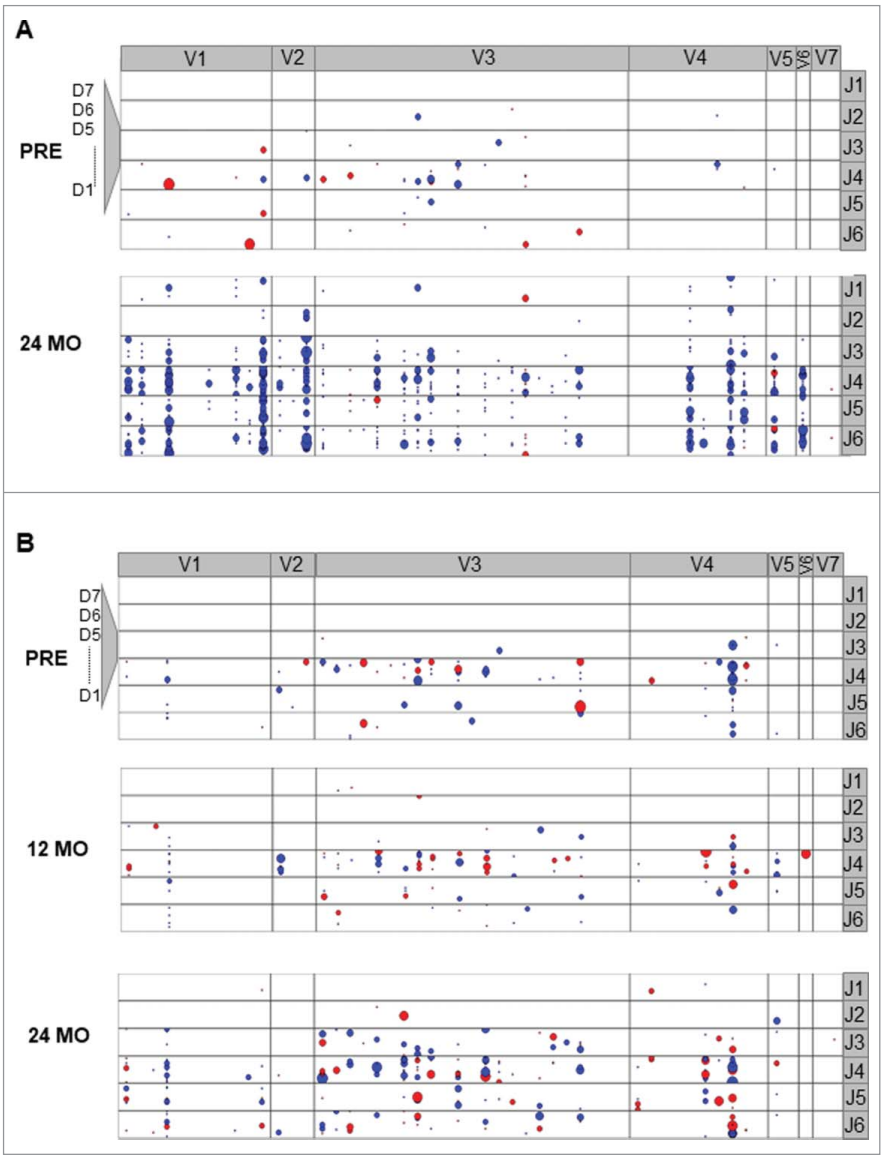


Figure 3. Exemplary *IGH* clonotype distribution of non-malignant B cell repertoires in the peripheral blood. A: Exemplary FCR treated patient. B: Exemplary ibrutinib treated patient. Coordinates of each dot are defined by the unique V_H, D_H and J_H gene rearrangement. V_H gene subgroups (*V1-2 – V7-81*) are shown from left to right, D_H gene subgroups (*D1-1 – D7-27*) are shown from bottom to top. Dot size corresponds to the frequency. Blue color: unmutated V_H gene sequence, red: hypermutated V_H gene sequence.

IgM expressing naïve B cells, while IgA and IgG repertoire diversities remained stable (Fig. 4C and 4D, p = 0.017). The IgM, IgA and IgG compartments from ibrutinib treated patients remained stable throughout treatment confirming our DNA-based sequencings (Fig. 4C and 4D).

Discussion

The adaptive immune system is severely impaired in CLL patients, with hypogammaglobulinemia and T cell dysfunction representing cardinal features.[2–4] This immune dysfunction is associated with ineffective immune responses towards the malignant B cells, autoimmune phenomena, and high susceptibility for infectious complications, which remain a leading cause for morbidity and mortality in CLL patients.[1] CIT with purine analogs, alkylating agents, and CD20 antibodies, such as FCR, remain a standard of care therapy option for the front-line treatment of younger low-risk patients, and are nowadays less commonly used for recurrent disease due to the advent of the kinase inhibitors targeting BCR signaling, such as ibrutinib, and the BCL-2 antagonist venetoclax.[18,19,34,35] The myelo- and lymphotoxicity of CIT is associated with infectious complications in a significant proportion of patients.[15,16,36] In contrast, ibrutinib generally lacks myelotoxicity, neutropenia is rarely seen with ibrutinib, and – although it can modulate T cell function – it is thought to lack direct toxicity towards T cells. However, the consequences of long-term inhibition of BTK in CLL patients on the adaptive immune system and especially the B cell compartment remain incompletely defined. Children with XLA carrying functional null BTK mutations lack peripheral blood B cells and have markedly decreased or absent serum immunoglobulins of all isotypes.[37] This is due to failed

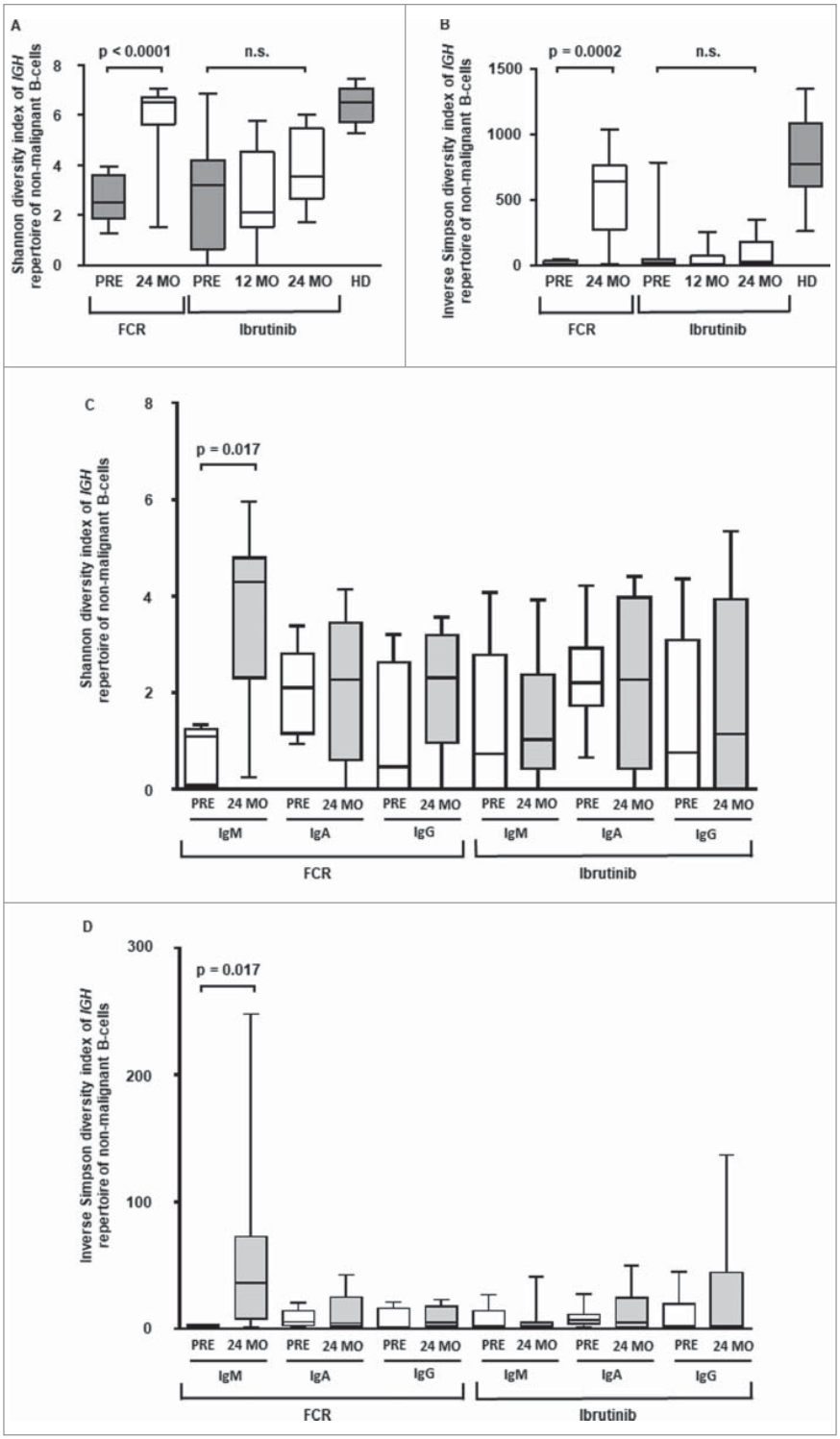


Figure 4. Diversity of the non-malignant B cell repertoire. A and B: Diversity of the non-malignant *IGH* repertoire based on genomic DNA with Shannon-Wiener and inverse Simpson diversity index. C and D: Isotype specific *IGH* repertoire diversity based on RNA sequencing with Shannon-Wiener and inverse Simpson diversity index. Horizontal lines show mean values and error bars show the SEM, n = 5-10 for each group. Statistical significance testing was performed using student's t-test.

development of B cell precursors in the bone marrow into mature B lymphocytes. In addition, BTK is also essential for germinal center reactions, as B cells from atypical XLA patients with mildly impaired BTK function show class switch defects and low frequencies of somatic hypermutation, while BTK overexpression increases germinal center formation.[38,39] CLL patients treated with ibrutinib have relatively stable levels of immunoglobulins and do not exhibit an increasing incidence of infections during the first years of treatment, presumably because, in contrast to children with XLA, these patients have an antigen-experienced B cell compartment. Moreover, BTK is downregulated in plasma cells and therefore ibrutinib may not

affect Ig production of preexisting plasma cells. However, the effect of ibrutinib on the normal B cell compartment and the composition of the B cell repertoire have not yet been studied.

Therefore, we examined the non-malignant B cell repertoire after or during treatment with CIT (FCR) or continuous BTK inhibition with ibrutinib using state-of-the-art next-generation immunosequencing. Interestingly, we noted strikingly different changes in the B cell compartment in both patient groups; FCR resulted in a major renewal of the B cell compartment with depletion of pre-existing B memory cells and emergence of large numbers of naïve antigen-inexperienced B cells. In contrast, ibrutinib treatment preserved, in part, the non-malignant pre-treatment B cell repertoire, but total B cell numbers declined and were not replenished by naïve B cells.

The declining numbers of B cells in ibrutinib treated patients, along with lack of replenishment by naïve B cells, are reminiscent of XLA patients, with the fundamental difference that CLL patients have a plasma cell pool to maintain Ig levels that is not affected by the treatment, which protects patients from frequent infections. However, our data also suggest that ibrutinib treated patients may have impaired responses towards neoantigens and consequently responses towards vaccines may be dampened. In XLA patients, this has been shown to be a characteristic feature that distinguishes XLA from other immunodeficiencies. In an elegant study, Ochs and colleagues explored responses to bacteriophage varphiX 174, and noted prolonged circulation of phage and no detectable antibody response as distinguishing characteristics of XLA.[40] In patients with CLL, vaccine studies might be complicated to conduct and interpret due to the pre-existing disease-inherent deficiencies in vaccine responses, but generally such studies are much-needed to refine our infection-preventive measures in this patient group.

Overall, comparisons between the two analyzed cohorts in this study need to be interpreted with caution, since patient characteristics differed, with ibrutinib treated patients having a less favorable genetic risk and being more heavily pretreated (median 2 versus 0 prior treatments). The effects of a drug on the immunocompetence through modulation of the B cell repertoire also needs to be seen in the context of baseline, disease-associated immunosuppression, as it is well established that not only more advanced CLL, but also a high number of pretreatments negatively impact immunocompetence.[22] Differences in the cohorts were also reflected by divergent levels of pretreatment ratios of naive versus antigen-experienced non-malignant B cells: While in the FCR cohort about equal numbers of naive and antigen-experienced B cells were seen, the percentage of naive B cells was higher in ibrutinib patients, who had received ibrutinib in a treatment line most often following CIT.

In summary, our study provides insight into the differential effects of CLL therapy on the normal B cell compartment. While FCR therapy depletes the experienced B cell compartment, followed by replenishment with naïve B cells, ibrutinib therapy over time results in a decline in normal B cell numbers and lack of replenishment with naïve B cells. This is consistent with the different mechanism of action of these types of therapy, acute lymphotoxicity in case of FCR and inhibition of BCR signaling-dependent growth and maturation of B cells in case of ibrutinib. In contrast, the data from the FCR treated cohort suggest that in these patients memory B cell responses may be more altered. Further studies about normal B cell development and function are warranted to address these questions.

Disclosure of potential conflicts of interest

A potential conflict of interest was declared: J.A.B. received research funding from Pharmacyclics. The remaining authors declare no potential conflicting interests.

Financial support

To J.A.B: Leukemia & Lymphoma Society Scholar Award in Clinical Research; MD Anderson's Moon Shot Program in CLL; and MD Anderson Cancer Center Support under Grant CA016672

To M.B.: Eppendorfer Krebs- und Leukämiehilfe e.V.; Annemarie Hilgemann Stiftung; Deutsche Krebshilfe under Grant 110906; and Hubertus Wald-Foundation.

Authorship contributions

M.B. designed the study, supervised the experiments, analyzed data and wrote the manuscript. J.B. designed the study, provided patient samples, analyzed data and wrote the manuscript. S.S. designed, performed and interpreted the experiments and wrote the manuscript. M.S and E.K. provided samples, analyzed clinical data and critically revised the manuscript. N.A. analyzed data and wrote the manuscript. L.W., B.T., C.F.-G., A.O. and D.S. analyzed data. T.T. and A.K.-G. provided the bioinformatics pipeline. C.B. interpreted data and critically revised the manuscript. N.J., Z.E., A.F., W.W. and M.K. provided samples and critically revised the manuscript. All authors reviewed the manuscript and approved the final version.

Acknowledgments

We thank Christiane Horn, Katrin Kluge and Annette Schäfer for expert technical assistance with multicolour flow cytometry. We also thank the healthy donors.

Funding

MD Anderson Cancer Center Support ID: (CA016672); Deutsche Forschungsgemeinschaft (DFG) ID: (110906).

ORCID

Clemens Falker-Gieske http://orcid.org/0000-0001-9160-1909

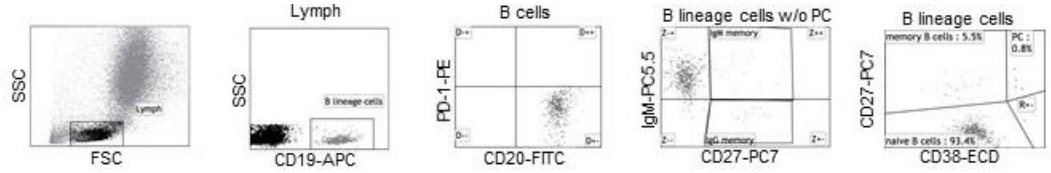
References

1. Morrison V. Infectious complications in patients with chronic lymphocytic leukemia: pathogenesis, spectrum of infection, and approaches to prophylaxis. Clin Lymphoma Myeloma. 2009;9:365–70. doi:10.3816/CLM.2009.n.071. PMID:19858055.
2. Forconi F, Moss P. Perturbation of the normal immune system in patients with CLL. Blood. 2015;126:573–81. doi:10.1182/blood-2015-03-567388. PMID:26084672.
3. Rissiek A, Schulze C, Bacher U, Schieferdecker A, Thiele B, Jacholkowski A, Flammiger A, Horn C, Haag F, Tiegs G, et al. Multidimensional scaling analysis identifies pathological and prognostically relevant profiles of circulating T-cells in chronic lymphocytic leukemia. Int J Cancer. 2014;135:2370–9. doi:10.1002/ijc.28884. PMID:24723150.
4. Ramsay AG, Johnson AJ, Lee AM, Gorgün G, Dieu R Le, Blum W, Byrd JC, Gribben JG. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with

an immunomodulating drug. J Clin Invest. 2008;118:2427–37. PMID:18551193.
5. Christopoulos P, Pfeifer D, Bartholomé K, Follo M, Timmer J, Fisch P, Veelken H. Definition and characterization of the systemic T-cell dysregulation in untreated indolent B-cell lymphoma and very early CLL. Blood. 2011;117:3836–46. doi:10.1182/blood-2010-07-299321. PMID:21270444.
6. Rozman C, Montserrat E, Viñolas N. Serum immunoglobulins in B-chronic lymphocytic leukemia. Natural history and prognostic significance. Cancer. 1988;61:279–83. doi:10.1002/1097-0142(19880115)61:2%3c279::AID-CNCR2820610215%3e3.0.CO;2-4. PMID:2446736.
7. Boughton BJ, Jackson N, Lim S, Smith N. Randomized trial of intravenous immunoglobulin prophylaxis for patients with chronic lymphocytic leukaemia and secondary hypogammaglobulinaemia. Clin Lab Haematol. 1995;17:75–80. doi:10.1111/j.1365-2257.1995.tb00322.x. PMID:7621634.
8. Hartkamp A, Mulder AH., Rijkers G, van Velzen-Blad H, Biesma D. Antibody responses to pneumococcal and haemophilus vaccinations in patients with B-cell chronic lymphocytic leukaemia. Vaccine. 2001;19:1671–7. doi:10.1016/S0264-410X(00)00409-6. PMID:11166890.
9. Sinsalo M, Aittoniemi J, Käyhty H, Vilpo J. Haemophilus influenzae type b (Hib) antibody concentrations and vaccination responses in patients with chronic lymphocytic leukaemia: predicting factors for response. Leuk Lymphoma. 2002;43:1967–9. doi:10.1080/1042819021000015916. PMID:12481893.
10. Keating MJ, O'Brien S, Albitar M, Lerner S, Plunkett W, Giles F, Andreeff M, Cortes J, Faderl S, Thomas D, et al. Early Results of a Chemoimmunotherapy Regimen of Fludarabine, Cyclophosphamide, and Rituximab As Initial Therapy for Chronic Lymphocytic Leukemia. J Clin Oncol. 2005;23:4079–88. doi:10.1200/JCO.2005.12.051. PMID:15767648.
11. Hallek M, Fischer K, Fingerle-Rowson G, Fink A, Busch R, Mayer J, Hensel M, Hopfinger G, Hess G, von Grünhagen U, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. Lancet. 2010;376:1164–74. doi:10.1016/S0140-6736(10)61381-5. PMID:20888994.
12. Burger JA, Tedeschi A, Barr PM, Robak T, Owen C, Ghia P, Bairey O, Hillmen P, Bartlett NL, Li J, et al. Ibrutinib as Initial Therapy for Patients with Chronic Lymphocytic Leukemia. N Engl J Med. 2015;373:2425–37. doi:10.1056/NEJMoa1509388. PMID:26639149.
13. O'Brien S, Furman RR, Coutre SE, Sharman JP, Burger JA, Blum KA, Grant B, Richards DA, Coleman M, Wierda WG, et al. Ibrutinib as initial therapy for elderly patients with chronic lymphocytic leukaemia or small lymphocytic lymphoma: an open-label, multicentre, phase 1b/2 trial. Lancet Oncol. 2014;15:48–58. doi:10.1016/S1470-2045(13)70513-8. PMID:24332241.
14. Byrd JC, Brown JR, O'Brien S, Barrientos JC, Kay NE, Reddy NM, Coutre S, Tam CS, Mulligan SP, Jaeger U, et al. Ibrutinib versus Ofatumumab in Previously Treated Chronic Lymphoid Leukemia. N Engl J Med. 2014;371:213–23. doi:10.1056/NEJMoa1400376. PMID:24881631.
15. Ysebaert L, Gross E, Kühlein E, Blanc A, Corre J, Fournié JJ, Laurent G, Quillet-Mary A. Immune recovery after fludarabine-cyclophosphamide-rituximab treatment in B-chronic lymphocytic leukemia: implication for maintenance immunotherapy. Leukemia. 2010;24:1310–6. doi:10.1038/leu.2010.89. PMID:20463751.
16. Tam CS, O'Brien S, Wierda W, Kantarjian H, Wen S, Do K-A, Thomas DA, Cortes J, Lerner S, Keating MJ. Long-term results of the fludarabine, cyclophosphamide, and rituximab regimen as initial therapy of chronic lymphocytic leukemia. Blood. 2008;112:975–80. doi:10.1182/blood-2008-02-140582. PMID:18411418.
17. Schliffke S, Akyüz N, Ford CT, Mährle T, Thenhausen T, Krohn-Grimberghe A, Knop S, Bokemeyer C, Binder M. Clinical response to ibrutinib is accompanied by normalization of the T-cell environment in CLL-related autoimmune cytopenia. Leukemia. 2016;30:2232–4. doi:10.1038/leu.2016.157. PMID:27220665.
18. Hallek M. Chronic lymphocytic leukemia: 2015 Update on diagnosis, risk stratification, and treatment. Am J Hematol. 2015;90:446–60. doi:10.1002/ajh.23979. PMID:25908509.
19. Eichhorst B, Fink A-M, Bahlo J, Busch R, Kovacs G, Maurer C, Lange E, Köppler H, Kiehl M, Sökler M, et al. 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. Lancet Oncol. 2016;17:928–42. doi:10.1016/S1470-2045(16)30051-1. PMID:27216274.
20. Dubovsky JA, Beckwith KA, Natarajan G, Woyach J, Jaglowski S, Zhong Y, Hessler JD, Liu T-M, Chang BY, Larkin KM, et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. Blood. 2013;122:2539–49. doi:10.1182/blood-2013-06-507947. PMID:23886836.
21. Sun C, Tian X, Lee Y, Gunti S, Lipsky A, Herman S, Salem D, Stetler-Stevenson M, Yuan C, Kardava L, et al. Partial reconstitution of humoral immunity and fewer infections in patients with chronic lymphocytic leukemia treated with ibrutinib. Blood. 2015;126:2213–9. doi:10.1182/blood-2015-04-639203. PMID:26337493.
22. Byrd JC, Furman RR, Coutre SE, Burger JA, Blum KA, Coleman M, Wierda WG, Jones JA, Zhao W, Heerema NA, et al. Three-year follow-up of treatment-naive and previously treated patients with CLL and SLL receiving single-agent ibrutinib. Blood. 2015;125:2497–506. doi:10.1182/blood-2014-10-606038. PMID:25700432.
23. Wierda WG, Kipps TJ, Mayer J, Stilgenbauer S, Williams CD, Hellmann A, Robak T, Furman RR, Hillmen P, Trneny M, et al. Ofatumumab As Single-Agent CD20 Immunotherapy in Fludarabine-Refractory Chronic Lymphocytic Leukemia. J Clin Oncol. 2010;28:1749–55. doi:10.1200/JCO.2009.25.3187. PMID:20194866.
24. Perkins JG, Flynn JM, Howard RS, Byrd JC. Frequency and type of serious infections in fludarabine-refractory B-cell chronic lymphocytic leukemia and small lymphocytic lymphoma: implications for clinical trials in this patient population. Cancer. 2002;94:2033–9. doi:10.1002/cncr.0680. PMID:11932906.
25. Ahn IE, Jerussi T, Farooqui M, Tian X, Wiestner A, Gea-Banacloche J. Atypical Pneumocystis jirovecii pneumonia in previously untreated patients with CLL on single-agent ibrutinib. Blood. 2016;128:1940–3. doi:10.1182/blood-2016-06-722991. PMID:27503501.
26. Strati P, Keating MJ, O'Brien SM, Burger J, Ferrajoli A, Jain N, Tambaro FP, Estrov Z, Jorgensen J, Challagundla P, et al. Eradication of bone marrow minimal residual disease may prompt early treatment discontinuation in CLL. Blood. 2014;123:3727–32. doi:10.1182/blood-2013-11-538116. PMID:24705492.
27. Akyüz N, Brandt A, Stein A, Schliffke S, Mährle T, Quidde J, Goekkurt E, Loges S, Haalck T, Ford CT, et al. T-cell diversification reflects antigen selection in the blood of patients on immune checkpoint inhibition and may be exploited as liquid biopsy biomarker. Int J Cancer. 2017;140:2535–44. doi:10.1002/ijc.30549. PMID:27925177.
28. van Dongen JJM, Langerak AW, Brüggemann M, Evans PAS, Hummel M, Lavender FL, Delabesse E, Davi F, Schuuring E, García-Sanz R, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17:2257–317. doi:10.1038/sj.leu.2403202. PMID:14671650.
29. Thiele B, Kloster M, Alawi M, Indenbirken D, Trepel M, Grundhoff A, Binder M. Next-generation sequencing of peripheral B-lineage cells pinpoints the circulating clonotypic cell pool in multiple myeloma. Blood. 2014;123:3618–21. doi:10.1182/blood-2014-02-556746. PMID:24753536.
30. Bolotin DA, Poslavsky S, Mitrophanov I, Shugay M, Mamedov IZ, Putintseva E V, Chudakov DM. MiXCR: software for comprehensive adaptive immunity profiling. Nat Methods. 2015;12:380–1. doi:10.1038/nmeth.3364. PMID:25924071.
31. Jost L. Partitioning diversity into independent alpha and beta components. Ecology. 2007;88:2427–39. doi:10.1890/06-1736.1. PMID:18027744.
32. Kirsch I, Vignali M, Robins H. T-cell receptor profiling in cancer. Mol Oncol. 2015;9:2063–70. doi:10.1016/j.molonc.2015.09.003. PMID:26404496.
33. Erkeller-Yuksel FM, Deneys V, Yuksel B, Hannet I, Hulstaert F, Hamilton C, Mackinnon H, Stokes LT, Munhyeshuli V, Vanlangendonck F. Age-related changes in human blood lymphocyte subpopulations. J Pediatr. 1992;120:216–22. doi:10.1016/S0022-3476(05)80430-5. PMID:1735817.

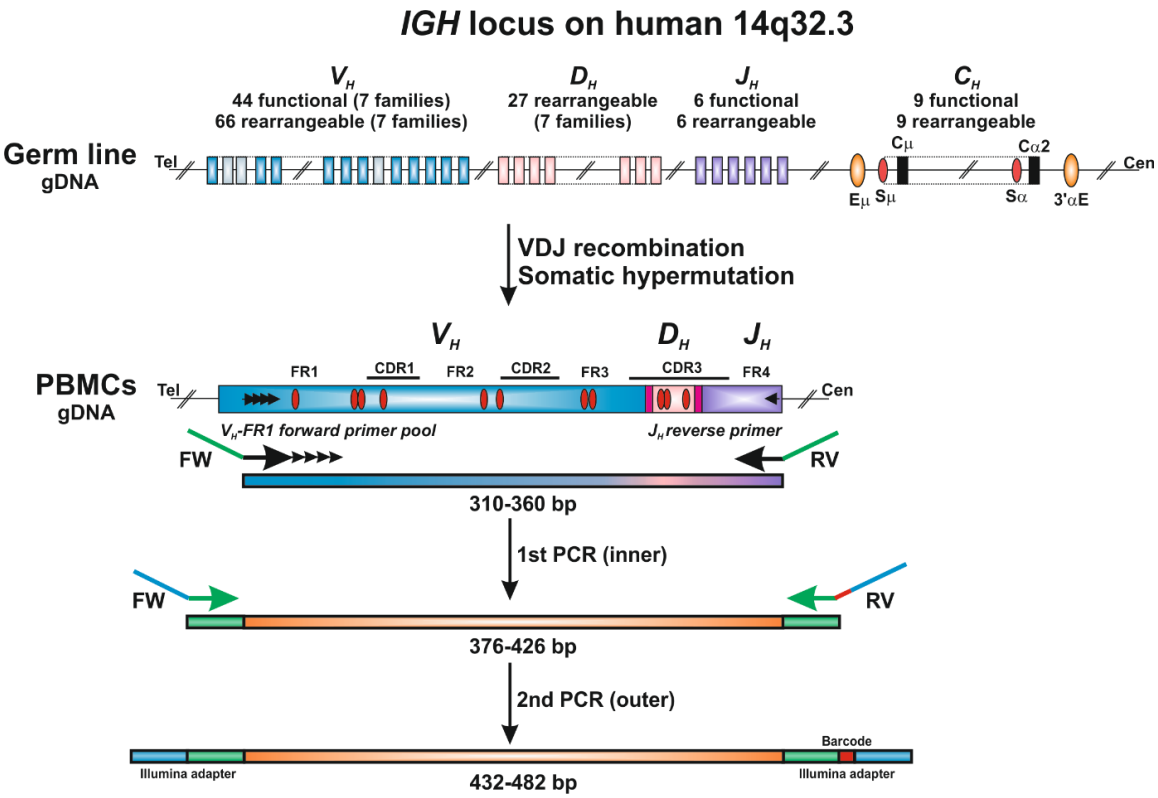
34. Jain N, O'Brien S. Initial treatment of CLL: integrating biology and functional status. Blood. 2015;126:463–70. doi:10.1182/blood-2015-04-585067. PMID:26065656.
35. Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD, Gerecitano JF, Kipps TJ, Anderson MA, Brown JR, Gressick L, et al. Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med. 2016;374:311–22. doi:10.1056/NEJMoa1513257. PMID:26639348.
36. García Muñoz R, Izquierdo-Gil A, Muñoz A, Roldan-Galiacho V, Rabasa P, Panizo C. Lymphocyte recovery is impaired in patients with chronic lymphocytic leukemia and indolent non-Hodgkin lymphomas treated with bendamustine plus rituximab. Ann Hematol. 2014;93:1879–87. doi:10.1007/s00277-014-2135-8. PMID:24951124.
37. Ponader S, Burger JA. Bruton's tyrosine kinase: from X-linked agammaglobulinemia toward targeted therapy for B-cell malignancies. J Clin Oncol. 2014;32:1830–9. doi:10.1200/JCO.2013.53.1046. PMID:24778403.
38. Mitsuiki N, Yang X, Bartol SJW, Grosserichter C. Mutations in Bruton' s tyrosine kinase impair IgA responses. Int J Hematol. 2015;101:305–13. doi:10.1007/s12185-015-1732-1. PMID:25589397.
39. Kil LP, de Bruijn MJW, van Nimwegen M, Corneth OBJ, van Hamburg JP, Dingjan GM, Thaiss F, Rimmelzwaan GF, Elewaut D, Delsing D, et al. Btk levels set the threshold for B-cell activation and negative selection of autoreactive B cells in mice. Blood. 2012;119:3744–56. doi:10.1182/blood-2011-12-397919. PMID:22383797.
40. Ochs HD, Davis SD, Wedgwood RJ. Immunologic responses to bacteriophage ϕX 174 in immunodeficiency diseases. J Clin Invest. 1971;50:2559–68. doi:10.1172/JCI106756. PMID:5129308.

Supplementary Figure S1



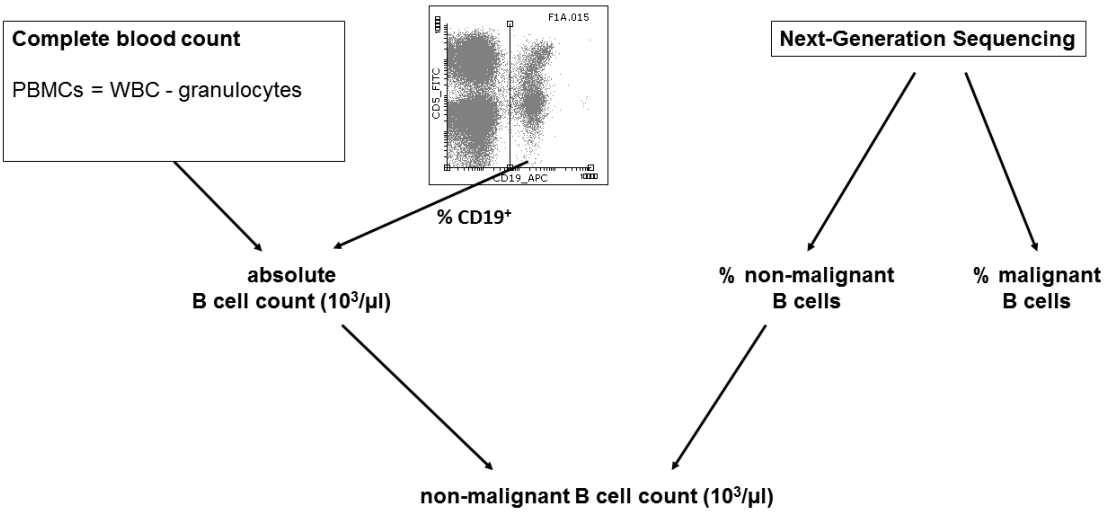
Supplementary Figure S1: Representative flow cytometry plots for B-cell phenotyping. Naïve B cells, memory B cells and plasma cells.

Supplementary Figure S2



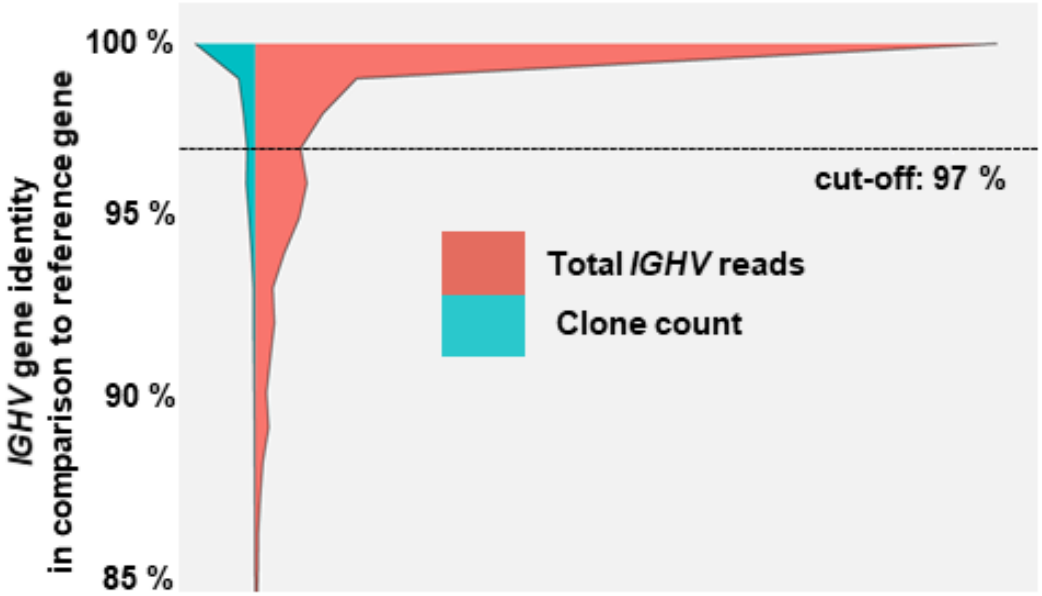
Supplementary Figure S2: Amplification scheme of the rearranged human *IGH* locus. Scheme of the human *IGH* locus consisting of variable (V_H, filled boxes in blue), diversity (D_H, pink), joining (J_H, purple) and constant (black) segments in the germ line (upper scheme) and after recombination events in peripheral blood mononuclear cells (PBMCs). Red circles within the *VDJ* schema after recombination indicate somatic hypermutation and filled boxes in magenta indicate nucleotides possibly added to the *V-D* and *D-J* junctions during the recombination process. Framework (FR1-4) and complementarity determining regions (CDR1-3) are indicated. Primers for the first PCR annealing to the *IGH* gene are indicated with black arrows. Illumina-compatible adapters are shown in green (fused after the first PCR) and blue (fused after the second PCR). The barcode consisting of 7 nucleotides is included within the reverse primer of the 2nd PCR and is shown in red. Cen: Centromere. FW: forward primer. RV: reverse primer. Tel: telomere.

Supplementary Figure S3



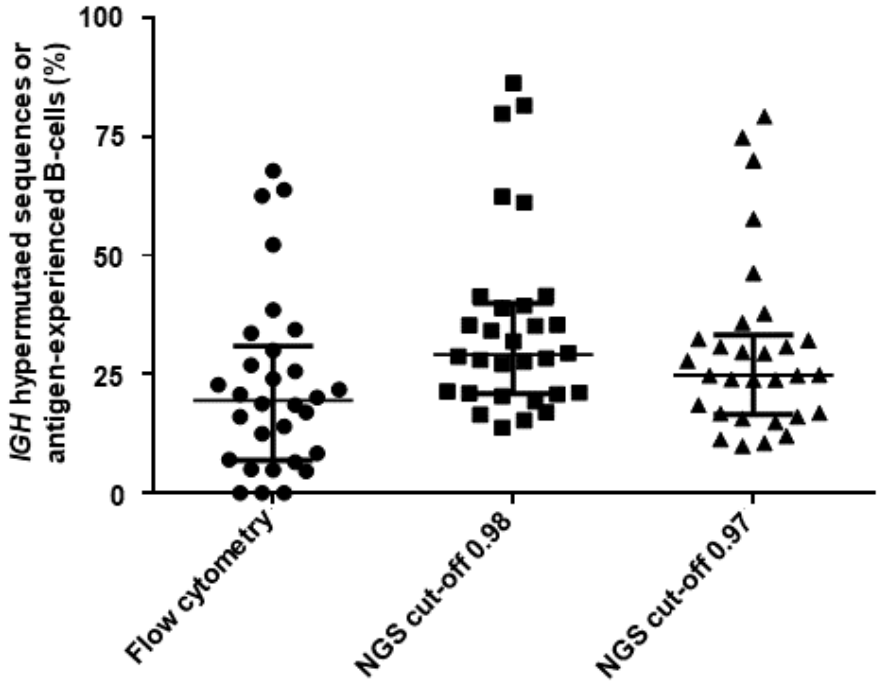
Supplementary Figure S3: Integration of flow cytometry and NGS data. The PBMC count is the white blood cell count (WBC) subtracted by the granulocyte count, which, after multiplication with the fraction of $CD19^+$ cells (measured with flow cytometry) equals the absolute B cell count. Using next generation sequencing of the human *IGH* locus, the fraction of non-malignant B cells can be determined to calculate the absolute non-malignant B cell count.

Supplementary Figure S4



Supplementary Figure S4: Distribution of reads or clonotypes according to relative V gene identity. Each *IGH* sequence from a cohort of 30 control patients was plotted with R statistical software tool according to the relative identity of the V gene in comparison to the reference gene. The shown pooled data comprises >360.000 successfully aligned *IGH* reads and >38.000 clones.

Supplementary Figure S5



Supplementary Figure S5: Comparison of *IGH* hypermutated sequences and flow cytometry to define antigen-experienced B-cells in a control patient cohort. PBMCs were stained for CD19, CD27 and CD38 to determine the ratio of naïve B-cells ($CD27^{low}$) versus antigen-experienced B-lineage cells ($CD27^{high}$; $CD38^{low}$ memory B-cells and $CD38^{high}$ plasma cells). The percentage of antigen-experienced B-cells measured with flow cytometry is shown in comparison to next-generation sequencing (NGS) with the cut-off at 98% or 97% V gene identity in comparison to germline as definition of hypermutated *IGH* sequences. Horizontal lines show median values and error bars show the interquartile range, n=30 for each group.

Donor	Total sequencing reads	Successfully aligned reads	Reads used in clonotypes (percent of total)	Reads used as a core (percent of used)	Mapped low quality reads (percent of used)	Reads dropped due to the lack of a clone sequence (percent of total)	Reads dropped due to failed mapping (percent of total)	Number of clonotypes	Hypermutated fraction (% of all clonotypes)
HD036	43104	28694	13948 (32.36%)	12796 (91.74%)	1152 (8.26%)	998 (2.32%)	13747 (31.89%)	1744	25,34
HD037	37839	19158	7628 (20.16%)	7131 (93.48%)	497 (6.52%)	497 (6.52%)	10923 (28.86%)	1248	19,79
HD039	44632	23762	11693 (26.2%)	10601 (90.66%)	1092 (9.34%)	851 (1.91%)	11218 (25.14%)	1174	37,22
HD051	28604	17537	5891 (20.6%)	5677 (96.37%)	214 (3.63%)	537 (1.88%)	11109 (38.84%)	880	34,66
HD052	46045	20978	9282 (20.16%)	8474 (91.29%)	808 (8.71%)	1904 (4.14%)	9792 (21.27%)	1228	71,58
HD074	47088	25757	9445 (20.06%)	8991 (95.19%)	454 (4.81%)	1192 (2.53%)	15120 (32.11%)	1447	43,12
HD075	51540	24002	12606 (24.46%)	11378 (90.26%)	1228 (9.74%)	819 (1.59%)	10577 (20.52%)	995	28,04
HD076	34232	18913	5939 (17.35%)	5803 (97.71%)	1228 (9.74%)	819 (1.59%)	10577 (20.52%)	718	39,69
HD077	36212	19924	10115 (26.41%)	9250 (91.45%)	865 (8.55%)	526 (1.37%)	9283 (24.24%)	1133	17,30

Donor	Total sequencing reads		Successfully aligned reads		Reads used in clonotypes (percent of total)		Reads used as a core (percent of used)		Mapped low quality reads (percent of used)		Reads dropped due to the lack of a clone sequence (percent of total)		Reads dropped due to failed mapping (percent of total)		Number of clonotypes		Hypermutated fraction (% of all clonotypes)	
	pre	24 months	pre	24 months	pre	24 months	pre	24 months	pre	24 months	pre	24 months	pre	24 months	pre	24 months	pre	24 months
F1	65182	19025	64312	18877	63810 (97.9%)	16943 (89.06%)	63449 (99.43%)	16802 (99.17%)	361 (0.57%)	141 (0.83%)	151 (0.23%)	212 (1.11%)	351 (0.54%)	1722 (9.05%)	62	849	51,61	3,42
F2	140434	12773	138432	12709	137306 (97.77%)	11098 (86.89%)	135847 (98.94%)	10944 (98.61%)	1459 (1.06%)	154 (1.39%)	198 (0.14%)	370 (2.9%)	928 (0.66%)	1241 (9.72%)	14	1380	42,86	8,41
F3	43578	11464	42988	11301	42391 (97.28%)	10092 (88.03%)	41823 (98.66%)	9916 (98.26%)	568 (1.34%)	176 (1.74%)	133 (0.31%)	250 (2.18%)	464 (1.06%)	959 (8.37%)	75	983	94,67	6,61
F4	184057	18102	182084	17677	181110 (98.4%)	15493 (85.59%)	179741 (99.24%)	15329 (98.94%)	1369 (0.76%)	164 (1.06%)	253 (0.14%)	261 (1.44%)	721 (0.39%)	1923 (10.62%)	11	576	18,18	2,78
F5	75568	18087	74726	16749	74189 (98.18%)	15423 (85.27%)	73622 (99.24%)	15228 (98.74%)	567 (0.76%)	195 (1.26%)	62 (0.08%)	329 (1.82%)	475 (0.63%)	997 (5.51%)	38	823	28,95	10,94
F6	86813	29027	86131	28853	85535 (98.53%)	28491 (98.15%)	84742 (99.07%)	28139 (98.76%)	793 (0.93%)	352 (1.24%)	43 (0.05%)	17 (0.06%)	553 (0.64%)	345 (1.19%)	3	23	66,67	17,39
F7	35921	12897	35296	12793	35007 (97.46%)	10367 (80.38%)	34803 (99.42%)	10331 (99.65%)	204 (0.58%)	36 (0.35%)	132 (0.37%)	305 (2.36%)	157 (0.44%)	2121 (16.45%)	48	836	52,08	4,07
F8	91660	15139	90788	14914	90430 (98.66%)	12437 (82.15%)	89850 (99.36%)	12349 (99.29%)	580 (0.64%)	88 (0.71%)	72 (0.08%)	300 (1.98%)	286 (0.31%)	2177 (14.38%)	4	680	50,00	4,71
F9	46152	15718	45683	15423	45404 (98.38%)	14807 (94.2%)	44979 (99.06%)	14557 (98.31%)	425 (0.94%)	250 (1.69%)	44 (0.1%)	91 (0.58%)	235 (0.51%)	525 (3.34%)	8	413	37,50	14,04
F10	63236	23879	62490	23684	61914 (97.91%)	23076 (96.64%)	61375 (99.13%)	22944 (99.43%)	539 (0.87%)	132 (0.57%)	100 (0.16%)	67 (0.28%)	476 (0.75%)	541 (2.27%)	28	162	53,57	6,79

Donor	Total sequencing reads			Successfully aligned reads			Reads used in clonotypes (percent of total)			Reads used as a core (percent of used)			Mapped low quality reads (percent of used)		
	pre	12 months	24 months	pre	12 months	24 months	pre	12 months	24 months	pre	12 months	24 months	pre	12 months	24 months
I1	50756	69702	92314	50000	68416	90833	49723 (97.96%)	68125 (97.74%)	90385 (97.91%)	49407 (99.36%)	67708 (99.39%)	89952 (99.52%)	316 (0.64%)	417 (0.61%)	433 (0.48%)
I2	38488	59637	58292	38146	59197	57669	37551 (97.57%)	58875 (98.72%)	57344 (98.37%)	37168 (98.98%)	58589 (99.51%)	57020 (99.43%)	383 (1.02%)	286 (0.49%)	324 (0.57%)
I3	59364	77436	27992	58597	76444	27534	58177 (98%)	75815 (97.91%)	27215 (97.22%)	57674 (99.14%)	75339 (99.37%)	26643 (97.9%)	503 (0.86%)	476 (0.63%)	572 (2.1%)
I4	99234	92662	110296	97756	91267	108760	97335 (98.09%)	90935 (98.14%)	108332 (98.22%)	96998 (99.65%)	90625 (99.66%)	107985 (99.68%)	337 (0.35%)	310 (0.34%)	347 (0.32%)
I5	64980	56799	54947	63775	56212	54158	63058 (97.04%)	55493 (97.7%)	53422 (97.22%)	62413 (98.98%)	54666 (98.51%)	52612 (98.48%)	645 (1.02%)	827 (1.49%)	810 (1.52%)
I6	80415	116989	52520	79156	115545	51918	78800 (97.99%)	114912 (98.22%)	51600 (98.25%)	78558 (99.69%)	114592 (99.72%)	51430 (99.67%)	242 (0.31%)	320 (0.28%)	170 (0.33%)
I7	43211	47457	40861	42757	46642	40437	42305 (97.9%)	46228 (97.41%)	39792 (97.38%)	42031 (99.35%)	46013 (99.53%)	39587 (99.48%)	274 (0.65%)	215 (0.47%)	205 (0.52%)
I8	19740	29776	30461	19073	29576	30324	17170 (86.98%)	28560 (95.92%)	29712 (97.54%)	16848 (98.12%)	28342 (99.24%)	29470 (99.19%)	322 (1.88%)	218 (0.76%)	242 (0.81%)
I9	124758	93926	71437	123091	92666	70518	121934 (97.74%)	91874 (97.82%)	69632 (97.47%)	120937 (99.18%)	91207 (99.27%)	68949 (99.02%)	997 (0.82%)	667 (0.73%)	683 (0.98%)
I10	118809	91026	36721	117252	90129	36033	116332 (97.92%)	89190 (97.98%)	34858 (94.93%)	115551 (99.33%)	88442 (99.16%)	34416 (98.73%)	781 (0.67%)	748 (0.84%)	442 (1.27%)

Donor	Reads dropped due to the lack of a clone sequence			Reads dropped due to failed mapping			Number of clonotypes			Hypermutated fraction (% of all clonotypes)		
	pre	12 months	24 months	pre	12 months	24 months	pre	12 months	24 months	pre	12 months	24 months
I1	83 (0.16%)	90 (0.13%)	191 (0.21%)	194 (0.38%)	201 (0.29%)	257 (0.28%)	32	8	15	43,75	37,50	46,67
I2	77 (0.2%)	89 (0.15%)	66 (0.11%)	518 (1.35%)	233 (0.39%)	259 (0.44%)	133	16	30	7,52	43,75	20,00
I3	142 (0.24%)	328 (0.42%)	109 (0.39%)	278 (0.47%)	301 (0.39%)	210 (0.75%)	3	2	6	0,00	0,00	33,33
I4	254 (0.26%)	196 (0.21%)	257 (0.23%)	167 (0.17%)	136 (0.15%)	171 (0.16%)	22	8	65	36,36	75,00	58,46
I5	372 (0.57%)	340 (0.6%)	335 (0.61%)	345 (0.53%)	379 (0.67%)	401 (0.73%)	1	1	22	0,00	0,00	4,55
I6	258 (0.32%)	479 (0.41%)	202 (0.38%)	98 (0.12%)	154 (0.13%)	116 (0.22%)	10	11	89	40,00	36,36	10,11
I7	112 (0.26%)	102 (0.21%)	216 (0.53%)	340 (0.79%)	312 (0.66%)	429 (1.05%)	88	126	246	29,55	40,48	43,09
I8	479 (2.43%)	335 (1.13%)	166 (0.54%)	1424 (7.21%)	681 (2.29%)	446 (1.46%)	1126	377	373	39,70	60,48	73,19
I9	234 (0.19%)	142 (0.15%)	228 (0.32%)	923 (0.74%)	650 (0.69%)	658 (0.92%)	1	24	159	0,00	66,67	32,70
I10	410 (0.35%)	452 (0.5%)	329 (0.9%)	510 (0.43%)	487 (0.54%)	846 (2.3%)	55	139	593	25,45	30,94	19,39

3. Discussion

Chronic lymphocytic leukemia represents a major challenge for clinicians as it affects numerous patients every year for whom the best available therapy has to be determined individually. Recent advantages in basic as well as clinical research added not only a large number of possible drugs but also an increasingly complex network of prognostic factors to consider. Starting with conventional chemotherapy to the addition of monoclonal antibodies up to recently introduced small molecule inhibitors targeting key components of pathways dysregulated in CLL there have been substantial changes in the treatment landscape over the past decades.[131] However, CLL is still considered incurable and therapy responses and prognosis vary immensely from patient to patient. Traditionally, the most important prognostic marker is the mutational status of the IGHV gene used in the assembly of the B cell receptor of the respective tumor cell.[82,132] Several studies have shown that M-CLL patients benefit from the classical CIT regimen fludarabine, cyclophosphamide and rituximab and a significant proportion of this subgroup achieves complete remission over a remarkably long time period. Nonetheless does this not apply to all M-CLL and the differences that lead to this intergroup variety are still insufficiently understood.[102,103,133] On the other hand, U-CLL cases show rather poor response to chemoimmunotherapy and often become refractory after early relapse.[134] For these patients, ibrutinib has proven to be a valuable option but as with most small molecule inhibitors resistance remains a major challenge .[135,136] Another important aspect are the side effects that come along with either therapy regimen and demand close monitoring of the patients. To identify novel biomarkers for a rational guided choice of the optimal treatment sequence a deeper understanding of the mechanisms that cause the great heterogeneity among CLL cases depending on their molecular background is urgently needed and the data presented in this thesis contributes to this aim.

3.1. Anergy in CLL

One main factor to discriminate between U- and M-CLL seems to be the reaction to BCR engagement which leads to a more anergic signature in CLL cells with a mutated IGHV.[137] At first, this seems to be advantageous but given that anergy is no permanent state anergic cells can ultimately be re-activated and become potentially harmful.[138] Knowing what drives M-CLL cells towards anergy could therefore be of great clinical significance.

Although the cellular signature of anergy has been clearly described in the past, the molecular mechanisms have remained unsatisfyingly elucidated but the first project for this thesis provides novel insights. Propagation of signals downstream of the BCR depends on proteins carrying SH2 domains that can bind to phosphorylated tyrosine residues. To further characterize the complex network of kinases and phosphatases that act in the signaling pathways after BCR engagement we conducted a far-western based profiling of primary CLL samples using SH2 domain probes that revealed SHP2 as a putative key regulator in pathological signaling. Following up on that we could clearly show that overexpression of SHP2 impaired proliferation and induced an anergic phenotype in our CLL cell model MEC-1 as seen in decreased sIgM expression and mitigated calcium flux after BCR engagement. Our data also links SHP2 to the constitutively enhanced basal phosphorylation of ERK1/2 as a hallmark of anergy in B cells that has until now remained enigmatic. Importantly, these effects can be attributed to the phosphatase domain of SHP2 since a catalytically inactive mutant version failed to induce them.

There is evidence that the degree of anergy also influences the response to BTK inhibition with the anergic CLL cells being more resistant to spontaneous apoptosis as opposed to physiological anergic B cells that eventually succumb to cell death.[39,139] This could explain the more pronounced lymphocytosis that is observed after ibrutinib treatment in M-CLL in comparison to U-CLL.[140] Our data shows that SHP2 overexpression enhances the anti-proliferative effects of ibrutinib. The anti-apoptotic protein BCL-2 is also often upregulated in CLL and this overexpression has been associated with a deletion on chromosome 13 that is generally considered a positive prognostic marker, especially when it occurs in patients with a mutated IGHV.[141,142] It is tempting to speculate that this comes along with an increased

sensitivity towards BCL2 inhibitor venetoclax but this is still a subject of ongoing trials and recent data suggests that the mutational status does not influence the response to this compound in different settings and combinations.[121,143,144] However, venetoclax seems to be superior to FCR or an alternative kinase inhibitor when given as second line therapy after ibrutinib failure.[145]

The anti-proliferative properties of SHP2 in CLL are in conflict with its previously proposed role as an proto-oncogene.[146] Activating mutations in the PTPN11 gene encoding for SHP2 are found in many childhood hematopoietic disorders the most prominent being juvenile myelomonocytic leukemia (JMML) with ca. 35% affected cases but also in adult acute myelogenous leukemia and solid malignancies albeit with lower frequency.[147] A study using whole exome sequencing of more than 500 CLL cases found activating PTPN11 mutations in around 1% of all cases, notably more frequent in U-CLL.[148] It has been shown that activating SHP2 mutations promote leukemogenesis in a lineage independent fashion in mice indicating a role in early tumorigenesis that is somehow switched in full blown CLL.[149] Albeit our data does not provide an explanation for this discrepancy, it highlights the versatility many signaling molecules display and supports the importance of disease specific research attempts taking in to account the individual cellular background.

3.2. The role of SLAMF receptors in CLL

Another project that contributed to this thesis was focused on the role of SLAMF1 and SLAMF7 in CLL biology. The above mentioned SH2 domain screen revealed a second cluster of CLL patients that had a favorable prognosis and were characterized by high EAT2 binding. Since EAT2 is a known downstream modulator of SLAMF signaling in several immune cells, we hypothesized that high expression of these receptors on CLL cells could identify patients belonging to this subset. As surface receptors they are eligible for flow cytometry staining that is routinely used in CLL diagnosis and could be incorporated in the clinical workup making them ideal biomarkers for prognosis. Of the nine members of the SLAM family of receptors we chose SLAMF1 and SLAMF7 because both are downregulated on CLL cells compared to their normal B cell counterparts which suggests anti-tumor characteristics.[92] They also carry a ITSM in their intracellular domain that can bind downstream mediators like EAT2.[52] We could

indeed show, that CLL patients who highly expressed one of these receptors on their tumor cells and were therefore considered SLAMF^{high} showed a prolonged time to first treatment as a surrogate marker for the aggressiveness of the disease. The SLAMF^{high} signature was also closely associated with a mutated IGHV but proved to be an independent positive prognostic factor.

3.2.1. BCR signaling and PHB2

Loss of SLAMF1 expression in CLL cells has already been linked in-vitro to impaired autophagy in response to fludarabine treatment and changes in chemokine induced migration.[93] Given the close association between the SLAMF and the mutational status of the CLL patient, we are proposing a second, independent mechanism that directly influences BCR signaling in CLL cells. Using the IGHV mutated MEC-1 CLL cell line, we could show that BCR signaling was attenuated by the overexpression of SLAMF1 or SLAMF7 and strengthened by the knock out of both receptors simultaneously. This data supports the clinical observation of intergroup heterogeneity among patients with a mutated IGHV gene and points to SLAMF1 or SLAMF7 expression as one putative underlying cause. However, this seems not to be mediated via the known SLAMF adaptor proteins SAP or EAT2 since we found them only weakly expressed or absent as reported by earlier studies and with no correlation to the SLAMF status of the respective CLL patient in primary samples from our cohort.[52] In line with this, direct targeting of SLAMF7 in our in vitro CLL cell line model with the SLAMF7 antibody Elotuzumab that is routinely used in treating Multiple Myeloma (MM) where the receptor is overexpressed on plasma cells showed no efficacy (data not shown).[150]

A biotinylation screen as an unbiased approach to finding novel interaction partners for SLAMF1 and SLAMF7 that could mediate the observed effects on CLL biology revealed prohibitin 2 (PHB2) as a likely candidate. It has initially been described as a B cell receptor associated protein of 37 kDa (BAP37) sharing sequence homology with the anti-proliferative prohibitin 1 (PHB1) that was also non-covalently bound to sIgM.[151] Both proteins are ubiquitously expressed and participate in a plethora of cellular processes depending on their localization inside the cell.[152] They could be linked to MAPK signaling and

shown as binding partners to p53 and Akt.[153-155] Several studies suggested a distinct role in BCR signaling including activation of the NFκB pathway, IgG antibody production and Syk mediated coupling of downstream effectors.[156,157] However, due to their versatility in different cellular settings, the precise functions of PHB1 and PHB2 are difficult to determine and our data adds even more branches to this complex network. PHB2 expression was comparable in all cell lines irrespective of the SLAMF status supporting the notion that the localization rather than total amount is important for its effects on BCR signaling. Hence, we hypothesized that the SLAMF receptors 1 and 7 recruit PHB2 away from the B cell receptor thereby detaining it from its role in physiological signaling. Subsequently, downstream signaling involving BTK seems to be inherently attenuated in the patients who are considered SLAMF^{high} and inhibition via ibrutinib has no additional effects. This observation argues for the expression of SLAMF1 and SLAMF7 as a potential biomarker that could help facilitate the choice of the most appropriate first-line treatment in young and fit M-CLL patients since latest data on FCR vs ibrutinib showed no significant benefit for either of the regimens in this subgroup.[158] Patients showing a downregulation of these receptors, as already reported for SLAMF1 in U-CLL, could be more susceptible to BTK inhibition while high SLAMF levels would qualify for upfront chemoimmunotherapy.[93]

It remains to be investigated if the response to other small molecule inhibitors like venetoclax or idelalisib that target other components of the BCR signaling cascade is also influenced by the SLAMF status of the patients. To address this, our group investigated the use of the Syk inhibitor entospletinib as another approach to directly target BCR signaling in-vitro in MEC-1 cells with different SLAMF background. Unfortunately, we could see no sensitization in the subline devoid of SLAMF1 and SLAMF7 as observed for ibrutinib (data not shown). This could be explained by the fact, that prohibitins have been found to directly bind to Syk and PHB2 might compete with entospletinib for the same binding site.[156]

The pharmacological targeting of PHBs has also been of great interest considering its suspected role in a variety of pathologies including different types of cancer.[159] Fluorizoline was identified during a small molecule screen to increase apoptosis rates in several cancer cell lines which was attributed to its ability to bind PHB1 and PHB2.[160] This could also be achieved by treating primary CLL cells and the effect was even bigger when fluorizoline was combined with ibrutinib.[161] However, the individual responses seem to vary greatly and it would be interesting to analyze if the mutational status of the respective CLL patient influences the treatment response since we found it to be closely connected to the SLAMF status on the tumor cells. In light of our data acquired for this thesis, it seems possible that fluorizoline blocks the binding of PHB2 to the SLAMF receptors thereby mimicking a $SLAMF^{low}$ signature as seen in most of the U-CLL cases and the associated increased sensitivity towards ibrutinib. Unfortunately, the study gives little to no information regarding this question. Follow up projects should include the question if $SLAMF^{high}$ patients can be sensitized to respond to ibrutinib in a similar fashion by combining it with fluorizoline.

It is currently also unclear, what connects the structure of the BCR and PHB2 activity since PHB2 seems to be indispensable for normal signaling solely in B cells expressing an immunoglobulin of the IgM subclass using a mutated IGHV gene. When switched to expressing sIgG instead of sIgM we could not find any of the SLAMF related detrimental effects on BCR signaling in our MEC-1 CLL cell model. This is in line with previous work that connects PHB2 to sIgM but not sIgD on B cells, presumably via the transmembrane domain of the BCR.[151] Although IgM and IgG BCRs are linked to identical CD79a/b dimers, our co-immunoprecipitation experiments suggest decreased PHB2 binding to CD79a in the IgG setting.[162] This argues for one or more additional linker molecules mediating this interaction either in a positive or negative fashion. Since the cytoplasmatic tail of the sIgG subclass BCR is considerably longer (this does not apply to the sIgD receptor), there would be putative binding sites for such molecules that could prevent an interaction between PHB2 and CD79a and explain the unique role of PHB2 in IgM signaling.[163]

3.2.2. Immunomodulatory functions

Another elusive topic regarding CLL and its treatment is the question if and how tumor cells are recognized and targeted by the immune system. In the last years, the concept of immune checkpoint inhibition emerged as a new approach to targeting cancer cells by re-activating the patient's own immune system and novel drugs based on this principle have been introduced in a wide variety of tumor entities.[164] The observation that T-cells in CLL patients show a dysregulated pattern of immune checkpoint markers, especially an upregulation of PD-1 and CTLA-4, and promising preclinical data led to the initiation of clinical trials for the assessment of safety and efficacy of monoclonal antibodies against these and other checkpoint inhibitors either alone or in combination with established compounds in high-risk or relapsed/refractory CLL.[126,165-168] However, the overall results are rather disappointing with exception only in the small subset of CLL patients with Richter's transformation after ibrutinib failure but no obvious benefit for other patients.

An important part of the innate immune system with a role in therapeutic applications are NK cells. Their most important feature is the ability to release cytotoxic granules that lead to the lysis of target cells. In CLL, the total number of NK cells is elevated and the amount relative to the leukemic burden was shown to be of prognostic value with a higher ratio indicating a longer time to first treatment.[169,170] However, the cytotoxic activity of CLL patient's NK cells is markedly decreased, largely attributed to the aberrant expression of inhibitory and activating receptors on the NK or tumor cells but comprehensive data is missing and the precise role of NK cells in CLL pathology is still insufficiently understood.[171,172]

Our data now suggests that SLAMF1 and SLAMF7 impact anti-CLL immunity by positively modulating the degranulation capacity of the patients' NK cells intrinsically with the ones deriving from CLL patients in the SLAMF^{high} group displaying increased activity in co-culture assays with tumor cells. Counterintuitively this is not mediated by elevated levels of these receptors on the CLL patients' NK cells since they were comparable to the ones on NK cells from healthy donors regardless whether the

patient was characterized as SLAMF^{high} or SLAMF^{low}. Moreover, the SLAMF receptor level on the target cells did not influence the degree of degranulation neither of NK cells from healthy donors nor those isolated from CLL patients and irrespective if they were confronted with primary CLL cells or our MEC-1 cellular model system. The majority of cytolytic NK cells resides in the peripheral blood where they are in constant encounter with CLL cells.[173] This close proximity together with the above outlined observations might indicate some kind of educational process that allows for more efficient immune control in SLAMF^{high} CLL patients. The concept of NK cell education has been of growing interest in the last couple of years. Briefly, the interaction with activating receptors on target cells during maturation is believed to cause hyporesponsive NK cells while the engagement of inhibitory receptors increases their reactivity.[174] The SLAMF receptors can act as both, inhibitory and activating depending on the presence of SAP adaptor proteins for downstream signaling.[51] In previous studies, SLAMF6 in the absence of SAP was shown to be responsible for an increased NK cell response towards non-hematopoietic target cells that do not express the receptor while homotypic interactions with hematopoietic cells triggered NK cell inhibition.[175] The mechanism by which SLAMF1 and SLAMF7 influence NK cell activity seems to be distinct in that the presence of the "educating" receptor on the target cells does not diminish the effects. However, our experimental model is admittedly very artificial and lacks many of the components of the CLL microenvironment that are most likely also involved in modulating NK cell activity. More research is clearly needed to better understand this process and if it can be exploited for therapeutic purposes.

Inducing so called antibody dependent cellular cytotoxicity (ADCC) by interacting via their Fc region with NK cells and causing them to degranulate is also one of the mechanisms by which therapeutic antibodies elicit their effects.[176] In CLL therapy anti-CD20 monoclonal antibodies and especially rituximab are widely used, mainly as part of chemoimmunotherapy. In light of the NK cell dysfunction observed in CLL patients it is assumed that the efficacy of the anti-CD20 antibody rituximab is mainly based on different NK cell independent processes like the intrinsic activation of apoptosis.[177] However, it might be worth investigating if the increased activity of NK cells from SLAMF^{high} CLL patients can be

further enhanced with the use of therapeutic antibodies and if this might even be part of the answer why a significant number CLL patients with an mutated IGHV (presumably the ones that are also SLAMF^{high}) especially benefit from the FCR regimen that contains rituximab. A second promising compound besides rituximab could be the anti-SLAMF7 antibody elotuzumab that has been shown not only to induce ADCC but also to directly activate NK cells in the therapy of multiple myeloma.[150]

Interestingly, while the majority of the CLL patients we examined for our studies were considered SLAMF^{high} for the expression of only one receptor, SLAMF1 or SLAMF7, there was a very small subset of about 2-3% that were positive for both and these patients showed an even better overall survival Given that our cell models suggest that overexpression of either SLAMF1 or SLAMF7 is sufficient to block proliferative BCR signals via PHB2 we hypothesize that the additional benefit is due to other unrelated mechanisms that might involve the autophagy related SLAMF1 functions or the interplay with cells of the CLL microenvironment including NK cells.[93] However, these questions could not be addressed using the applied cell models and need further investigation.

3.3. Implications of CLL therapy on immunity

Despite all research attempts to improve not only the efficacy but also decrease side effects CLL patients still suffer from therapy related issues. Of special importance is the patient's immunocompetence that is already dampened representing a hallmark of the disease that manifests primarily as a lack of circulating antibodies and T cell dysfunction.[178] As a result, infectious complications are a leading cause of morbidity and mortality already among untreated CLL patients and conventional chemotherapy as well as novel approaches directly targeting the tumor cells further impairs their immune system.[179] Depending on the treatment strategy, distinct patterns of immune modulation become apparent that physicians need to be aware of to closely monitor the patients. The chemoimmunotherapy regimen FCR for example causes a depletion of T cells that persist after the end of treatment whereas the BTK inhibitor ibrutinib is generally non-toxic for normal hematopoiesis.[180] Opportunistic infections occur in patients of both treatment groups but are less common when ibrutinib is given as a first-line treatment. However, in pre-treated patients the number of infectious

complications with ibrutinib is similar to other regimens.[135,181] Important for the management of infections is the capability of the patient's healthy B cells to fight the invading pathogens. Successful infection prophylaxis by vaccination is also dependent on an intact immune system and has therefore been challenging in CLL patients. Since respiratory tract infections are a common source for complications, pneumococcal immunization is of special interest and the response to vaccines has been studied by several groups. The reported response rates vary greatly and seem to be mainly dependent on the timepoint, the vaccine was given. Early stage CLL patients without the need for treatment develop higher immunoglobulin titers than patients already undergoing treatment of any kind.[182-184] However, earlier studies suggest that the response to recall- or neoantigens in patients who achieved remission after lymphoma treatment was partly recovered but this was before anti-CD20 antibodies were introduced in CLL therapy.[185] It is therefore essential to understand the influence of different treatment options on the normal B cell repertoire of the respective CLL patient especially considering that ibrutinib is supposed to be a long-term medication that permanently inhibits BTK function in pathological but also healthy B cells. In the last project for this thesis, we aimed to explore the changes in this compartment in FCR and ibrutinib treated patients with the help of immunosequencing, a technique that is based on massive parallel sequencing of CDR3 regions of individual B cell receptors and allows to study the composition of patient specific repertoires.[186]

Dysfunctional BTK due to mutations is known as the underlying cause of X-linked agammaglobulinemia (XLA), a hereditary disease that leads to a block in B cell differentiation followed by the absence of mature B cells and antibody producing plasma cells and patients suffer from frequent infections.[187] Our data shows a declining number of B cells and the lack of replenishment with newly generated antigen naïve B cells in ibrutinib treated CLL patients that closely resembles the XLA phenotype. However, we also saw the preservation of the pretreatment, antigen experienced B cell repertoire which could explain the less frequent infections in those patients. This presumably also includes plasma cell pools that maintain antibody levels in CLL but are innately absent in XLA. Contrary to this,

chemoimmunotherapy with FCR led to a repertoire renewal where total B cell counts recovered to baseline levels but the proportion of antigen experienced cells significantly declined.

Besides these novel insights into B cell repertoire dynamics under CLL therapy, our study has several limitations. Foremost, the differences between the two patient groups are to be interpreted with caution since the clinical characteristics lack resemblance in terms of genetic risk factors or the number of pretreatments, where a higher number has been shown to negatively influence immunocompetence.[188] Our sequencing data is also primarily suitable to picture global changes in the B cell compartment and conclusions about individual B cell clones are difficult to draw due the nature of CLL as a B cell disorder where the tumor clone takes up most of the repertoire and the coverage of healthy B cells is technically demanding and resources, patient material as well as consumables for sequencing, are usually limited.

Taken together, although therapies targeting components of the B cell receptor signaling pathway are a promising approach for the treatment of CLL they are most likely to be accompanied by a distinct profile of side effects. More research is needed to not only better understand the underlying mechanisms but also develop novel strategies to manage them.

4. Summary

The data presented in this thesis arises from three projects that addressed different aspects of pathological B cell receptor signaling in chronic lymphocytic leukemia.

We were able to add valuable insights into the molecular mechanisms that shape the differential signaling response of the two main patient subgroups, U- and M-CLL, downstream of the BCR and that translate to a clinically observed heterogeneity. Our data opens up perspectives for the development of novel therapeutic approaches with SHP2, SLAMF1, SLAMF7 and PHB2 as promising targets and at the same time might help to improve established therapy regimens with the implementation of SLAMF1 and SLAMF7 expression as biomarkers for a personalized choice of the most beneficial treatment option (chemoimmunotherapy vs BTK inhibitor ibrutinib) especially for M-CLL patients. On

the other hand, it also helps in understanding the specific side effects that come along with already established therapies including those that directly target the BCR pathway.

In conclusion, our research further pursues the positive developments in the field of CLL biology and, if successfully translated from bench to bedside, could provide clinicians and patients with hopeful perspectives for an improved prognosis and disease control.

Abbreviations

ADCC	antibody dependent cellular cytotoxicity
AID	activation-induced cytidine deaminase
BCAP	B cell adaptor protein
BCR	B cell receptor
Blk	B lymphocyte kinase
BLNK	B cell linker protein
BTK	Bruton's tyrosine kinase
C region	constant region
CART cells	chimeric antigen receptor T cells
CDR	complementary determining region
CIT	chemoimmunotherapy
CLL	chronic lymphocytic leukemia
CR	complete remission
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAG	diacylglycerol
DLBCL	Diffuse large B cell lymphoma
EAT2	Ewing sarcoma activated transcript 2
ERK1/2	extracellular signal-regulated kinase 1/2
FCR	Fludarabin cyclophosphamide rituximab chemoimmunotherapy
FR	framework region
GC	germinal centers
HSC	hematopoietic stem cell
Ig	immunoglobulin
IGHV	immunoglobulin heavy chain variable region gene
IP_3	inositoltriphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
iwCLL	international Workshop on Chronic Lymphocytic Leukemia
JMML	juvenile myelomonocytic leukemia
Lyn	Lck/Yes novel tyrosine kinase
MAPK	mitogen-activated protein kinase
MBL	monoclonal B cell lymphocytosis

M-CLL	CLL with mutated IGHV
MHC	major histocompability complex
miRNA	micro RNA
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NK cell	natural killer cells
NKT cells	natural killer T cells
OR	overall response
PD-1	programmed death 1 receptor
PH domain	pleckstrin homology domain
PHB1	prohibitin 1
PHB2	prohibitin 2
PI3K	phosphoinositide 3-kinases
PKC	protein kinase C
PLC-γ2	phospholipase C gamma 2
PSF	progression free survival
PTEN	phosphatase and tensin homolog
PTPN11	protein Tyrosine Phosphatase Non-Receptor Type 11
RAG1/2	recombination activating gene 1/2
RSS	recombination signal sequences
SAP	SLAM associated protein
SH2 domain	src homology 2 domain
SHIP1	src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1
SHP1	src homology 2 domain-containing protein tyrosine phosphatase 1
SHP2	src homology region 2 domain-containing phosphatase-2
sIg	surface immunoglobulin
siRNA	small interferring RNA
SLAMF	signaling lymphocytic activation molecule family
Syk	spleen tyrosine kinase
TCR	T cell receptor
TTFT	time to first treatment
U-CLL	CLL with unmutated IGHV
V region	variable region
XLA	X-linked agammaglobulinemia

References

1. Murphy K, Travers P, Walport M, Janeway C. Janeway's immunobiology (ed 8th). New York: Garland Science; 2012.
2. Tonegawa S. Somatic generation of antibody diversity. *Nature*. 1983;302(5909):575-581.
3. Watson CT, Breden F. The immunoglobulin heavy chain locus: genetic variation, missing data, and implications for human disease. *Genes & Immunity*. 2012;13(5):363-373.
4. Early P, Huang H, Davis M, Calame K, Hood L. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. *Cell*. 1980;19(4):981-992.
5. Collins AM, Watson CT. Immunoglobulin Light Chain Gene Rearrangements, Receptor Editing and the Development of a Self-Tolerant Antibody Repertoire. *Frontiers in Immunology*. 2018;9(2249).
6. Swanson PC, Kumar S, Raval P. Early Steps of V(D)J Rearrangement: Insights from Biochemical Studies of RAG-RSS Complexes. In: Ferrier P, ed. V(D)J Recombination. New York, NY: Springer New York; 2009:1-15.
7. Malu S, Malshetty V, Francis D, Cortes P. Role of non-homologous end joining in V(D)J recombination. *Immunol Res*. 2012;54(1-3):233-246.
8. Pilzecker B, Jacobs H. Mutating for Good: DNA Damage Responses During Somatic Hypermutation. *Frontiers in Immunology*. 2019;10(438).
9. Victora GD, Nussenzweig MC. Germinal Centers. *Annual Review of Immunology*. 2012;30(1):429-457.
10. Hombach J, Tsubata T, Leclercq L, Stappert H, Reth M. Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature*. 1990;343(6260):760-762.
11. Johnson SA, Pleiman CM, Pao L, Schneringer J, Hippen K, Cambier JC. Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. *J Immunol*. 1995;155(10):4596-4603.
12. Saijo K, Schmedt C, Su IH, et al. Essential role of Src-family protein tyrosine kinases in NF-kappaB activation during B cell development. *Nat Immunol*. 2003;4(3):274-279.
13. Bohnenberger H, Oellerich T, Engelke M, Hsiao HH, Urlaub H, Wienands J. Complex phosphorylation dynamics control the composition of the Syk interactome in B cells. *Eur J Immunol*. 2011;41(6):1550-1562.
14. Rowley RB, Burkhardt AL, Chao HG, Matsueda GR, Bolen JB. Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Ig alpha/Ig beta immunoreceptor tyrosine activation motif binding and autophosphorylation. *J Biol Chem*. 1995;270(19):11590-11594.
15. Fu C, Turck CW, Kurosaki T, Chan AC. BLNK: a central linker protein in B cell activation. *Immunity*. 1998;9(1):93-103.
16. Pal Singh S, Dammeijer F, Hendriks RW. Role of Bruton's tyrosine kinase in B cells and malignancies. *Molecular Cancer*. 2018;17(1):57.
17. Aiba Y, Kameyama M, Yamazaki T, Tedder TF, Kurosaki T. Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase. *Blood*. 2008;111(3):1497-1503.
18. Park H, Wahl MI, Afar DE, et al. Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity*. 1996;4(5):515-525.
19. Kim YJ, Sekiya F, Poulin B, Bae YS, Rhee SG. Mechanism of B-cell receptor-induced phosphorylation and activation of phospholipase C-gamma2. *Mol Cell Biol*. 2004;24(22):9986-9999.
20. Scharenberg AM, Humphries LA, Rawlings DJ. Calcium signalling and cell-fate choice in B cells. *Nature reviews Immunology*. 2007;7(10):778-789.
21. Craxton A, Jiang A, Kurosaki T, Clark EA. Syk and Bruton's tyrosine kinase are required for B cell antigen receptor-mediated activation of the kinase Akt. *J Biol Chem*. 1999;274(43):30644-30650.
22. Werner M, Hobeika E, Jumaa H. Role of PI3K in the generation and survival of B cells. *Immunol Rev*. 2010;237(1):55-71.

23. Nitschke L, Carsetti R, Ocker B, Köhler G, Lamers MC. CD22 is a negative regulator of B-cell receptor signalling. *Curr Biol*. 1997;7(2):133-143.
24. Nishizumi H, Horikawa K, Mlinaric-Rascan I, Yamamoto T. A double-edged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. *J Exp Med*. 1998;187(8):1343-1348.
25. Franks SE, Cambier JC. Putting on the Brakes: Regulatory Kinases and Phosphatases Maintaining B Cell Anergy. *Front Immunol*. 2018;9:665.
26. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science*. 2003;301(5638):1374-1377.
27. Nemazee DA, Bürki K. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature*. 1989;337(6207):562-566.
28. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. *The Journal of experimental medicine*. 1993;177(4):1009-1020.
29. Gay D, Saunders T, Camper S, Weigert M. Receptor editing: an approach by autoreactive B cells to escape tolerance. *The Journal of experimental medicine*. 1993;177(4):999-1008.
30. Goodnow CC, Crosbie J, Adelstein S, et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature*. 1988;334(6184):676-682.
31. Eris JM, Basten A, Brink R, Doherty K, Kehry MR, Hodgkin PD. Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions. *Proceedings of the National Academy of Sciences of the United States of America*. 1994;91(10):4392-4396.
32. Gauld SB, Benschop RJ, Merrell KT, Cambier JC. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol*. 2005;6(11):1160-1167.
33. Yarkoni Y, Getahun A, Cambier JC. Molecular underpinning of B-cell anergy. *Immunological reviews*. 2010;237(1):249-263.
34. Cooke MP, Heath AW, Shokat KM, et al. Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. *J Exp Med*. 1994;179(2):425-438.
35. Pao LI, Famiglietti SJ, Cambier JC. Asymmetrical phosphorylation and function of immunoreceptor tyrosine-based activation motif tyrosines in B cell antigen receptor signal transduction. *J Immunol*. 1998;160(7):3305-3314.
36. O'Neill SK, Getahun A, Gauld SB, et al. Monophosphorylation of CD79a and CD79b ITAM motifs initiates a SHIP-1 phosphatase-mediated inhibitory signaling cascade required for B cell anergy. *Immunity*. 2011;35(5):746-756.
37. Pao LI, Lam KP, Henderson JM, et al. B cell-specific deletion of protein-tyrosine phosphatase Shp1 promotes B-1a cell development and causes systemic autoimmunity. *Immunity*. 2007;27(1):35-48.
38. Getahun A, Beavers NA, Larson SR, Shlomchik MJ, Cambier JC. Continuous inhibitory signaling by both SHP-1 and SHIP-1 pathways is required to maintain unresponsiveness of anergic B cells. *J Exp Med*. 2016;213(5):751-769.
39. Fulcher DA, Basten A. Reduced life span of anergic self-reactive B cells in a double-transgenic model. *J Exp Med*. 1994;179(1):125-134.
40. Oliver PM, Vass T, Kappler J, Marrack P. Loss of the proapoptotic protein, Bim, breaks B cell anergy. *J Exp Med*. 2006;203(3):731-741.
41. Calpe S, Wang N, Romero X, et al. The SLAM and SAP gene families control innate and adaptive immune responses. *Adv Immunol*. 2008;97:177-250.
42. Davis SJ, van der Merwe PA. The structure and ligand interactions of CD2: implications for T-cell function. *Immunol Today*. 1996;17(4):177-187.
43. Tangye SG, Phillips JH, Lanier LL. The CD2-subset of the Ig superfamily of cell surface molecules: receptor-ligand pairs expressed by NK cells and other immune cells. *Semin Immunol*. 2000;12(2):149-157.
44. Kingsbury GA, Feeney LA, Nong Y, et al. Cloning, expression, and function of BLAME, a novel member of the CD2 family. *J Immunol*. 2001;166(9):5675-5680.

45. Fraser CC, Howie D, Morra M, et al. Identification and characterization of SF2000 and SF2001, two new members of the immune receptor SLAM/CD2 family. *Immunogenetics*. 2002;53(10-11):843-850.
46. Latchman Y, McKay PF, Reiser H. Cutting Edge: Identification of the 2B4 Molecule as a Counter-Receptor for CD48. *The Journal of Immunology*. 1998;161(11):5809-5812.
47. Brown MH, Boles K, van der Merwe PA, Kumar V, Mathew PA, Barclay AN. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med*. 1998;188(11):2083-2090.
48. Staunton DE, Fisher RC, LeBeau MM, et al. Blast-1 possesses a glycosyl-phosphatidylinositol (GPI) membrane anchor, is related to LFA-3 and OX-45, and maps to chromosome 1q21-23. *J Exp Med*. 1989;169(3):1087-1099.
49. Yokoyama S, Staunton D, Fisher R, Amiot M, Fortin JJ, Thorley-Lawson DA. Expression of the Blast-1 activation/adhesion molecule and its identification as CD48. *The Journal of Immunology*. 1991;146(7):2192-2200.
50. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. *Annu Rev Immunol*. 2011;29:665-705.
51. Wu N, Veillette A. SLAM family receptors in normal immunity and immune pathologies. *Curr Opin Immunol*. 2016;38:45-51.
52. Veillette A. The SAP family: a new class of adaptor-like molecules that regulates immune cell functions. *Sci STKE*. 2002;2002(120):pe8.
53. Veillette A. SLAM-family receptors: immune regulators with or without SAP-family adaptors. *Cold Spring Harb Perspect Biol*. 2010;2(3):a002469.
54. Detre C, Keszei M, Romero X, Tsokos GC, Terhorst C. SLAM family receptors and the SLAM-associated protein (SAP) modulate T cell functions. *Semin Immunopathol*. 2010;32(2):157-171.
55. De Salort J, Sintes J, Llinas L, Matesanz-Isabel J, Engel P. Expression of SLAM (CD150) cell-surface receptors on human B-cell subsets: from pro-B to plasma cells. *Immunol Lett*. 2011;134(2):129-136.
56. Shachar I, Barak A, Lewinsky H, Sever L, Radomir L. SLAMF receptors on normal and malignant B cells. *Clin Immunol*. 2019;204:23-30.
57. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375-2390.
58. Rozman C, Montserrat E. Chronic lymphocytic leukemia. *N Engl J Med*. 1995;333(16):1052-1057.
59. Nennecke A, Wienecke A, Kraywinkel K. Inzidenz und Überleben bei Leukämien in Deutschland nach aktuellen standardisierten Kategorien. *Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz*. 2014;57(1):93-102.
60. Robert Koch Institut, Zentrum für Krebsregisterdaten. 17. December 2019. https://www.krebsdaten.de/Krebs/DE/Content/Krebsarten/Leukaemien/leukaemien_inhalt.html. Accessed: 09. November 2020.
61. Landau DA, Tausch E, Taylor-Weiner AN, et al. Mutations driving CLL and their evolution in progression and relapse. *Nature*. 2015;526(7574):525-530.
62. Kikushige Y, Ishikawa F, Miyamoto T, et al. Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia. *Cancer Cell*. 2011;20(2):246-259.
63. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic leukaemia. *Nature Reviews Cancer*. 2016;16(3):145-162.
64. Rossi D, Gaidano G. Richter syndrome: molecular insights and clinical perspectives. *Hematological Oncology*. 2009;27(1):1-10.
65. Strati P, Shanafelt TD. Monoclonal B-cell lymphocytosis and early-stage chronic lymphocytic leukemia: diagnosis, natural history, and risk stratification. *Blood*. 2015;126(4):454-462.

66. Rawstron AC, Kreuzer KA, Soosapilla A, et al. Reproducible diagnosis of chronic lymphocytic leukemia by flow cytometry: An European Research Initiative on CLL (ERIC) & European Society for Clinical Cell Analysis (ESCCA) Harmonisation project. *Cytometry B Clin Cytom*. 2018;94(1):121-128.
67. Foon KA, Rai KR, Gale RP. Chronic Lymphocytic Leukemia: New Insights into Biology and Therapy. *Annals of Internal Medicine*. 1990;113(7):525-539.
68. Ghia P, Stamatopoulos K, Belessi C, et al. ERIC recommendations on IGHV gene mutational status analysis in chronic lymphocytic leukemia. *Leukemia*. 2007;21(1):1-3.
69. Seifert M, Sellmann L, Bloehdorn J, et al. Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J Exp Med*. 2012;209(12):2183-2198.
70. Rossi D. Epigenetic Trajectories of the Premalignant-to-Malignant Transition of Chronic Lymphocytic Leukemia. *Blood Cancer Discovery*. 2021;2(1):6-8.
71. Agathangelidis A, Darzentas N, Hadzidimitriou A, et al. Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood*. 2012;119(19):4467-4475.
72. Vardi A, Agathangelidis A, Sutton L-A, Ghia P, Rosenquist R, Stamatopoulos K. Immunogenetic Studies of Chronic Lymphocytic Leukemia: Revelations and Speculations about Ontogeny and Clinical Evolution. *Cancer Research*. 2014;74(16):4211.
73. Binder M, Léchenne B, Ummanni R, et al. Stereotypical chronic lymphocytic leukemia B-cell receptors recognize survival promoting antigens on stromal cells. *PloS one*. 2010;5(12):e15992-e15992.
74. Ten Hacken E, Burger JA. Microenvironment interactions and B-cell receptor signaling in Chronic Lymphocytic Leukemia: Implications for disease pathogenesis and treatment. *Biochimica et biophysica acta*. 2016;1863(3):401-413.
75. Dühren-von Minden M, Übelhart R, Schneider D, et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature*. 2012;489(7415):309-312.
76. Contri A, Brunati AM, Trentin L, et al. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J Clin Invest*. 2005;115(2):369-378.
77. D'Avola A, Drennan S, Tracy I, et al. Surface IgM expression and function are associated with clinical behavior, genetic abnormalities, and DNA methylation in CLL. *Blood*. 2016;128(6):816-826.
78. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. *Blood*. 1975;46(2):219-234.
79. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer*. 1981;48(1):198-206.
80. Rai K. A critical analysis of staging in CLL. *Chronic Lymphocytic Leukemia, Recent Progress and Future Directions*. 1987:253-264.
81. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*. 1999;94(6):1848-1854.
82. Damle RN, Wasil T, Fais F, et al. Ig V Gene Mutation Status and CD38 Expression As Novel Prognostic Indicators in Chronic Lymphocytic Leukemia: Presented in part at the 40th Annual Meeting of The American Society of Hematology, held in Miami Beach, FL, December 4-8, 1998. *Blood*. 1999;94(6):1840-1847.
83. Amaya-Chanaga CI, Rassenti LZ. Biomarkers in chronic lymphocytic leukemia: Clinical applications and prognostic markers. *Best Pract Res Clin Haematol*. 2016;29(1):79-89.
84. Baliakas P, Agathangelidis A, Hadzidimitriou A, et al. Not all IGHV3-21 chronic lymphocytic leukemias are equal: prognostic considerations. *Blood*. 2015;125(5):856-859.
85. Döhner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med*. 2000;343(26):1910-1916.
86. Calin GA, Dumitru CD, Shimizu M, 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(24):15524-15529.

87. Klein U, Lia M, Crespo M, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer Cell*. 2010;17(1):28-40.
88. Zenz T, Mertens D, Küppers R, Döhner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nat Rev Cancer*. 2010;10(1):37-50.
89. Tsimberidou AM, Tam C, Abruzzo LV, et al. Chemoimmunotherapy may overcome the adverse prognostic significance of 11q deletion in previously untreated patients with chronic lymphocytic leukemia. *Cancer*. 2009;115(2):373-380.
90. Seiffert M, Dietrich S, Jethwa A, Glimm H, Lichter P, Zenz T. Exploiting biological diversity and genomic aberrations in chronic lymphocytic leukemia. *Leuk Lymphoma*. 2012;53(6):1023-1031.
91. Lynne VA, Carmen DH, George AC, et al. Trisomy 12 chronic lymphocytic leukemia expresses a unique set of activated and targetable pathways. *Haematologica*. 2018;103(12):2069-2078.
92. Coma M, Tothova E, Guman T, Hajikova M, Giertlova M, Sarissky M. Altered expression pattern of SLAM family receptors on pathological B cells of patients with chronic lymphocytic leukemia. *Leukemia & Lymphoma*. 2017;58(7):1726-1729.
93. Bologna C, Buonincontri R, Serra S, et al. SLAMF1 regulation of chemotaxis and autophagy determines CLL patient response. *The Journal of Clinical Investigation*. 2016;126(1):181-194.
94. Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. *American Journal of Hematology*. 2019;94(11):1266-1287.
95. Rai KR, Peterson BL, Appelbaum FR, et al. Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. *N Engl J Med*. 2000;343(24):1750-1757.
96. Knauf WU, Lissichkov T, Aldaoud A, et al. Phase III randomized study of bendamustine compared with chlorambucil in previously untreated patients with chronic lymphocytic leukemia. *J Clin Oncol*. 2009;27(26):4378-4384.
97. Eichhorst BF, Busch R, Hopfinger G, et al. Fludarabine plus cyclophosphamide versus fludarabine alone in first-line therapy of younger patients with chronic lymphocytic leukemia. *Blood*. 2006;107(3):885-891.
98. Flinn IW, Neuberg DS, Grever MR, et al. Phase III trial of fludarabine plus cyclophosphamide compared with fludarabine for patients with previously untreated chronic lymphocytic leukemia: US Intergroup Trial E2997. *J Clin Oncol*. 2007;25(7):793-798.
99. Hainsworth JD, Litchy S, Barton JH, et al. Single-Agent Rituximab as First-Line and Maintenance Treatment for Patients With Chronic Lymphocytic Leukemia or Small Lymphocytic Lymphoma: A Phase II Trial of the Minnie Pearl Cancer Research Network. *Journal of Clinical Oncology*. 2003;21(9):1746-1751.
100. Hallek M, Fischer K, Fingerle-Rowson G, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet*. 2010;376(9747):1164-1174.
101. Robak T, Dmoszynska A, Solal-Céligny P, et al. Rituximab plus fludarabine and cyclophosphamide prolongs progression-free survival compared with fludarabine and cyclophosphamide alone in previously treated chronic lymphocytic leukemia. *J Clin Oncol*. 2010;28(10):1756-1765.
102. Fischer K, Bahlo J, Fink AM, et al. Long-term remissions after FCR chemoimmunotherapy in previously untreated patients with CLL: updated results of the CLL8 trial. *Blood*. 2016;127(2):208-215.
103. Thompson PA, Tam CS, O'Brien SM, et al. Fludarabine, cyclophosphamide, and rituximab treatment achieves long-term disease-free survival in IGHV-mutated chronic lymphocytic leukemia. *Blood*. 2016;127(3):303-309.
104. Wiestner A. Emerging role of kinase-targeted strategies in chronic lymphocytic leukemia. *Blood*. 2012;120(24):4684-4691.
105. Herman SE, Gordon AL, Hertlein E, et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood*. 2011;117(23):6287-6296.

106. Advani RH, Buggy JJ, Sharman JP, et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J Clin Oncol*. 2013;31(1):88-94.
107. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013;369(1):32-42.
108. Burger JA, Tedeschi A, Barr PM, et al. Ibrutinib as Initial Therapy for Patients with Chronic Lymphocytic Leukemia. *New England Journal of Medicine*. 2015;373(25):2425-2437.
109. Woyach JA, Ruppert AS, Heerema NA, et al. Ibrutinib Regimens versus Chemoimmunotherapy in Older Patients with Untreated CLL. *N Engl J Med*. 2018;379(26):2517-2528.
110. Shanafelt TD, Wang V, Kay NE, et al. Ibrutinib and Rituximab Provides Superior Clinical Outcome Compared to FCR in Younger Patients with Chronic Lymphocytic Leukemia (CLL): Extended Follow-up from the E1912 Trial. *Blood*. 2019;134(Supplement_1):33-33.
111. Woyach JA, Furman RR, Liu TM, et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N Engl J Med*. 2014;370(24):2286-2294.
112. Woyach JA, Ruppert AS, Guinn D, et al. BTK(C481S)-Mediated Resistance to Ibrutinib in Chronic Lymphocytic Leukemia. *J Clin Oncol*. 2017;35(13):1437-1443.
113. Maddocks KJ, Ruppert AS, Lozanski G, et al. Etiology of Ibrutinib Therapy Discontinuation and Outcomes in Patients With Chronic Lymphocytic Leukemia. *JAMA Oncology*. 2015;1(1):80-87.
114. Hoellenriegel J, Meadows SA, Sivina M, et al. The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood*. 2011;118(13):3603-3612.
115. Furman RR, Sharman JP, Coutre SE, et al. Idelalisib and Rituximab in Relapsed Chronic Lymphocytic Leukemia. *New England Journal of Medicine*. 2014;370(11):997-1007.
116. Sharman JP, Coutre SE, Furman RR, et al. Final Results of a Randomized, Phase III Study of Rituximab With or Without Idelalisib Followed by Open-Label Idelalisib in Patients With Relapsed Chronic Lymphocytic Leukemia. *J Clin Oncol*. 2019;37(16):1391-1402.
117. Souers AJ, Leverson JD, Boghaert ER, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med*. 2013;19(2):202-208.
118. Stilgenbauer S, Eichhorst B, Schetelig J, et al. Venetoclax in relapsed or refractory chronic lymphocytic leukaemia with 17p deletion: a multicentre, open-label, phase 2 study. *Lancet Oncol*. 2016;17(6):768-778.
119. Eyre TA, Kirkwood AA, Gohill S, et al. Efficacy of venetoclax monotherapy in patients with relapsed chronic lymphocytic leukaemia in the post-BCR inhibitor setting: a UK wide analysis. *Br J Haematol*. 2019;185(4):656-669.
120. Seymour JF, Kipps TJ, Eichhorst B, et al. Venetoclax–Rituximab in Relapsed or Refractory Chronic Lymphocytic Leukemia. *New England Journal of Medicine*. 2018;378(12):1107-1120.
121. Fischer K, Al-Sawaf O, Bahlo J, et al. Venetoclax and Obinutuzumab in Patients with CLL and Coexisting Conditions. *N Engl J Med*. 2019;380(23):2225-2236.
122. Hillmen P, Rawstron AC, Brock K, et al. Ibrutinib Plus Venetoclax in Relapsed/Refractory Chronic Lymphocytic Leukemia: The CLARITY Study. *J Clin Oncol*. 2019;37(30):2722-2729.
123. Jain N, Keating M, Thompson P, et al. Ibrutinib and Venetoclax for First-Line Treatment of CLL. *New England Journal of Medicine*. 2019;380(22):2095-2103.
124. Burger JA, Gribben JG. The microenvironment in chronic lymphocytic leukemia (CLL) and other B cell malignancies: insight into disease biology and new targeted therapies. *Semin Cancer Biol*. 2014;24:71-81.
125. Wodarz D, Garg N, Komarova NL, et al. Kinetics of CLL cells in tissues and blood during therapy with the BTK inhibitor ibrutinib. *Blood*. 2014;123(26):4132-4135.
126. Ding W, LaPlant BR, Call TG, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. *Blood*. 2017;129(26):3419-3427.
127. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric Antigen Receptor–Modified T Cells in Chronic Lymphoid Leukemia. *New England Journal of Medicine*. 2011;365(8):725-733.

128. Turtle CJ, Hay KA, Hanafi LA, et al. 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(26):3010-3020.
129. Chemotherapeutic options in chronic lymphocytic leukemia: a meta-analysis of the randomized trials. CLL Trialists' Collaborative Group. *J Natl Cancer Inst*. 1999;91(10):861-868.
130. Langerbeins P, Bahlo J, Rhein C, et al. IBRUTINIB VERSUS PLACEBO IN PATIENTS WITH ASYMPTOMATIC, TREATMENT-NAÏVE EARLY STAGE CLL: PRIMARY ENDPOINT RESULTS OF THE PHASE 3 DOUBLE-BLIND RANDOMIZED CLL12 TRIAL. *Hematological Oncology*. 2019;37:38-40.
131. Burger JA. Treatment of Chronic Lymphocytic Leukemia. *New England Journal of Medicine*. 2020;383(5):460-473.
132. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig VH Genes Are Associated With a More Aggressive Form of Chronic Lymphocytic Leukemia. *Blood*. 1999;94(6):1848-1854.
133. Rossi D, Terzi-di-Bergamo L, De Paoli L, et al. Molecular prediction of durable remission after first-line fludarabine-cyclophosphamide-rituximab in chronic lymphocytic leukemia. *Blood*. 2015;126(16):1921-1924.
134. Zenz T, Busch R, Fink A, et al. Genetics of Patients with F-Refractory CLL or Early Relapse After FC or FCR: Results From the CLL8 Trial of the GCLLSG. *Blood*. 2010;116(21):2427-2427.
135. O'Brien S, Furman RR, Coutre S, et al. Single-agent ibrutinib in treatment-naïve and relapsed/refractory chronic lymphocytic leukemia: a 5-year experience. *Blood*. 2018;131(17):1910-1919.
136. Woyach JA, Johnson AJ. Targeted therapies in CLL: mechanisms of resistance and strategies for management. *Blood*. 2015;126(4):471-477.
137. Mockridge CI, Potter KN, Wheatley I, Neville LA, Packham G, Stevenson FK. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood*. 2007;109(10):4424-4431.
138. Coelho V, Krysov S, Steele A, et al. Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function. *Blood*. 2013;122(15):2664-2672.
139. Apollonio B, Scielzo C, Bertilaccio MT, et al. Targeting B-cell anergy in chronic lymphocytic leukemia. *Blood*. 2013;121(19):3879-3888, s3871-3878.
140. Woyach JA, Smucker K, Smith LL, et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood*. 2014;123(12):1810-1817.
141. Gladstone DE, Swinnen LJ, Gocke C, Blackford A, Griffin CA, Jones RJ. IgVH Mutational Status Is Prognostic In 13q Deletion (del) Chronic Lymphocytic Leukemia (CLL). *Blood*. 2010;116(21):2419-2419.
142. Robertson LE, Plunkett W, McConnell K, Keating MJ, McDonnell TJ. Bcl-2 expression in chronic lymphocytic leukemia and its correlation with the induction of apoptosis and clinical outcome. *Leukemia*. 1996;10(3):456-459.
143. Tausch E, Schneider C, Robrecht S, et al. Prognostic and predictive impact of genetic markers in patients with CLL treated with obinutuzumab and venetoclax. *Blood*. 2020;135(26):2402-2412.
144. Mato AR, Thompson M, Allan JN, et al. Real-world outcomes and management strategies for venetoclax-treated chronic lymphocytic leukemia patients in the United States. *Haematologica*. 2018;103(9):1511-1517.
145. Mato AR, Hill BT, Lamanna N, et al. Optimal sequencing of ibrutinib, idelalisib, and venetoclax in chronic lymphocytic leukemia: results from a multicenter study of 683 patients. *Ann Oncol*. 2017;28(5):1050-1056.
146. Chan RJ, Feng G-S. PTPN11 is the first identified proto-oncogene that encodes a tyrosine phosphatase. *Blood*. 2007;109(3):862-867.
147. Bentires-Alj M, Paez JG, David FS, et al. Activating Mutations of the Noonan Syndrome-Associated SHP2/PTPN11 Gene in Human Solid Tumors and Adult Acute Myelogenous Leukemia. *Cancer Research*. 2004;64(24):8816-8820.

148. Puente XS, Beà S, Valdés-Mas R, et al. Non-coding recurrent mutations in chronic lymphocytic leukaemia. *Nature*. 2015;526(7574):519-524.
149. Xu D, Liu X, Yu WM, et al. Non-lineage/stage-restricted effects of a gain-of-function mutation in tyrosine phosphatase Ptpn11 (Shp2) on malignant transformation of hematopoietic cells. *J Exp Med*. 2011;208(10):1977-1988.
150. Trudel S, Moreau P, Touzeau C. Update on elotuzumab for the treatment of relapsed/refractory multiple myeloma: patients' selection and perspective. *OncoTargets and therapy*. 2019;12:5813-5822.
151. Terashima M, Kim KM, Adachi T, et al. The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. *The EMBO Journal*. 1994;13(16):3782-3792.
152. Thuaud F, Ribeiro N, Nebigil CG, Désaubry L. Prohibitin ligands in cell death and survival: mode of action and therapeutic potential. *Chem Biol*. 2013;20(3):316-331.
153. Rajalingam K, Wunder C, Brinkmann V, et al. Prohibitin is required for Ras-induced Raf-MEK-ERK activation and epithelial cell migration. *Nat Cell Biol*. 2005;7(8):837-843.
154. Sun L, Liu L, Yang XJ, Wu Z. Akt binds prohibitin 2 and relieves its repression of MyoD and muscle differentiation. *J Cell Sci*. 2004;117(Pt 14):3021-3029.
155. Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S. Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. *J Biol Chem*. 2003;278(48):47853-47861.
156. Paris LL, Hu J, Galan J, et al. Regulation of Syk by phosphorylation on serine in the linker insert. *J Biol Chem*. 2010;285(51):39844-39854.
157. Lucas CR, Cordero-Nieves HM, Erbe RS, et al. Prohibitins and the cytoplasmic domain of CD86 cooperate to mediate CD86 signaling in B lymphocytes. *Journal of immunology (Baltimore, Md : 1950)*. 2013;190(2):723-736.
158. Shanafelt TD, Wang XV, Kay NE, et al. Ibrutinib-Rituximab or Chemoimmunotherapy for Chronic Lymphocytic Leukemia. *N Engl J Med*. 2019;381(5):432-443.
159. Mishra S, Murphy LC, Nyomba BLG, Murphy LJ. Prohibitin: a potential target for new therapeutics. *Trends in Molecular Medicine*. 2005;11(4):192-197.
160. Pérez-Perarnau A, Preciado S, Palmeri CM, et al. A Trifluorinated Thiazoline Scaffold Leading to Pro-apoptotic Agents Targeting Prohibitins. *Angewandte Chemie International Edition*. 2014;53(38):10150-10154.
161. Cosialls AM, Pomares H, Iglesias-Serret D, et al. The prohibitin-binding compound fluorizoline induces apoptosis in chronic lymphocytic leukemia cells through the upregulation of NOXA and synergizes with ibrutinib, 5-aminoimidazole-4-carboxamide riboside or venetoclax. *Haematologica*. 2017;102(9):1587-1593.
162. Reth M. Antigen receptors on B lymphocytes. *Annu Rev Immunol*. 1992;10:97-121.
163. Xu Y, Xu L, Zhao M, et al. No receptor stands alone: IgG B-cell receptor intrinsic and extrinsic mechanisms contribute to antibody memory. *Cell Res*. 2014;24(6):651-664.
164. Darvin P, Toor SM, Sasidharan Nair V, Elkord E. Immune checkpoint inhibitors: recent progress and potential biomarkers. *Experimental & Molecular Medicine*. 2018;50(12):1-11.
165. Younes A, Brody J, Carpio C, et al. Safety and activity of ibrutinib in combination with nivolumab in patients with relapsed non-Hodgkin lymphoma or chronic lymphocytic leukaemia: a phase 1/2a study. *Lancet Haematol*. 2019;6(2):e67-e78.
166. McClanahan F, Hanna B, Miller S, et al. PD-L1 checkpoint blockade prevents immune dysfunction and leukemia development in a mouse model of chronic lymphocytic leukemia. *Blood*. 2015;126(2):203-211.
167. Sagiv-Barfi I, Kohrt HE, Czerwinski DK, Ng PP, Chang BY, Levy R. Therapeutic antitumor immunity by checkpoint blockade is enhanced by ibrutinib, an inhibitor of both BTK and ITK. *Proc Natl Acad Sci U S A*. 2015;112(9):E966-972.
168. Palma M, Gentilcore G, Heimersson K, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. *Haematologica*. 2017;102(3):562-572.

169. Palmer S, Hanson CA, Zent CS, et al. Prognostic importance of T and NK-cells in a consecutive series of newly diagnosed patients with chronic lymphocytic leukaemia. *British journal of haematology*. 2008;141(5):607-614.
170. Huergo-Zapico L, Acebes-Huerta A, Gonzalez-Rodriguez AP, et al. Expansion of NK cells and reduction of NKG2D expression in chronic lymphocytic leukemia. Correlation with progressive disease. *PLoS One*. 2014;9(10):e108326.
171. Ziegler HW, Kay NE, Zarling JM. Deficiency of natural killer cell activity in patients with chronic lymphocytic leukemia. *Int J Cancer*. 1981;27(3):321-327.
172. MacFarlane AW, Jillab M, Smith MR, et al. NK cell dysfunction in chronic lymphocytic leukemia is associated with loss of the mature cells expressing inhibitory killer cell Ig-like receptors. *OncoImmunology*. 2017;6(7):e1330235.
173. Moretta L. Dissecting CD56dim human NK cells. *Blood*. 2010;116(19):3689-3691.
174. He Y, Tian Z. NK cell education via nonclassical MHC and non-MHC ligands. *Cellular & Molecular Immunology*. 2017;14(4):321-330.
175. Wu N, Zhong MC, Roncagalli R, et al. A hematopoietic cell-driven mechanism involving SLAMF6 receptor, SAP adaptors and SHP-1 phosphatase regulates NK cell education. *Nat Immunol*. 2016;17(4):387-396.
176. Lo Nigro C, Macagno M, Sangiolo D, Bertolaccini L, Aglietta M, Merlano MC. NK-mediated antibody-dependent cell-mediated cytotoxicity in solid tumors: biological evidence and clinical perspectives. *Annals of translational medicine*. 2019;7(5):105-105.
177. Farag SS, Flinn IW, Modali R, Lehman TA, Young D, Byrd JC. FcγRIIIa and FcγRIIa polymorphisms do not predict response to rituximab in B-cell chronic lymphocytic leukemia. *Blood*. 2004;103(4):1472-1474.
178. Forconi F, Moss P. Perturbation of the normal immune system in patients with CLL. *Blood*. 2015;126(5):573-581.
179. Morrison VA. Infectious complications of chronic lymphocytic leukaemia: pathogenesis, spectrum of infection, preventive approaches. *Best Practice & Research Clinical Haematology*. 2010;23(1):145-153.
180. Ysebaert L, Gross E, Kühlein E, et al. Immune recovery after fludarabine-cyclophosphamide-rituximab treatment in B-chronic lymphocytic leukemia: implication for maintenance immunotherapy. *Leukemia*. 2010;24(7):1310-1316.
181. Tam CS, O'Brien S, Wierda W, et al. Long-term results of the fludarabine, cyclophosphamide, and rituximab regimen as initial therapy of chronic lymphocytic leukemia. *Blood*. 2008;112(4):975-980.
182. Hartkamp A, Mulder AH, Rijkers GT, van Velzen-Blad H, Biesma DH. Antibody responses to pneumococcal and haemophilus vaccinations in patients with B-cell chronic lymphocytic leukaemia. *Vaccine*. 2001;19(13-14):1671-1677.
183. Pasiarski M, Rolinski J, Grywalska E, et al. Antibody and plasmablast response to 13-valent pneumococcal conjugate vaccine in chronic lymphocytic leukemia patients--preliminary report. *PloS one*. 2014;9(12):e114966-e114966.
184. Mauro FR, Giannarelli D, Galluzzo CM, et al. Response to the conjugate pneumococcal vaccine (PCV13) in patients with chronic lymphocytic leukemia (CLL). *Leukemia*. 2020.
185. King GW, Grozea PC, Eyre HJ, LoBuglio AF. Neoantigen response in patients successfully treated for lymphoma. A Southwest Oncology Group study. *Ann Intern Med*. 1979;90(6):892-895.
186. van Dongen JJM, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17(12):2257-2317.
187. Winkelstein JA, Marino MC, Lederman HM, et al. X-Linked Agammaglobulinemia: Report on a United States Registry of 201 Patients. *Medicine*. 2006;85(4):193-202.
188. Byrd JC, Furman RR, Coutre SE, et al. Three-year follow-up of treatment-naïve and previously treated patients with CLL and SLL receiving single-agent ibrutinib. *Blood*. 2015;125(16):2497-2506.

Statutory declaration/Eidesstattliche Versicherung

I hereby declare that I have written the present thesis unassisted solely with the resources and references indicated. The content of this work has not been previously submitted for a degree in any educational institution.

Hiermit erkläre ich, dass die vorliegende Arbeit von mir selbst ohne fremde Hilfe und nur mit den angegebenen Quellen und Hilfsmitteln verfasst wurde. Der Inhalt wurde noch nicht an einer anderen Einrichtung für die Erlangung eines Doktorgrades eingereicht.

Hamburg, den 25.03.2021 Lisa von Wenserski

Danksagung

Auf dem Weg der bis zum Verfassen dieser Doktorarbeit nun hinter mir liegt, haben mich zahlreiche Menschen begleitet, denen ich an dieser Stelle Danke sagen möchte.

Mascha, die mir die Möglichkeit gegeben hat Teil ihres Labors und damit vieler spannender Projekte zu sein. Vielen Dank für deinen ansteckenden Optimismus, deine Begeisterung für die Forschung und das Vertrauen mich trotz vieler Sackgassen immer neue Wege gehen zu lassen, um so am Ende die Nadel im Heuhaufen zu finden.

Meiner Mutter und meinem Bruder, die mich immer bedingungslos unterstützen, in dunklen Momenten aufheitern und die ich jederzeit an meiner Seite weiß.

Meinen Freunden, die mich teilweise schon seit dem Studium oder noch länger begleiten, immer ein offenes Ohr für mich haben, mich die Welt außerhalb des Labors nicht vergessen lassen und den Alltag so oft weniger grau machen.

Meinen Kollegen am UKE in Hamburg, die mich mit offenen Armen empfangen haben und mir stets mit Rat und Tat zur Seite standen. Danke für die vielen gemeinsamen Stunden innerhalb und außerhalb des Labors, für die schönen Erinnerungen und dafür, dass ich euretwegen immer gern zur Arbeit gekommen bin.

Ein besonderer Dank geht an Dona, die mich nicht nur als Wissenschaftlerin, sondern auch als Person während unserer gemeinsamen Zeit geprägt und inspiriert hat. Vor allem durch die Herausforderungen unseres Umzugs nach Halle und darüber hinaus waren unsere Gespräche für mich eine große Bereicherung und Motivation zugleich.

Christoph, der mit seinem trockenen Humor und unerschütterlicher Ruhe ebenfalls dazu beigetragen hat, im Umzugschaos oder Verwaltungslabyrinth nicht die Nerven zu verlieren und der auch für jedes Problem im Labor eine Lösung parat hatte (oft aber nicht ausschließlich in Form von Keksen).

Der „neuen“ AG Binder und allen Menschen in Halle, die meine Zeit dort auf vielen Ebenen zu einer unvergesslichen und lehrreichen Erfahrung gemacht haben, die ich nicht missen möchte.

Robert, für dein Verständnis und deine Unterstützung, für zahllose Stunden in Zügen und an Bahnhöfen, für das geduldige Zuhören, für alle Umzüge, fürs Zurückstecken, fürs Aufbauen, Ermutigen und Anschubsen, fürs Rücken freihalten, für deinen unerschütterlichen Optimismus, für alle Excel-Listen und für das, was am Ende wirklich zählt.

Curriculum vitae

Lisa von Wenserski, M.Sc.

Date of birth	07.01.1991
Place of birth	Hennigsdorf
nationality	german

Education/Employment

10/2018-07/2020	University hospital Halle (Saale)
	Research associate/PhD student in the lab of Mascha Binder
01/2017-09/2018	University hospital Hamburg Eppendorf
	Research associate/PhD student in the lab of Mascha Binder
10/2014-09/2016	Philipps Universität Marburg
	Biomedical Science/Tumor biology M.Sc. (final grade: 1.2)
	Master thesis: Characterization of the synthetic lethal interactions between ATR and PRIM1/POLD1 for the identification of novel approaches to tumor therapy (1.2)
10/2011-09/2014	Philipps Universität Marburg
	Biomedical Science B.Sc. (final grade: 1.7)
	Bachelor thesis: Biochemical characterization of the small isoform of dCoREST
09/2003-06/2010	Louise-Henriette-Gymnasium, Oranienburg Abitur (final grade: 1.2)

Hamburg, 25.03.2021 Lisa von Wenserski

Publications

von Wenserski L, Schultheiß C, Bolz S, Schliffke S, Simnica D, Willscher E, Gerull H, Wolters-Eisfeld G, Riecken K, Fehse B, Altfeld M, Nollau P, Binder M. SLAMF receptors negatively regulate B cell receptor signaling in chronic lymphocytic leukemia via recruitment of prohibitin-2. Leukemia. 2020 Aug 21. doi: 10.1038/s41375-020-01025-z. Online ahead of print.

Schultheiß C, Paschold L, Simnica D, Mohme M, Willscher E, **von Wenserski L**, Scholz R, Wieters I, Dahlke C, Tolosa E, Sedding DG, Ciesek S, Addo M, Binder M. Next-Generation Sequencing of T and B Cell Receptor Repertoires from COVID-19 Patients Showed Signatures Associated with Severity of Disease. Immunity. 2020 Aug 18;53(2):442-455.e4. doi: 10.1016/j.immuni.2020.06.024. Epub 2020 Jun 30.

Simnica D, Akyüz N, Schliffke S, Mohme M, **von Wenserski L**, Mährle T, Fanchi LF, Lamszus K, Binder M. T cell receptor next-generation sequencing reveals cancer-associated repertoire metrics and reconstitution after chemotherapy in patients with hematological and solid tumors. Oncoimmunology. 2019 Jul 25;8(11):e1644110. doi: 10.1080/2162402X.2019.1644110.

Tintelnot J, Metz S, Trentmann M, Oberle A, **von Wenserski L**, Schultheiß C, Braig F, Kriegs M, Fehse B, Riecken K, Bokemeyer C, Stein A, Binder M. Cancer Cells Expressing Oncogenic Rat Sarcoma Show Drug-Addiction Toward Epidermal Growth Factor Receptor Antibodies Mediated by Sustained MAPK Signaling. Front Oncol. 2020 Jan 21;9:1559. doi: 10.3389/fonc.2019.01559. eCollection 2019.

Job A, Schmitt LM, **von Wenserski L**, Lankat-Buttgereit B, Gress TM, Buchholz M, Gallmeier E. Inactivation of PRIM1 Function Sensitizes Cancer Cells to ATR and CHK1 Inhibitors. Neoplasia. 2018 Nov;20(11):1135-1143

Brandt A, Matschke J, Fehrle W, **von Wenserski L**, Bokemeyer C, Illerhaus G, Binder M. A significant proportion of patients with primary central nervous system lymphoma harbor clonal bone marrow B-cells. Leuk Lymphoma. 2018 Jul 22:1-7

Schliffke S, Buhs S, Bolz S, Gerull H, **von Wenserski L**, Rieken K, Fehse B, Nollau P, Binder M. The phosphotyrosine phosphatase SHP2 promotes anergy in chronic lymphocytic leukemia. Blood. 2018 Feb 23. pii: blood-2017-06-788166.

Schliffke S, Sivina M, Kim E, **von Wenserski L**, Thiele B, Akyüz N, Falker-Gieske C, Statovci D, Oberle A, Thenhausen T, Krohn-Grimberghe A, Bokemeyer C, Jain N, Estrov Z, Ferrajoli A, Wierda W, Keating M, Burger J.A, Binder M. Dynamic changes of the normal B lymphocyte repertoire in chronic lymphocytic leukemia in response to ibrutinib or FCR chemo-immunotherapy. Oncoimmunology. 2018 Jan 15;7(4):e1417720.